The role of γδ T cells in peritoneal dialysis-associated bacterial infection

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Dedication

This thesis is dedicated to my family, especially my wife Yoke-Kuen and parents who have believed in me and supported me throughout all my studies.

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Summary

Despite advances in treatment, peritoneal dialysis (PD)-associated peritonitis remains a major cause of morbidity and mortality in PD patients. Given that peritonitis can be the proximate cause of technique failure and cause ultrafiltration failure at a later time, it is important to understand the peritoneal immune response, microbiology and outcomes of these infections.

Data presented in this thesis have shown that leukocytes are recruited to the peritoneal cavity, starting with a rapid accumulation of neutrophils, which are later replaced by a population of mononuclear cells, including monocytes/macrophages and T cells during acute peritonitis. Of note, Vγ9/Vδ2 T cells are also recruited to the peritoneal cavity in the early stage, which implies a significant role of Vγ9/Vδ2 T cells as early responders in acute peritonitis.

In patients with acute peritonitis, the capacity of the causative pathogen to produce (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), together with the infiltration of activated Vγ9/Vδ2 T cells are important risk factors and possible predictors of patient outcomes from infection. By performing a detailed immunological and microbiological analysis in PD patients on the first day of peritonitis, our findings provide proof of concept that acute bacterial infections indeed leave characteristic disease-specific '*immune fingerprints*' of diagnostic and prognostic value. Local fingerprints not only discriminated between episodes of culture-negative and culture-positive PD-associated peritonitis but also predicted infections caused by $Gram^-$ or $Gram^+$ bacteria.

HPMC play an important role in maintaining homeostasis of the peritoneal immunity. Our data revealed the regulation of Vγ9/Vδ2 T cells by HPMC and demonstrated that resting HPMC were potent suppressors of Vγ9/Vδ2 T-cell cytokine production and proliferation in the presence of HMB-PP.

Collectively, these findings improve our insight into the complex cellular interactions in PD-associated peritonitis and peritoneal homeostasis, identify novel biomarkers of possible diagnostic and predictive value and highlight new avenues for therapeutic intervention.

Publications and Presentations

Publication

Davey MS*, Lin CY*, Roberts GW, Heuston S, Brown AC, Chess JA, Toleman MA, Gahan CG, Hill C, Parish T, Williams JD, Davies SJ, Johnson DW, Topley N, Moser B, Eberl M. Human neutrophil clearance of bacterial pathogens triggers anti-microbial γδ T cell responses in early infection. PLoS Pathog. 2011 May; 7(5): e1002040. Epub 2011 May 12. **(*equal contribution to manuscript)**

Oral presentations arising from this thesis

Peritoneal dialysis research day, Sheffield, UK, 18th January 2011

"The HMB-PP producing capacity of the causative pathogen predict clinical outcome in PD-associated peritonitis"

The 10th European Peritoneal Dialysis Meeting, Birmingham, UK, 21st-24th October 2011

"The HMB-PP producing capacity of the causative pathogen predict outcome in peritoneal dialysis-associated peritonitis"

Poster presentations arising from this thesis

The 9th European Peritoneal Dialysis Meeting, Palais des Congrés, Strasbourg, France, 9th - 12th October 2009

"The HMB-PP producing capacity of the causative pathogen can predict early outcome in peritoneal dialysis-related bacterial peritonitis"

4th International gamma/delta T-Cell Conference, Kiel, Germany, 19th - 21th May 2010

"The HMBPP-producing capacity of the causative pathogen predicts early outcome in first-time episodes of bacterial peritonitis"

I3-IRG Annual Meeting at St Donat's Castle, South Wales, UK, 8th -9th July 2010

"The HMBPP-producing capacity of the causative pathogen predicts early outcome in first-time episodes of bacterial peritonitis"

Annual congress of the British Society of Immunology, Liverpool, UK, 6th -9th December 2010

"The HMB-PP producing capacity of the causative pathogen and local γδ T cell numbers predict clinical outcome from bacterial peritonitis"

BSI Summer School 2011, St Donat's Castle, South Wales,UK, 5th - 8th July 2011

"The HMB-PP producing capacity of the causative pathogen predict outcome in peritoneal dialysis-associated peritonitis"

The 10th European Peritoneal Dialysis Meeting, Birmingham, UK, 21st - 24th October 2011

"Peritoneal dialysis-associated infection: limited inflammatory responses and superior clinical outcome from culture-negative peritonitis"

Awarded prize for best poster presentation

5th International gamma/delta T-Cell Conference, Freiburg, Germany, 31th May - 2nd June 2012

"A regulatory crosstalk between γδ T cells and mesothelial cells"

Abbreviations

ALK	Activin receptor-like kinase
ANZDATA	Australia and New Zealand Dialysis Transplant Registry
APC	Antigen presenting cells
APD	Automated peritoneal dialysis
AUROC	Area under the receiver operating characteristic curve
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CAPD	Continuous ambulatory peritoneal dialysis
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CKD	Chronic kidney disease
CTL	Cytotoxic T cells
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ESRD	End-stage renal disease
FCS	Foetal calf serum
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HD	Haemodialysis
HLA	Human leukocyte antigen
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HPMC	Human peritoneal mesothelial cells
IFN-γ	Interferon gamma
IL	Interleukin
IPP	Isopentenyl pyrophosphate
LPS	Lipopolysaccharides
mAb	monoclonal antibody
MC	Mesothelial cells
MCP-1	Monocyte chemoattractant protein-1

MHC	Major histocompatibility complex
MMP-3	Matrix metalloproteinase-3
NKF-K/DOQI	National Kidney Foundation - Kidney Disease Outcomes Quality Initiative
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Peritoneal dialysis
PET	Peritoneal equilibration test
PGE-2	Prostaglandin-E2
PMA	Phorbol myristate acetate
RANTES	Regulated upon activation, normal T-cell expressed and secreted
SEM	Standard error of the mean
SD	Standard deviation
SIL-6R	Soluble interleukin-6 receptor
T_{CM}	Central memory T cells
TCR	T cell receptors
T_{EM}	Effector memory T cells
T_{EMRA}	$CD45RA^{+}$ effector memory T cells
TGF-β	Transforming growth factor beta
T_H	T helper cells
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Treg	Regulatory T cells
WBC	White blood cells

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Chapter 1

Introduction

1.1 Chronic kidney disease

1.1.1 Definition and staging system

Chronic kidney disease (CKD) is a worldwide public health problem. The National Kidney Foundation - Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) workgroup has defined CKD as the following (1):

1) The presence of markers of kidney damage for ≥ 3 months, as defined by structural or functional abnormalities of the kidney with or without decreased glomerular filtration rate (GFR), that can lead to decreased GFR, manifest by either pathological abnormalities or other markers of kidney damage, including abnormalities in the composition of blood or urine, or abnormalities in imaging tests; or 2) the presence of GFR < 60 ml/min/1.73 m2 for ≥ 3 months, with or without other signs of kidney damage as described above.

The staging system (Table 1.1) for CKD is intended to aid clinicians in the management of patients with CKD by identifying those with the most severe disease who are, therefore, at greatest risk for progression and complications.

Table 1.1 Stages of chronic kidney disease

Stage	Description	GFR (ml/min/1.73 m^2)
1	Kidney damage with normal or ↑GFR	≥ 90
2	Kidney damage with mild ↓GFR	60~89
3	Moderate ↓GFR	30~59
4	Severe ↓GFR	15~29
5	Kidney failure	<15 (or dialysis)

Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies. Adapted from (1).

Once the patient has reached the stage of near end-stage renal disease (stage 5 with GFR less than 15 ml/min), signs and symptoms related to uraemia begin to occur, such as malnutrition, anorexia, nausea, vomiting, fatigue, sexual dysfunction, platelet dysfunction, pericarditis, and neuropathy. When these problems reach a critical stage, renal replacement therapy will be required.

1.1.2 Renal Replacement Therapy

The 2006 K/DOQI guidelines recommend that patients with a GFR less than 30 ml/min per 1.73 m^2 should be educated about kidney failure and options for its treatment, including renal transplantation, peritoneal dialysis (PD) and haemodialysis (HD) (2).

Renal transplantation is one of the treatment choices for end-stage renal disease (ESRD). A successful renal transplant improves the quality of life and reduces the mortality risk for most patients, when compared with maintenance dialysis (3). However, not all patients are appropriate candidates for a kidney allograft. Furthermore, there is a shortage of donated organs and the waiting list for transplantation is growing (4). As a consequence, most patients with ESRD receive maintenance dialysis to support their renal function.

There are two types of dialysis: PD and HD. The choice between PD and HD is influenced by a number of considerations such as availability and convenience, medical factors, and socioeconomic and dialysis centre factors. Starting patients on PD as their initial treatment modality seems appropriate for many reasons. One reason is to take potential advantage of the better survival of PD patients compared with HD patients during the first 2 years of dialysis treatment (5). Other reasons to offer PD as the first-choice modality include the lower cost of therapy, convenience of home therapy, a flexible schedule, and increased freedom from the patient's perspective (6).

1.2 Peritoneal Dialysis

1.2.1 General consideration

Over the last thirty years, PD has become an alternative to HD for the treatment of ESRD that is widespread around the world. Its popularity has increased greatly, mainly because of its simplicity, convenience, and offering patients a home-based therapy. Studies comparing differences in patient mortality between PD and HD have shown conflicting results. The relative long-term outcome with PD compared to HD remains uncertain, but peritoneal dialysis may offer a slight advantage in younger, nondiabetic patients (7, 8).

1.2.2 Peritoneal dialysis technique

PD requires a peritoneal dialysis catheter as the access to permit clean dialysis solution to enter the patient's peritoneal cavity and let wastes and fluid drain out later (Figure 1.1). The transport of solutes and water between circulation and the dialysis solution in the peritoneal space is based on the ability of the peritoneal membrane to function as a dialyzing membrane. The dialysis solution in the peritoneal space typically contains sodium, chloride, calcium, and lactate or bicarbonate and is made hyperosmolar by the inclusion of a high concentration of glucose (9).

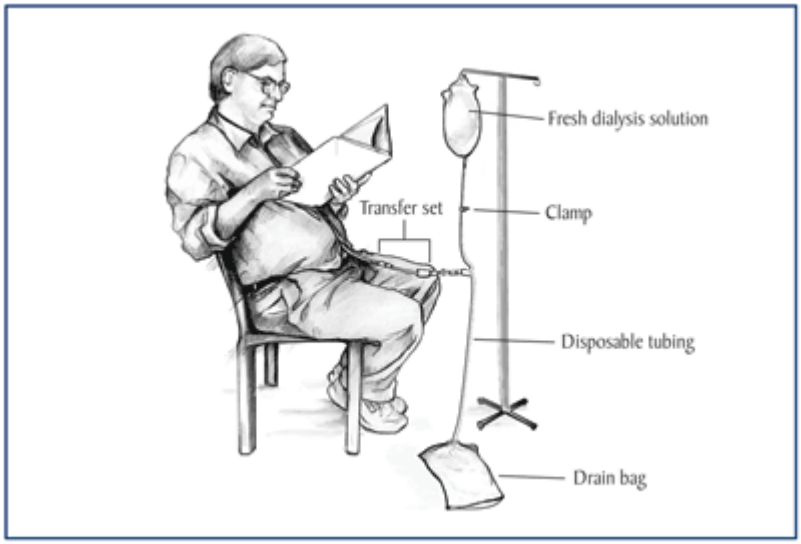

Figure 1.1 This drawing shows a person having peritoneal dialysis.
Taken from: Treatment Methods for Kidney Failure: Peritoneal Dialysis. National Institute of Diabetes and Digestive and Kidney Diseases. Available at: *http://kidney.niddk.nih.gov/kudiseases/pubs/peritoneal*.

1.2. 3 Physiology of peritoneal transport

The peritoneal membrane is a complex membrane composed of the capillary endothelium, the interstitium, and the mesothelium (9). The transport of water and solutes across this membrane best fits the three-pore model that describes the presence of three different sized pores in the peritoneal membrane. Large pores that constitute less than 0.01% of the total number of pores are thought to represent clefts between endothelial cells that allow the transport of macromolecules such as proteins. Numerous small pores are responsible for the transport of lower molecular weight solutes such as urea and creatinine, while ultra-small pore water channels (aquaporin-1) allow the movement of water only (10).

In PD, solute transport across the peritoneum occurs by the processes of diffusion and ultrafiltration. Diffusion varies directly with the magnitude of the concentration gradient and inversely with the size of the solute. During the course of a PD dwell, smaller solutes such as creatinine and urea diffuse down a concentration gradient from the peritoneal capillary blood into the PD solution. The concentration gradient across the membrane is highest in the beginning of the dwell. Thus, the removal of small solutes such as urea is maximal in the beginning, and falls progressively during the course of the dwell. Diffusion becomes more restricted as molecular weight increases. Glucose, lactate, and calcium will diffuse in the opposite direction, from dialysate to the blood.

Ultrafiltration occurs as a result of an osmotic pressure gradient between the dialysis solution and the peritoneal capillary blood. The dialysis solution is made hypertonic usually by the addition of high concentrations of glucose. Ultrafiltration is maximal at the beginning of a dwell when the osmotic pressure gradient is highest. The osmotic pressure gradient will decrease over time due to the dilution of the glucose by ultrafiltrate and by the diffusion of glucose from the peritoneal cavity into the bloodstream. Fluid removal is maximized by using more hypertonic dialysis solutions or by doing more frequent exchanges.

During ultrafiltration, solutes present in body fluids will be transported by solvent drag, which is called convection, contributing to overall solute clearance. Water and solutes are also constantly being absorbed from the peritoneal cavity into the lymphatic system and this will counteract both solute and fluid removal (9).

1.2.4 Peritoneal equilibration test (PET)

In clinical practice, a patient's peritoneal membrane transport function can be assessed by measuring the creatinine equilibration curve and the glucose absorption curve during a standardized peritoneal equilibration test (9). The standardized four-hour PET involves a 2-L 2.5% dextrose dwell with dialysate samples taken at 0, 2, and 4 hours and a plasma sample at 2 hours. Net fluid removal is also measured along with the ratio of dialysate glucose at 4 hours to dialysate glucose at time zero. Patients are classified principally into one of four transport categories: high, high-average, low-average, and low.

High transporters will have the fastest equilibration of creatinine, but ultrafiltration will not be as great due to rapid absorption of glucose and dissipation of the osmotic gradient. High transporters also tend to have higher dialysate protein losses. In contrast, low transporters will have less complete equilibration of creatinine, but will have good net ultrafiltration due to the slower absorption of glucose into the bloodstream. Thus, high transporters tend to do better on regimens that have frequent, short-dwell exchanges, such as automated peritoneal dialysis (APD), whereas low transporters tend to do better on regimens with long-dwell exchange, such as continuous ambulatory peritoneal dialysis (CAPD) or continuous cyclic peritoneal dialysis. Patients with average transport rates can effectively be treated with either short- or long-dwell exchange techniques.

1.2.5 Modalities of PD

Chronic peritoneal dialysis is divided into CAPD and APD. CAPD typically involves four 2.0- to 2.5L dwells daily, with each lasting 4–8 hours. In APD, 3-10 dwells are instilled nightly using an automated cycler. In the daytime, the patient usually carries a dwell, which is drained each night before cycling recommences.

1.3 Acute peritonitis

PD associated peritonitis remains a major cause of morbidity and mortality in PD patients. Although less than 4% of peritonitis episodes result in death, peritonitis is a "contributing factor" to death in 16% of deaths on PD. Moreover, severe and prolonged peritonitis can lead to peritoneal membrane failure. It is a major cause for the switch from PD to HD, especially within the first 1 to 2 years of initiating PD (6, 11-15).

1.3.1 Diagnosis

Peritonitis is thought to occur most often by touch contamination at the time of catheter connection, but may also occur associated with exit site/tunnel infection or by transmural migration of bacteria across the intestinal wall. Patients with peritonitis usually present with cloudy peritoneal fluid and abdominal pain. When a patient presents with these complaints, the peritoneal fluid should be drained and the effluent sent for white blood cell (WBC) count and differential, Gram stain, and culture. The diagnosis of peritonitis is supported by the presence of WBC count more than 100/μl of PD effluent with at least 50% polymorphonuclear neutrophil cells (14).

1.3.2 Microbiology

Infections due to Gram-positive bacteria are most common (50-70% of episodes) compared to infections with Gram-negative bacteria (20–25%) or fungi (2–3%). Infection with mycobacteria is rare (16, 17). The proportion of Gram-negative peritonitis is increasing and such infections are becoming more severe with poor outcome (18). Among Gram-positive bacteria, coagulase-negative staphylococcus is the most common species, followed by *Staphylococcus aureus* and *Streptococcus* species. *Enterococcus* and *Corynebacterium* are less common. The most common Gram-negative organisms are *Pseudomonas, Escherichia coli*, and *Klebsiella* (13, 15). Peritonitis with multiple organisms or anaerobes should raise the concern of intra-abdominal pathology and lead to abdominal computed tomography scans and surgical evaluation.

1.3.3 Culture-negative peritonitis

Culture-negative peritonitis is defined as the presence of cloudy dialysate and/or abdominal pain in the presence of a WBC count of more than 100/µl of PD effluent with at least 50% polymorphonuclear neutrophil cells, with no microbiological growth at 72 hours. Most cases of culture-negative peritonitis result from recent antibiotic exposure, suboptimal sample collection/culture methods or unusual organism (*e.g.*, *mycobacteria*, or *mycoplasm*). However, noninfectious causes such as chemical peritonitis or chylous ascites or effluent eosinophilia (19) should also be considered, especially when peritonitis fails to respond to empirical antibiotic therapy (14, 20).

In keeping with the International Society for Peritoneal Dialysis recommendations, the rate of culture-negative peritonitis in a centre should not be greater than 20% of episodes when standard culture technique using blood-culture bottles is adopted (14). Using large-volume culture (*e.g.*, culturing the sediment after centrifuging 50 mL of effluent) could further improve the recovery of micro-organisms (21). There is not enough evidence for recommending the use of novel techniques such as broad-spectrum polymerase chain reaction with RNA sequencing for the diagnosis of peritonitis (22, 23).

1.3.4 Treatment of PD peritonitis

Patients who present with signs and symptoms of peritonitis are usually treated empirically with antibiotics covering both Gram-positive and Gram-negative organisms. Gram-positive organisms may be covered by vancomycin or a cephalosporin, and Gram-negative organisms by a third-generation cephalosporin or aminoglycoside. The choice of antibiotics should take into account the patient's infection history and the centre's history of resistant organisms (14). Antibiotics are usually administered by the intra-peritoneal route and can be given by intermittent or continuous dosing. Most patients will show considerable clinical improvement within 48 hours of starting antibiotic therapy. Final antibiotic therapy is guided by culture results and sensitivities. Treatment is continued for a total of 2 weeks, while more severe infections due to *Staphylococcus aureus*, *Pseudomonas*, or multiple

Gram-negative organisms are treated for 3 weeks. If there is no clinical improvement after 48 hours, cell counts and cultures should be repeated.

Refractory peritonitis is defined as failure to respond to appropriate antibiotics within 5 days and is usually managed by catheter removal. Other indications for catheter removal are fungal peritonitis, relapsing peritonitis, peritonitis in the setting of severe exit site or tunnel infection, and infection due to multiple enteric organisms in the setting of a surgical abdomen (14).

1.4 Inflammation in peritoneal dialysis

1.4.1 Anatomy of the peritoneal cavity

The peritoneum is a serosal membrane that lines the peritoneal cavity. It has a surface area that is found to correlate with body surface area and typically ranges from 1 to 2 m^2 in an adult (24). It is divided into two portions: (a) the visceral peritoneum, which lines the gut and other viscera, and (b) the parietal peritoneum, which lines the walls of the abdominal cavity (9). During peritoneal dialysis, the parietal peritoneum takes the major part of peritoneal transport, since only about 20~30% of the visceral peritoneum is in contact with the dialysis solution at a given time (25).

1.4.2 Peritoneal membrane histology

The peritoneal membrane is lined by a monolayer of mesothelial cells (MC). Mesothelial cells are active cells involving the structural and functional alteration of the peritoneum. They can coordinate inflammation, host defence, contributing to solute transport, and wound healing (26). Beneath the mesothelium is the interstitium, which contains collagen, fibroblasts, peritoneal capillaries and lymphatics (27, 28).

During PD, peritoneal cells are repeatedly exposed to a non-physiological hyperosmotic and hyperglycemic solutions with low pH environment. Long-term exposure to the bio-incompatible PD solutions results in denudation of the mesothelium, submesothelial compact zone thickening, hyalinizing vasculopathy, and an epithelial-to-mesenchymal transition (EMT) of MC (26, 29-31). The fibrotic process in the peritoneal membrane develops following acute and chronic release of

PD-related inflammatory mediators.

1.4.3 Chronic inflammation in PD patients

Chronic inflammation is common in PD patients. The inflammatory status of PD is affected by reduced renal excretion of cytokines, dialysis procedure and comorbidities including chronic heart failure and coronary heart disease (32). Inflammation in PD patients is associated with a poor prognosis. Noh *et al.* demonstrated that elevated serum C-reactive protein level is associated with increased mortality of CAPD patients (33). Continuing exposure of bio-incompatible PD solutions, glucose degradation products, advanced glycation end-products, peritonitis, and uraemia all contribute to peritoneal inflammation (30, 34), which is characterized by increased vascular permeability, activation and expansion of the peritoneal macrophage population, recruitment of infiltrating cells to sites of injury, release of pro- and anti-inflammatory mediators, and increased matrix protein synthesis and tissue remodelling (26).

Major cell types involving the inflammation in PD patients include macrophages and mast cells, together with mesothelial cells, fibroblasts, and endothelial cells. Figure 1.2 summarises the cellular system known to contribute to PD-induced changes and includes the cytokines and chemokines produced.

1.4.4 Peritoneal Macrophages

Macrophages are the predominant cell type found in peritoneal cavity and play an important role in the first line of defence against invading microorganisms. During PD, reported peritoneal macrophage numbers are variable ranging from 20% to 95% (35, 36). Peritoneal macrophages from CAPD patients are activated with increased expression of Fc and C5a receptors, and human leukocyte antigen (HLA)-DR, which are important in host defence (37). Macrophages can release prostaglandin-E2 (PGE-2), interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and tumour necrosis factor (TNF)-α following stimulation of bacteria and thereby modulate the intraperitoneal inflammation (34, 38). In addition, $CD206^+$ and $CD163^+$ M2 macrophages were found in peritoneal effluent, which may participate in human peritoneal fibrosis through the stimulation of fibroblast cell growth and CCL18 production (39).

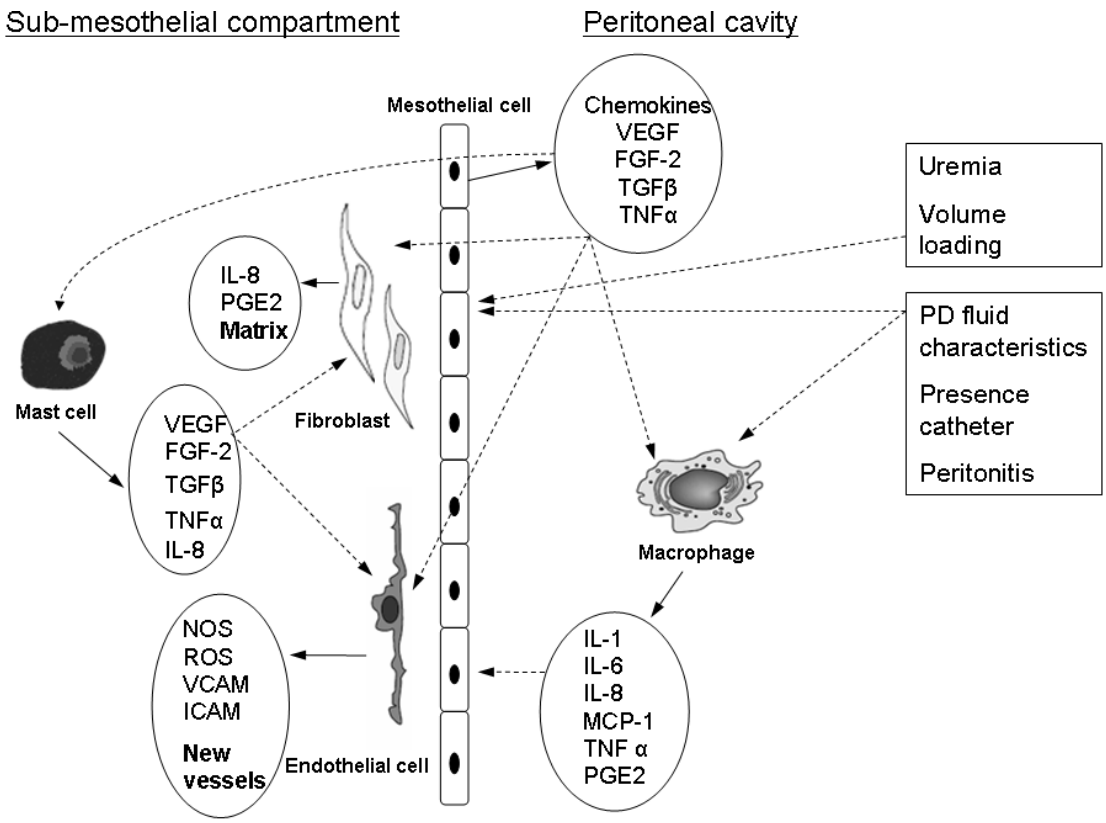

Figure 1.2 Cellular system of inflammatory response, including production of cytokines and chemokines, upon exposure to peritoneal dialysis fluid.

Dotted lines indicate activation of peritoneal cells; straight lines indicate production of cytokines and growth factors. IL = interleukin; PGE2 =prostaglandin E2; VEGF = vascular endothelial growth factor; FGF-2 = basic fibroblast growth factor; TGF-β = transforming growth factor beta; TNF-α = tumour necrosis factor alpha; NOS = nitric oxide synthase; ROS = reactive oxygen species; VCAM = vascular cell adhesion molecule; ICAM = intercellular adhesion molecule; MCP-1 = monocyte chemoattractant protein 1. Taken from (40).

1.4.5 Mesothelial Cells

By secreting numerous cytokines/chemokines, growth factors, matrix proteins, and intracellular adhesion molecules, along with their ability to present antigens to lymphocytes, mesothelial cells play a critical role as immunomodulators during peritoneal injury and inflammation (26). Mesothelial cells express HLA-DR and intercellular adhesion molecules and secrete IL-6, IL-8, RANTES (regulated upon activation, normal T-cell expressed and secreted), and MCP-1 on exposure to bacterial products and inflammatory cytokines (41-45).

Toll-like receptors (TLR) play a critical role in innate immune responses by specifically recognizing molecular patterns from a wide range of microorganisms, including bacteria, viruses, parasites and fungi (46). Recognition of bacterial pathogens by the peritoneum is mediated in part by TLR that induce inflammation by triggering nuclear factor kappa B and signalling transduction pathways, leading to the induction of inflammatory cytokines (46-48). Although human peritoneal mesothelial cells (HPMC) respond to TLR1/2, TLR2/6 and TLR5 ligands in a similar manner to murine cells, there are no cell surface TLR4 or responses to lipopolysaccharides (LPS) detected in it, unlike reports from murine cells (47-49).

Chronic exposure of human mesothelial cells to glucose degradation products resulted in a dose-dependent inhibition of cell growth, cell viability, IL-6 and fibronectin release, and increased synthesis of transforming growth factor beta (TGF-β) and vascular endothelial growth factor (30, 34). During PD, HPMC can undergo EMT with a down-regulation of E-cadherin and an induction of *snail* expression. In addition, profibrotic factor TGF-β and inflammatory cytokines may induce a complete transition of mesothelial cells, which may play an important role in peritoneal fibrosis leading to failure of peritoneal membrane function (29). Treating the cultured HPMC with bone morphogenic protein 7 (BMP-7), an intrinsic antifibrotic protein and member of the TGF-β superfamily, blocks high glucose or TGF-β1-induced EMT (50, 51). Aguilera *et al.* also demonstrated that EMT reversal of mesothelial cells by rapamycin, a mammalian target of rapamycin (mTOR) inhibitor (52).

In summary, extrinsic factors and PD fluid characteristics activate macrophages and mesothelial cells, which produce inflammatory cytokines and growth factors. Thereafter, these factors activate fibroblasts, endothelial cells, and mast cells, which secrete angiogenic and fibrotic cytokines and growth factors, playing an important role in tissue remodelling after long-term PD (Figure 1.2) (40).

1.5 Immune responses in acute peritonitis

1.5.1 Progression from innate to adaptive immunity

Peritonitis is an inflammatory process that involves all the components of the immune system (Figure 1.3). Immune responses are frequently categorized as innate and adaptive responses on the basis of the time course kinetics and contributing cells and mediators. The innate response involves mainly monocytes, neutrophils, or natural killer cells, and are readily activated following engagement of non-clonal receptors referred to as pattern recognition receptors such as TLR, which rapidly initiate cytokine production upon recognition of conserved microbial or endogenous danger signals.

Adaptive immune cells have functions that are similar to those of innate cells, but they can give stronger responses after repeated exposure to a given eliciting agent due to immunological memory. T cells and B cells are characterized by the expression of clonally variable antigen receptors that are built up from rearranged gene segments (53). The establishment of immunological memory implies expansion of T and B cells, specific for the eliciting antigen, after engagement of clonally distributed T cell receptors (TCR) and B cell receptors. Clonally expanded T and B cell populations acquire a restricted set of effector functions that are presumably best suited for clearance of the eliciting agent at a given time point in a given tissue (54).

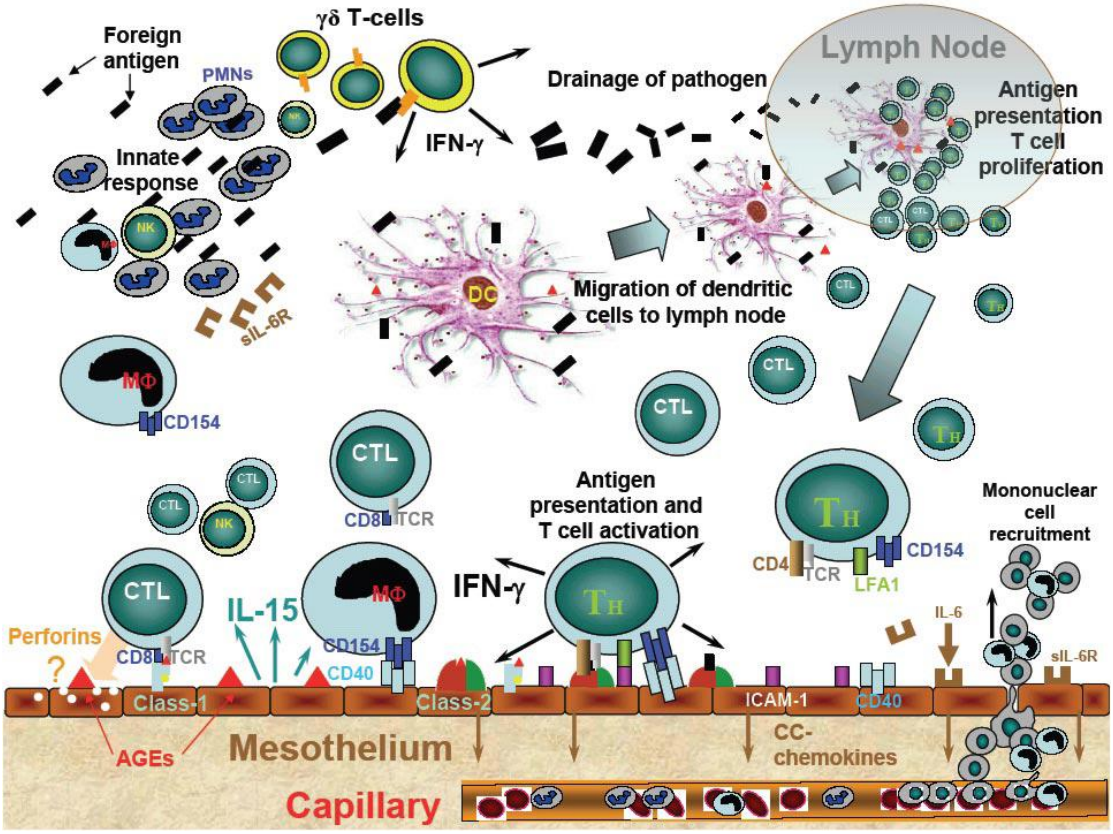

Figure 1.3 Immune responses in acute peritonitis.

The initial reaction against invading pathogens is usually made by cells of innate immunity (upper left). Unlike other T cells, γδ T cells are able to react against antigens without presentation on antigen presenting cells. Pathogens in the peritoneal fluid are drained to the lymphatic system and accumulate in lymph nodes, where their antigens are presented to T cells by dendritic cells (DC) (upper left). DC may migrate from the peritoneum to the lymph node to present antigens that they have processed. Stimulated T helper (T_H) cells and cytotoxic T lymphocytes (CTL) differentiate, proliferate, and migrate from lymph nodes to the peritoneum. Taken from (55).

1.5.2 Recruitment of mononuclear cells during peritonitis

During acute peritonitis, leukocytes are recruited to the peritoneum, starting with a rapid accumulation of neutrophils, which are later replaced by a population of mononuclear cells, including macrophages and lymphocytes (Figure 1.4) (56). This represents the transition from the innate immunity to adaptive immunity, which play an important role in the clearance of infection. IL-6 and its soluble receptor (sIL-6R) have been showed to be involved in controlling this pattern of leukocyte recruitment. The CXC chemokines, such as IL-8 (CXCL8), can stimulate the recruited neutrophils to release sIL-6R. In turn, the sIL-6R/IL-6 complex attenuates IL-8 production by HPMC, ensuring clearance of neutrophils, and simultaneously promoting the secretion of the CC chemokines, such as MCP-1 and RANTES, which increase the recruitment of monocytes and lymphocytes (57).

In addition to the effects of sIL-6R/IL-6 complex in regulation of the transition to mononuclear cell dominance during peritonitis, mononuclear cells positively regulate their recruitment by secretion of interferon gamma (IFN-γ) and by expression of CD154 (CD40 ligand). The expression of CD40, a member of the tumour necrosis factor receptor family, was shown to be present on HPMC (Figure 1.3) and was elevated on exposure to inflammatory cytokines. The ligation of CD40 and IFN-γ synergize to up-regulate RANTES secretion from HPMC (58). In a mouse model, CD40 in peritoneal mesothelial cells increased and CD154 expression was induced in peritoneal leukocytes after induction of peritonitis. Peritoneal macrophages were the main peritoneal leukocyte population to express CD154 (59). In a human study, a positive correlation between CD154 levels and mononuclear dominance, suggest that CD40–CD154 ligation plays an important role in the transition to mononuclear predominance in the late phase of peritonitis (60).

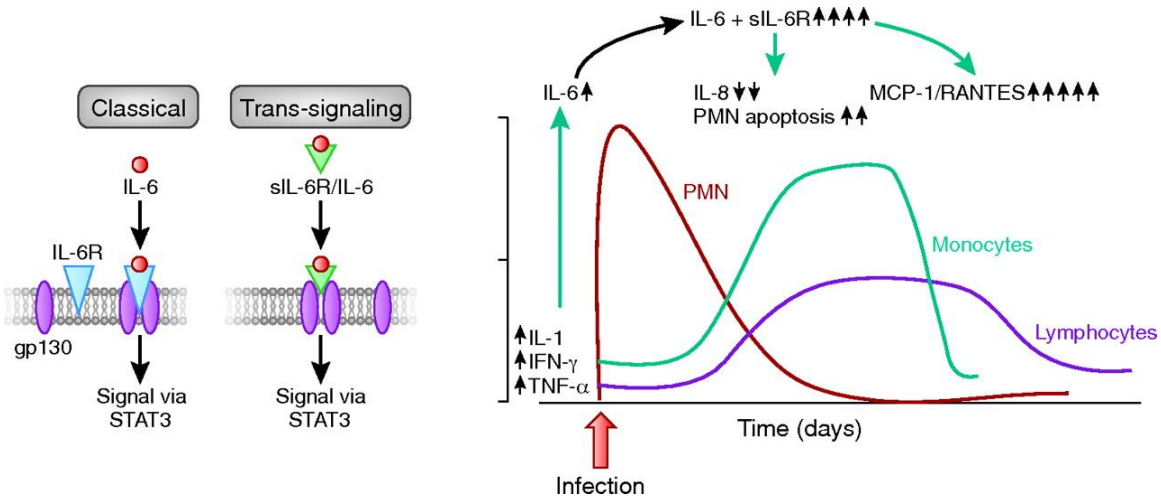

Figure 1.4 IL-6 and sIL-6R signalling in the regulation of leukocyte trafficking.

The regulation of leukocyte trafficking in the peritoneal cavity is mediated by proinflammatory cytokine– driven activation of IL-6/sIL-6R trans-signalling mediated through control of STAT3 activation that results in differential control of chemokine secretion and polymorphonuclear neutrophils (PMN) apoptosis. Taken from (56)

1.6 The role of peritoneal αβ T cells

In the normal peritoneal cavity, 5-10% of the leukocytes are lymphocytes. In PD patients, wide ranges of lymphocyte percentages in PD effluent have been found between 2% and 84% (35, 36). The majority of lymphocytes in both patients' peripheral blood and peritoneal effluent are T cells. Compared to patients' peripheral blood, more B cells are found in PD effluent (61). Several studies from PD effluent have reported the presence of activated lymphocytes, which suggests that some degree of local activation exists continuously in a patient's peritoneum (35, 61, 62). Although peritoneal lymphocytes are activated, they appear to be functionally impaired, with a significantly lower IL-2 production than is seen with peripheral lymphocytes (61).

1.6.1 TCR signal transduction and T cell subsets

Most mature T cells express T cell receptors composed of α/β protein heterodimers which are non-covalently linked to the signal-transducing CD3 complex. Engagement of TCR leads to the activation of Src family protein tyrosine kinases resulting in the phosphorylation of CD3 immunoreceptor tyrosine-based activation motifs and recruitment of Zeta-chain-associated protein kinase 70 (ZAP-70) followed by numerous downstream signals and leading to T cell activation (53). αβ T cells are further divided into subsets based on expressed co-receptors or distinct patterns of cytokine production. $CD4^+$ T cells recognize via their TCR peptides derived from exogenous antigens and presented in the context of major histocompatibility complex (MHC) class II molecules. In contrast, $CD8^+$ T cells recognize their cognate antigenic peptides generated endogenously, *e.g.* during viral infection via presentation by MHC class I molecules (63). It is obvious that $CD4^+$ T cells are functionally heterogeneous and comprise distinct subpopulations (Figure 1.5). There are at least 4 well-characterized $CD4^+$ T cell populations that can be distinguished on the basis of cytokines and the expression of specific transcription factors: T helper 1 (T_H1) cells produce IFN-γ as a key cytokine and express the transcription factor T-bet; T helper 2 (T_H2) cells produce IL-4, IL-5 and IL-13 under the control of the transcription factor GATA-3; T helper 17 cells produce IL-17(T_H17) and express the transcription factor RORγt; and regulatory T cells (Treg) which inhibit immune responses and express the transcription factor FoxP3.

However, there is substantial plasticity in the lineage commitment of $CD4^+$ T cells, pointing to the importance of the local microenvironment for the functional $CD4^+$ T cell development (64). Another T cell subpopulation is the γδ T cells, which contribute to anti-infective and tumour immune responses and regulate local immune surveillance (65).

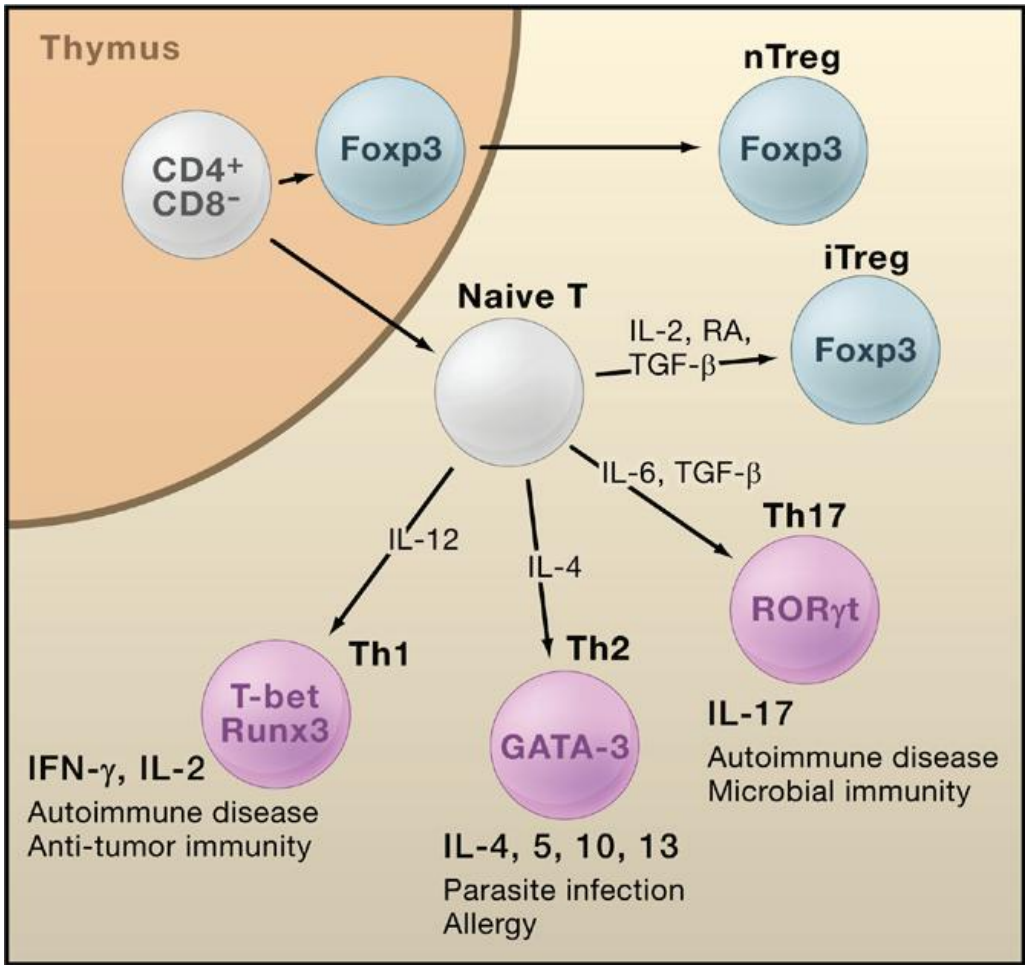

Figure 1. 5 Differentiation of naive $CD4^+$ T cells into Tregs or Effector T cells.

Cytokines and transcription factors that promote the differentiation of naive T cells into Tregs or effector T cells are shown. The transcription factors T-bet, GATA3, or RORγt are required for the differentiation of naive T cells into Th1, Th2, or Th17 cells, respectively. nTreg, natural Treg; iTreg, induced Treg; RA = retinoic acid. Taken from (66)

1.6.2 T_H1 and T_H2

T Helper cells provide help to B cells, $CD8^+$ cytotoxic T cells, and activating the cells of the innate immune system. They also play critical roles in the pathogenesis of autoimmunity, asthma, allergy and cancer (64). The differentiation of the $CD4^+$ T cell lineage into effector cells underlies successful adaptive immune responses aimed at distinct categories of pathogens. The first paradigm for this functional diversification was the description of T_H1 and T_H2 $CD4^+$ effector subsets by Mosmann and Coffman in 1986 (67). T_H1 cells were thought to be responsible for delayed type hypersensitivity, activating macrophages via release of IFN-γ and enabling them to kill intracellular pathogens. T_H2 cells were considered the classical helper T cells providing help to B cells to generate class switched antibodies (68).

In the peritoneum, T_H cells are one of the most important cell populations. PD associated peritonitis is predominantly the type 1 immune response, characterized by early induction of TNF-α, IL-1β, IL-12, and IL-18, followed by elevation of IFN-γ (69, 70). Wang et al. demonstrated that local IL-12 and IL-18 production is part of a protective early immune to PD associated peritonitis. High levels of IL-12 and IL-18 in PD effluent during the early phase of peritonitis correlated with a predominant type 1 immune response and favourable outcome (70).

1.6.3 Regulatory T cells

$CD4^+CD25^+$ Treg are vital for the preservation of immune tolerance. It has been shown that these cells suppress proliferation and cytokine production of naive $CD4^+CD25^-$ T cells, differentiated $CD4^+$ T cells, $CD8^+$ T cells, and natural killer cells *in vitro* and *in vivo*. They suppress immune responses through several mechanisms including the secretion of immunosupressive cytokines, cell-cell contact, and by modulating the function of antigen presenting cells (71). Besides thymic-derived Treg (natural or nTreg), it was shown that naive T cells in the periphery could acquire immunosuppressive properties and become induced Treg (iTreg). Absence of Foxp3, a forkhead/winged-helix transcriptional factor, results in widespread autoimmunity and absence of Treg. However, over-expression of Foxp3 results in immunosuppressive functions. Accordingly, Foxp3 was designated as the Treg master regulator (64, 66, 71).

Thymic-derived Treg (natural or nTreg): The $CD4^+CD25^+$ Treg represent 5-10% of all peripheral $CD4^+$ T cells. These cells develop in the thymus and then enter the peripheral tissue. It was observed that $CD4^+CD25^+$ T cells remained fairly constant in the peripheral blood and significantly increased in the peritoneal fluid 24 hours and 48 hours after elective surgery of the gastrointestinal tract (72). In a mouse model, adoptive transfer of in vitro stimulated $CD4^+CD25^+$ T cells significantly improved survival of infected mice. Furthermore, the effect on survival was accompanied by improved peritoneal bacterial clearance, enhanced peritoneal mast cell recruitment, and TNF-α production (73).

Induced Treg: Naive $CD4^+$ T cells in the periphery can acquire Foxp3 expression and Treg function in several experimental settings such as in vitro antigenic stimulation of naive T cells in the presence of TGF-β (74-76). It remains to be determined whether Treg induced from naive T cells in the periphery are functionally stable *in vivo* and to what extent they contribute to the peripheral pool of $Foxp3^+$ Treg (66).

1.6.4 T_H17

T_H17 cells are a subset of $CD4^+$ helper T cells characterized by producing IL-17 and express the lineage specific transcription factors, RORγt (77). In healthy individuals, less than 1% of $CD4^+$ T cells in peripheral blood are Th17 cells (78, 79). T_H17 cells might be actively recruited to and/or expanded in the pathological site, and their tissue localization could be important for T_H17 cell-associated pathology. Supporting the idea of active recruitment, the chemokine receptor CCR6 and the integrin CD49, which are molecules implicated in T_H17 tissue trafficking, are highly expressed on T_H17 cells in human blood and tissues (79, 80). Although T_H17 cells form a distinct T_H lineage under specific conditions *in vitro*, it is emerging that T_H17 cells exhibit plasticity in some *in vivo* settings (68) and the cytokine profile of T_H17 cells may be altered in tissues. For example, human T_H17 cells may express IL-4 (81), IFN-γ (78) and Foxp3 (82) in different pathological environments. Although the precise role and underlying mechanisms of T_H17 cells in tissues in human disease are unclear relative to other effector T cell subsets, it is thought that the plasticity could be important for T_H17 cell-associated pathology and -mediated immunity (68).

1.6.5 $CD8^+$ Cytotoxic T cells (CTL)

Cytotoxic T cells that express the CD8 co-receptor and recognize peptide–MHC class I complexes have a key role in clearing viral infections. During a primary immune response to pathogens, naive $CD8^+$ T cells expressing pathogen-specific T cell receptors clonally expand and differentiate into effector $CD8^+$ T cells that control the primary infection. This differentiation process produces effector CTL that can destroy virally infected cells through the targeted secretion of perforin and granzymes from lytic granules. After the primary infection is cleared there is a contraction phase when most of the effector CTL die by apoptosis. However, an effective immune response also produces a stable population of antigen-specific long lived memory $CD8^+$ T cells that can respond rapidly to clear secondary infections (83). T cells from the normal human peritoneum contain high frequencies of T_H2-type $CD8^+$ T cells (84). A previous study showed that a progressively decreasing CD4/CD8 ratio in PD effluent correlates with the persistent expression of TGF-β1 in the dialysate and may play a pathogenetic role in the evolution of peritonitis, peritoneal equilibration test deterioration and peritoneal fibrosis. The pattern of the CD4/CD8 T cell ratio in PD effluent may predict the outcome of peritonitis in CAPD patients (85).

1.7 The role of peritoneal γδ T Cells

Many reports indicate that γδ T cells play an important role in anti-infective and anti-tumour immune responses and regulate chronic inflammation (65). γδ T cells, which express γδ TCR instead of the αβ TCR found on classic T cells, constitute considerable variation (0.2-20%) of the $CD3^+$ T cells in human blood. Vγ9/Vδ2 T cells, Vγ2/Vδ2 T cells according to alternative nomenclature, are the major subset (50-95%) of the peripheral blood γδ T cells population (86-88). Unlike classical αβ T cells, Vγ9/Vδ2 T cells recognize nonpeptide ligands such as pyrophosphate antigens produced in the course of isoprenoid synthesis (89), alkylamines (90) and several synthetic aminobisphosphonate (91). The pyrophosphate antigens may be autologous (isopentenyl pyrophosphate, IPP) or microbial ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, HMB-PP) (87). This recognition is mediated by the TCR and is not restricted by MHC molecules (92).

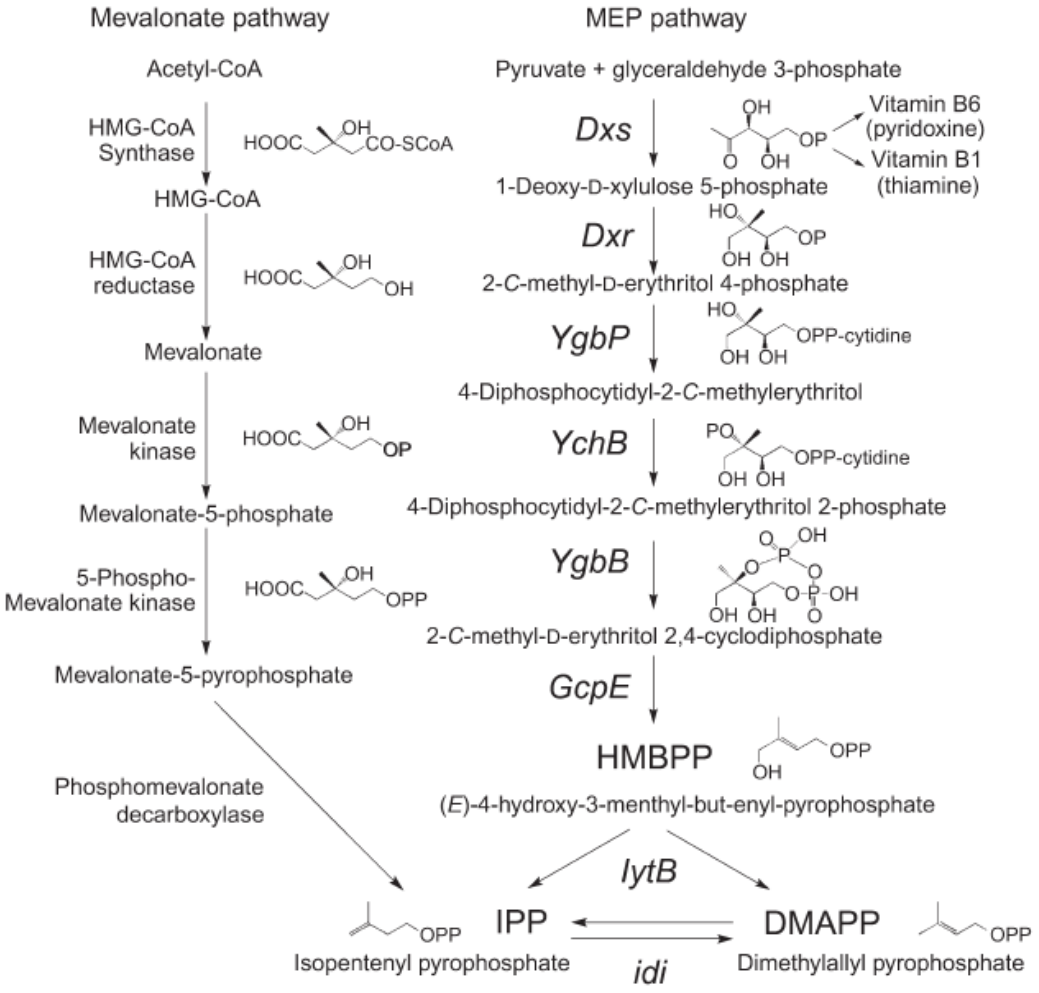

Figure 1.6 2-*C*-methyl-D-erythritol 4-phosphate (MEP) and mevalonate pathways for isoprenoid biosyntheiss.

Genes for MEP enzymes are also termed *ispC* (*dxr*), *ispD* (*ygbP*), *ispE* (*ychB*), *ispF* (*ygbB*), *ispG* (*gcpE*), and *ispH* (*lytB*). Taken from (89).

1.7.1 Antigen presentation to γδ T Cells: HMB-PP

HMB-PP is an intermediate of the non-mevalonate pathway of isoprenoid biosynthesis (Figure 1.6) that is found in many microbes, but not in higher eukaryotes including humans, and triggers Vγ9/Vδ2 T-cell responses at sub-nanomolar concentrations. Bacteria that possess the non-mevalonate pathway and hence produce HMB-PP represent some of the most detrimental human pathogens, such as the causative agents of tuberculosis, diphtheria, typhoid, plague and cholera (Table 1.2). HMB-PP is also produced by numerous commensal species in the mucosal flora and in faeces (87). Notable exceptions of clinical relevance that do not produce HMB-PP are Gram-positive *enterococci*, *streptococci* and *staphylococci* as well as Gram-negative *Legionella*.

Although Vγ9/Vδ2 T cells also recognize multiple compounds of highly variable structure, HMB-PP is 10,000 times more potent than any other physiological compound such as IPP and dimethylallyl pyrophosphate, substances often used in γδ T cell culture experiments and which might derive locally from necrotic or transformed host tissues (93). This allows efficient detection by human Vγ9/Vδ2 T cells of infected tissues, which produce minute amounts of microbial phosphoantigens, and prevents them from being activated by normal tissue cells that express basal levels of the weakly stimulatory mammalian metabolites. (54, 87, 94)

1.7.2 HMB-PP: an unconventional pathogen-associated molecular pattern

HMB-PP resembles classical pathogen-associated molecular patterns (PAMP) (95). It is: (1) an invariant metabolite produced by numerous Gram-positive and Gram-negative bacteria; (2) an essential metabolite in bacteria that synthesise isoprenoid precursors exclusively via non-mevalonate pathway, but not by higher eukaryocytes; and (3) specifically recognized by human and non-human primate γδ T cells (94). The molecular mechanism for the recognition of HMB-PP by Vγ9/Vδ2 T cells remains unclear. However, cell–cell contact with accessory cells is required for efficient Vγ9/Vδ2 T cell activation, and basically any nucleated human cell type is capable of acting as feeder cells (96, 97). Although the Vγ9/Vδ2 TCR is essential for the recognition of HMB-PP (and related compounds) (98), there is no evidence for a direct binding of soluble HMB-PP to the TCR (94).

Table 1.2 HMB-PP-producing capacities across a variety of microbial pathogens. Adapted from (94).

HMB-PP$^+$		
Gram$^-$ bacteria	Gram$^+$ bacteria	Other bacteria
Acinetobacter baumannii	*Bacillus anthracis*	*Ehrlichia chaffeensis*
Enterobacter aerogenes	*Clostridium difficile*	*Mycoplasma penetrans*
Escherichia coli	*Corynebacterium diphtheriae*	*Treponema pallidum*
Francisella tularensis	*Listeria monocytogenes*	
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 cholera		
Yersinia pestis		
HMB-PP$^-$		
Gram$^-$ bacteria	Gram$^+$ bacteria	Other bacteria
Legionella pneumophila	*Enterococcus faecalis*	*Borrelia burgdorferi*
	Staphylococcus aureus	*Mycoplasma genitalium*
	Streptococcus pneumoniae	*Rickettsia prowazekii*

1.7.3 Effector functions of γδ T cells

γδ T cells have a broad array of effector functions that reflect their involvement in diverse physiopathological processes. They can kill infected, activated or transformed cells, through pathways that involve the engagement of death-inducing receptors, such as CD95 (also known as FAS) and TNF-related apoptosis-inducing ligand receptors (TRAIL-R), and the release of cytotoxic effector molecules, such as perforin and granzymes (99, 100). Moreover, they contribute to pathogen clearance directly through the production of bacteriostatic or lytic molecules, such as granulysin and defensins (100, 101).

1.7.4 Differentiation of Effector/Memory γδ T cells

It is well established that circulating naive αβ T cells use CD62L and CCR7 to migrate to lymph nodes (102). They also express the CD45RA isoform and the costimulatory receptor CD27, which are switched off after primary antigen encounter (103). Conversely, experienced T cells consist of central memory (T_{CM}) ($CD45RA^{-}CCR7^{+}$), effector memory (T_{EM}) ($CD45RA^{-}CCR7^{-}$), and $CD45RA^{+}$ effector memory cells (T_{EMRA}) ($CD45RA^{+}CCR7^{-}$) (102, 104).

Similarly, Francesco *et al.* showed that the expression of CD45RA and CD27 antigens defines four subsets of human Vδ2 T cells with distinctive compartmentalisation routes (105). $CD45RA^{+}CD27^{+}$ Vδ2 T cells showed characteristics of naive cells with expression of $CD45RO^{-}$ and expressed the lymph node homing receptors CCR7 and CD62L (106). These cells did not express receptors for inflammatory chemokines such as CCR2, CCR5, CCR6, and CXCR3 (107). $CD45RA^{-}CD27^{+}$ Vδ2 T cells shared chemokine receptor expression with naive cells since they still express high levels of CD62L and CCR7, but about 25% of these cells also expressed CCR5 and CXCR3. Despite expression of CCR7 and CD62L, $CD45RA^{-}CD27^{+}$ Vδ2 T cells had the $CD45RO^{+}$ memory phenotype, thus resembling central memory αβ T cells (102). $CD45RA^{-}CD27^{-}$ Vδ2 T cells showed an effector phenotype, having down-regulated CD62L and CCR7. But they expressed high levels of receptors for inflammatory chemokines and were $CD45RO^{+}$. These results identify three subsets of human peripheral blood Vδ2 carrying distinct homing receptors. Of note, in inflammatory sites only two Vδ2 subsets were detected,

which were effector $CD45RA^{-}CD27^{-}$ subset and $CD45RA^{+}CD27^{-}$ phenotype. The latter one resembles a subset of terminally differentiated CD8 memory T cells (105, 108). Additionally, Vδ2 T cells collected from these inflammatory fluids expressed CCR5 and CXCR3, but were virtually negative for CCR7 and CD62L expression.

1.7.5 Antigen-Presenting γδ T Cells (γδ T-APC)

Antigen-stimulated human Vγ9/Vδ2 T cells take up and process antigen, and either present it on MHC-II, or cross-present it on MHC Class-I. These cells activated via the TCR and certain cytokines upregulate co-stimulatory molecules (CD80, CD86, CD40) and adhesion receptors (CD11a, CD18, CD54) and can turn naïve $CD4^{+}$ and $CD8^{+}$ αβ T cells into effector cells (Figure 1.7). αβ T cells differentiation induced by γδ T cells led to T helper cell response with a predominant pro-inflammatory cytokine (IFN-γ, TNF-α) profile (109, 110).

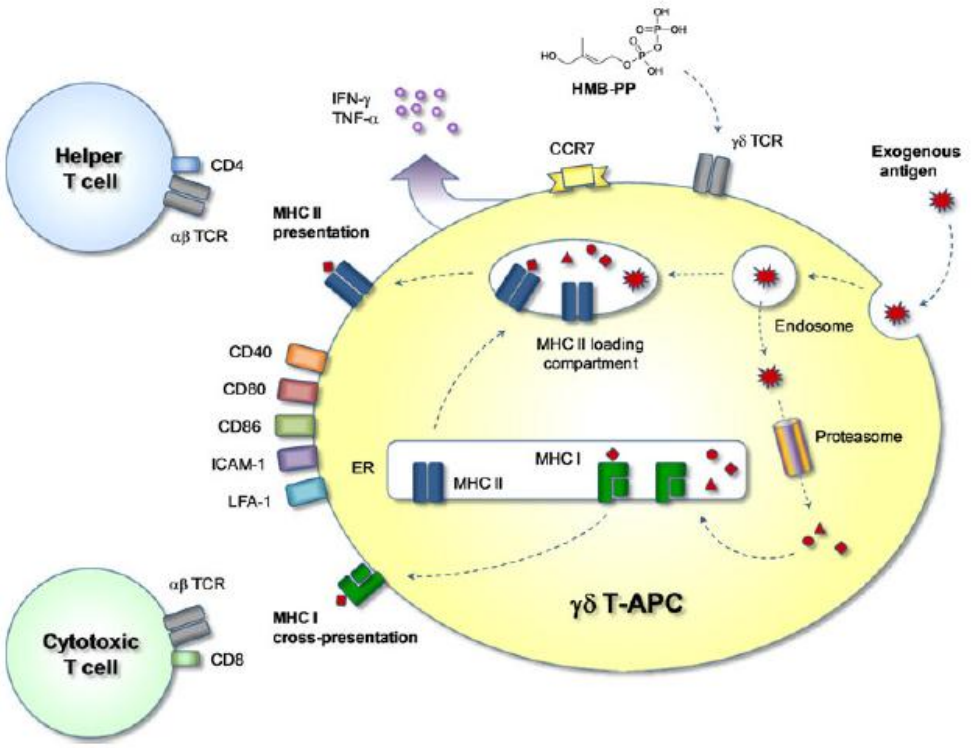

Figure 1.7 Antigen processing and presentation in Antigen-Presenting γδ T Cells (γδ T-APC). Exogenous antigens are taken up by endocytosis and are directed to MHC I or MHC II loading pathways. Activation of γδ T cells by HMB-PP leads to upregulation of costimulatory molecules, adhesion molecules, and the lymphoid homing chemokine receptor CCR7. Activated cells release cytokines such as IFN-γ and TNF-α, thus creating a proinflammatory microenvironment favouring the generation of $CD4^{+}$ Th1 cells and cytotoxic $CD8^{+}$ T cells. Taken from (111).

1.7.6 HMB-PP dependent interaction between γδ T cells, neutrophils and monocytes in acute microbial infection

Human blood Vγ9/Vδ2 T cells, monocytes and neutrophils share a responsiveness toward inflammatory chemokines and are rapidly recruited to sites of infection. Monocytes provide 'feeder' qualities for optimum stimulation of Vγ9/Vδ2 T cells with HMB-PP but that they also receive reciprocal differentiation signals. In an *in vitro* model, monocytes activated by HMB-PP stimulated γδ T cells undergo a rapid and substantial differentiation program toward an APC phenotype (112). Further study from the same group demonstrated that Vγ9/Vδ2 T cells also provide potent survival signals resulting in neutrophil activation and the release of the neutrophil chemoattractant CXCL8 (IL-8). In turn, Vγ9/Vδ2 T cells readily respond to neutrophils harbouring phagocytosed bacteria, as evidenced by expression of CD69, IFN-γ and TNF-α. This response is strictly dependent on the ability of these bacteria to produce the microbial metabolite HMB-PP and requires cell-cell contact of Vγ9/Vδ2 T cells with accessory monocytes through lymphocyte function-associated antigen-1, and results in a TNF-α dependent proliferation of Vγ9/Vδ2 T cells (113). The antibiotic fosmidomycin, which inhibits the first enzymatic step in the non-mevalonate pathway, not only has a direct antibacterial effect on most HMB-PP producing bacteria but also possesses immediate anti-inflammatory properties by inhibiting Vγ9/Vδ2 T cell responses *in vitro* (Figure 1.8) (113).

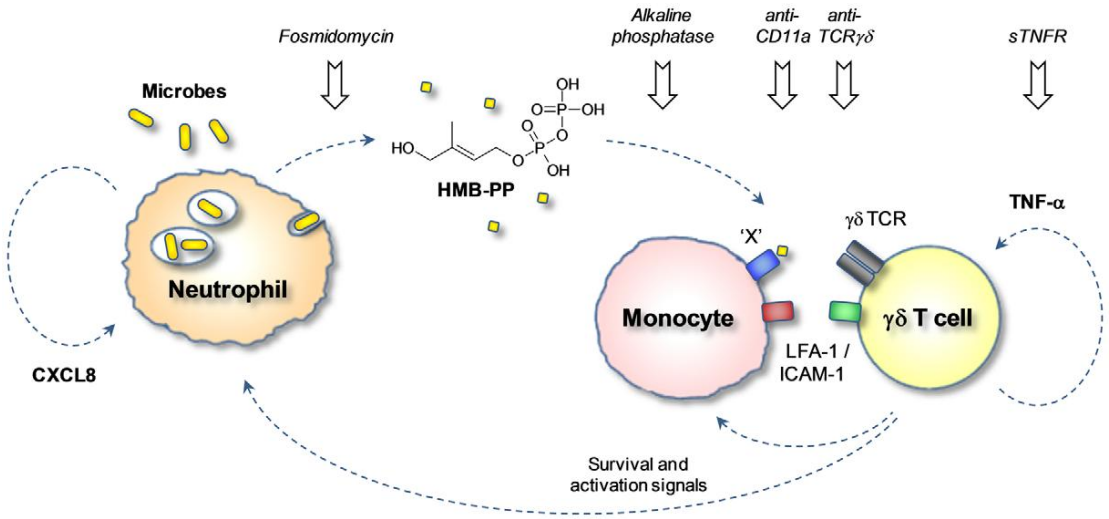

Figure 1. 8 HMB-PP dependent interaction between γδ T cells, neutrophils and monocytes in acute microbial infection.

Local secretion of inflammatory chemokines leads to extravasation of neutrophils, monocytes and γδ T cells toward the site of infection. Upon phagocytosis of invading microbes, neutrophils release traces of HMB-PP into the microenvironment where it becomes 'visible' to γδ T cells. γδ T cells recognize HMBPP in the context of a yet unidentified presenting molecule 'X' and contact-dependent signals provided by monocytes. Crosstalk between the three different cell types triggers the production of pro-inflammatory cytokines such as TNF-α, which drives local γδ T cell expansion, and chemokines such as CXCL8, which recruits further neutrophils to the site of infection. Activated γδ T cells also provide survival and activation signals such as TNF-α for newly arriving neutrophils and monocytes. This γδ T cell-driven inflammatory reaction can be interrupted at various check-points as demonstrated in the present study. Taken from (113).

Collectively, γδ T cells play a unique role in the modulation of innate and adaptive immunity, protective immunity against extracellular and intracellular pathogens, tumour surveillance, tissue healing and epithelial cell maintenance (86). Vγ9/Vδ2 T cells are rapidly activated by microbial phosphoantigens and produce cytokines including TNF-α and IFN-γ. Soon after activation, γδ T cells acquire the capacity to take up and process antigen and to present it to $CD4^+$ and $CD8^+$ T cells, and thereby help to initiate peptide-specific αβ T cell responses (110). In general, it appears that human Vγ9/Vδ2 T cells display a wide range of functional activities, referred to pro-inflammatory cytokine production, potent killer cell activity, regulatory (suppressive) functions (114), and antigen-processing and -presenting capacity.

1.8 Scope of this thesis

Peritoneal infection and associated inflammation remain frequent complications in PD patients. Key to developing improved approaches for infection prevention and therapy is a detailed understanding of the peritoneal immune response that contributes to infection resolution and long-term membrane damage and treatment failure. Peritonitis is a local inflammatory disorder, characterized by the recruitment and infiltration of leukocytes as essential elements of the immune response. Although T cells are present in high numbers and are recognized as likely major regulators of peritoneal inflammation, a comprehensive analysis of peritoneal T cell subsets has not been attempted, and little is known with respect to the functional characteristics of αβ and γδ T cell subsets and the correlation with clinical outcome in PD patients.

The specific aims for each investigative chapter are as follows:

Chapter 3: Clinical outcome analysis and characterisation of local immune responses including γδ T cells, $CD4^+$ T cells, and $CD8^+$ T cells in PD-associated bacterial peritonitis.

Chapter 4: To identify immunological signatures in PD-associated peritonitis to predict the nature of the causative pathogen and to discriminate among culture-negative, $Gram^+$ and $Gram^-$ episodes.

Chapter 5: Identification of the regulation of human γδ T-cell responses to HMB-PP by methothelial cells through an *in vitro* cell-culture model.

Chapter 2
Materials and Methods

2.1 Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines (Bro Taf Health Authority, Wales). Patient sampling was approved by the South East Wales Local Ethics Committee under reference numbers 04WSE04/27 (PD effluent) and 96/1730 (omentum). All patients provided written informed consent for the collection of samples and subsequent analysis.

2.2 Patient information and data collection

The Cardiff study population included 43 adult patients with end stage renal disease, who were receiving PD at the University Hospital of Wales, Cardiff, UK, and were treated with acute peritonitis (52 episodes) between September 2008 and January 2012. 15 stable patients without infection in the previous 3 months were included in this study as non-infected controls. In addition, microbiological and survival data were obtained from all 739 adult patients who received PD between 1987 and 2008 at the University Hospital of North Staffordshire, Stoke-on-Trent, UK; and all 2,542 Australian adult patients from the Australia and New Zealand Dialysis Transplant (ANZDATA) Registry who received PD between 2003 and 2008. Diagnosis of acute peritonitis was based on the presence of abdominal pain and cloudy peritoneal effluent with > 100 WBC/mm3. The day of the first appearance of leukocytes in the effluent was defined as day 1 post-infection. Infections were grouped into culture-positive and culture-negative episodes, according to the result of the microbiological analysis of the effluent. Bacteria identified in culture-positive infections were grouped into $Gram^+$ and $Gram^-$ species, or into $HMB\text{-}PP^+$ and $HMB\text{-}PP^-$ species, based on the presence or absence of HMB-PP in the microbial metabolism (89, 94). Cases of fungal infection or unrecorded culture results were excluded and endpoints of outcome analyses were 90^{th} day mortality and technique failure (catheter removal, transfer to haemodialysis, and/or patient death).

2.3 Leukocyte isolation from PD fluid

All general reagents, unless otherwise stated, were analytical grade and purchased from BD Bioscience (Oxford, UK), Beckman Coulter Ltd. (High Wycombe, UK), eBioscience (Hatfield, UK), Fisher Scientific UK Ltd. (Loughborough, UK), Invitrogen/Life Technologies Ltd (Paisley, UK), Nalgene/Thermo Fisher Scientific (Roskilde, Denmark), Miltenyi Biotec Ltd. (Bisley, UK), R&D Systems (Abingdon, UK), Sigma-Aldrich Co. Ltd. (Dorset, UK).

2.3.1 Total leukocyte isolation

PD effluent (mean drained volume 2146 ± 632 ml, range 930-3540 ml) was collected from patients after 4-10 hour dwell and total bag weight was recorded. Samples were collected on ice to reduce adhesion of cells to the PD bag and excess cellular changes. Following collection, the PD effluent was aliquoted into 500 ml centrifuge tube (Corning Inc., New York, USA) and centrifuged at 2000 rpm (953*g*) at 4℃ for 20 minutes. The cell-free supernatant was subsequently stored at -70℃ until assayed. The cell pellets were re-suspended in 50 ml phosphate buffered saline (PBS) (Invitrogen). Cell counts were performed using a dual chamber Neubauer haemocytometer (Assistent, Sondheim, Germany). The suspension was re-centrifuged at 1300 rpm (403*g*) at 4℃ for 8 minutes and the pellet was re-suspended in PBS at 2-3 $\times 10^7$ cells/ml.

2.3.2 Mononuclear cells isolation from total leukocytes

Mononuclear cells were isolated by discontinuous Percoll (Sigma-Aldrich) gradient. Percoll was adjusted to isotonicity by the addition of 9 parts (v/v) of Percoll to one part (v/v) of 1.5 M NaCl. Two concentrations of Percoll in medium were prepared (54% and 79%, prepared with 0.9% saline) (115, 116). 10 ml 79% Percoll was placed in the bottom of a 50 ml centrifuge tube (Corning Inc., New York, USA), then layered with 20 ml 54% Percoll, followed by 10 ml of total leukocyte suspension (up to 300 x 10^6 cells). The 50 ml tube was centrifuged at 1100 rpm (294*g*), at 18℃ for 20 minutes with minimal acceleration and no brake. The mononuclear cell layer formed at the interface of the 54% Percoll layer was aspirated in to a 50 ml tube and washed with PBS (x 2 times), which was centrifuged at 1300 rpm (403*g*), at 4℃ for 8 minutes. The cell pellet was re-suspended in PBS at 1-2 ×

10^7 cells/ml. Some cells were resuspended in freezing buffer (section 2.13.1) at 5-10 × 10^6 cells/ml in cryo-tubes (Greiner Bio-One Ltd., Gloucestershire, UK), which were immediately frozen in an isopropanol freezing container (Nalgene, Thermo Fisher Scientific) and stored at –70℃ overnight. Frozen cells were transferred to liquid nitrogen containment.

2.4 Leukocyte isolation from peripheral blood

2.4.1 Peripheral blood mononuclear cells (PBMC)

Venous blood was collected from consenting healthy laboratory volunteers in a tube containing 20U/ml of Heparin and 15mM of EDTA (Fisher Scientific UK Ltd). PBMC were isolated by layering 20 ml of whole blood over 20 ml of Ficoll-Paque (Axis-Shield, Oslo, Norway) and centrifuged at 1680 rpm (687*g*), at 18℃ for 20 minutes with minimal acceleration and no brake. The mononuclear cell layer formed at the interface of Ficoll-Paque was aspirated into a 50 ml tube and washed twice with PBS, which was centrifuged at 1300 rpm (403*g*), at 4℃ for 8 minutes. The cell pellet was re-suspended in PBS or medium depending on the experiment design.

2.4.2 Purified leukocytes using magnetic column

PBMC were re-suspended in 1 ml MACS buffer (section 2.13.2) in a 15 ml Falcon tube and blocked with 1% human normal immunoglobulin (KIOVIG, Baxter, Thetford, UK) for 10 minutes on ice. Following incubation, 2.5 µl anti-Vγ9 TCR PE-Cy5 monoclonal antibody (mAb) (1:400) (Immu360; Beckman-Coulter) was added and incubated for 15 minutes on ice in the dark. Following incubation, the cells were washed once with MACS buffer and re-suspended in 400 µl MACS buffer. 80 µl Anti-PE microbeads (1:5) (Miltenyi Biotec) were added and the cells were incubated for 15 minutes at 4℃ in the dark. Following incubation, the cells were washed once with MACS buffer and re-suspended to 2 ml. The cells were subjected to magnetic separation using LS columns (Miltenyi Biotec). $V\gamma9^+$ T cells (>98%) were purified from PBMC using positive selection. Thereafter, the pass-through fraction (non-$V\gamma9^+$ leukocytes) was used to purify Monocytes (>95%) with anti-CD14 microbeads (Miltenyi Biotec). Again, the pass-through fraction ($V\gamma9^-CD14^-$ leukocytes) was used to purify αβ T cells (90%) using pan T-Cell isolation kit (Miltenyi Biotec).

2.5 Leukocyte isolation from omental specimens

Omental tissues from consenting patients undergoing elective abdominal surgery, but not undergoing CAPD, were collected from surgery in a sterile plastic receptacle containing approximately 50ml of sterile PBS solution and stored at 4℃. The tissue (about 10 g) was processed immediately to obtain single-cell suspensions. Omental samples were minced and incubated with 10 ml Krebs Ringer bicarbonate buffer (section 2.13.3) with 2 mg/mL collagenase D (Roche, Welwyn, UK) and 4% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich), shaking in a water bath at 180 rpm, at 37℃ for 60 min. Samples were washed with Krebs Ringer bicarbonate buffer with 1% BSA (washing buffer), which was centrifuged at 1300 rpm (403*g*), at 4℃ for 8 minutes. After centrifugation, the cells were re-suspended in 5 ml washing buffer and passed twice through a 40 μm nylon mesh (BD Bioscience). The cell suspensions were centrifuged at 1300 rpm (403*g*), at 4℃ for 8 minutes and the cell pellet was lysed with 10 ml RBC lysis buffer (eBioscience) for 5 min at room temperature. After adding 10ml of complete RPMI medium (section 2.13.4), the cell suspensions were centrifuged at 1300rpm for 8 minutes. The cell pellet was then re-suspended in 1 ml complete RPMI.

2.6 Mesothelial cell culture

Human peritoneal mesothelial cell isolation and culture were performed with the assistance of Dr. Ann Kift-Morgan (Institute of Infection & Immunity, Cardiff University) as previously described (117). In brief, omental tissues from consenting patients undergoing elective abdominal surgery, but not undergoing CAPD, were collected from surgery in a sterile plastic receptacle containing approximately 50ml of sterile PBS solution and stored at 4℃. The tissue was gently manipulated to remove as much blood and unwanted surface material as possible. Twenty ml of 0.05% trypsin-EDTA (Invitrogen) solution was aliquoted into 50 ml tubes. The tissue was dissected into pieces by two scalpels using a crossover motion. Each piece of tissue was placed into a tube with 20 ml of 0.05% trypsin-EDTA solution. The tube was sealed with a screw cap and placed on a rotating wheel in the 37℃ incubator for 15 minutes. The tubes were than centrifuged at 1300 rpm (403*g*) for 8 minutes at 15℃. Supernatant was carefully removed using a syringe and the bottom of the tube was tapped vigorously to loosen the pellet and make re-suspension easier and more efficient. Twenty ml of 10% foetal calf serum (FCS) supplemented M-199

medium (Invitrogen, section 2.13.5) was added and rinsed around the tube to re-suspend and wash all the pelleted cells. The tubes were centrifuged at 1300 rpm (403*g*) for 8 mins at 15℃. The supernatants were removed and replaced with an appropriate volume of fresh 10% FCS supplemented M-199 medium. Cells were detached and re-suspended gently using a syringe. 5 ml of cells were syringed into a fresh T25 flask (NUNC, Thermo Scientific) and left to stand for 1 min. Cells were incubated at 37℃ and observed frequently. After 1-2 days, cells were pooled into a T75 flask (NUNC, Thermo Scientific) with 10% FCS supplemented M-199 medium (digestion 1). Medium was replaced every 3 days (passage 1) and incubated to reach confluence. HPMC cultures were incubated at 37℃ in a humidified 5% CO_2 atmosphere. All experiments performed with cells of the second passage cultured in a 96 well plate (NUNC, Thermo Scientific). Prior to assays, HPMC from a second passage were growth arrested for 48 h by culture in serum-free M-199 medium (49).

The culture supernatants of HPMC were collected from HPMC (~ 6×10^3 cells/well) cultured in a 96 well plate using complete RPMI (200µl/well) for 20 hours. For exosome-depletion experiments, the culture supernatants of HPMC were subjected to serial centrifugations: 2,000 *g*, 20 min, to remove dead cells; 10,000 *g*, 20 min, to remove cell debris; 100,000 *g*, 1 hour, to remove most exosomes (Optima-Max ultracentrifuge, Beckman Coulter); 200,000 *g*, 1 hour, to remove all exosomes (Optima-Max ultracentrifuge, Beckman Coulter).

2.7 Co-cultures of γδ T cells, monocytes and mesothelial cells

Unless indicated otherwise, γδ T cells (0.5×10^6 cells/well) stimulated with 0-100 nM HMB-PP (Table 2.3) were co-cultured for 20 hours in complete RPMI-1640 medium with autologous monocytes or allogeneic mesothelial cells at the indicated ratios in an incubator maintained at 37℃ with a humidified environment containing 5% CO_2. Proliferation assays (Section 2.10) using γδ T cells that had been pre-labelled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes/Invitrogen) were incubated for 4 days. Controls included cultures of γδ T cells alone in the absence or presence of HMB-PP (93).

Cell-free supernatants derived from mesothelial cells cultured in complete RPMI-1640 medium after 24 hours were incubated with γδ T cells alone or a co-culture of

monocyte-γδ T cells (1:1) at a dilution of 1 in 2 (50%). Blocking reagents used were 10 μM SB431542 (Sigma-Aldrich), 10 ng/ml TGF-β1–neutralizing antibody (clone 141322, R&D Systems), and 10 ng/ml IL-10 receptor α-mAb (R&D Systems). Recombinant human TGF-β1 (R&D Systems), IL-10 (Miltenyi Biotec), activin A (R&D Systems) and follistatin-288 (R&D Systems) were used at indicated concentrations (Table 2.3).

2.8 Culture of $CD3^+$ T cells with supernatants of primary mesothelial cells

$CD3^+$ T cells (90%) were purified using Pan-T Cell isolation kit (Miltenyi Biotec) and magnetic column (section 2.4.2). $CD3^+$ T cells were cultured in the presence of a 1 in 2 (50%) dilution of supernatants derived from mesothelial-cell culture and complete RPMI-1640 or further stimulated with anti-CD3/CD28 beads (Invitrogen) at a cell-to-bead ratio of 4:1 for 20 hours.

2.9 Flow cytometry

Cells were acquired on an eight-colour FACSCanto II (BD Biosciences) and analyzed with FloJo 7.6 (TreeStar).

2.9.1 Staining for surface markers

Prior to staining, the cells were washed once with PBS. The cell pellet (up to 3×10^6 Cells) was stained by adding 3 μl live/dead stain (fixable Aqua; Invitrogen) and incubated at room temperature for 15 minutes in the dark. Following incubation, the cells were washed with PBS and re-suspended in FACS buffer (section 2.13.6). 1% human normal immunoglobulin (KIOVIG, Baxter) was added and the cells were incubated for 15 minutes on ice in the dark. Following incubation, the cells were washed with FACS buffer and stained for surface markers by adding cocktails of mAbs (Table 2.1), together with appropriate isotype controls, for 20 minutes on ice in the dark.

Cells of interest were gated based on their appearance in side scatter and forward scatter area/height, exclusion of live/dead staining (fixable Aqua; Invitrogen) and surface staining: $CD3^-$ $CD14^-$ $CD15^+$ neutrophils, $CD3^-$ $CD14^+$ $CD15^-$ monocytes, and $CD3^+$ $CD14^-$ $CD15^-$ T cells. T cell subsets were identified as $CD3^+$ $CD4^+$ $CD8^-$

helper T cells, $CD3^+$ $CD4^-$ $CD8^+$ cytotoxic T cells, and $CD3^+$ $V\gamma9^+$ or $CD3^+$ $V\delta2^+$ $\gamma\delta$ T cells (Figure 2.1).

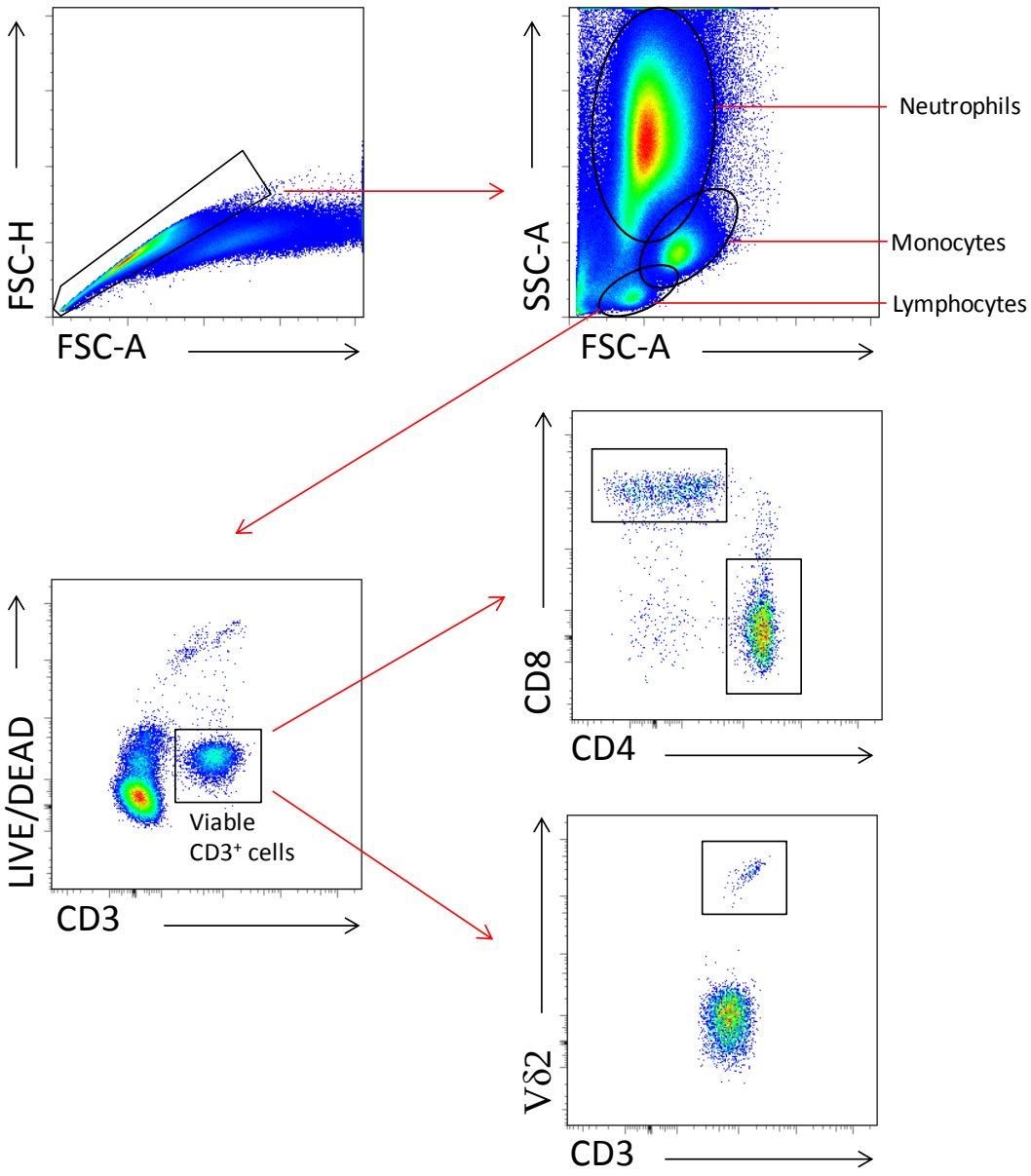

Figure 2.1 Flow diagram of gating strategies.

SSC/FSC-A/H: side scatter and forward scatter area/height; LIVE/DEAD: live/dead staining (fixable Aqua; Invitrogen).

2.9.2 Staining for intracellular cytokines

For detection of intracellular cytokines, 10 μg/ml brefeldin A (Sigma) was added to cell cultures 4 hours prior to harvesting. To stimulate global cytokine production, cells were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml) (both from Sigma). Cells were then stained with live/dead stain and for surface marker expression (Section 2.9.1). After washing in PBS (x 2 times), surface-stained cells were incubated in 100 μl fixation buffer (eBioscience) for 15 minutes at room temperature in the dark. Cells were subsequently washed once with PBS and once with permeabilisation buffer (eBioscience) and then re-suspended in 50 μl of permeabilisation buffer containing fluorochrome-conjugated mAbs directed against various cytokines. Cells were incubated with antibodies for 15 minutes at room temperature in the dark, then washed twice with PBS, re-suspended in 200 μl of FACS buffer and analyzed on a flow cytometer.

2.9.3 Staining for transcription factors

For detection of transcript factors, cells were stained for surface marker expression (Section 2.9.1). After washing twice in FACS buffer, surface-stained cells were incubated in 100 μl Fixation/Permeabilisation solution (eBioscience) for 30 minutes at 4℃ in the dark. Cells were subsequently washed once with FACS buffer and once with permeabilisation buffer (eBioscience) and then re-suspended in 50 μl of permeabilisation buffer containing 2% mouse serum (Jackson Immunoresearch, Newmarket Suffolk, UK) for 10 minutes at 4℃ in the dark. Fluorochrome-conjugated mAbs directed against various transcription factors (Table 2.2) were incubated with cells for 30 minutes at 4℃ in the dark. Cells were subsequently washed once with Fixation/Permeabilisation solution (eBioscience) and once with FACS buffer, re-suspended in 200 μl of FACS buffer and analysed by flow cytometry.

2.10 Assessment of γδ T-cell proliferation

Purified γδ T cells were suspended in PBS to a concentration of 1-2 × 10^6 cells/ml. Cells were labelled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes/Invitrogen) at 37℃ for 10 minutes in dark. Thereafter, cells were washed 3 times in cold RPMI. Labelled γδ T cells were incubated in different experimental conditions for 4 days and were analyzed by flow cytometry.

2.11 Culture supernatants and effluent samples

All samples were measured in duplicate. Cell-free peritoneal effluents were analyzed on a SECTOR Imager 600 (Meso Scale Discovery, Maryland, USA) for TNF-α, GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, CXCL8 (IL-8), soluble IL-6 receptor (sIL-6R), and MMP3. In addition, IL-17, IL-22 and CXCL10 in peritoneal effluents were measured in duplicate on a Dynex MRX II reader, using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). TGF-β1 was measured in duplicate on a Dynex MRX II reader, using a commercial ELISA kits (eBioscience).

2.12 Statistical analysis

Statistical analyses were performed using SPSS 16.0 and GraphPad Prism 4.0 software. Descriptive statistics are expressed as means ± standard error of the mean (SEM) unless otherwise stated. All variables were tested for normal distributions using the Kolmogorov-Smirnov test. Differences between unpaired groups were analyzed using the Student's *t*-test for normally distributed data or Mann-Whitney *U*-test for non-parametric data. Differences between paired groups were analyzed using the paired *t*-test or Wilcoxon signed-rank test, as indicated in the tables. Categorical data were tested using the Chi-square test. Discrimination was assessed using the area under the receiver operating characteristic curve (AUROC), which was compared using a nonparametric approach. The AUROC analysis was also performed to calculate cutoff values, sensitivity, and specificity. Finally, cut-off points were calculated by acquiring the best Youden index (sensitivity + specificity

− 1). Cumulative survival curves as a function of time were generated using the Kaplan-Meier approach and compared using the log-rank test. Predictive biomarkers were assessed using univariate analysis; statistically significant (p<0.05) variables from the univariate analysis were included in a multivariate analysis. Multiple logistic regression analyses were conducted based on forward and/or backward elimination of data, as indicated in the tables. 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.

2.13 Reagents

2.13.1 Freezing medium

50% v/v foetal calf serum and 10% dimethylsulfoxide were added to RPMI-1640, and stored at 4℃.

2.13.2 MACS buffer

5 mM EDTA and 2% v/v foetal calf serum were added to sterile PBS, and stored at 4℃.

2.13.3 Krebs Ringer bicarbonate buffer

118 mM NaCl (6.9g/L), 24.8 mM $NaHCO_3$ (2.08g/L), 1.2 mM KH_2PO_4 (0.16g/L), 4.8 mM KCl (0.358g/L), 1.25 mM $CaCl_2$ (0.139g/L), 1.2 mM $MgSO_4$ (0.14g/L), 10 mM HEPES (2.3831g/L) (all from Sigma-Aldrich), dissolved in distilled water and adjusted to pH 7.35-7.45.

2.13.4 Complete RPMI medium

2 mM L-glutamine, 1% sodium pyruvate, 50 mg/ml penicillin/streptomycin and 10% foetal calf serum were added to RPMI-1640 (Invitrogen).

2.13.5 FCS supplemented M-199 medium

Earle's buffered Medium 199 (Invitrogen) was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM; Invitrogen), transferrin (5μg/ml), insulin (5μg/ml), hydrocortisone (0.4μg/ml; all from Sigma-Aldrich) and 10% v/v foetal calf serum (Invitrogen).

2.13.6 FACS buffer

2% v/v foetal calf serum (Invitrogen), 0.04% v/v sodium azide (Fisher Scientific) were added to sterile PBS, and stored at 4℃.

Table 2.1 Fluorochrome conjugated antibodies for surface marker staining

Antigen	Conjugate	Clone	Isotype	Company	Final concentration
CD3	Pacific blue	UCHT1	Mouse IgG1,κ	BD Bioscience	1:100
CD3	FITC	SK7	Mouse IgG1,κ	BD Bioscience	1:50
CD3	PE	SK7	Mouse IgG1,κ	BD Bioscience	1:50
CD3	APC	HIT3a	Mouse IgG2a,κ	BD Bioscience	1:20
CD3	APC-H7	SK7	Mouse IgG1,κ	BD Bioscience	1:100
CD4	APC-H7	SK3	Mouse IgG1,κ	BD Bioscience	1:60
CD8	APC	RPA-T8	Mouse IgG1,κ	BD Bioscience	1:40
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
CD19	APC	SJ25C1	Mouse IgM	eBioscience	1:40
CD25	Cy	M-A251	Mouse IgG1,κ	BD Bioscience	1:10
CD25	PE-Cy7	M-A251	Mouse IgG1,κ	BD Bioscience	1:40
CD27	FITC	M-T271	Mouse IgG1,κ	BD Bioscience	1:40
CD40	PE	mAB89	Mouse IgG1,κ	Beckman Coulter	1:20
CD45	FITC	HI30	Mouse IgG1,κ	BD Bioscience	1:10
CD45RA	APC	HI100	Mouse IgG1,κ	eBioscience	1:10
CD56	PE-Cy7	B159	Mouse IgG1,κ	BD Bioscience	1:50
CD62L	Cy	DREG-56	Mouse IgG1,κ	BD Bioscience	1:15
CD69	FITC	FN50	Mouse IgG1,κ	BD Bioscience	1:20
CD86	FITC	2331; FUN1	Mouse IgG1,κ	BD Bioscience	1:40
Vδ2	PE	B6.1	Mouse IgG1,κ	BD Bioscience	1:100
Vγ9	PC5	Immu360	Mouse IgG1,κ	Beckman Coulter	1:400
HLA-DR	APC-H7	L243	mouse IgG2a,	BD Bioscience	1:40
CCR4	PE-Cy7	1G1	Mouse IgG1,κ	BD Bioscience	1:20
CCR5	PE	2D7	Mouse IgG2a,κ	BD Bioscience	1:10
CCR6	PE	11A9	Mouse IgG1,κ	BD Bioscience	1:80
CCR7	PE-Cy7	3D12	Rat IgG2a, κ	BD Bioscience	1:100
CCR9	APC	248601	Mouse IgG1,κ	R&D systems	1:25
CXCR3	FITC	49801.111	Mouse IgG1,κ	R&D systems	1:20

Table 2.2 Fluorochrome conjugated antibodies for intracellular marker staining

Antigen	Conjugate	Clone	Isotype	Company	Final concentration
IFN-γ	FITC	B27	Mouse IgG1	BD Bioscience	1:100
IFN-γ	eFluor 450	4S.B3	Mouse IgG1	eBioscience	1:100
TNF-α	APC	6401.1111	Mouse IgG1	BD Bioscience	1:20
IL-4	PE	3010.211	Mouse IgG1	BD Bioscience	1:20
IL-17	Alexa488	64DEC17	Mouse IgG1	eBioscience	1:10
FoxP3	FITC	236A/E7	Mouse IgG1	eBioscience	1:10

Table 2.3 Soluble mediators and blocking antibodies

Reagent	Supplier
SB431542	Sigma-Aldrich
TGF-β1–neutralizing antibody	clone 141322, R&D Systems
Recombinant Human TGF-β1	R&D Systems
Recombinant Human IL-10	Miltenyi Biotec
Recombinant Human Activin A	R&D Systems
Recombinant Human Follistatin-288	R&D Systems
human IL-10 receptor α mAb	R&D Systems
HMB-PP	A gift from Dr. Hassan Jomaa, Universitätsklinikum Giessen und Marburg, Germany

Chapter 3

Clinical outcome analysis and characterisation of the local immune responses in PD-associated peritonitis

3.1 Introduction

Bacterial infection remains a leading cause of morbidity and mortality worldwide, not the least due to the alarming spread of antibiotic-resistant pathogens that is posing an enormous challenge on clinical practice, biomedical research and public healthcare (118). In urgent cases such as acute PD-associated peritonitis in patients, antimicrobial treatment is largely empirical since therapeutic intervention has to commence before the nature of the causative pathogen is known, and microbiological culture results usually only become available within 1-3 days. Moreover, in many cases no organism can be identified, for instance if a patient is already on antibiotics, if sample collection and/or culture techniques are inadequate, or if the type of organism is unusual or fastidious (*e.g*. *Mycobacterium* spp.) (14, 19, 119). Depending on the centre, rates of culture-negative peritonitis may vary from 0-50 % (20, 120).

Positive identification of the causative pathogen is not only crucial in order to guide and refine patient management with respect to the choice of antibiotic treatment; it also provides important clues as to the underlying inflammatory mechanisms and how to manipulate them to resolve infection. The innate immune system has evolved to survey the body constantly for potentially hazardous structures, and responds to PAMP that are invariant among a broad range of organisms and allow self/non-self discrimination (121, 122). However, different types of pathogens carry different types of PAMP, and hence interact with different components of the immune system. One such PAMP is bacterial endotoxin or LPS that is present in Gram-negative organisms but absent from Gram-positive organisms, and it is well-established that episodes of Gram-negative and Gram-positive sepsis present with different clinical signs and may have different clinical outcomes (123). As recently highlighted by Llewelyn and Cohen it is becoming increasingly clear that the nature of the infection is a major determinant of outcome, and future interventions may well have to focus on subgroups of patients with different forms of infection (124). Key to developing effective treatment strategies is therefore a more detailed understanding of the inflammatory response in acute infection. Such an understanding may then facilitate the use of appropriate biomarkers to both aid diagnosis and predict treatment outcome (57, 112, 125, 126).

In the present study, in order to identify potentially useful diagnostic and prognostic biomarkers of inflammation severity and outcomes from bacterial infection, we have performed a detailed analysis of immune responses in PD patients on the first day of presentation with acute peritonitis. The peritoneal cavity in PD serves as unique window to inflammatory scenarios that can be prospectively observed *in vivo*. It affords easy, continuous access to all relevant cellular components and humoral mediators, and allows us to examine how treatment and the type of infection modulate these processes. We know of no other experimental model that gives such direct insight into human immune responses in a similarly clinically relevant and non-invasive manner. Our findings indicate that a combination of microbiological characteristics and immunological parameters predict clinical outcome, and also suggest novel targets for of therapeutic intervention in acute bacterial peritonitis.

3.2 Aims

The aims of this Chapter were:

1) To characterize local immune responses in PD associated peritontitis including the phenotype, effector/memory status, cytokine profile and migration properties of peritoneal T cell subsets
2) To investigate whether the dichotomy between HMB-PP$^+$ and HMB-PP$^-$ pathogens in their potential to trigger γδ T cells occurs under physiological conditions *in vivo*.
3) To investigate whether the occurrence of clinical complications in PD patients depends on the capacity of the causative pathogen to produce HMB-PP.
4) To identify potentially useful prognostic biomarkers of inflammation severity and outcomes from PD-associated peritonitis

3.3 Results

In order to study local immune response, we recruited 52 episodes of acute peritonitis from PD patients who were admitted at the University Hospital of Wales, Cardiff, UK. Pre-treatment effluent samples from day 1 of the infection (*i.e.* the day of presentation with a 'cloudy bag') were subjected to a contemporary immunological analysis by multi-colour flow cytometry and multiplex ELISA. Effluent samples from 15 non-infected age and sex-matched stable PD patients served as the control group.

3.3.1 Acute peritonitis is characterized by a significant peritoneal influx of immune cells

To assess the distribution of immune cells during acute peritonitis, we collected serial samples of peritoneal effluent from PD-associated peritonitis patients. During acute peritonitis, leukocytes are recruited to the peritoneal cavity, starting with a rapid accumulation of $CD15^+$ neutrophils, which are later replaced by a population of mononuclear cells, including $CD14^+$ monocytes/macrophages and $CD3^+$ T cells. While peritoneal leukocytes in stable patients comprised mainly of monocytes/macrophages and T cells, acute peritonitis was dominated by a massive recruitment of neutrophils. Of note, γδ T cells were also recruited to the peritoneal cavity in the early stage (Figure 3.1).

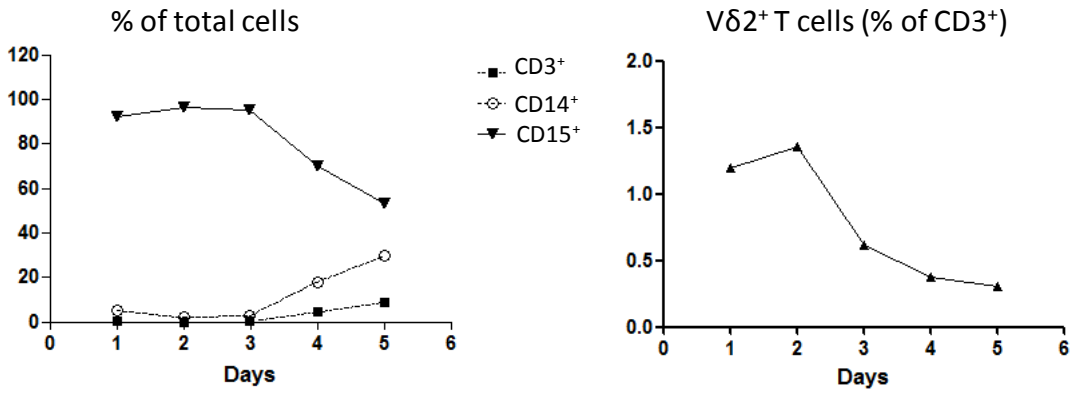

Figure 3.1 Dynamic changes of peritoneal leukocyte subpopulations during acute peritonitis. Serial samples of peritoneal effluents were collected from patients with acute peritonitis. Data are shown as frequencies of $CD15^+$ neutrophils, $CD14^+$ monocytes/macrophages and $CD3^+$ T cells in total cells, and frequencies of $V\delta2^+$ T cells in $CD3^+$ T cells. Data shown are representative from 3 independent peritonitis episodes.

3.3.2 Monocyte/Macrophage activation

Macrophages are the predominant cell type found in the peritoneal cavity and play an important role in the first line of defence against invading microorganisms. To examine the activation status of monocytes/macrophages in different conditions, we isolated leukocytes from peripheral blood of age-matched healthy donors or PD effluents of non-infected (stable) PD patients or PD patients with acute peritonitis on day 1 (Table 3.1). The expression of the APC markers CD40, CD86 and HLA-DR on monocytes/macrophages ($CD14^+$) was analyzed. Data are shown in Figure 3.2.

Of note, the expression levels of CD86 on peritoneal monocytes from stable and peritonitis patients were lower compared to circulating monocytes. Further analysis of peritonitis samples on day 1 showed that the expression levels of CD86 on peritoneal monocytes were lower in HMB-PP^+ peritonitis compared to HMB-PP^- peritonitis (Figure 3.2B).

Table 3.1 Characteristics of samples analyzed in the present study.

	PBMC from healthy donors	**Stable**	**Peritonitis**
Total number	5	15	52
Age (mean ± SD)	53.8 ± 2.7	60.4 ± 17.8	65.5 ± 14.0
Women (%)	20	40	35.5

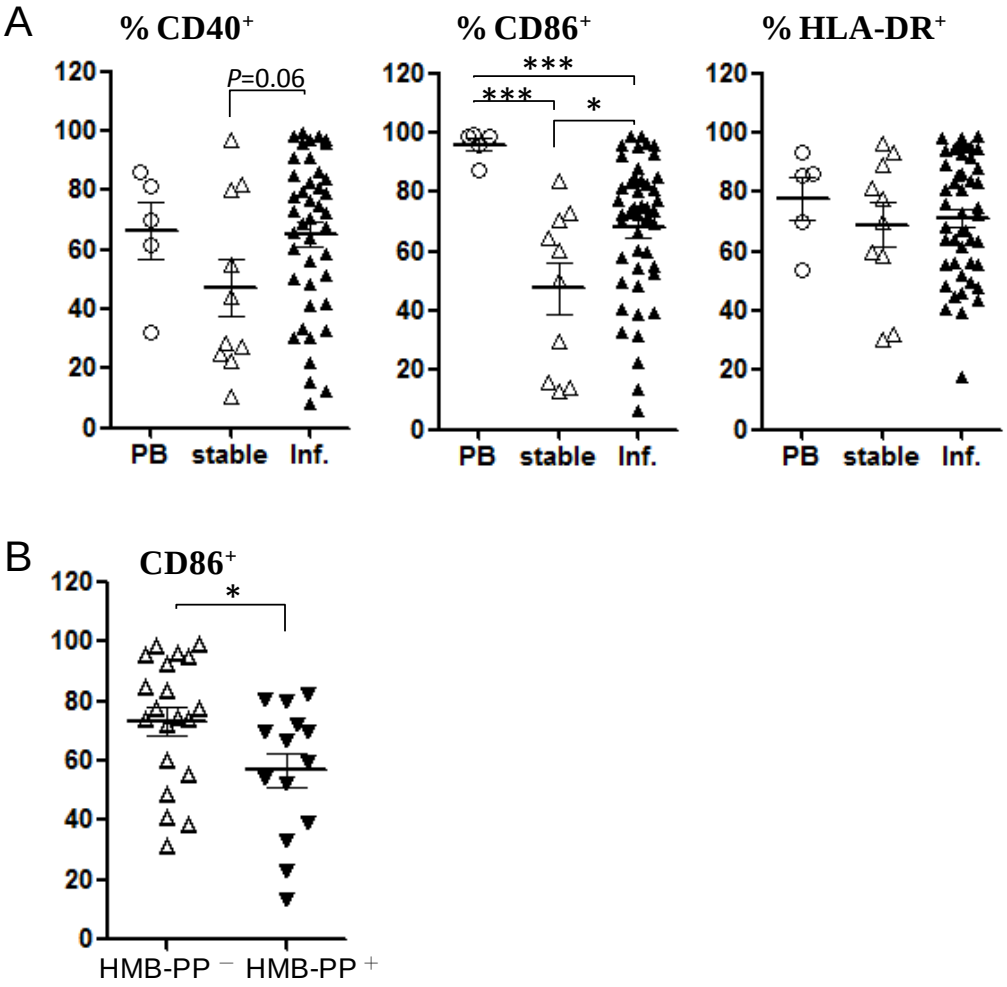

Figure 3.2 Expression of APC markers on monocytes/macrophages.

(A) Expression of $CD40^+$, $CD86^+$ or $HLA\text{-}DR^+$ on monocytes/macrophages ($CD14^+$) in peripheral blood of healthy donors (PB) or stable PD effluents (stable), or PD effluents of peritonitis patients on day 1 (Inf.). (B) Expression of $CD86^+$ on monocytes/macrophages in PD effluents of PD-associated peritonitis on day 1, depending on whether or not the causative pathogen was capable of producing the microbial metabolite HMB-PP. Statistical differences between patient groups were analyzed using the Student's *t*-test. Error bars indicate SEM. *, $P<0.05$; ***, $P< 0.001$.

3.3.3 Peritoneal T-cell activation

To define the activation properties of peritoneal T cells, leukocytes were isolated from PD effluents of stable PD patients or PD patients with acute peritonitis on day 1. Peripheral blood of age-matched healthy donors was used as controls. Cells were analyzed for the expression of CD25 (a late activation marker) and CD69 (an early activation marker) on $CD4^+$, $CD8^+$ and γδ T cells. Data are shown in Figure 3.3.

Our results demonstrate that all three subsets of peritoneal T cells from PD effluents of stable patients expressed higher levels of CD69 in comparison with circulating T cells. Furthermore, the expression of CD25 on peritoneal γδ T cells was significantly higher compared to circulating γδ T cells. When we looked at the activation status of peritoneal T cells from peritonitis samples, both CD25 and CD69 expression levels were higher compared to circulating T cells. The peritoneal $CD4^+$ T cells from peritonitis patients expressed higher levels of CD25 than stable PD patients. Interestingly, the expression of CD69 on peritoneal $CD8^+$ T cells was lower in peritonitis patients compared to stable PD patients.

Further analysis of peritonitis samples on day 1 demonstrated no significant difference in the expression of these markers on $CD4^+$, $CD8^+$ or γδ T-cell between culture-negative and culture-positive peritonitis, or between HMB-PP^+ and HMB-PP^- peritonitis (data not shown).

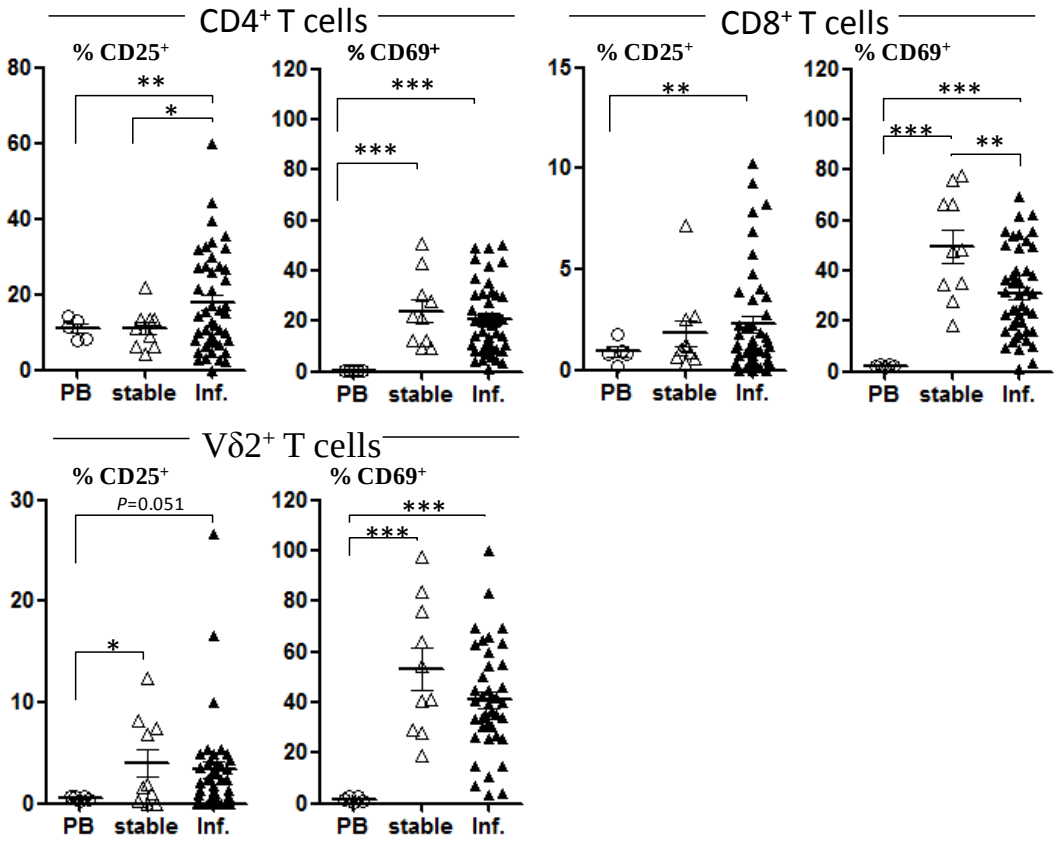

Figure 3.3 Expression of the activation markers CD25 and CD69 on T cells.

Expression of CD25 or CD69 on $CD4^+$, $CD8^+$ or γδ T cells ($V\delta2^+$) in peripheral blood of healthy donors (PB) or stable PD effluents (stable), or PD effluents of peritonitis patients on day 1 (Inf.). Statistical differences between patient groups were analyzed using the Student's *t*-test. Error bars indicate SEM. *, P<0.05; **, P<0.01, ***, P< 0.001.

3.3.4 Peritoneal T-cell memory subsets

A previous study demonstrated that the peritoneal cavity is enriched in cells with effector/memory T cells, which were able to mount a T_H1-polarized response to recall antigens, and that these responses were greater in peritoneal T cells compared with circulating T cells (126). To investigate the potential role of peritoneal memory T cells in peritoneal inflammation in more detail, we characterized the phenotype of peritoneal T-cell subsets.

$CD4^+$ T cells were identified as naïve T cells ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector/memory ($CD45RA^-CCR7^-$), and $CD45RA^+$ effector/memory cells ($CD45RA^+CCR7^-$) (102, 104). Figure 3.4A illustrates the different subsets of $CD4^+$ T cells in peripheral blood of healthy donors or PD effluents of stable or peritonitis on day 1 samples. In comparison with circulating $CD4^+$ T cells, the frequencies of peritoneal naïve $CD4^+$ T cells decreased and effector/memory $CD4^+$ T cells increased significantly in both stable and peritonitis patients. Effector/memory $CD4^+$ T cells were the most predominant subset in the peritoneal cavity of either stable or peritonitis patients. No significant differences in the proportion of $CD4^+$ T-cell memory subsets between stable and peritonitis patients were noted. Further analysis of peritonitis samples demonstrated no significant difference in the proportion of $CD4^+$ T-cell subsets between culture-negative and culture-positive peritonitis, or between HMB-PP^+ and HMB-PP^- peritonitis (data not shown).

We also characterized the potential memory status of γδ T cells with antibodies against the surrogate memory markers CD45RA and CD27 (105). In comparison with circulating γδ T cells, the frequencies of peritoneal $CD45RA^+CD27^+$ $V\delta2^+$ T cells decreased in peritonitis patients and $CD45RA^-CD27^-V\delta2^+$ T cells increased in both stable and peritonitis patients. $CD45RA^-CD27^+V\delta2^+$ T cells were the most enriched subsets in the peritoneal cavity of either stable or peritonitis patients (Figure 3.4B). No significant differences in the proportion of γδ T-cell memory subsets between stable and peritonitis patients were noted. Further analysis of peritonitis samples demonstrated no significant differences in the proportion of γδ T-cell

subsets between culture-negative and culture-positive peritonitis, or between HMB-PP$^+$ and HMB-PP$^-$ peritonitis (data not shown).

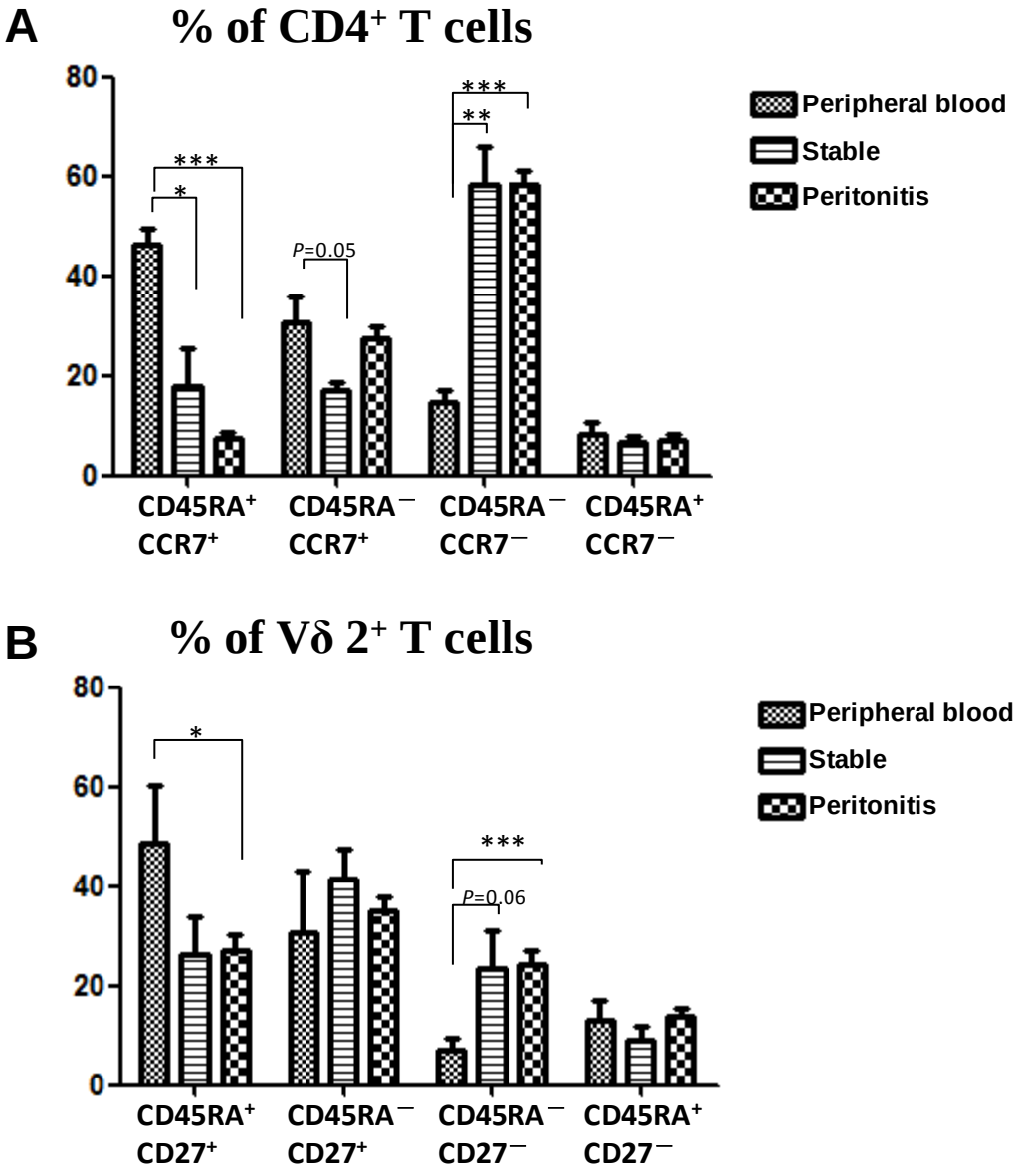

Figure 3.4 Expression of memory markers on CD4$^+$ or Vδ2$^+$ T cells.

(A) CD4$^+$ T-cell memory subsets in peripheral blood of healthy donors or PD effluents of stable or peritonitis samples on day 1. (B) Vδ2$^+$ T-cell memory subsets in peripheral blood of healthy donors or PD effluents of stable or peritonitis samples on day 1. Statistical differences between patient groups were analyzed using the Student's *t*-test. Error bars indicate SEM. *, P<0.05; **, P<0.01, ***, P< 0.001.

3.3.5 APC markers on γδ T cells

Since antigen-stimulated human Vγ9/Vδ2 T cells can take up and process antigen, and up-regulate co-stimulatory molecules (109), we also examined the expression of APC markers CD40, CD86 and HLA-DR on γδ T cells. Although peritoneal γδ T cells expressed low level of CD40 and CD86 in either stable or peritonitis patients, the expression levels of these two markers were not significantly higher in comparison with circulating γδ T cells in the present study. The expression of HLA-DR on peritoneal γδ T cells from stable or peritonitis patients was higher in comparison with circulating γδ T cells.

Further analysis of peritonitis samples on day 1 demonstrated no significant differences in the expression of these markers on γδ T-cells between culture-negative and culture-positive peritonitis, or between HMB-PP$^+$ and HMB-PP$^-$ peritonitis (data not shown).

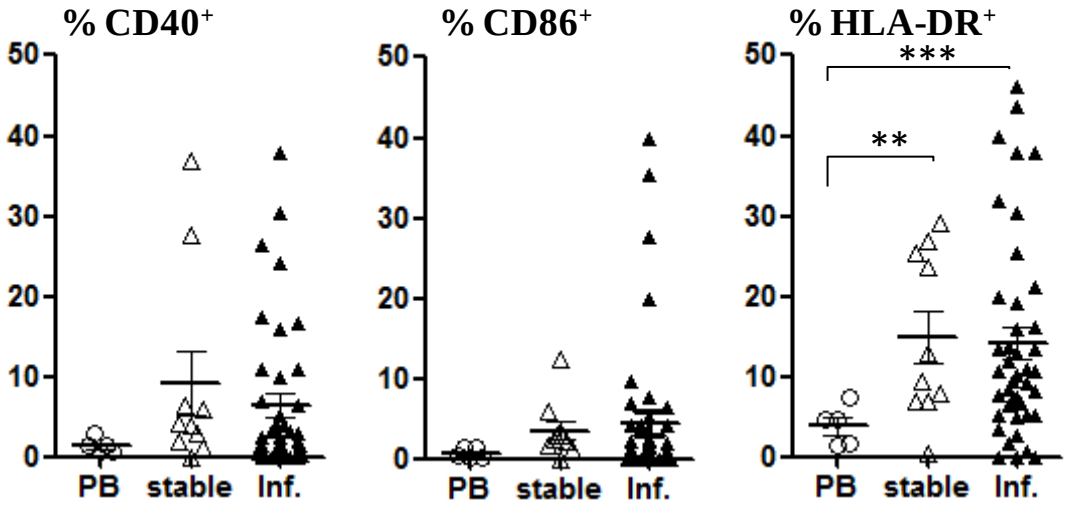

Figure 3.5 Expression of APC markers on γδ T cells.

(A) Expression of CD40$^+$, CD86$^+$ or HLA-DR$^+$ on γδ T cells (Vγ9$^+$) from peripheral blood of healthy donors (PB) or stable PD effluents (stable), or PD effluents of peritonitis patients on day 1 (Inf.). Statistical differences between patient groups were analyzed using the Student's *t*-test. Error bars indicate SEM. **, P<0.01; ***, P< 0.001.

3.3.6 Migration properties of peritoneal T cells

Infections trigger the local production of inflammatory chemokines, which control the composition of the cellular infiltrate. A change in local chemokines is an essential factor in the transition from the neutrophil-driven immediate response to the T and B cell-driven later response to infection. Effector T cells in the peripheral blood express distinct combinations of receptors for inflammatory chemokines such as CXCR3 and CCR5. Enhanced expression of these receptors enable effector memory T cells to enter inflammatory sites (127). CCR6 mediates the homing of both helper T cells and dendritic cells to the gut mucosal lymphoid tissue (128). Previous studies have also shown that CCR6 is a specific marker for Th17 cells and regulatory T cells distinguishing them from other helper T cells (129, 130). To define the migration properties of peritoneal T cells, cells were isolated from peripheral blood of healthy donors or PD effluents of stable patients, or peritonitis patients on day 1, and were analyzed for the expression of different chemokine receptors on $CD4^+$, $CD8^+$ and γδ T cells. Data are shown in Figure 3.6.

The peritoneal γδ T cells from peritonitis patients expressed similar levels of CCR5 and showed up-regulation of CCR6 and down-regulation of CXCR3 in comparison with circulating γδ T cells. The peritoneal $CD4^+$ and $CD8^+$ from peritonitis samples also had similar expression patterns of these markers. Further analysis of peritonitis samples demonstrated no significant differences in the expression of these markers on $CD4^+$ or $CD8^+$ or γδ T cells between culture-negative and culture-positive peritonitis, or between HMB-PP^+ and HMB-PP^- peritonitis (data not shown).

Effector T-cell entry into intestinal mucosae requires cellular adhesion receptors on T cells such as CCR9 and integrin α4β7 (131, 132). T cells measured in this study did not express CCR9 (data not shown).

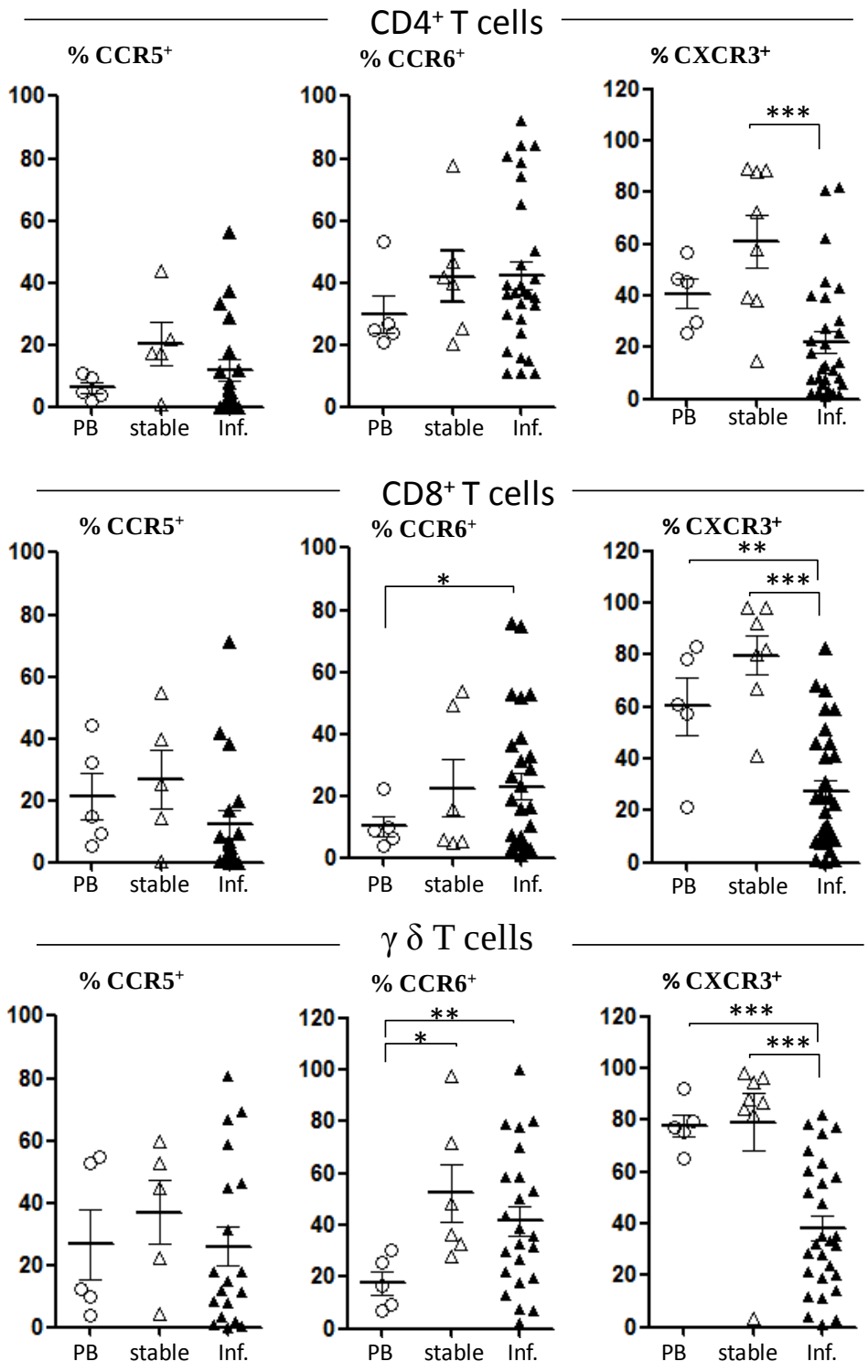

Figure 3.6 Migration properties of CD4$^+$, CD8$^+$ and γδ T cells.

Cells were taken from peripheral blood of healthy donors (PB) or stable PD effluents (stable), or PD effluents of peritonitis patients on day 1 (Inf.). The expression of CCR5, CCR6 and CXCR3 on CD4$^+$, CD8$^+$ and γδ T cells (Vγ9$^+$) were analyzed. Statistical differences between patient groups were analyzed using the Student's *t*-test. Error bars indicate SEM. *, P<0.05; **, P<0.01, ***, P< 0.001.

3.3.7 Characterization of helper T-cell subsets in PD patients

To characterize different helper T-cell subsets in PD patients, cells from peripheral blood and PD effluents of stable PD patients or PD effluents of peritonitis patients on day 1 were isolated by centrifugation. Cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin-A (10 μg/ml) for 4 hours before intracellular cytokine staining. Immunofluorescent staining of intracellular cytokines was used to detect the percentage of T cells producing IFN-γ (T_H1), IL-4 (T_H2) or IL-17 (T_H17). Regulatory T cells (Treg) were identified by anti-CD25 and anti-Foxp3 antibodies.

The $CD4^+$ T cells showed a predominant IFN-γ production, manifested as 37.7 ± 4.9 % positive intracellular staining (Figure 3.7 upper panels, stable patients), indicating that the majority of peritoneal $CD4^+$ T cells of stable PD patients were T_H1 cells. This pattern of cytokine production was the same in $CD8^+$ T cells and γδ T cells in stable PD patients. Peritoneal T cells in peritonitis patients were characterized by an increase in the percentage of T_H2 cells, as well as T_H2-type $CD8^+$ T cells and IL-4 producing γδ T cells, without a significantly different percentage of T_H1 cells in comparison with T cells in stable PD effluents (Figure 3.7).

In order to evaluate the pattern of T-cell subsets in acute PD peritonitis patients, we further divided peritonitis samples into culture-negative or culture-positive peritonitis. Higher frequencies of T_H1 and lower frequencies of T_H2 and Treg were found in culture-positive peritonitis on day 1 (Figure 3.8). Further analysis of culture positive peritonitis samples demonstrated no significant differences in the proportion of helper T-cell subsets between $HMB\text{-}PP^+$ and $HMB\text{-}PP^-$ peritonitis (data not shown).

Low frequencies of $IL\text{-}17^+$ T cells could be identified within peritoneal T lymphocytes yet without significant differences among different subgroups.

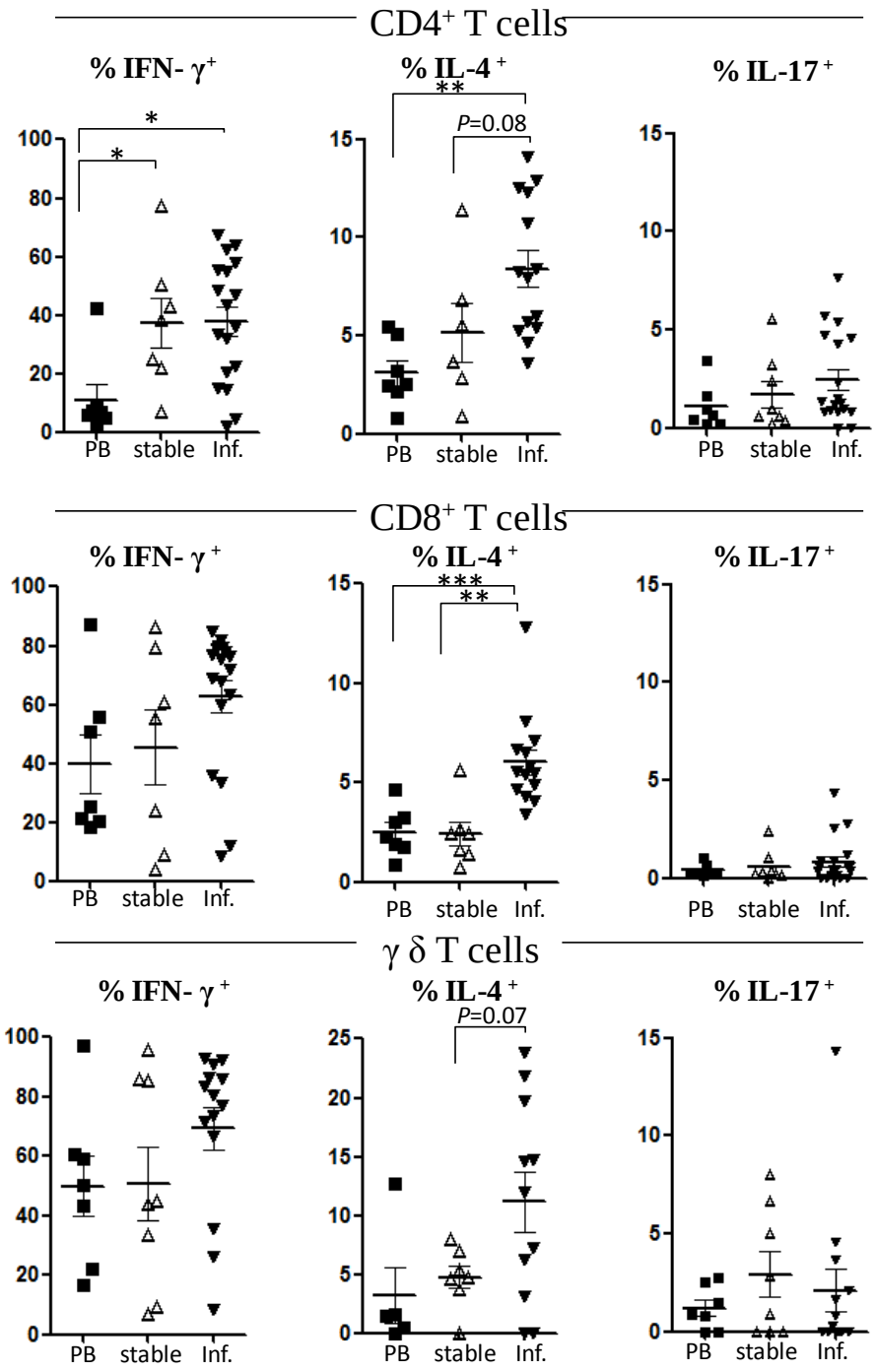

Figure 3.7 Helper T-cell subsets in PD patients.

Frequency (%) of IFN-γ^+ (T_H1), IL-4^+ (T_H2), or IL-17^+ (T_H17) T cells in peripheral blood (PB) and PD effluents of stable PD patients (stable), or PD effluents of peritonitis patients on day 1 (Inf.). Cells from peripheral blood and PD effluents of stable PD patients were processed freshly. Data from peritonitis samples were obtained from frozen samples. Statistical differences between patient groups were analyzed using the Student's *t*-test or the Mann-Whitney *U* test, depending on normal distribution test using the Kolmogorov-Smirnov test. Error bars indicate SEM. *, $P<0.05$; **, $P<0.01$, ***, $P< 0.001$.

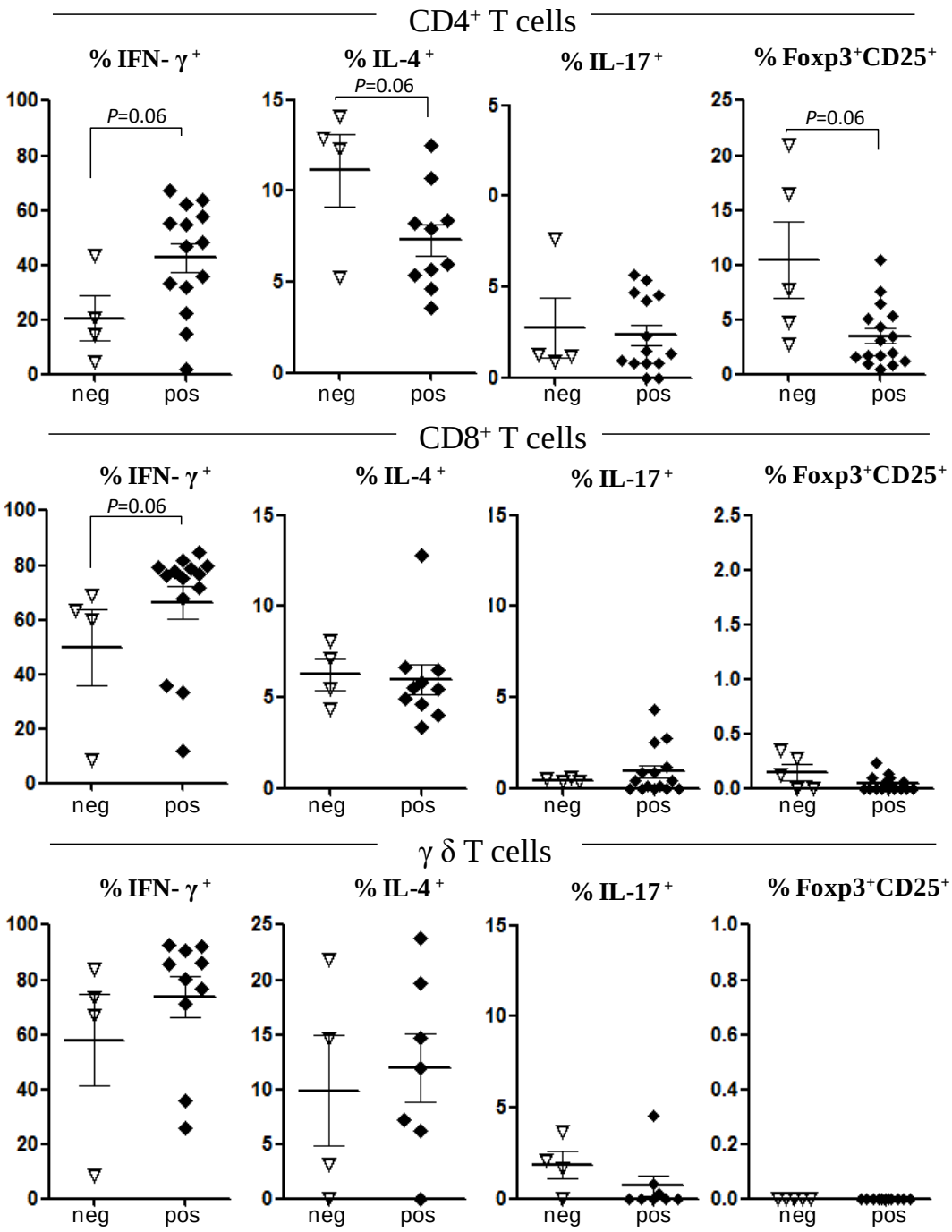

Figure 3.8 Helper T-cell subsets in acute peritonitis patients.

Frequency (%) of IFN-γ^+ (T_H1), IL-4^+ (T_H2), IL-17^+ (T_H17) or Foxp3^+CD25^+ (Treg) T cells in PD effluents from PD-associated culture-negative (neg) or culture-positive peritonitis (pos) on day 1. Cells were processed from frozen samples. Statistical differences between patient groups were analyzed using the Student's *t*-test or Mann-Whitney *U* test, depending on normal distribution test using the Kolmogorov-Smirnov test. Error bars indicate SEM.

3.3.8 Culture-positive episodes of peritonitis are associated with poorer clinical outcome.

We next assessed the clinical outcome after episodes of acute peritonitis. Two episodes in the above study were not included due to unavailable data of the 90th day outcome when analyzing the data (Table 3.2). Although not reaching statistical significance due to the relatively small number of patients recruited to this study, cumulative survival curves as a function of time suggested that episodes of culture-positive peritonitis were associated with poorer outcome compared with culture-negative cases (Figure 3.9, top panels). In order to validate this pattern of clinical outcome observed in the Cardiff cohort, we analyzed patient and technique survival with respect to the infecting organism in two larger and entirely independent, well-defined patient cohorts (Table 3.2). Out of all patients from the ANZDATA registry who were receiving PD in Australia between 2003 and 2008, a total of 2,424 patients with first-time episodes of peritonitis were included in the present analysis and grouped according to the result of organism culture into culture-negative and culture-positive infection, excluding cases of fungal infection. A parallel analysis was conducted in a total of 412 patients who were treated at the University Hospital of North Staffordshire, Stoke-on-Trent, UK, between 1987 and 2008, among whom 385 cases of first-time peritonitis occurred, again excluding cases of fungal infection. In both cases, and in agreement with the data from Cardiff, episodes of culture-positive peritonitis were associated with poorer outcome compared with culture-negative peritonitis (Figure 3.9, middle and bottom panels).

Table 3.2 Characteristics of PD patients with acute peritonitis for outcome analysis.

	Cardiff (UK)	**Stoke-on-Trent (UK)**	**ANZDATA (Australia)**
Total number	50	385	2,424
Age (mean ± SD)	65.5 ± 14.2	57.0 ± 16.0	58.5 ± 16.8
Women (%)	38.0	43.1	44.8
Days on PD (mean ± SD)	1163.8 ± 949.1	532.0 ± 581.9	738.8 ± 502.5
90^{th} day mortality (%)	6.0	8.8	1.8
90^{th} day technique failure (%)	22.0	19.0	16.8
Culture-positive infections (%)	72.0	61.5	85.8
HMB-PP^{+} organisms among positively identified species (%)	41.7	26.4	38.6

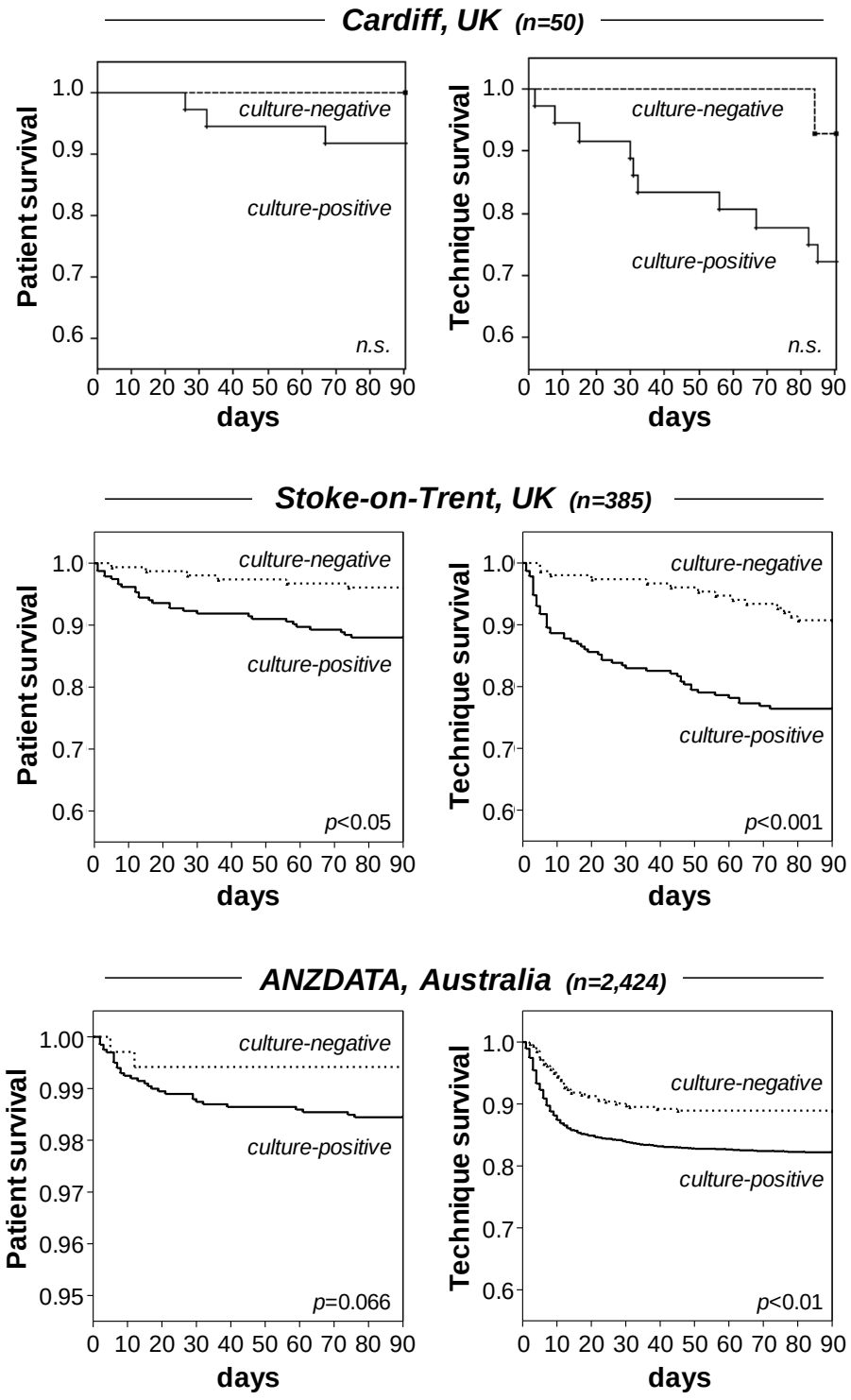

Figure 3.9 Culture-positive peritonitis are associated with poor clinical outcome.

Cumulative patient survival (*left*) and cumulative technique survival (*right*) of patients with acute peritonitis, grouped into culture-negative (dotted lines) or culture-positive infections (solid lines). *Top*, PD patients admitted at the University Hospital of Wales, Cardiff, with acute peritonitis. *Middle*, Australian PD patients from the ANZDATA registry with first-time peritonitis. *Bottom*, PD patients with first-time peritonitis treated at the University Hospital of North Staffordshire, Stoke-on-Trent, UK. Comparisons were made using log-rank tests.

3.3.9 Culture-positive episodes of peritonitis are associated with more severe peritoneal inflammation.

In order to identify a possible correlation between inflammatory responses and the ensuing clinical outcome, we measured several pro-inflammatory cytokines/chemokines. Peritoneal levels of TNF-α, IL-1β, IL-2, IL-6, IL-10, IL-22, sIL-6R, and MMP-3 in culture-positive patients were significantly or marginally significantly higher compared with culture-negative patients and stable non-infected PD patients (Figure 3.10), indicative of a local inflammatory response in those patients (57, 112, 125). This was confirmed by a detailed leukocyte subset analysis revealing that during culture-positive peritonitis a larger proportion of the infiltrating immune cells were $CD15^+$ neutrophils whereas a smaller proportion were $CD14^+$ monocytes/macrophages (Figure 3.11). Total numbers of all major peritoneal leukocyte populations (neutrophils, monocytes/macrophages, and $CD4^+$, $CD8^+$ and $V\delta2^+$ T cells) were elevated in culture-positive when compared to culture-negative peritonitis, revealing that in general this patient group experienced a more severe peritoneal inflammatory response.

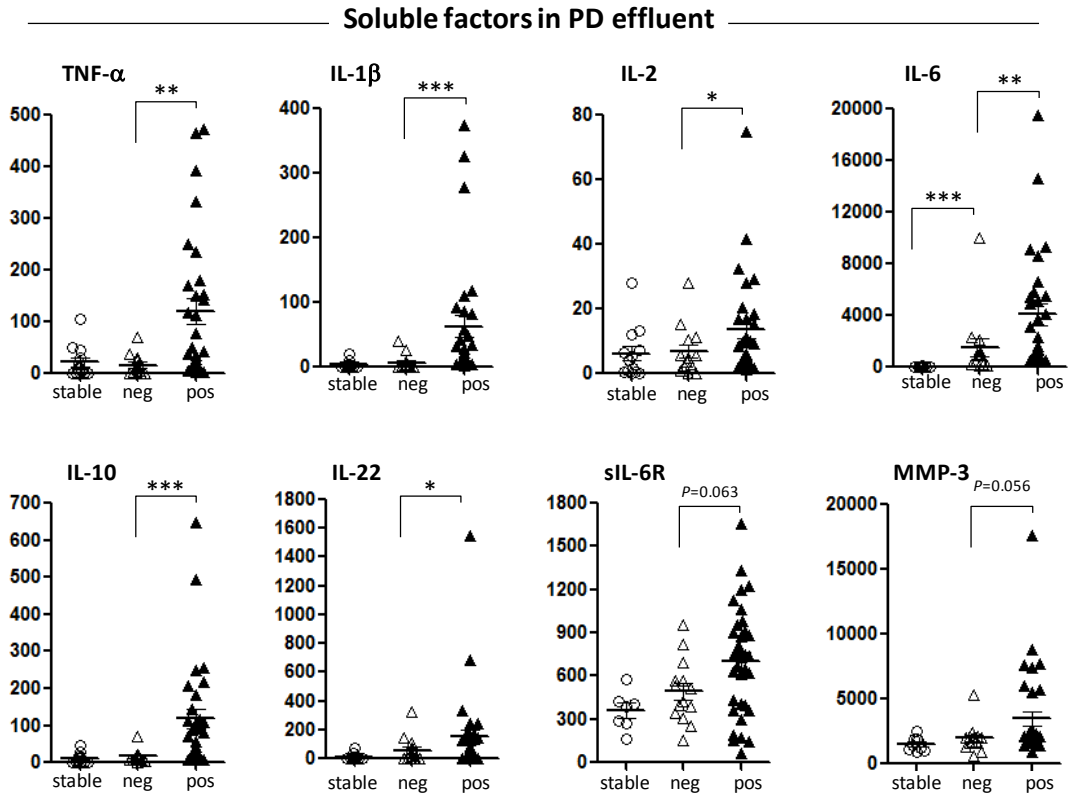

Figure 3.10 Culture-positive episodes of peritonitis are associated with higher levels of pro-inflammatory cytokines/chemokines.

Peritoneal levels (pg/ml) of TNF-α, IL-1β, IL-2, IL-6, IL-10, IL-22, sIL-6R, and MMP-3 in stable PD patients and in patients with PD-associated culture-negative (neg) or culture-positive peritonitis (pos) on day 1. Statistical differences between patient groups were analyzed using the Student's *t*-test or Mann-Whitney *U* test, depending on normal distribution test using the Kolmogorov-Smirnov test. Error bars indicate SEM. *, P<0.05; **, P<0.01, ***, P< 0.001.

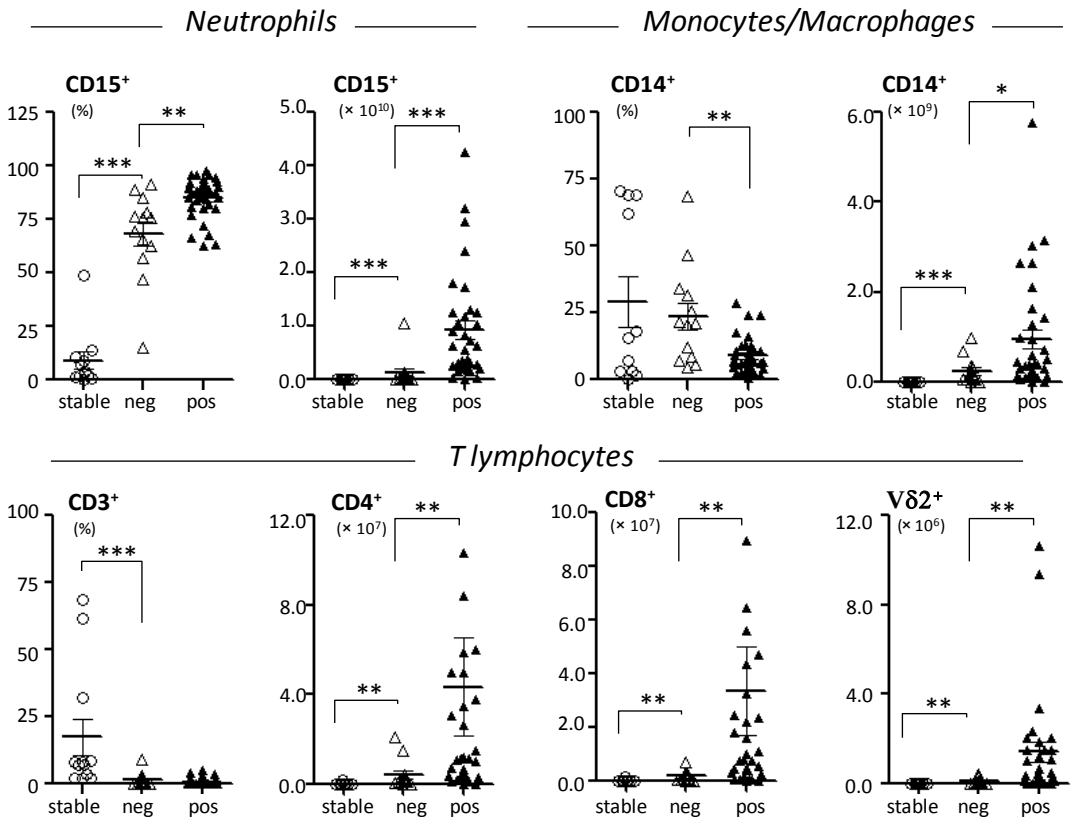

Figure 3.11 Culture-positive peritonitis are associated with more infiltrating leukocytes.

Frequency (%) and total numbers of peritoneal neutrophils ($CD15^+$), frequency and total number of peritoneal monocytes/macrophages ($CD14^+$), frequency of peritoneal T cells ($CD3^+$), and total numbers of helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), and γδ T cells ($V\delta2^+$), in stable PD patients and in patients with PD-associated culture-negative (neg) or culture-positive peritonitis (pos) on day 1. Statistical differences between patient groups were analyzed using the Student's *t*-test or Mann-Whitney *U* test, depending on normal distribution test using the Kolmogorov-Smirnov test. Error bars indicate SEM. *, $P<0.05$; **, $P<0.01$, ***, $P< 0.001$.

3.3.10 Episodes of peritonitis caused by HMB-PP producing bacteria are associated with poor clinical outcome.

We speculated that patient and technique survival might depend on the capacity of the causative pathogen to produce the Vγ9/Vδ2 T-cell activator HMB-PP. Our analysis of the three independent patient cohorts demonstrated that infections with HMB-PP$^+$ bacteria were indeed associated with worse outcomes, while HMB-PP$^-$ bacteria (*Enterococcus, Staphylococcus, Streptococcus*) caused milder symptoms (Figure 3.12, top panels).

In order to rule out that the pattern observed in our study was not just due to differences in the Gram staining, as reported by previous investigators (15, 18, 119, 133-137), we divided the group of HMB-PP$^+$ bacteria further into Gram$^+$ and Gram$^-$ species. Our outcome analysis demonstrated that even within the Gram$^+$ group, bacteria capable of producing HMB-PP were associated with worse outcomes compared to HMB-PP$^-$ pathogens, suggesting that the HMB-PP producing capacity of the causative pathogen might be of predictive value for the clinical outcome from bacterial peritonitis (Figure 3.12).

Infections caused by HMB-PP$^-$ pathogens had only marginally increased odds ratios in relation to culture-negative infection. In contrast, infections caused by HMB-PP$^+$ pathogens were characterized by odds ratios of 3.4-7.4 in relation to culture-negative infections, for 90th day technique failure and 90th day mortality (Table 3.3). Of note, both Gram$^+$ HMB-PP$^+$ and Gram$^-$ HMB-PP$^+$ bacteria contributed to the increased risk of technique failure although the number of episodes caused by Gram$^+$ HMB-PP$^+$ species such as *Bacillus* and *Corynebacterium* was too low to reach statistical significance in the Stoke-on-Trent database.

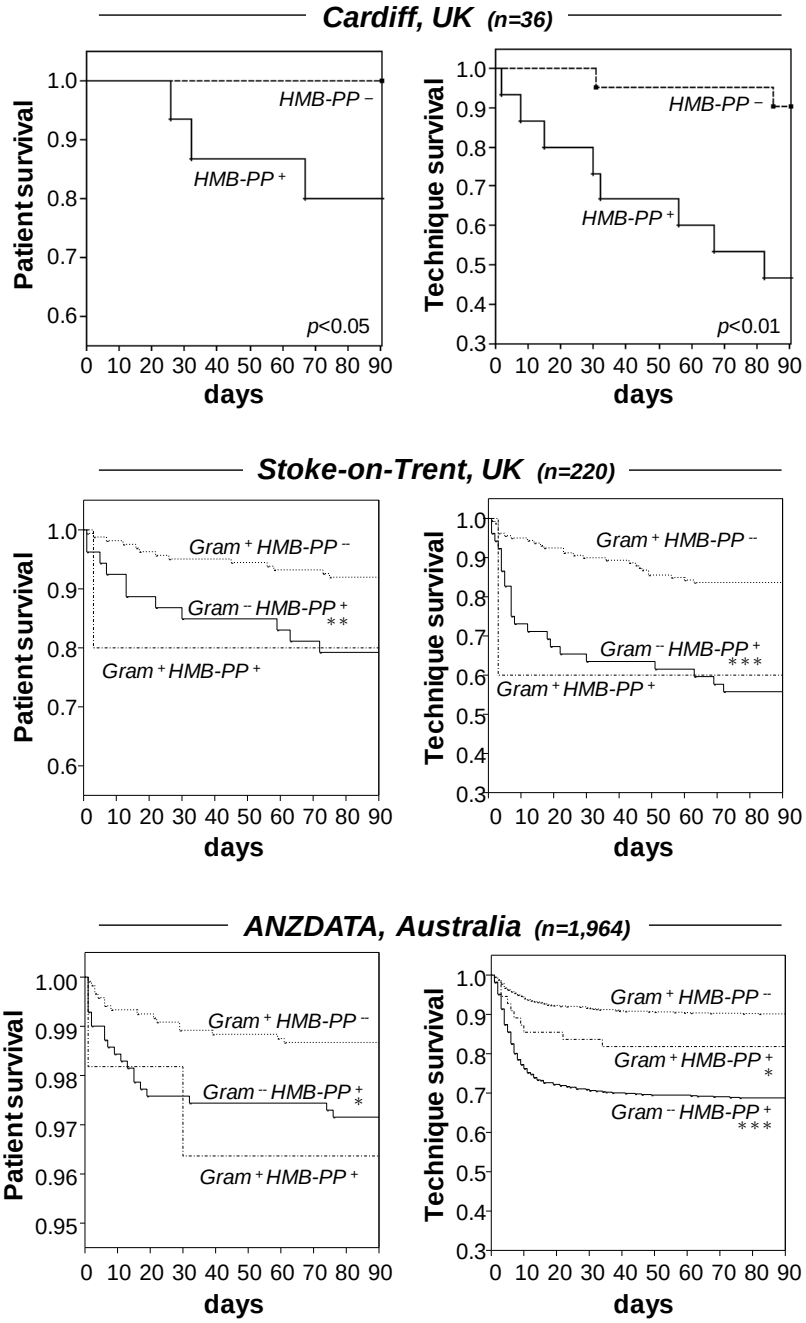

Figure 3.12 Episodes of peritonitis caused by HMB-PP producing bacteria are associated with poor clinical outcome.

Cumulative patient survival (*left*) and cumulative technique survival (*right*) of patients with acute bacterial peritonitis, grouped into infections with HMB-PP deficient (dotted lines) or HMB-PP producing pathogens (solid lines). *Top*, PD patients admitted at the University Hospital of Wales, Cardiff, with acute peritonitis. *Middle*, Australian PD patients from the ANZDATA registry with first-time peritonitis. *Bottom*, PD patients with first-time peritonitis treated at the University Hospital of North Staffordshire, Stoke-on-Trent, UK. Comparisons were made using log-rank tests. *, P<0.05; **, P<0.01, ***, P< 0.001, compared to $Gram^{+}HMB\text{-}PP^{-}$ peritonitis.

Table 3. 3 Odds ratios for risk of technique failure and patient mortality within 90 days after infection, depending on the causative pathogen. Comparisons were made using multinomial logistic regression.

90th day technique failure	**Stoke-on-Trent (UK)**	***p* value**	**ANZDATA (Australia)**	***p* value**	**ANZDATA + Stoke-on-Trent combined**	***p* value**
Reference: culture-negative	**1.0**		**1.0**		**1.0**	
HMB-PP$^-$	2.0	*	0.8	*n.s.*	1.0	*n.s.*
HMB-PP$^+$	7.4	***	3.4	***	4.1	***
Reference: HMB-PP$^-$	**1.0**		**1.0**		**1.0**	
Gram$^+$ HMB-PP$^+$	3.1	*n.s.*	2.0	0.066	2.1	*
Gram$^-$ HMB-PP$^+$	3.8	***	4.3	**	4.0	***
90th day mortality						
Reference: culture-negative	**1.0**		**1.0**		**1.0**	
HMB-PP$^-$	2.1	*n.s.*	2.3	*n.s.*	1.3	*n.s.*
HMB-PP$^+$	6.3	***	5.4	*	2.8	**
Reference: HMB-PP$^-$	**1.0**		**1.0**		**1.0**	
Gram$^+$ HMB-PP$^+$	2.9	*n.s.*	2.8	*n.s.*	2.4	*n.s.*
Gram$^-$ HMB-PP$^+$	3.0	*	2.4	**	2.2	**

3.3.11 Infections caused by HMB-PP producing bacteria are characterized by elevated frequencies of local γδ T cells.

Vγ9/Vδ2 T cell specifically respond to the microbial metabolite HMB-PP, an essential metabolite in the majority of Gram$^+$ (*Bacillus*, *Clostridium*, *Corynebacterium*, *Mycobacterium*) and Gram$^-$ bacteria (*E. coli*, *Klebsiella*, *Pseudomonas*, *Salmonella*) (87, 94). We therefore speculated that infections by HMB-PP producing bacteria directly lead to increased infiltration and activation of local γδ T cells. In a total of 33 peritonitis episodes examined on the first day of infection, we did not see any significant differences in the numbers and frequencies of peritoneal neutrophils, monocytes/macrophages, and CD4$^+$ or CD8$^+$ T cells, regardless of the HMB-PP status of the causative pathogen. However, the frequencies of peritoneal Vγ9/Vδ2 T cells were elevated in HMB-PP$^+$ infections compared to HMB-PP$^-$ infections (Figure 3.13), suggesting increased infiltration and/or proliferation in response to HMB-PP released by bacteria. Although CD69 expression on peritoneal Vγ9/Vδ2 T cells was higher compared to circulating counterparts, there were no significant difference in the expression of CD69 on Vγ9/Vδ2 T cells between HMB-PP$^+$ and HMB-PP$^-$ peritonitis (Figures 3.3 and 3.13).

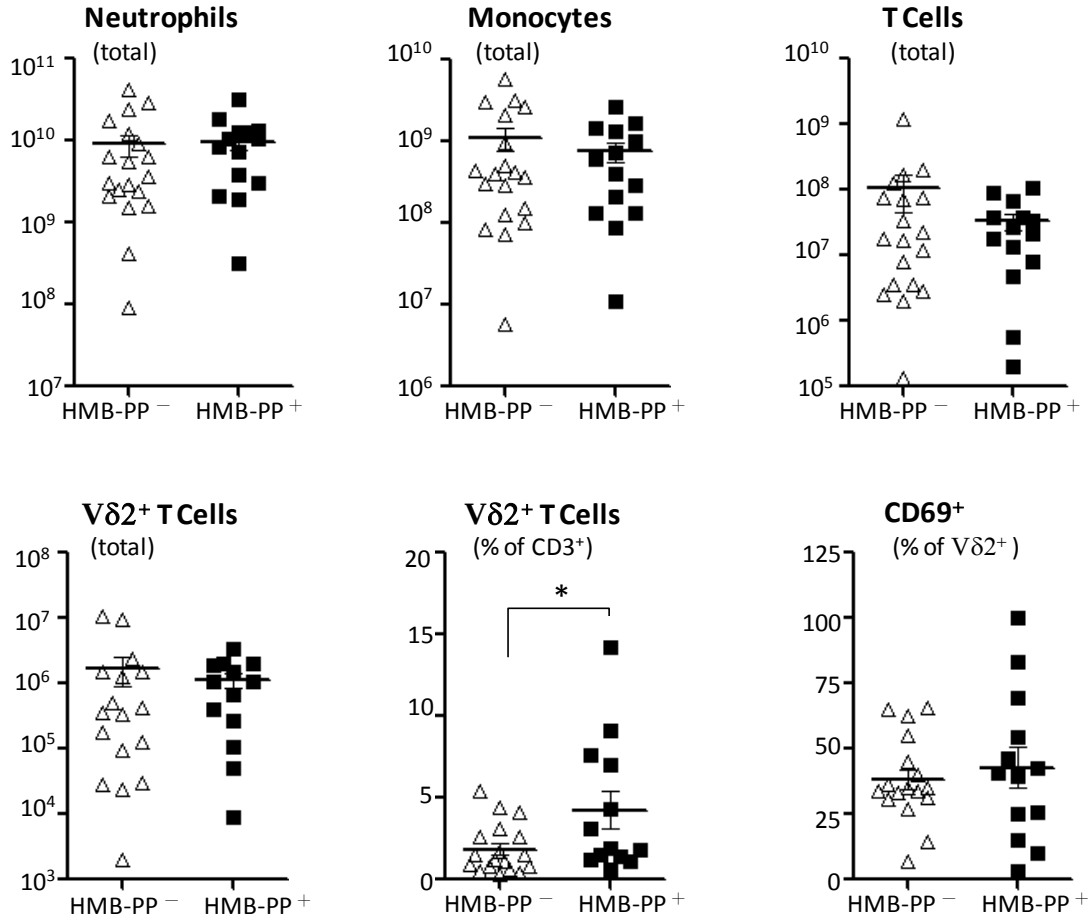

Figure 3.13 Infections caused by HMB-PP producing bacteria are characterized by elevated frequencies of local γδ T cells.

Total numbers of peritoneal neutrophils ($CD15^+$), monocytes/macrophages ($CD14^+$), and T cells ($CD3^+$), and total numbers and frequencies of γδ T cells ($V\delta2^+$), and expression of CD69 on peritoneal γδ T cells in patients with PD-associated peritonitis on day 1, depending on whether or not the causative pathogen is capable of producing the microbial metabolite HMB-PP. Statistical differences between patient groups were analyzed using the Student's *t*-test. Error bars indicate SEM. *, $P<0.05$.

3.3.12 Frequencies of peritoneal γδ T cells predict clinical outcome in peritonitis patients.

Analysis of all immunological parameters determined in the present study identified elevated frequencies of peritoneal Vγ9/Vδ2 T cells were associated with technique failure within 30 and 90 days after infection (Table 3.4). Similarly, expression of the activation marker HLA-DR by peritoneal Vγ9/Vδ2 T cells on the day of admission might be associated with increased 90^{th} day mortality. Other parameters such as numbers and frequencies of neutrophils, monocytes, $CD4^+$ or $CD8^+$ T cells, the activation status of monocytes, or soluble factors in the effluent did not reach statistical significance (data not shown).

Although some subsets of $CD4^+$ (T_{EM} and T_{EMRA}) or γδ T cells (naïve) reached significant differences in the outcome analysis, inconsistent results among different markers (CCR7, CD27 or CD62L vs. CD45RA) made these data difficult to interpret. Moreover, low expression of CD25 on $CD8^+$ T cells makes CD25 a poor marker for predicting outcome, even though its expression on $CD8^+$ T cells reached a significant difference.

While preliminary in nature due to the limited number of patient samples analyzed, our observations imply that positive identification of the causative pathogen to produce HMB-PP together with elevated frequencies of γδ T cells might be negative predictors of clinical outcome.

Table 3.4 Identification of day 1 predictive immune markers for clinical outcome in PD-associated peritonitis patients (means ± SEM).

30th day technique failure	**Survivors (46)**	**Non-survivors (4)**	***p* value**
$V\delta2^+$ (% of $CD3^+$ T cells)	2.6 ± 0.4	7.0 ± 1.4	**
$CCR7^+CD45RA^+$(% of $V\delta2^+$ T cells)	2.5 ± 0.7	8.7 ± 4.4	*
$CD62L^-CD45RA^-$ (% of $CD4^+$ T cells)	51.1 ± 3.1	25.6 ± 6.6	*

90th day technique failure	**Survivors (39)**	**Non-survivors (11)**	***p* value**
$V\delta2^+$ (% of $CD3^+$ T cells)	2.1 ± 0.2	5.9 ± 1.4	*
$V\delta2^+$ T cells ($\cdot10^6$)	0.7 ± 0.3	1.3 ± 0.4	*
$CCR7^-CD45RA^+$(% of $CD4^+$ T cells)	8.2 ± 1.8	3.3 ± 1.0	*
$CD27^-CD45RA^+$(% of $CD4^+$ T cells)	5.5 ± 1.3	2.0 ± 0.8	*
$CD25^+$(% of $CD8^+$ T cells)	2.6 ± 0.5	1.2 ± 0.2	*

90th day mortality	**Survivors (47)**	**Non-survivors (3)**	***p* value**
$V\delta2^+$ T cells ($\cdot10^6$)	0.9 ±0.3	0.16 ± 0.12	*
HLA-DR^+ (% of $V\delta2^+$ T cells)	12.1 ± 1.7	40.6 ± 4.3	***
$CD62L^-CD45RA^+$ (% of $CD4^+$ T cells)	3.0 ± 1.2	6.1 ± 0.4	*

3.4 Discussion

The number of patients developing end stage renal failure is increasing year upon year, and in the UK is expected to double over the next ten years (138, 139). Patients generally benefit most if they commence treatment with PD as the most efficacious and cost-effective part of an integrated program of renal replacement therapy. Currently however, the median treatment time on PD is only 2.5-3.5 years, with the major reason for cessation remaining recurrent or severe episodes of peritonitis (140). If we are to limit the susceptibility to infection and the detrimental impact of prolonged inflammation on peritoneal membrane longevity, we need to better understand the processes causing deleterious alterations to the local immune response.

To realize the activation status of monocytes/macrophages, we measured the expression of APC markers including CD40, CD86 and HLA-DR. Peritoneal monocytes/macrophages from peritonitis patients on day 1 were unable to express high levels of HLA-DR or the costimulatory molecules CD40 and CD86 compared to circulating monocytes from healthy donors. In addition, the expression of CD86 on peritoneal monocytes/macrophages from stable and peritonitis patients was significantly lower than that on circulating monocytes. Further analysis of peritonitis samples showed that the expression of CD86 was even lower in HMB-PP^{+} peritonitis compared to HMB-PP^{-} peritonitis. Clinical observations have shown that HLA-DR expression was significantly decreased both in patients with severe sepsis and septic shock (141). Down regulation of HLA-DR and CD86 on circulating monocytes were also showed in patients with indeterminate clinical form of Chagas disease (142). Compared to cells from controls, CD40L-activated monocytes from septic patients with Gram-negative organisms showed significantly reduced production of TNF-α, IL-1β, and IL-12 and were unable to express high levels of CD80 and CD86 molecules (143). This could be the explanation for our observation of down-regulation of CD86 on peritoneal monocyes in HMB-PP^{+} peritonitis, which were mostly Gram-negative infections in this study. Pathogens such as *Salmonella* (144) have been showed to develope immune evasion strategies (e.g. ubiquitination) to down-regulate expression of MHC class II molecules on APCs.

Consistent with previous studies (35, 61, 62), our data show that some degree of local activation is present in the peritoneal cavity of stable PD patients evidenced by enhanced expression of CD69 on $CD4^+$, $CD8^+$ and γδ T cells and higher CD25 expression on γδ T cells in comparison with their counterparts in blood. The peritoneal T cells from peritonitis patients on day 1 were also activated with enhanced expression of both CD25 and CD69 on all three T cell subsets compared to circulating T cells. Of note, the levels of CD69 expression were much higher than those of CD25 expression on activated T cells. Since CD69 is generally regarded as an early activation surface maker expressed on lymphocytes and CD25 is a relatively late marker (145, 146), the collecting time point of peritonitis samples might be one of the reasons for this difference. On the other hand, CD25 is an important marker of some Treg sub-populations. Many of the peritoneal $CD4^+CD25^+$ cells may be Tregs rather than activated T cells.

Because CCR7 and CD62L are essential for homing to lymph nodes (106) and human naïve T cells can be identified by the expression of the CD45RA isoforms (147), staining of T cells with antibodies to CD45RA and CCR7 revealed three subsets of $CD4^+$ T cells: one naïve $CD45RA^+CCR7^+$; and two memory subsets, $CD45RA^-CCR7^+$ (T_{CM}) and $CD45RA^-CCR7^-$ (T_{EM}). Both naïve and T_{CM} cells expressed high levels of CD62L, whereas the T_{EM} cells expressed lower CD62L. Within $CD8^+$ T cells, an extra subset of $CD45RA^+CCR7^-$ cells (T_{EMRA}) was identified (102). Accordingly, our results confirmed that effector/memory ($CD45RA^-CCR7^-$) $CD4^+$ T cells were the most predominant subsets in the peritoneal cavity in stable PD effluents as shown before (126). In parallel, analysis of γδ T-cell memory subsets characterized with antibodies against CD45RA and CD27 (105) demonstrated that $CD45RA^-CD27^+$ $V\delta2^+$ T cells were the most enriched subsets in peritoneal cavity either in stable or peritonitis samples on day 1. However, in comparison with circulating γδ T cells, the frequencies of peritoneal effector/memory $CD4^+$ T cells and $CD45RA^-CD27^-V\delta2^+$ T cells increased in peritonitis patients. Conversely, the frequencies of naïve $CD4^+$ T cells and $CD45RA^+CD27^+$ $V\delta2^+$ T cells decreased in peritonitis patients.

There are a number of chemokine receptors and adhesion molecules involved in

lymphocyte migration to secondary lymphoid organs or tissues under homeostatic or inflammatory conditions (102). The peritoneal γδ T cells from peritonitis patients on day 1 expressed similar level of CCR5 but showed up-regulation of CCR6 and down-regulation of CXCR3 in comparison with their counterparts in blood. The peritoneal $CD4^+$ and $CD8^+$ T cells from peritonitis samples expressed these markers in a similar pattern. It has been reported that CCR5 and CXCR3 are predominantly expressed on T_H1 cells in peripheral blood (148). Of note, higher levels of CXCL10, one of the ligands of CXCR3, were found in peritonitis samples (514.1 ± 82.7 pg/ml) compared to stable PD effluents (43.2 ± 22.5 pg/ml), suggesting a possible down-regulation of CXCR3 on peritoneal T cells as a result of chemokine-mediated receptor internalization (149, 150).

Sine $CD4^+$ helper T cells can differentiate into T_H1, T_H2, T_H17 or Treg cells in response to various stimuli and the different cytokines secreted by these cells mediate distinct effector functions (151), we evaluated different subsets of T cells and observed the predominant $CD4^+$ T cells subsets in stable PD effluents to be T_H1 cells. This pattern of cytokine production was the same in $CD8^+$ T cells and γδ T cells in stable PD patients. T lymphocytes in the peritoneal cavity of peritonitis patients on day1 were characterized by an increase in the percentage of T_H2 cells, as well as T_H2-type $CD8^+$ T cells and IL-4 producing γδ T cells, without a significantly different percentage of T_H1 cells in comparison with these cells in stable PD effluents. High numbers of T_H2-type $CD8^+$ T cell clones have been generated from HIV-infected patients (152). In the normal human peritoneum, T_H2-type $CD8^+$ T cells exhibited reduced cytolytic activity and provide B cell helper function (84). By measuring the expression patterns of cytokine mRNAs, Wang et al. demonstrated that peritoneal $CD8^+$ T cells cloned from the PD effluents of new patients and during peritonitis on day 2 were associated with a T_H2-type pattern (85). The role of peritoneal T_H2-type $CD8^+$ T cell in acute peritonitis still needs further evaluation.

In this study, our data demonstrate that culture-positive peritonitis carries a worse prognosis in terms of technique and patient survival, in keeping with previous studies (20, 153-156). As compared to culture-negative infections, these episodes have a different inflammatory signature characterized by higher numbers (and

proportions) of infiltrating neutrophils and more severe peritoneal inflammation. Moreover, culture-positive peritonitis is skewed toward higher frequencies of T_H1 cells and lower frequencies of T_H2 and Treg cells, according to the expressions of cytokine and transcription factor profiles. We then extended these observations by identifying that the capacity of the causative pathogen to produce HMB-PP, together with the infiltration of activated γδ T cells are important risk factors and possible predictors of patient outcomes from infection.

Re-analysis of peritonitis data from validated registry data confirms that HMB-PP$^+$ bacteria (such as *E. coli*, *Corynebacterium spp.*, and *Pseudomonas spp.*) caused clinically more severe infection, with higher rates of non-resolving infection, peritoneal catheter removal, and patient mortality (15, 18, 119, 133-137). These findings emphasize the importance of shifting the focus away from a simple classification into Gram-negative and Gram-positive species, and the need to pay more attention to detailed host-pathogen interactions. These interactions are clearly dependent on the nature of the causative pathogens and might impact on treatment outcomes. In this respect, the correct identification of the causative pathogen is of pivotal relevance for our understanding of the underlying inflammatory response and possible ways of improving patient management and refining/targeting treatment. Further improvements in culture techniques and the introduction of rapid molecular diagnostic methods such as 16S and 23S rDNA sequencing are therefore urgently needed (22, 23, 157, 158).

There is a necessity for more research into the role of different leukocyte populations and soluble mediators during acute inflammatory responses, in order to dissect the interplay between neutrophils, monocytes/macrophages, and T cells, and the potentially detrimental consequences for clinical outcome (57, 112, 125, 126). One such interaction may lead to aberrant activation and expansion of γδ T cells and the perpetuation of inflammatory responses during acute peritonitis. γδ T cells clearly play a beneficial role in bridging innate and adaptive immune responses in infection and inflammation (87, 89, 94). However, their excessive activation in the peritoneal cavity of a vulnerable patient group, where the continuous exchange of PD effluent and thus the repeated removal of all infiltrating leukocytes and soluble mediators does not allow the natural progression and resolution of an anti-microbial immune

response, may ultimately contribute both to irreversible damage on the peritoneal membrane and to detrimental outcomes.

Chapter 4

Local immune fingerprints in PD associated peritonitis

4.1. Introduction

Infection-related morbidity and mortality remains unacceptably high, indicating that conventional research solutions have not made sufficient impact (159). Effective infection control is hampered by the poor performance of standard diagnostics and hence inadequate choice of treatments, and a deep lack of appreciation how the body senses and fights bacterial pathogens.

Antimicrobial treatment is largely empirical as therapeutic intervention usually commences before the nature of the causative pathogen is known. Microbiological identification is often delayed and in many cases no organism can be identified. Rates of culture-negative infections in PD peritonitis may vary from 0-50 % (20), in sepsis they typically exceed >50% (160). Molecular tests that identify microorganisms in biological fluids often suffer from lack of specificity and unacceptably high rates of false positivity. Point-of-care methods that direct therapy especially in cases where the virulence of an organism is a determinant of outcome are urgently required (161-164). The failure in improving outcomes across diverse patient groups demonstrates that the physiological and pathophysiological events driving early inflammatory responses and pathogen clearance remain poorly understood. It is becoming increasingly clear that the nature of the infection is a major determinant of outcome (124), and detailed knowledge about the intricate relationships between immunological, microbiological and clinical parameters in disease is needed. This is especially the case in particularly vulnerable individuals such as patients with sepsis or PD associated peritonitis, where overshooting host responses have detrimental consequences (57, 165). Positive identification of the causative pathogen is not only crucial in order to guide and refine patient management with respect to the choice of antibiotic treatment, it also provides important clues as to the underlying inflammatory mechanisms and how to manipulate them to resolve infection.

Different types of pathogens carry different types of pathogen-associated molecular patterns, and hence interact with different components of the immune system. Each infection is therefore likely to leave a distinct immunological signature that can be assessed quantitatively and qualitatively (166-168). However, aside from theoretical

considerations, to our knowledge no experimental attempt has been made so far to translate the notion of pathogen-specific immune responses into the clinic and use it for diagnostic purposes.

In the present study we performed a detailed immunological and microbiological analysis in PD patients on the first day of presentation with acute peritonitis. Our findings provide proof of concept that acute bacterial infections indeed leave characteristic disease-specific '*immune fingerprints*' of diagnostic and prognostic value. Local fingerprints not only discriminated between episodes of culture-negative and culture-positive PD associated peritonitis but also predicted infections caused by $Gram^-$ or $Gram^+$ bacteria. As such, our results have far-reaching implications for differential diagnosis of patients with suspected infections and may help guide patient management through faster biomarker-based diagnostics, better predictive risk modeling and improved targeting of therapy.

4.2 Aims

The aims of this Chapter were:

1) To identify local 'immune fingerprints' of culture-negative, $Gram^-$ and $Gram^+$ PD-associated peritonitis.
2) To demonstrate the discrimination power and cut-off values of biomarkers with diagnostic potential.
3) To demonstrate potential biomarkers for the immune signature of individual bacteria.

4.3 Results

4.3.1 Acute episodes of peritonitis are associated with severe peritoneal inflammation.

Following the analysis of Chapter 3, we tried to identify potential biomarkers with characteristic disease-specific '*immune fingerprints*' of diagnostic value using the data collected from the 52 episodes of acute peritonitis on day1.

As mentioned in Section 3.3.1, patients presenting with acute peritonitis were characterized by a significant peritoneal influx of immune cells, predominantly $CD15^+$ neutrophils, $CD14^+$ monocytes/macrophages and $CD3^+$ T cells. While peritoneal leukocytes in stable patients comprised mainly of monocytes/macrophages and T cells, acute peritonitis was dominated by a massive recruitment of neutrophils, at times reaching >95% of all peritoneal cells and $>10^{11}$ cells in total per PD bag. Within the peritoneal T cell population, there was a preferential increase in the frequency of $V\delta2^+$ T cells within the T cell population in acute peritonitis, while the percentages of $CD4^+$ and $CD8^+$ T cells remained virtually unchanged (Table 4.1).

Soluble mediators significantly increased in acute peritonitis included IL-1β, IL-6, sIL-6R, IL-10, IL-22, CXCL8, CXCL10, TNF-α, TGF-β and MMP-3; at the same time levels of cytokines such as IL-2, IL-12p70, IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were not elevated above baseline or, in the case of IFN-γ, not significantly (Table 4.1). Taken together, these measurements identified a broad range of cellular and soluble biomarkers that indicate acute inflammatory responses in PD patients, some of which might be of diagnostic value.

Table 4.1 Immunological biomarkers in stable patients and patients presenting with a 'cloudy bag' on day 1 (mean ± SEM). *n.s.*, not significant.

	Stable PD	**Cloudy bag**	***p***
Gender (male/female)	9/6	32/20	*n.s.*
Age (years)	60.4 ± 4.9	65.5 ± 2.0	*n.s.*
Days on PD (days)	1,058 ± 219.2	1,142 ± 131.1	*n.s.*
Neutrophils ($\cdot 10^6$)	0.6 ± 0.3	7,048 ± 1,384	***
Monocytes ($\cdot 10^6$)	2.8 ± 1.5	753.9 ± 166.0	***
$CD4^+$ T cells ($\cdot 10^6$)	0.3 ± 0.2	32.3 ± 15.7	***
$CD8^+$ T cells ($\cdot 10^6$)	0.3 ± 0.2	24.5 ± 12.0	***
$V\delta2^+$ T cells ($\cdot 10^6$)	0.01 ± 0.003	1.1 ± 0.4	***
Neutrophils (% of total)	9.0 ± 4.2	80.3 ± 2.2	***
Monocytes (% of total)	28.9 ± 9.4	12.9 ± 1.9	*n.s.*
T cells (% of total)	17.5 ± 6.8	1.0 ± 0.3	***
$CD4^+$ (% of T cells)	52.2 ± 3.3	50.6 ± 2.1	*n.s.*
$CD8^+$ (% of T cells)	40.0 ± 3.8	37.6 ± 2.1	*n.s.*
$V\delta2^+$ (% of T cells)	1.3 ± 0.3	2.9 ± 0.4	*
IL-1β (pg/ml)	3.7 ± 1.6	45.1 ± 12.6	***
IL-2 (pg/ml)	6.2 ± 2.2	11.4 ± 2.1	*n.s.*
IL-6 (pg/ml)	37.1 ± 7.3	3,249 ± 623.6	***
sIL-6R (pg/ml)	360.1 ± 50.8	639.6 ± 50.3	**
IL-10 (pg/ml)	10.9 ± 3.8	85.6 ± 19.4	***
IL-12p70 (pg/ml)	4.2 ± 1.5	4.5 ± 1.0	*n.s.*
IL-17 (pg/ml)	1.5 ± 1.0	6.7 ± 2.7	*n.s.*
IL-22 (pg/ml)	13.9 ± 7.7	121.8 ± 36.5	**
CXCL8 (pg/ml)	18.1 ± 3.2	699.3 ± 191.7	***
CXCL10 (pg/ml)	43.2 ± 22.5	514.1 ± 82.7	***
IFN-γ (pg/ml)	52.7 ± 16.6	123.0 ± 39.7	*n.s.*
TNF-α (pg/ml)	21.8 ± 8.4	86.8 ± 18.8	*
GM-CSF (pg/ml)	11.3 ± 4.4	15.5 ± 3.8	*n.s.*
TGF-β (pg/ml)	78.4 ± 10.8	151.4 ± 15.0	**
MMP-3 (pg/ml)	1,540 ± 184.9	3,029 ± 427.3	**

4.3.2 Culture-negative and culture-positive episodes of peritonitis display distinct immune signatures.

We next stratified patients presenting with a 'cloudy bag' into distinct subgroups, according to the microbiological culture results. Together with Section 3.3.9, these analyses revealed that immune signatures were markedly different between patients with confirmed culture-positive infection and those with culture-negative infection. Patients with culture-positive peritonitis had significantly higher numbers of neutrophils and monocytes/macrophages as well as $CD4^+$, $CD8^+$ and $V\delta2^+$ T cells (Figure 3.11 and Table 4.2). However, besides these quantitative differences we also noted pronounced qualitative differences. In culture-positive patients, a larger proportion of the infiltrating immune cells were neutrophils whereas a smaller proportion were monocytes/macrophages, suggesting that the neutrophil:monocyte ratio might be of relevance for diagnostic purposes (Table 4.2). In contrast, the proportion of T cells in the infiltrate and the relative composition of $CD4^+$, $CD8^+$ and $V\delta2^+$ T cells within the T cell population did not show any difference between culture-positive and culture-negative infection. In line with differences on the cellular level, the two patient groups could also be distinguished based on the peritoneal levels of inflammatory markers, with higher levels of IL-1β, IL-2, IL-6, IL-10, IL-22 and TNF-α (as well as sIL-6R and MMP-3 with borderline significance) in culture-positive patients, whereas IL-12p70, IL-17, CXCL8, CXCL10, IFN-γ, GM-CSF and TGF-β showed no such differences (Figure 3.10 and Table 4.2). Taken together, these findings confirm earlier reports that patients with culture-positive peritonitis experience a more severe peritoneal inflammatory response than those with culture-negative peritonitis, and identify immune signatures that might have discriminatory power between the two groups.

Table 4.2 Immunological biomarkers in patients presenting with a 'cloudy bag' on day 1, depending on the microbiological culture results (mean ± SEM). *n.s.*, not significant.

	Culture-negative	**Culture-positive**	***p***
Gender (male/female)	10/5	22/15	*n.s.*
Age (years)	66.7 ± 3.7	65.0 ± 2.3	*n.s.*
Days on PD (days)	1,185 ± 230.0	1,140 ± 164.9	*n.s.*
Neutrophils ($\cdot 10^6$)	1,225 ± 772.9	9,342 ± 1,756	***
Monocytes ($\cdot 10^6$)	245.5 ± 81.4	954.1 ± 220.4	*
$CD4^+$ T cells ($\cdot 10^6$)	4.3 ± 1.9	43.5 ± 21.7	**
$CD8^+$ T cells ($\cdot 10^6$)	1.8 ± 0.5	33.6 ± 16.5	**
$V\delta2^+$ T cells ($\cdot 10^6$)	0.1 ± 0.04	1.4 ± 0.5	**
Neutrophils (% of total)	68.2 ± 5.6	85.0 ± 1.6	**
Monocytes (% of total)	23.6 ± 5.1	8.8 ± 1.1	**
T cells (% of total)	1.4 ± 0.7	0.8 ± 0.2	*n.s.*
$CD4^+$ (% of T cells)	54.0 ± 4.2	49.3 ± 2.5	*n.s.*
$CD8^+$ (% of T cells)	36.6 ± 3.8	37.9 ± 2.6	*n.s.*
$V\delta2^+$ (% of T cells)	2.4 ± 0.4	2.9 ± 0.6	*n.s.*
IL-1β (pg/ml)	7.2 ± 3.1	62.9 ± 17.5	***
IL-2 (pg/ml)	6.7 ± 2.0	13.6 ± 2.8	*
IL-6 (pg/ml)	1,502 ± 677.8	4,064 ± 823.2	**
sIL-6R (pg/ml)	493.9 ± 58.9	699.6 ± 64.5	*0.063*
IL-10 (pg/ml)	16.4 ± 4.5	117.9 ± 26.4	***
IL-12p70 (pg/ml)	2.4 ± 0.7	5.5 ± 1.4	*n.s.*
IL-17 (pg/ml)	4.7 ± 3.4	7.8 ± 3.7	*n.s.*
IL-22 (pg/ml)	55.1 ± 24.5	151.0 ± 50.7	*
CXCL8 (pg/ml)	470.0 ± 296.9	806.3 ± 245.5	*n.s.*
CXCL10 (pg/ml)	323.1 ± 123.6	603.6 ± 104.3	*n.s.*
IFN-γ (pg/ml)	59.4 ± 20.6	152.6 ± 57.0	*n.s.*
TNF-α (pg/ml)	16.3 ± 5.3	119.7 ± 25.5	**
GM-CSF (pg/ml)	8.7 ± 2.9	18.7 ± 5.4	*n.s.*
TGF-β (pg/ml)	115.2 ± 16.8	166.1 ± 19.6	*n.s.*
MMP-3 (pg/ml)	1,969 ± 291.8	3,479 ± 581.4	*0.056*

4.3.3 A combination of immunological biomarkers accurately predicts culture-negative episodes of peritonitis on day 1.

Given the distinct immune signatures observed in the different patient groups, we tested whether specific immunological biomarkers on day 1 could predict the microbiological culture results. AUROC calculations identified a number of parameters with discriminatory power, such as the proportion of neutrophils amongst peritoneal cells as well as the levels of IL-1β, IL-10, TNF-α and IL-6 (Table 4.3). A simple model for the discriminatory potential between culture-negative and culture positive peritonitis was developed by combination of biomarkers according to the cut-off values determined by Youden index (Table 4.4). For culture-positive prediction, IL-10 (≥ 23.3 pg/ml) (one point), IL-1β (≥ 4.1 pg/ml) (one point), TNF-α (≥ 19.5 pg/ml) (one point), and IL-6 (≥ 1,035 pg/ml) (one point) were used to calculate AUROC values. Conversely, IL-10 (< 23.3 pg/ml) (one point) and IL-1β (< 4.1 pg/ml) (one point) and proportion of peritoneal monocytes/macrophages (≥19.1%) (one point) were used for culture-negative prediction. Combination of the parameters with the highest AUROC values yielded an excellent discriminatory potential for IL-1β and IL-10 levels to predict cases of culture-positive infection. Similarly, combination of the proportion of peritoneal monocytes/macrophages with IL-1β and IL-10 levels resulted in an excellent discriminatory potential to predict cases of culture-negative infection (Table 4.3), as illustrated in Figure 4.1A.

Table 4.3 Ability of immunological biomarkers to discriminate between culture-negative and culture-positive episodes of peritonitis on day 1.

	AUROC ± SEM	95% CI	*p*
For culture-positive prediction			
Neutrophils (% of total)	0.805 ± 0.073	0.663 - 0.948	**
IL-1β (pg/ml)	0.886 ± 0.059	0.770 - 1.000	***
IL-10 (pg/ml)	0.857 ± 0.057	0.745 - 0.970	***
TNF-α (pg/ml)	0.814 ± 0.065	0.687 - 0.941	**
IL-6 (pg/ml)	0.755 ± 0.082	0.594 - 0.916	**
IL-1β + IL-10[a]	0.924 ± 0.050	0.825 - 1.000	***
IL-1β + IL-10 + TNF-α[a]	0.908 ± 0.054	0.802 - 1.000	***
IL-1β + IL-10 + TNF-α + IL-6 [a]	0.888 ± 0.059	0.773 - 1.000	***
For culture-negative prediction			
Monocytes (% of total) [a]	0.787 ± 0.078	0.634 - 0.941	**
Monocytes + IL-1β + IL-10 [a]	0.927 ± 0.052	0.825 - 1.000	***
Monocytes + IL-1β[a]	0.857 ± 0.076	0.708 - 1.000	***

[a], The unit is point(s).

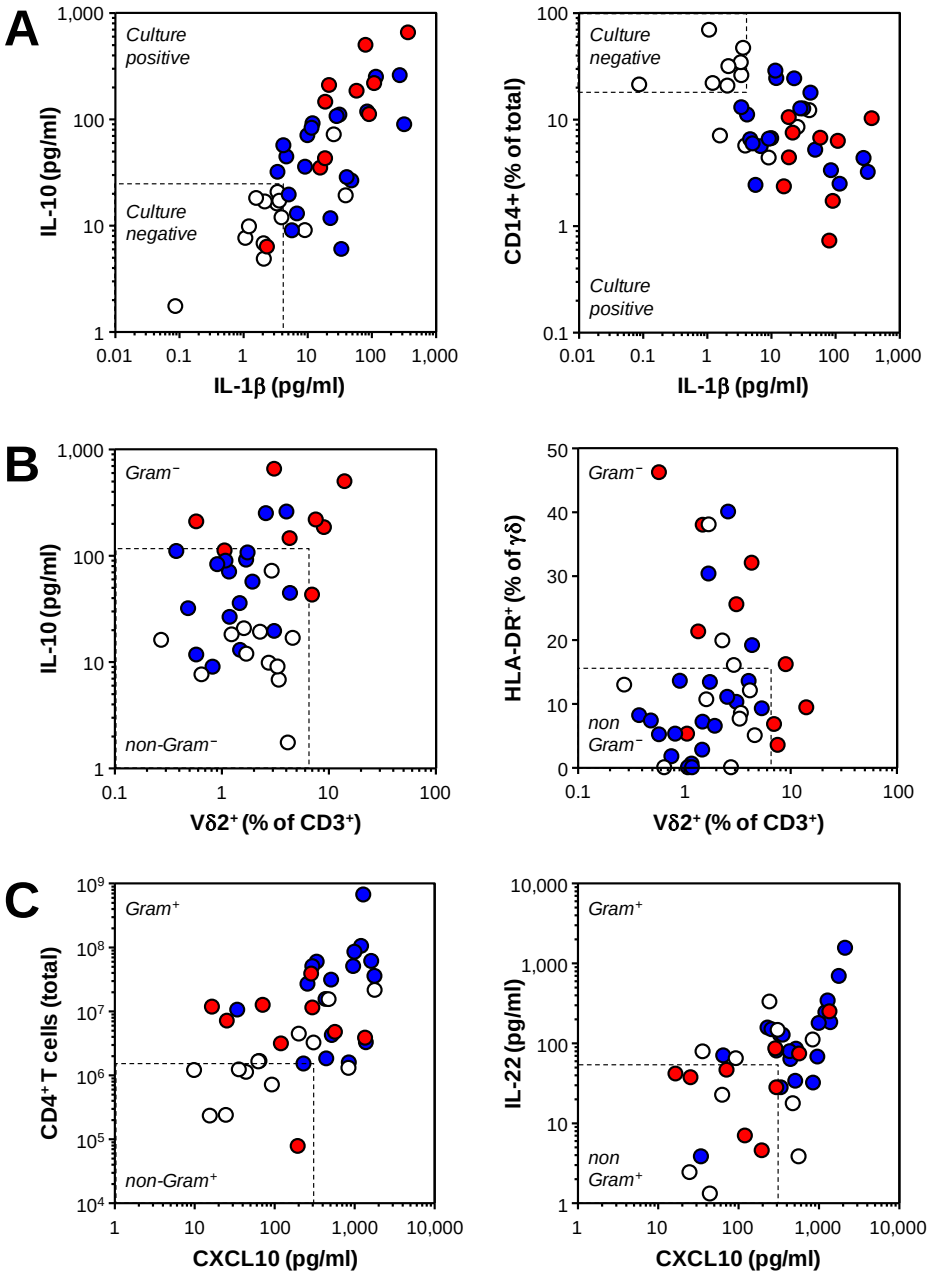

Figure 4.1 Examples of immunological biomarkers in patients presenting with a 'cloudy bag' on day 1, depending on the microbiological culture results.

(**A**) Discrimination between culture-negative and culture-positive infections. (**B**) Discrimination between Gram$^-$ and non-Gram$^-$ infections. (**C**) Discrimination between Gram$^+$ and non-Gram$^+$ infections. Data points represent individual episodes (white, culture-negative; blue, confirmed Gram$^+$ infection; red, confirmed Gram$^-$ infection. Dashed lines indicate calculated cut-off values for positive or negative discrimination.

Levels of IL-1β and IL-10 gave the best Youden Index and highest overall correctness as individual parameters, for prediction of either culture-positive or culture-negative peritonitis (Table 4.4). When combined together, IL-1β (≤ 4.1 pg/ml) and IL-10 (≤ 23.3 pg/ml) resulted in a 79% sensitivity and 97% specificity for the correct prediction of culture-negative peritonitis. Univariate analyses indicated that the proportion of neutrophils and monocytes/macrophages amongst total peritoneal cells as well as the levels of IL-1β and IL-10 had prognostic value on day 1. Further multivariate analyses of these four parameters and combinations of parameters listed in Table 4.4 confirmed that combination of IL-1β and IL-10 had independent prognostic significance for the correct prediction of culture-negative peritonitis on day 1 (Table 4.5).

Table 4.4 Prediction of culture-negative and culture-positive infection on the first day of presentation with acute peritonitis.

	Cut-off Point	Youden Index	Sensitivity (%)	Specificity (%)	Overall Correctness (%)
For culture-positive prediction					
Neutrophils (% of total)	79.0	0.59	82	77	79.5
IL-1β (pg/ml)	4.1	0.72	93	79	86
IL-10 (pg/ml)	23.3	0.73	80	93	86.5
TNF-α (pg/ml)	19.5	0.48	77	71	74
IL-6 (pg/ml)	1,035	0.45	73	71	72
IL-1β + IL-10[a]	0.5	0.75	97	79	88
IL-1β + IL-10 + TNF-α[a]	1.5	0.69	83	86	84.5
IL-1β + IL-10 + TNF-α + IL-6[a]	0.5	0.68	97	71	84
For culture-negative prediction					
Monocytes (% of total)	19.1	0.53	62	91	76.5
IL-1β + IL-10[a]	1.5	0.75	79	97	88
Monocytes + IL-1β + IL-10[a]	1.5	0.73	77	96	86.5
IL-1β + IL-10 + TNF-α[a]	1.5	0.69	86	83	84.5
Monocytes + IL-1β[a]	0.5	0.63	77	86	81.5

[a], The unit is point(s).

Table 4.5 Immunological biomarkers showing diagnostic significance for discrimination between culture-negative and culture-positive episodes of peritonitis on day 1.

	β Coefficient	Standard error	Odds ratio (95%CI)	*p*
Univariate logistic regression				
Monocytes (% of total)	-0.121	0.042	0.886 (0.816 - 0.963)	**
Neutrophils (% of total)	0.096	0.034	1.101 (1.030 - 1.177)	**
IL-1β	0.079	0.038	1.082 (1.006 - 1.165)	*
IL-10	0.052	0.023	1.053 (1.007 - 1.101)	*
Multivariate logistic regression (backward)				
IL-1β + IL-10 [a]	3.404	1.019	30.086 (4.082 - 221.765)	**
Constant	-3.057	1.225	–	–

[a], The unit is point(s).

4.3.4 Distinct immune fingerprints in patients with Gram$^-$ infections have diagnostic potential on day 1.

We next tested whether pathogen-specific immune fingerprints exist that could predict infections by certain groups of bacteria. Given the importance of Gram$^-$ bacteria in the clinic and their association with worse outcomes we initially concentrated on the prediction of Gram$^-$ infections. Amongst all patients presenting with a cloudy bag on day 1, patients with confirmed Gram$^-$ infections displayed larger numbers of infiltrating neutrophils and consequently lower proportions of monocytes/macrophages and T cells than the rest of the patients, *i.e*. individuals with culture-negative or Gram$^+$ infections (Table 4.6). Within the T cell population, V$\delta2^+$ T cells were significantly increased and expressed higher levels of the activation marker HLA-DR in Gram$^-$ infections. In return, peritoneal monocytes/macrophages expressed lower levels of CD86 in Gram$^-$ infections, compared to the rest of the patients. Inflammatory markers significantly increased in Gram$^-$ infections included IL-1β, IL-10 and TNF-α (Figure 4.1B; Table 4.6). AUROC calculations identified the combination of V$\delta2^+$ T cell frequencies and peritoneal levels of IL-10 as excellent discriminator to predict Gram$^-$ infections (Table 4.7). This combination also had the highest overall correctness, with a 100% sensitivity and 93% specificity for the correct prediction of Gram$^-$ peritonitis (Table 4.8). The prognostic value of the proportion of monocytes/macrophages amongst all cells (≤ 10.7%) and their expression of CD86 (≤ 67.9%), the frequency of V$\delta2^+$ T cells with the T cell population (≥ 6.3%) and their expression of HLA-DR (≥ 16.1%), and peritoneal IL-10 levels (≥ 110.1 pg/ml) were all confirmed by univariate analyses. Multivariate analysis of these parameters identified the V$\delta2^+$ T cell frequency as independent prognostic biomarker for the prediction of Gram$^-$ peritonitis in all patients presenting with a cloudy bag on day 1 (Table 4.9).

Table 4.6 Immunological biomarkers in patients presenting with a 'cloudy bag' on day 1, depending on the absence or presence of a confirmed Gram$^-$ infection (mean ± SEM). *n.s.*, not significant.

	Other	**Gram$^-$**	***p***
Gender (male/female)	25/15	7/5	*n.s.*
Age (years)	64.1 ± 2.4	70.1 ± 2.9	*n.s.*
Days on PD (days)	1,234 ± 156.2	899.3 ± 248.3	*n.s.*
Neutrophils ($\cdot 10^6$)	5,875 ± 1,580	10,779 ± 2,686	*
Monocytes ($\cdot 10^6$)	754.7 ± 206.1	751.1 ± 242.9	*n.s.*
CD4$^+$ T cells ($\cdot 10^6$)	39.3 ± 20.5	9.9 ± 3.4	*n.s.*
CD8$^+$ T cells ($\cdot 10^6$)	29.5 ± 15.6	8.6 ± 2.8	*n.s.*
Vδ2$^+$ T cells ($\cdot 10^6$)	1.1 ± 0.4	1.1 ± 0.4	*n.s.*
Neutrophils (% of total)	78.1 ± 2.7	87.7 ± 2.3	*n.s.*
Monocytes (% of total)	15.1 ± 2.3	5.8 ± 1.0	*
T cells (% of total)	1.3 ± 0.3	0.2 ± 0.03	*
CD4$^+$ (% of T cells)	52.7 ± 2.3	43.7 ± 4.6	*n.s.*
CD8$^+$ (% of T cells)	37.8 ± 2.4	36.6 ± 5.0	*n.s.*
Vδ2$^+$ (% of T cells)	2.0 ± 0.2	5.0 ± 1.4	*
HLA-DR$^+$ (% of γδ T cells)	10.8 ± 1.8	22.5 ± 4.7	*
CD86$^+$ (% of monocytes)	72.6 ± 3.6	53.1 ± 7.2	*
IL-1β (pg/ml)	35.0 ± 12.5	79.5 ± 34.7	*
IL-2 (pg/ml)	12.2 ± 2.6	8.8 ± 1.9	*n.s.*
IL-6 (pg/ml)	2,561 ± 538.8	5,586 ± 1,942	*n.s.*
sIL-6R (pg/ml)	606.3 ± 57.6	751.4 ± 100.9	*n.s.*
IL-10 (pg/ml)	49.4 ± 10.6	208.6 ± 65.5	**
IL-12p70 (pg/ml)	4.6 ± 1.3	4.1 ± 1.4	*n.s.*
IL-17 (pg/ml)	8.4 ± 3.7	2.4 ± 1.3	*n.s.*
IL-22 (pg/ml)	139.8 ± 45.9	56.8 ± 22.9	*n.s.*
CXCL8 (pg/ml)	742.6 ± 237.5	552.3 ± 257.8	*n.s.*
CXCL10 (pg/ml)	570.1 ± 97.7	306.9 ± 130.2	*n.s.*
IFN-γ (pg/ml)	144.5 ± 50.9	50.0 ± 11.6	*n.s.*
TNF-α (pg/ml)	65.3 ± 17.9	159.7 ± 51.8	*
GM-CSF (pg/ml)	16.6 ± 4.8	12.1 ± 3.6	*n.s.*
TGF-β (pg/ml)	143.0 ± 15.0	179.3 ± 42.6	*n.s.*
MMP-3 (pg/ml)	2,804 ± 359.2	3,765 ± 1,429	*n.s.*

Table 4.7 Ability of immunological biomarkers to discriminate between Gram$^-$ and non-Gram$^-$ episodes of peritonitis on day 1.

	AUROC ± SEM	95% CI	*p*
For Gram$^-$ prediction			
Monocytes (% of total)	0.745 ± 0.075	0.597 - 0.893	*
Vδ2$^+$ (% of T cells)	0.710 ± 0.106	0.501 - 0.918	*
HLA-DR$^+$ (% of $\gamma\delta$ T cells)	0.720 ± 0.097	0.530 - 0.911	*
CD86$^+$ (% of monocytes)	0.740 ± 0.083	0.578 - 0.903	*
IL-10 (pg/ml)	0.803 ± 0.092	0.622 - 0.984	**
IL-1β (pg/ml)	0.738 ± 0.086	0.569 - 0.907	*
TNF-α (pg/ml)	0.735 ± 0.091	0.557 - 0.914	*
IL-10 + Vδ2$^+$ [a]	0.976 ± 0.022	0.934 - 1.000	***
IL-10 + Vδ2$^+$ + HLA-DR$^+$ [a]	0.959 ± 0.031	0.899 - 1.000	***
IL-1β + IL-10 + TNF-α[a]	0.844 ± 0.077	0.693 - 0.995	**

[a], The unit is point(s).

Table 4.8 Prediction of Gram$^-$ infection on the first day of presentation with acute peritonitis.

	Cut-off Point	Youden Index	Sensitivity (%)	Specificity (%)	Overall Correctness (%)
For Gram$^-$ prediction					
Monocytes (% of total)	10.7	0.50	50	100	75
Vδ2$^+$ (% of T cells)	6.3	0.46	46	100	73
HLA-DR$^+$ (% of $\gamma\delta$ T cells)	16.1	0.48	64	84	74
CD86$^+$ (% of monocytes)	67.9	0.47	74	73	73.5
IL-10 (pg/ml)	110.1	0.61	70	91	80.5
IL-1β (pg/ml)	14.0	0.55	90	65	77.5
TNF-α (pg/ml)	32.8	0.45	80	65	72.5
IL-10 + Vδ2$^+$ [a]	0.5	0.93	100	93	96.5
IL-10 + Vδ2$^+$ + HLA-DR$^+$ [a]	0.5	0.78	100	78	89
IL-1β + IL-10 + TNF-α[a]	2.5	0.61	70	91	80.5

[a], The unit is point(s).

Table 4.9 Immunological biomarkers showing diagnostic significance for prediction of Gram$^-$ episodes of peritonitis on day 1.

	β Coefficient	Standard error	Odds ratio (95%CI)	*p*
Univariate logistic regression				
Monocytes (% of total)	-0.189	0.093	0.828 (0.690 - 0.994)	*
Vδ2$^+$ (% of T cells)	0.385	0.159	1.469 (1.075 - 2.008)	*
HLA-DR$^+$ (% of γδ T cells)	0.072	0.029	1.075 (1.014 - 1.139)	*
CD86$^+$ (% of monocytes)	-0.037	0.016	0.964 (0.934 - 0.995)	*
IL-10	0.012	0.005	1.012 (1.003 - 1.022)	*
Multivariate logistic regression (backward)				
Vδ2$^+$ (% of T cells)	0.571	0.246	1.770 (1.093 - 2.866)	*
Constant	-4.591	1.376	–	–

4.3.5 Distinct immune fingerprints in patients with Gram$^+$ infections have diagnostic potential on day 1.

In an analogous way to the prediction of Gram$^-$ infections, we next attempted to identify predictors of Gram$^+$ infections. Amongst all patients presenting with a cloudy bag on day 1, patients with confirmed Gram$^+$ infections displayed larger numbers of infiltrating CD4$^+$ and CD8$^+$ T cells than the rest of the patients, *i.e.* individuals with culture-negative or Gram$^-$ infections (Table 4.10), while the proportion of Vδ2$^+$ T cells within the T cell population was significantly lower in Gram$^+$ infections. Inflammatory markers significantly increased in Gram$^+$ infections included IL-22 and CXCL10 (Figure 4.1C; Table 4.10). AUROC calculations identified the combination of CXCL10 ($\geq$ 301.2 pg/ml) and IL-22 levels ($\geq$ 54.3 pg/ml) and Vδ2$^+$ T cell frequencies ($\leq$ 2.7%) as excellent discriminator to predict Gram$^+$ infections (Table 4.11). This combination also had the highest overall correctness, with a 89% sensitivity and 67% specificity for the correct prediction of Gram$^+$ peritonitis. In return, CXCL10 levels ($\geq$ 301.2 pg/ml) combined with total CD4$^+$ T cell counts ($\geq 15.2 \cdot 10^6$) had a sensitivity of only 53% yet at a specificity of 95% for the prediction of Gram$^+$ peritonitis (Table 4.12). CD4$^+$ T cell counts as well as Vδ2$^+$ T cell frequencies and CXCL10 levels had prognostic value as confirmed by univariate analyses. Multivariate analysis of these parameters and combinations of parameters listed in Table 4.11 identified combination of CD4$^+$ T cell counts and CXCL10 levels as independent biomarkers for the prediction of Gram$^+$ peritonitis in all patients presenting with a cloudy bag on day 1 (Table 4.13).

Table 4.10 Immunological biomarkers in patients presenting with a 'cloudy bag' on day 1, depending on the absence or presence of a confirmed Gram$^+$ infection (mean ± SEM). *n.s.*, not significant.

	Other	**Gram$^+$**	***p***
Gender (male/female)	17/10	15/10	*n.s.*
Age (years)	68.2 ± 2.4	62.4 ± 3.1	*n.s.*
Days on PD (days)	1,058 ± 167.9	1,266 ± 213.9	*n.s.*
Neutrophils ($\cdot 10^6$)	5,604 ± 1,610	8,623 ± 2,293	*n.s.*
Monocytes ($\cdot 10^6$)	477.2 ± 128.0	1,056 ± 309.1	*n.s.*
CD4$^+$ T cells ($\cdot 10^6$)	6.8 ± 1.9	60.2 ± 32.2	**
CD8$^+$ T cells ($\cdot 10^6$)	4.9 ± 1.5	46.1 ± 24.5	*
Vδ2$^+$ T cells ($\cdot 10^6$)	0.6 ± 0.2	1.6 ± 0.7	*n.s.*
Neutrophils (% of total)	77.2 ± 3.8	83.7 ± 2.1	*n.s.*
Monocytes (% of total)	15.4 ± 3.3	10.3 ± 1.5	*n.s.*
T cells (% of total)	0.9 ± 0.4	1.1 ± 0.3	*n.s.*
CD4$^+$ (% of T cells)	49.3 ± 3.2	51.9 ± 2.9	*n.s.*
CD8$^+$ (% of T cells)	36.6 ± 3.0	38.6 ± 3.1	*n.s.*
Vδ2$^+$ (% of T cells)	3.6 ± 0.7	1.8 ± 0.3	*
HLA-DR$^+$ (% of γδ T cells)	17.2 ± 3.0	10.3 ± 2.1	*n.s.*
CD86$^+$ (% of monocytes)	62.9 ± 5.3	73.0 ± 4.1	*n.s.*
IL-1β (pg/ml)	37.3 ± 16.0	54.5 ± 20.1	*n.s.*
IL-2 (pg/ml)	7.6 ± 1.4	16.0 ± 4.0	*n.s.*
IL-6 (pg/ml)	3,204 ± 970.8	3,303 ± 753.0	*n.s.*
sIL-6R (pg/ml)	607.2 ± 60.0	674.8 ± 83.2	*n.s.*
IL-10 (pg/ml)	96.5 ± 33.1	72.5 ± 16.0	*n.s.*
IL-12p70 (pg/ml)	3.1 ± 0.7	6.2 ± 2.0	*n.s.*
IL-17 (pg/ml)	3.7 ± 1.9	12.1 ± 6.5	*n.s.*
IL-22 (pg/ml)	55.8 ± 16.8	193.8 ± 71.8	**
CXCL8 (pg/ml)	504.3 ± 199.9	933.3 ± 346.0	*n.s.*
CXCL10 (pg/ml)	316.6 ± 88.8	738.4 ± 131.4	**
IFN-γ (pg/ml)	55.5 ± 12.7	204.0 ± 83.6	*n.s.*
TNF-α (pg/ml)	76.0 ± 25.8	99.7 ± 28.0	*n.s.*
GM-CSF (pg/ml)	10.1 ± 2.2	22.0 ± 7.8	*n.s.*
TGF-β (pg/ml)	143.7 ± 21.6	159.8 ± 21.3	*n.s.*
MMP-3 (pg/ml)	2,760 ± 658.1	3,336 ± 532.7	*n.s.*

Table 4.11 Ability of immunological biomarkers to discriminate between Gram$^+$ and non-Gram$^+$ episodes of peritonitis on day 1.

	AUROC ± SEM	95% CI	*p*
For Gram$^+$ prediction			
CD4$^+$ T cells ($\cdot 10^6$)	0.741 ± 0.079	0.587 - 0.895	**
CD8$^+$ T cells ($\cdot 10^6$)	0.723 ± 0.083	0.561 - 0.885	*
Vδ2$^+$ (% of T cells)	0.699 ± 0.079	0.543 - 0.854	*
CXCL10 (pg/ml)	0.744 ± 0.075	0.596 - 0.891	**
IL-22 (pg/ml)	0.736 ± 0.074	0.591 - 0.881	**
CXCL10 + Vδ2$^+$ [a]	0.821 ± 0.066	0.691- 0.951	***
CXCL10 + IL-22 + Vδ2$^+$ [a]	0.816 ± 0.069	0.681- 0.951	**
CXCL10 + CD4$^+$ [a]	0.797 ± 0.073	0.653 - 0.941	**
CXCL10 + IL-22 [a]	0.794 ± 0.068	0.661 - 0.926	**

[a], The unit is point(s).

Table 4.12 Prediction of Gram$^+$ infection on the first day of presentation with acute peritonitis.

	Cut-off Point	Youden Index	Sensitivity (%)	Specificity (%)	Overall Correctness (%)
For Gram$^+$ prediction					
CD4$^+$ T cells ($\cdot 10^6$)	15.2	0.46	55	91	73
CD8$^+$ T cells ($\cdot 10^6$)	7.8	0.46	60	86	73
CXCL10 (pg/ml)	301.2	0.49	77	72	74.5
IL-22 (pg/ml)	54.3	0.39	73	67	70
CXCL10 + IL-22 + Vδ2$^+$ [a]	1.5	0.561	89	67	78
CXCL10 + Vδ2$^+$ [a]	0.5	0.524	100	52	76
CXCL10 + CD4$^+$ [a]	1.5	0.48	53	95	74
CXCL10 + IL-22 [a]	0.5	0.49	91	55	73

[a], The unit is point(s).

Table 4.13 Immunological biomarkers showing diagnostic significance for prediction of Gram$^+$ episodes of peritonitis on day 1.

	β Coefficient	Standard error	Odds ratio (95%CI)	*p*
Univariate logistic regression				
$CD4^+$ T cells ($\cdot 10^6$)	0.062	0.026	1.064 (1.011 - 1.119)	*
$CD8^+$ T cells ($\cdot 10^6$)	0.077	0.035	1.080 (1.008 - 1.156)	*
$V\delta2^+$ (% of T cells)	-0.388	0.185	0.679 (0.472 - 0.976)	*
CXCL10 (pg/ml)	0.002	0.001	1.002 (1.000 - 1.003)	*
Multivariate logistic regression (backward)				
CXCL10 + $CD4^+$ (points)	1.932	0.603	6.901 (2.117 – 22.492)	**
Constant	-1.530	0.579	–	–

4.3.6 Pathogen-specific immune fingerprints in acute peritonitis reveal fundamentally different immune responses to distinct bacterial infections.

The above immune fingerprint analyses of patients presenting with a 'cloudy bag' identified a number of immunological biomarkers that could predict culture-negative, culture-positive, $Gram^{+}$ and/or $Gram^{-}$ infections (Table 4.14). However, being preliminary in nature and so far limited to a single hospital, these findings will have to be confirmed in independent centres. The performance of diagnostic laboratories and their ability to positively identify bacteria by microbiological culture varies greatly between different centres, as well as the spectrum of micro-organisms patients become infected with (20, 169-171). In this respect we noted pronounced pathogen-specific differences in certain immunological parameters that may bias the sensitivity/specificity of future biomarker-based diagnostic methods. Our data imply that the fingerprints outlined so far only represent only the first level of an in-depth analysis of the immune response in acute infection. In fact, the immune responses to different bacteria differed greatly, suggesting that the identification of pathogen-specific fingerprints is feasible.

Table 4.14 Biomarkers of potential diagnostic value in patients presenting with a 'cloudy bag' on day 1, depending on the microbiological culture results.

	Culture-negative	**Culture-positive**	**Gram$^+$**	**Gram$^-$**
Monocytes (% of total)	≥ 19.1	< 19.1		< 10.7
CD4$^+$ T cells ($\cdot 10^6$)			≥ 15.2	
Vδ2$^+$ (% of T cells)			≤ 2.7	≥ 6.3
HLA-DR$^+$ (% of γδ T cells)				≥ 16.1
CD86$^+$ (% of monocytes)				< 67.9
IL-1β (pg/ml)	< 4.1	≥ 4.1		≥ 14.0
IL-10 (pg/ml)	< 23.3	≥ 23.3		≥ 110.1
IL-22 (pg/ml)			≥ 54.3	
CXCL10 (pg/ml)			≥ 301.2	
TNF-α (pg/ml)	< 19.5	≥ 19.5		≥ 32.8

Within the Gram$^+$ group, patients with streptococcal infections were characterized by a higher proportion of infiltrating neutrophils (not shown) than patients with confirmed *Staphylococcus aureus* infections (Figure 4.2). The dominant role of neutrophils in the immune responses was mirrored by high levels of IL-1β and CXCL8 during streptococcal infections while levels of IFN-γ, IL-22 and CXCL10 were very low. In striking contrast, *S. aureus* infections showed a higher proportion of monocytes/macrophages and T cells and higher levels of IFN-γ, IL-22 and CXCL10, compared to streptococcal infections, while IL-1β and CXCL8 were virtually absent. Within the Gram$^-$ group, patients with infections caused by *E. coli* or other coliform bacteria (*Enterobacter*, *Klebsiella*, *Proteus*, *Morganella*) were characterized by higher levels of sIL-6R, IL-10 and TNF-α, compared to infections caused by pseudomonad species (Figure 4.2 and Table 4.15).

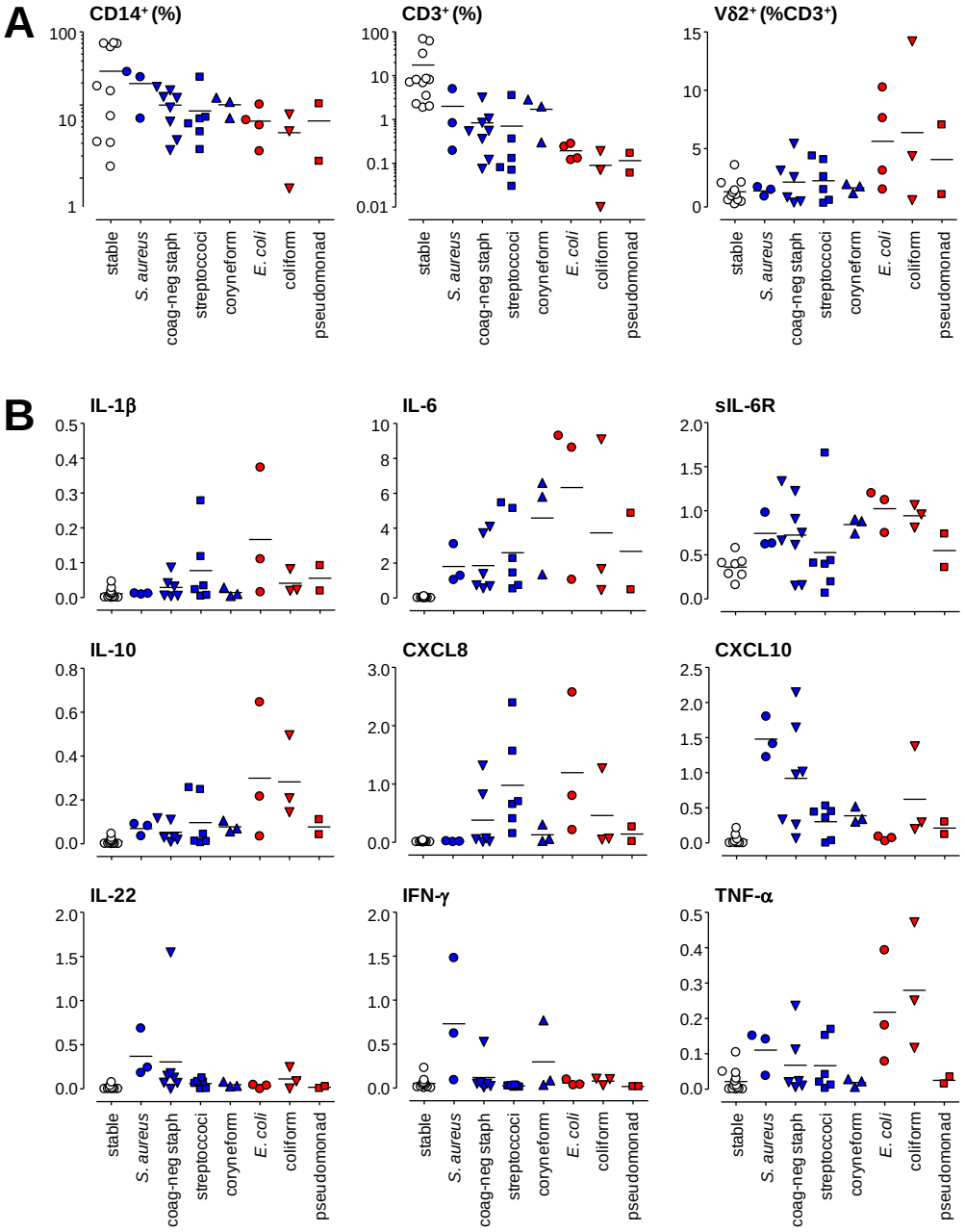

Figure 4.2 Pathogen-specific immune fingerprints in acute peritonitis.

(**A**) Relative frequency of peritoneal monocytes/macrophages ($CD14^+$) and T cells ($CD3^+$) and proportion of $V\delta2^+$ T cells within the peritoneal $CD3^+$ T cell population in stable and infected PD patients on day 1 of acute peritonitis. (**B**) Peritoneal levels (in ng/ml) of IL-1β, IL-6, sIL-6R, IL-10, IL-22, CXCL8, CXCL10, IFN-γ and TNF-α in stable and infected PD patients on day 1 of acute peritonitis. Causative pathogens identified by microbiological culture results were grouped into $Gram^+$ organisms shown in blue (circles, *Staphylococcus aureus*; downward triangles, coagulase-negative *Staphylococcus spp.*; squares, *Streptococcus spp.*; upward triangles, coryneform bacteria) and $Gram^-$ organisms in red (circles, *E. coli*; downward triangles, other coliform bacteria; squares, *Pseudomonas spp.*). Data points represent individual episode, horizontal lines mean values per group.

Table 4.15 Pathogen-specific immune fingerprints in patients with confirmed culture-positive infection, depending on the microbiological culture results. −, absent or low levels; +, present; ++, elevated; +++, highly elevated.

	IL-1β	IL-6	sIL-6R	IL-10	IL-22	CXCL8	CXCL10	IFN-γ	TNF-α
Gram$^+$ bacteria									
Staphylococcus aureus	−	+	++	+	++	−	+++	++	+
Coag.-neg. staphylococci	+	+	++	+	+	++	++	+	+
Streptococcus spp.	++	++	+	+	−	+++	+	−	+
Corynebacterium spp.	−	+++	++	+	−	+	+	+	−
Gram$^-$ bacteria									
E. coli	++	+++	++	++	−	+++	−	+	+++
Other Enterobacteriaceae	+	++	+++	+++	+	++	+	+	+++
Pseudomonas spp.	+	+	+	+	−	+	+	−	−

4.4 Discussion

Biomarkers can assist in the care of patients for diagnostic, prognostic and predictive purposes. Fundamental for the use of biomarkers in these situations is their accuracy and their ability to discriminate correctly one condition from another (172). However, given the huge biological variability between individual patients and the underlying causes of disease no biomarker offers 100% performance with optimum specificity and sensitivity. Sensitivity defines how good a test is at identifying patients with a certain condition, with a high sensitivity indicating a low false-negative rate. Specificity on the other hand defines how good a test is at correctly identifying patients without this condition, with a high specificity required for diagnostic tests with a low false-positive rate (172, 173). The utility of biomarkers therefore depends heavily on the clinical needs, for instance in the context of PD, whether correct identification of truly culture-negative (i.e. non-infectious) episodes of peritonitis or rather accurate prediction of the causative pathogen is sought. It is worth noting that at present even the two most widely used pro-inflammatory biomarkers remain of limited use in the clinic. Both C-reactive protein and procalcitonin are associated with early inflammatory responses, however they do not reach satisfying discriminatory power to distinguish infectious from non-infectious inflammation due to relatively poor sensitivity and specificity (174, 175). Moreover, both proteins have questionable relevance as diagnostic and prognostic biomarkers in patients with PD-associated peritonitis (176). Our research highlights the importance of combining humoral and cellular parameters to establish accurate 'immune fingerprints' in patients with a suspected infection. Our results reveal striking differences in the quality of local immune responses in culture-negative infections and to $Gram^-$ and $Gram^+$ bacteria. We are aware that in order to establish robust pathogen-specific immune fingerprints, accurate microbiological identification of the causative agents is absolutely critical. The fact that the majority of culture-negative and culture-positive episodes in the present study could easily be distinguished based on a range of immune parameters may indicate that the microbiological analysis at the University Hospital of Wales only yielded few false-positive and/or false-negative culture results. However, for optimum stratification in future studies and to circumvent the grave limitations of conventional culture techniques at a point in time

when the sequence information of >1,000 bacterial genomes is readily available, state-of-the-art molecular diagnostic methods such as 16S and 23S rDNA amplification, MALDI-ToF (Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer) analysis and/or whole-sample sequencing and metagenomics should be employed as 'gold standard' (158, 160-163, 177).

Our data (as shown in Chapter 3) and other studies showed that culture-negative peritonitis in PD patients is associated with a fairly benign course with low rates of technique failure (20, 153-156). Our results demonstrate that episodes of culture-negative peritonitis have significantly different immune fingerprints characterized by lower numbers (and proportions) of infiltrating neutrophils and less severe peritoneal inflammation as indicated by lower cytokine levels, compared to culture-positive infections. In this respect, the cytokines IL-1β, IL-10, TNF-α, and the ratio of neutrophils to monocytes/macrophages are particularly promising candidates for a future biomarker-based diagnosis of culture-negative peritonitis. Such a prediction of culture-negative episodes would have important clinical implications as it would allow the identification of low-risk patients and patients which may not require antimicrobial therapy, thereby reducing unnecessary exposure to antibiotics (19, 119, 178). In return, accurate prediction of culture-positive episodes with higher risk of treatment failure or even mortality would allow closer monitoring and refined management of affected patients.

The present study identified distinct immune fingerprints characteristic of $Gram^-$ and $Gram^+$ infections. While it is well-established that patients with $Gram^-$ and $Gram^+$ infections present with different clinical signs and outcomes (15, 18, 119, 123, 133, 136, 137), a differential diagnosis is missing that would guide treatment and allow predictive risk modelling at the time of presentation with a 'cloudy bag'. Here, on day 1 of acute peritonitis, $Gram^-$ infections (particularly those caused by coliform bacteria) were dominated by large numbers of infiltrating neutrophils and elevated levels of cytokines like IL-1β, IL-10 and TNF-α. It is likely that a large proportion of the immune fingerprints in $Gram^-$ infections were due to pronounced responses to bacterial LPS which is present in the outer cell wall of $Gram^-$ organisms but absent from $Gram^+$ organisms. LPS is a powerful inducer of pro-inflammatory cytokines and chemokines by monocytes/macrophages and neutrophils including IL-1β, TNF-α

and CXCL8. At the same time, LPS stimulation also leads to secretion or large amounts of IL-6 and IL-10 by monocytes/macrophages (but not human neutrophils) (179, 180). Another powerful microbial factor present in the vast majority of $Gram^-$ bacteria (including the causative pathogens identified in the present study) is HMB-PP, the natural activator of human Vγ9/Vδ2 T cells. In contrast, most $Gram^+$ bacteria do not produce HMB-PP and consequently do not activate Vγ9/Vδ2 T cells (94, 113). Despite the very low proportion of T cells in acute $Gram^-$ infections, Vγ9/Vδ2 T cells were selectively enriched among peritoneal T cells in those patients, thereby singling out Vγ9/Vδ2 T cells as highly sensitive and selective responder cells of potential diagnostic and prognostic value in acute peritonitis.

Immune fingerprints in $Gram^+$ infections (especially those caused by staphylococci) were markedly different from those in $Gram^-$ infections and were indicative of a relatively large underlying T cell component with higher numbers of $CD4^+$ and $CD8^+$ T cells and elevated levels of CXCL10, IFN-γ and IL-22. Of note, immune fingerprints in streptococcal infections were more biased toward innate, neutrophil-driven responses with pronounced levels of IL-1β and CXCL8. This indicates fundamental differences in the immune response to distinct groups of bacteria, which merit further investigation. Previous study demonstrated the presence of functional effector memory $CD4^+$ T cells in the peritoneal cavity of PD patients, which mounted more substantial T_H1 responses to recall antigens than their counterparts from peripheral blood (126). Why such recall T cell responses should play a larger role in staphylococcal infections compared to infections caused by other bacteria such as streptococci or *E. coli* is not clear. In more general terms, our results emphasize the importance of shifting the focus away from a simple classification into $Gram^-$ and $Gram^+$ (i.e. LPS containing and LPS deficient) species, and the need to pay more attention to detailed host-pathogen interactions that are dependent on the nature of the causative organism and might impinge on treatment outcomes.

The notion of diagnostic and/or prognostic immune fingerprints has far-reaching basic as well as clinical implications for our view of acute inflammatory responses in infection. First, because this pilot study provides for the first time proof-of-concept for disease-specific immune responses which are triggered by unique combinations of pathogen-associated molecular patterns interacting with distinct aspects of the

human immune system. Second, because it suggests that differential biomarker-based diagnosis of non-infectious and infectious syndromes is feasible and may extend to the discrimination of bacterial, viral and fungal causes of disease. Third, because our findings lay the foundations for targeted therapies that modulate disease-specific pathways to fight infection and reduce inflammation-related tissue damage. Besides episodes of acute peritonitis in PD patients, similar local and/or systemic immune fingerprints are likely to be found in other infectious scenarios such as urinary tract infections (181), lower respiratory tract infection including pneumonia and cystic fibrosis (182, 183), complicated skin and soft tissue infections (184), and sepsis (164, 185-187), where early diagnostic and prognostic biomarkers are urgently needed.

Chapter 5

Regulation of Human γδ T-cell Responses to HMB-PP by Mesothelial cells

5.1 Introduction

Human γδ T cells reactive to HMB-PP contribute to the development of microbe-specific immunity in the peritoneal cavity (113). The majority of circulating Vγ9/Vδ2 T cells shows migration properties similar to monocytes (188), suggesting that these two cell types are co-recruited to the site of inflammation and interact with each other at early stages of infection (94). Vγ9/Vδ2 T cell responses in vitro are greatly facilitated by contact with monocytes as 'feeder cells', which most likely act by 'presenting' HMB-PP to Vγ9/Vδ2 T cells and by providing contact-dependent signals (94). We have shown that peritoneal infections with HMB-PP producing bacteria are associated with higher frequencies of peritoneal γδ T cells and morbidity/mortality compared to HMB-PP negative infections, implying that γδ T cells may be of diagnostic, prognostic and therapeutic value in peritoneal dialysis (PD) associated peritonitis (as shown in Chapter 3).

The peritoneal membrane is lined with a monolayer of mesothelial cells, which are an integral part of the peritoneal immune response. By secreting numerous cytokines/chemokines, growth factors, matrix proteins, and intracellular adhesion molecules, and their ability to present antigens to lymphocytes, HPMC play a critical role in peritoneal homeostasis (26). HPMC isolated from omental specimens possess morphologic and biochemical properties identical to the properties identified in peritoneal mesothelial stem cells. Therefore, cultured HPMC provide a suitable tool for the study of methothelial cells (26, 117, 189).

The regulation of γδ T cells by HPMC has not been investigated so far. By co-culturing freshly isolated Vγ9/Vδ2 T cells and HPMC from primary culture, we demonstrate in this Chapter that resting HPMC are potent suppressors of γδ T cell cytokine production and proliferation in the presence of HMB-PP, suggesting an important homeostatic function of HPMC in the peritoneal cavity.

5.2 Aims

The aims of this Chapter were:

1) To examine the responses of γδ T cells from peripheral blood, omental tissue and peritoneal cavity to HMB-PP.

2) To investigate γδ T-cell responses to HMB-PP when co-cultured with mesothelial cells or in the presence of culture supernatants of mesothelial cells.

3) To explore possible mechanisms involved in the regulation of human γδ T cells by mesothelial cells.

5.3 Results

5.3.1 Impaired responses to HMB-PP in peritoneal γδ T cells

To examine the responses of human γδ T cells to HMB-PP, we collected PBMC from healthy donors, leukocytes from omental specimens of patients undergoing elective abdominal surgery and leukocytes from stable PD effluents. Cells were cultured (0.5×10^6 cells/well) in the presence of 100 nM HMB-PP for 20 hours. Thereafter, cells were stimulated with PMA (50 ng/ml) and Ionomycin (1μg/ml) in the presence of brefeldin-A (10μg/ml) for 4 hours before intracellular cytokine staining. γδ T cells from these three compartments could response to HMB-PP, as demonstrated by the percentage of γδ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α. Of note, the responses of peritoneal γδ T cells were significantly lower than their counterparts in peripheral blood or omental specimens (Figure 5.1).

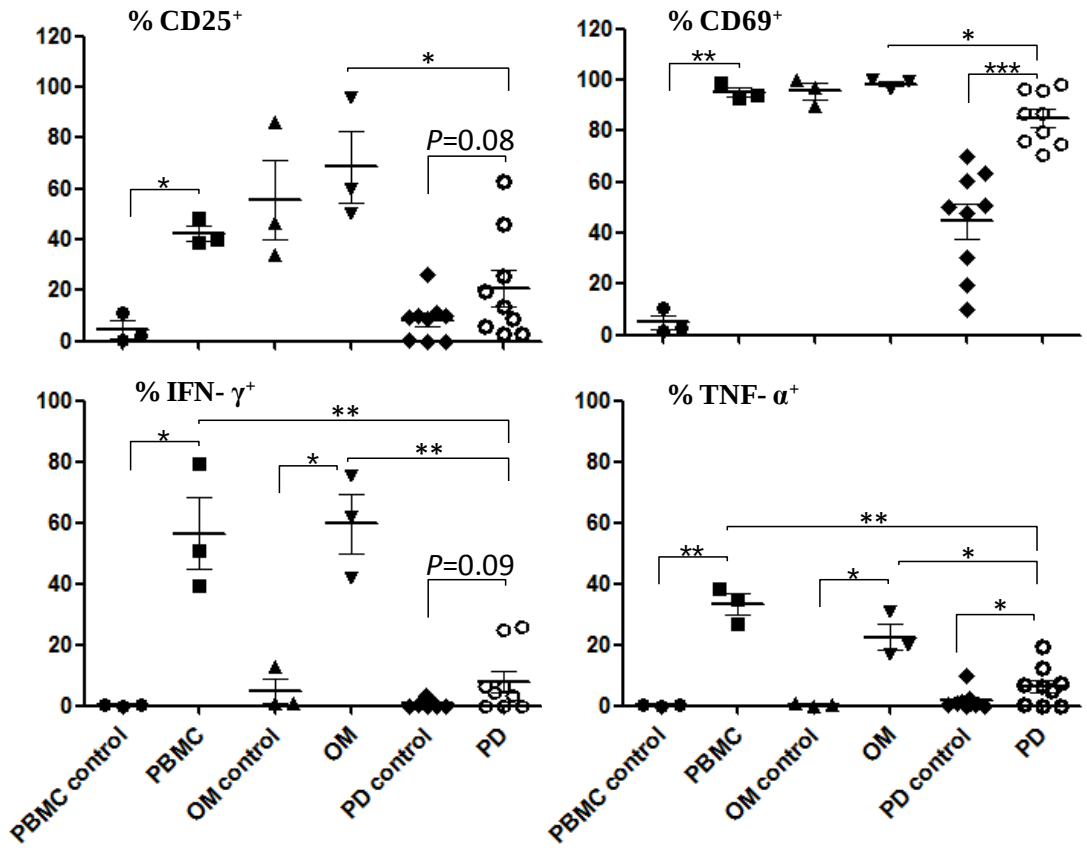

Figure 5.1 Peritoneal γδ T cells respond to HMB-PP with impaired activation.

Leukocytes were collected from peripheral blood of health donors (PBMC), omental specimens (Om) and stable PD effluents (PD) and cultured in the presence of 100 nM HMB-PP for 20 hours. Leukocytes were also cultured in the absence of HMB-PP for 20 hours as negative control. γδ T cell responses are shown as % of $V\gamma9^+$ (PBMC) or $V\delta2^+$ T cells (Om and PD) expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α. Statistical differences between different groups were analyzed using the Mann-Whitney *U* test for unpaired samples and the paired *t* test for paired samples. Error bars indicate SEM. *, P<0.05; **, P<0.01.

5.3.2 Mesothelial cells inhibit the HMB-PP-induced responses and proliferation of γδ T cells

Previous studies pointed out that although peritoneal lymphocytes display an activated phenotype, they appear to be functionally impaired, with a significantly lower IL-2 production in comparison with circulating lymphocytes (35, 61, 62). Continuous exposure to non-physiologic peritoneal dialysis fluid may result in an impairment of the local peritoneal host defense mechanisms (190). Apart from immune cells, HPMC, the largest resident cell population, take part in peritoneal host defense and can be harvested from PD effluents with cell number around 25,000 per PD bag (29). These detached HPMC are still viable (191) and might contribute to the impaired γδ T-cell responses to HMB-PP in the above experiments. Yet, the interplay between γδ T cell and HPMC has not been addressed. We therefore examined the interaction between these two cell types in co-cultures.

$V\gamma9^+$ T cells were purified from PBMC of healthy donors using positive selection and monocytes were purified using anti-CD14 microbeads (Miltenyi Biotec). Freshly purified γδ T cells were cultured alone or co-cultured with autologous monocytes as accessory cells or co-cultured with mesothelial cells for 20 hours at different ratios and in the absence or presence of HMB-PP. In the co-cultures, monocytes provided potent stimulation for production of IFN-γ and TNF-α as shown by intracellular staining of $V\gamma9^+$ T cells. In contrast, HPMC strongly inhibited the production of IFN-γ and TNF-α (Figure 5.2). After 4 days of co-culture in a CFSE dilution assay, γδ T cells alone did not proliferate even in the presence of 100 nM HMB-PP. The presence of HPMC resulted in decreased frequencies of divided $V\gamma9^+$ T cells in the co-cultures of γδ T cells and monocytes (Figure 5.3).

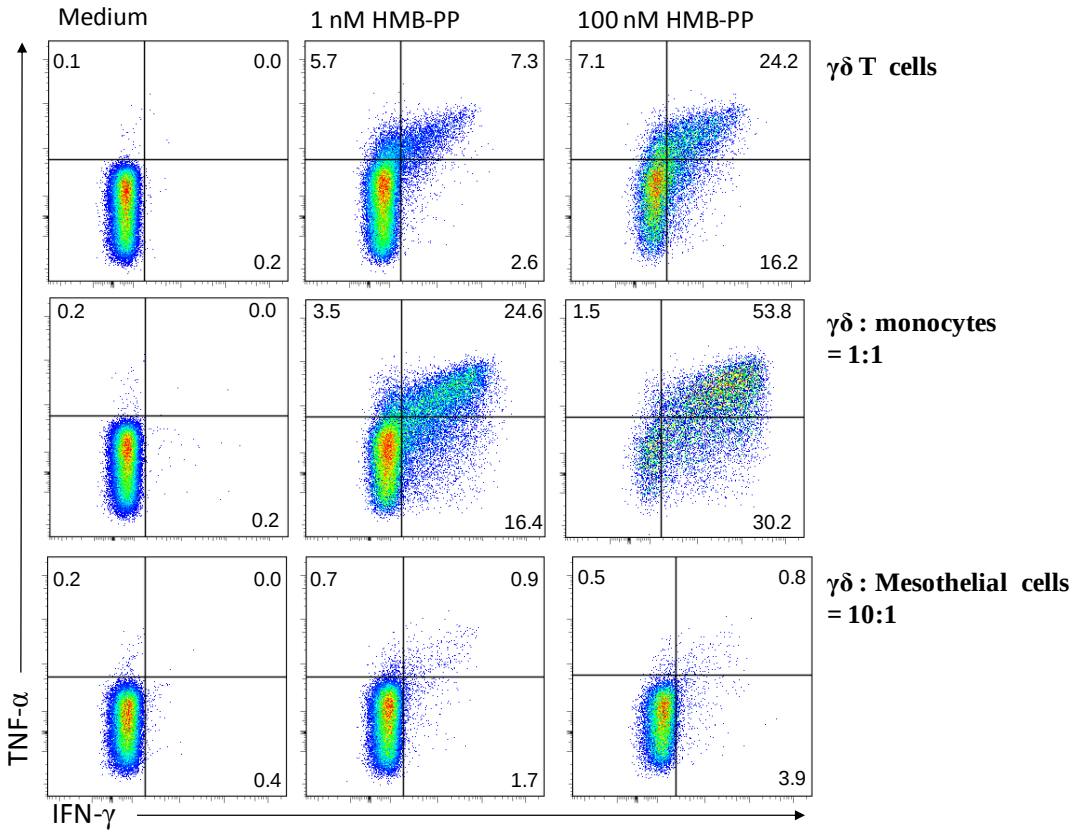

Figure 5.2 HPMC inhibit the HMB-PP-induced cytokine secretion by γδ T cells.

γδ T cells ($V\gamma9^+$) were cultured alone (top) or co-cultured with monocytes (middle) or co-cultured with mesothelial cells (bottom) for 20 hours at the indicated ratios, in the absence (medium) or presence of HMB-PP at different concentrations. γδ T cell responses are shown as frequencies of intracellular IFN-γ and TNF-α in $V\gamma9^+$ T cells. Data shown are representative from experiments using one blood donor and two different omental specimens.

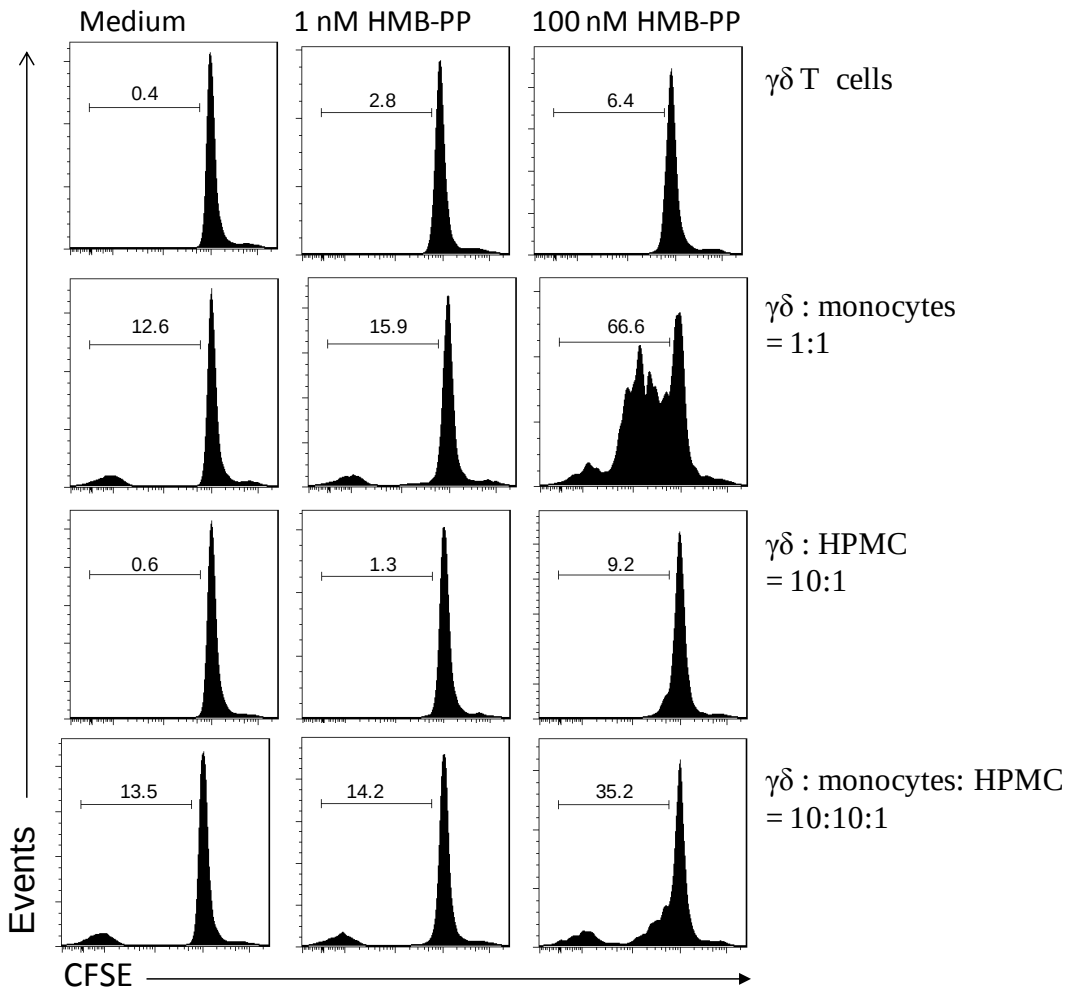

Figure 5.3 HPMC inhibit the HMB-PP-induced proliferation of γδ T cell.

γδ T cells ($V\gamma9^+$) were cultured alone or co-cultured with monocytes or mesothelial cells or co-cultured with both monocytes and HPMC at the indicated ratios for 4 days, in the absence (medium) or presence of HMB-PP at different concentrations. Data shown are frequencies of divided CFSE-labelled γδ T cells. Data shown are representative from experiments using one blood donor and two different omental specimens.

5.3.3 The inhibition of γδ T cell responses is mediated by soluble factors released by primary HPMC.

To determine whether the inhibition of γδ T cell response was mediated by soluble factors, we repeated the above experiments using culture supernatants of HPMC. Under these conditions, supernatants of HPMC inhibited the HMB-PP stimulated expression of surface CD25 and CD69 and cytokine production of γδ T cells (Figure 5.4). A CFSE dilution assay showed a dose-dependent inhibition of γδ T-cell proliferation by supernatants of HPMC (Figure 5.5). A similar inhibition of cytokine production was also noted in anti-CD3/CD28-stimulated $CD4^+$ and $CD8^+$ T cells (Figure 5.6).

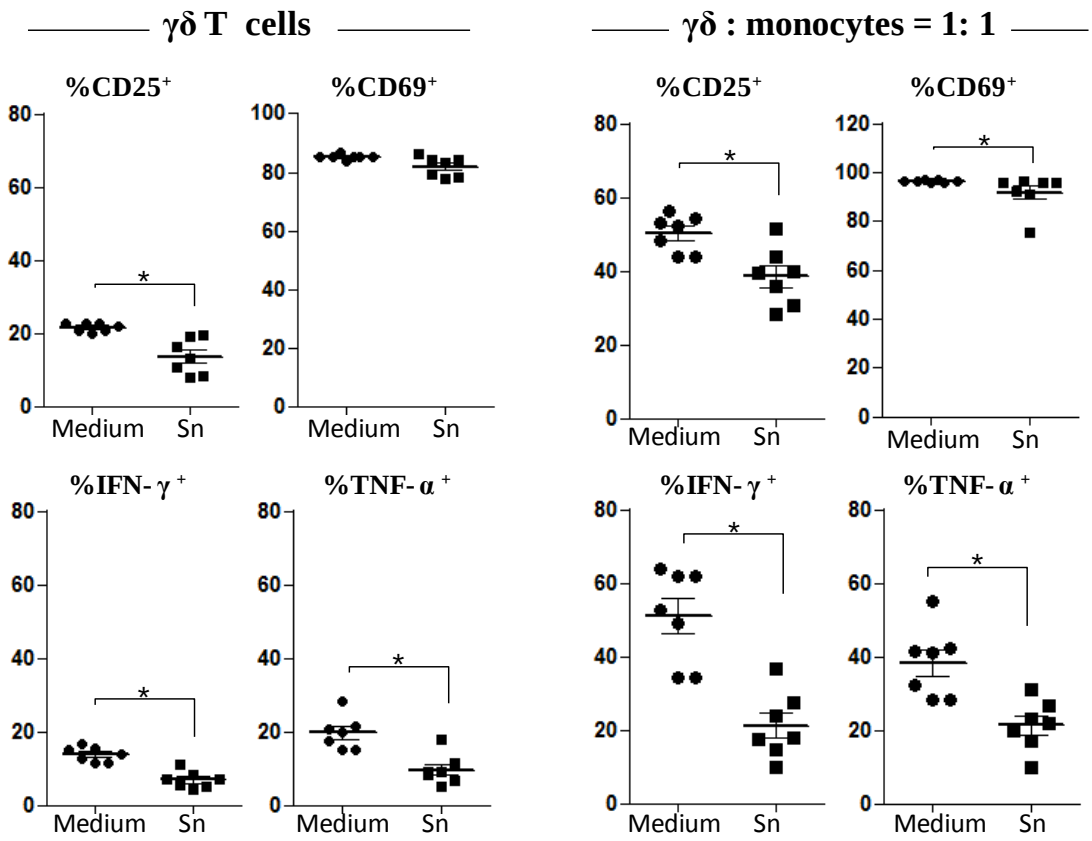

Figure 5.4 Culture supernatants of mesothelial cells inhibit γδ T cell responses.

γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and in the absence (medium) or presence of 50% HPMC supernatants (Sn). Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined in three independent experiments using one blood donor and seven different omental specimens. Statistical differences between groups were analyzed using the Wilcoxon signed-rank test. Error bars indicate SEM. *, $P<0.05$; **, $P<0.01$.

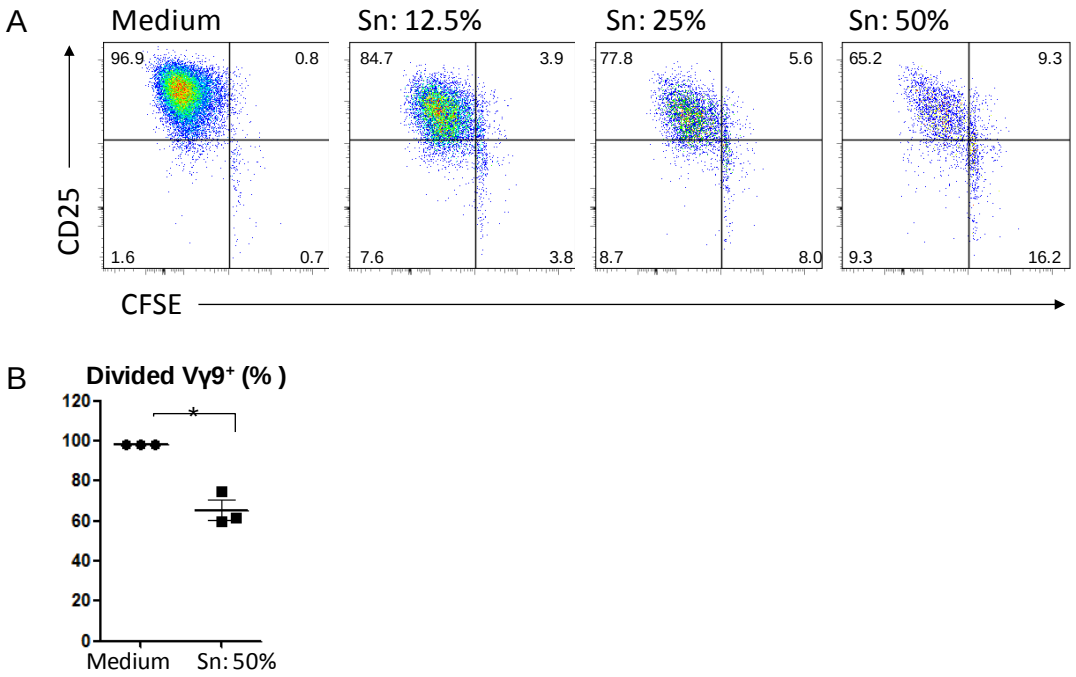

Figure 5.5 Culture supernatants of mesothelial cells inhibit γδ T cell proliferation.

(A) γδ T cells ($V\gamma9^+$) were co-cultured with monocytes at a ratio of 1:1 for 4 days, in the presence of 100 nM HMB-PP and in the absence (medium) or presence of the indicated proportions of HPMC supernatants (Sn). Data shown are proliferation and CD25 expression of CFSE-labelled γδ T cells (representative of three experiments). (B) Data shown are proportions of divided $V\gamma9^+$ T cells, as determined in two independent experiments using one blood donor and three different omental specimens. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

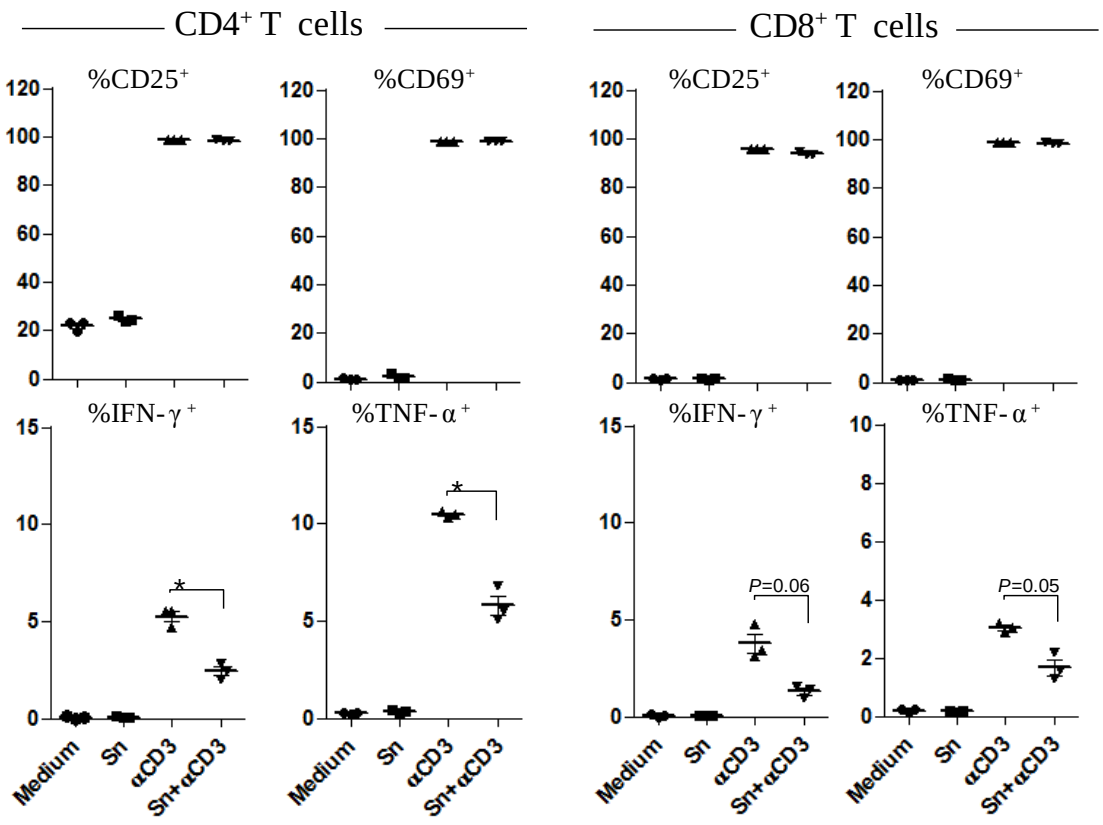

Figure 5.6 Culture supernatants of mesothelial cells inhibit $CD4^+$ and $CD8^+$ T cell responses.
$CD3^+$ T cells were cultured in the absence (medium) or presence of anti-CD3/CD28 beads, or 50% HPMC supernatants (Sn). Data shown are frequencies of $CD3^+CD4^+$ (left) or $CD3^+CD8^+$ (right) T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α using one blood donor and three different omental specimens. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

5.3.4 The inhibition of γδ T-cell responses by supernatants of HPMC can be abrogated by SB-431542

To identify the soluble mediator(s) responsible for HPMC suppression of γδ T-cell responses, we first tested SB-431542, which is a selective inhibitor of TGF-β and/or activin signalling via activin receptor-like kinase (ALK)-4, ALK-5 and ALK-7 (192). In the presence of 10 μM SB-431542, inhibition of γδ T-cell cytokine production by HPMC could be rescued, but not the expression of surface CD25 and CD69 (Figure 5.7). A CFSE dilution assay in the presence of monocytes also demonstrated that SB431542 significantly rescued the suppressive activity of HPMC supernatants on γδ T-cells (Figure 5.8). We also tried similar blocking experiments with $CD4^+$ and $CD8^+$ T cells but did not manage to get consistent results (data not shown).

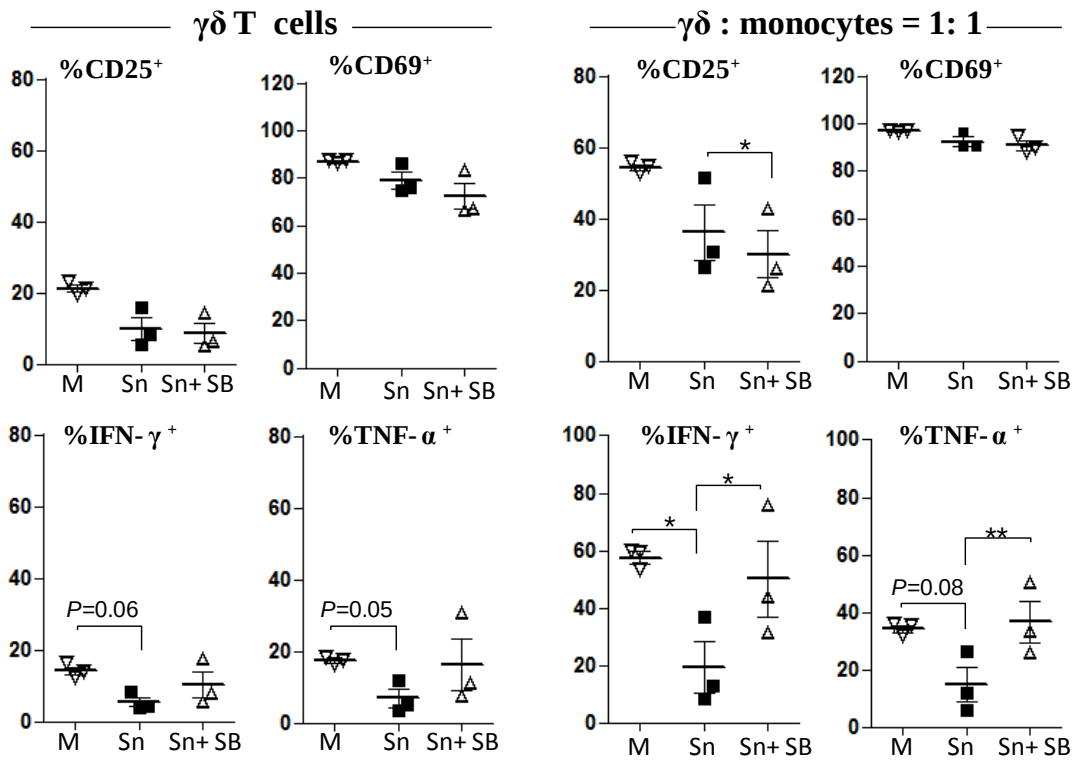

Figure 5.7 Inhibition of γδ T-cell cytokine production can be rescued by SB-431542.

γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and in the absence (M) or presence of 50% HPMC supernatants (Sn), or in the absence or presence of 10 μM SB431542 (SB). Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined in two independent experiments using one blood donor and three different omental tissues. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$; **, $P<0.01$.

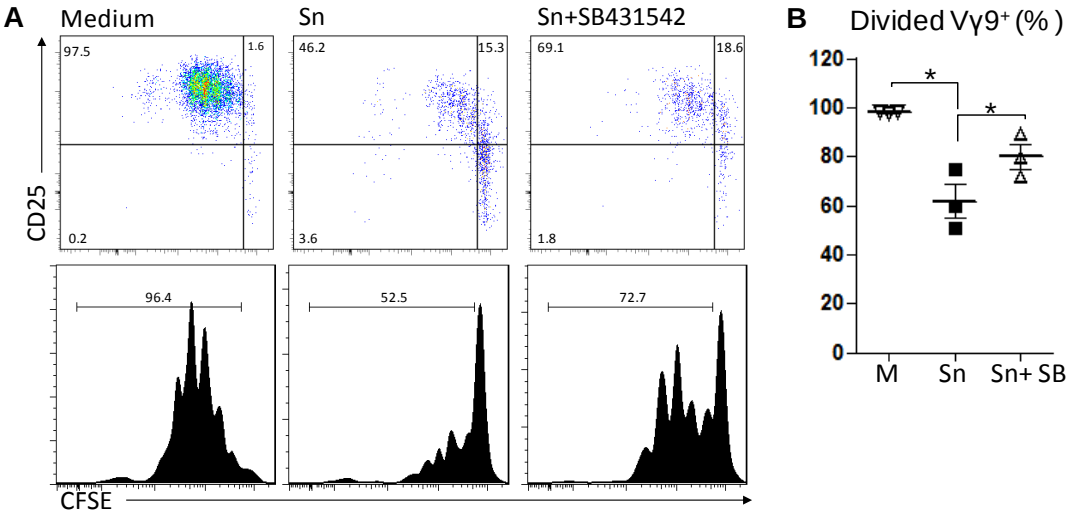

Figure 5.8 Inhibition of γδ T cell proliferation can be rescued by SB-431542.

(A) γδ T cells ($V\gamma9^+$) were co-cultured with monocytes at a ratio of 1:1 for 4 days, in the presence of 100 nM HMB-PP and the absence (medium) or presence of 50% HPMC supernatants (Sn), or in the presence of 10 μM SB-431542 (SB). Data shown are proliferation and CD25 expression of CFSE-labeled γδ T cells (representative of three experiments). (B) Data shown are proportions of divided $V\gamma9^+$ T cells, as determined in three experiments using one blood donor and three different omental specimens. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

5.3.5 TGF-β1 but not activin-A mimics the HPMC-mediated inhibition of γδ T cell responses to HMB-PP

The above data suggested that TGF-β and/or activin A could play a role in the suppressive activity of HPMC. Therefore, we tested the effects of recombinant TGF-β1 and activin-A addition in γδ T-cell activation and proliferation experiments. We also tested the effects of monoclonal anti-TGF-β1 antibody and follistatin, an antagonist of activin A, in experiments using supernatants of HPMC. Figures 5.9 and 5.10 show that recombinant TGF-β1 inhibited γδ T cell responses and proliferation. The effect of neutralizing antibodies against TGF-β1 was inconsistent (Figure 5.11). In contrast, recombinant activin A did not inhibit γδ T cell responses and proliferation (Figure 5.10 and 5.12), nor did follistatin rescue the suppressive activity of HPMC supernatants. Altogether, recombinant TGF-β1 but not activin A mimicked the HPMC-mediated inhibition of γδ T cell responses to HMB-PP.

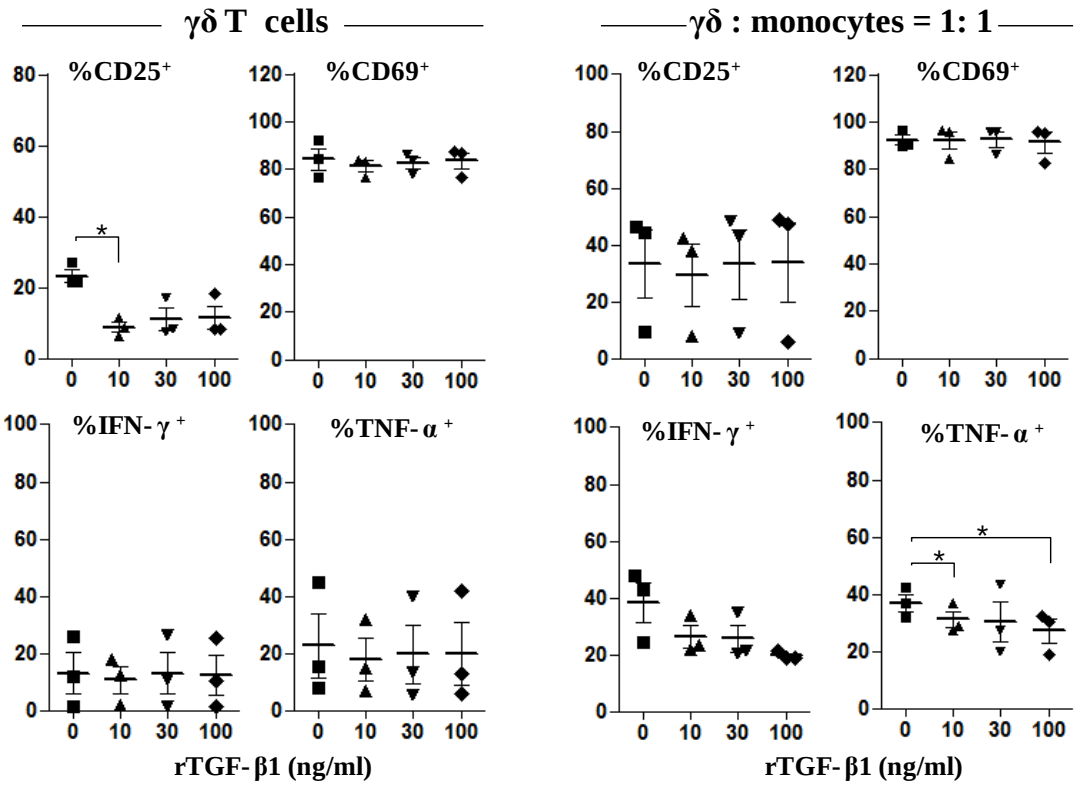

Figure 5.9 Recombinant TGF-β1 mimics the HPMC-mediated inhibition of γδ T cell responses. γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and different concentrations of recombinant TGF-β1 (ng/ml). Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined in three independent experiments using three blood donors. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, P<0.05.

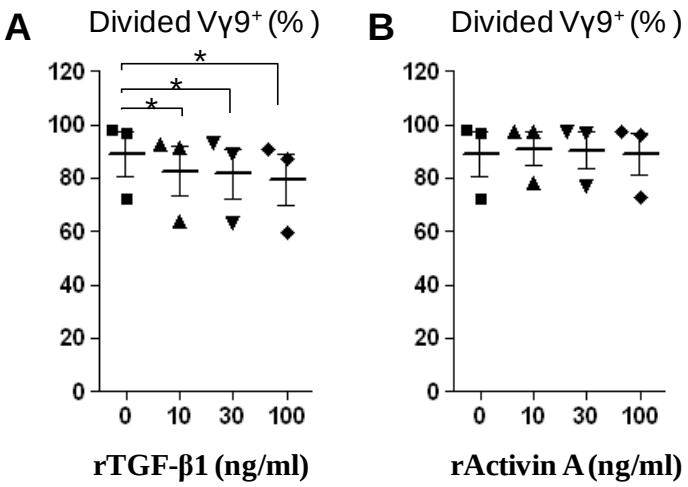

Figure 5.10 Recombinant TGF-β1 mimics the HPMC-mediated inhibition of γδ T cell proliferation.

γδ T cells ($V\gamma9^+$) were co-cultured with monocytes at a ratio of 1:1 for 4 days, in the presence of 100 nM HMB-PP and different concentrations of (A) recombinant TGF-β1 (ng/ml) or (B) activin A (ng/ml). Data shown are proportions of divided CFSE-labeled $V\gamma9^+$ T cells, as determined in three experiments using three blood donors. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

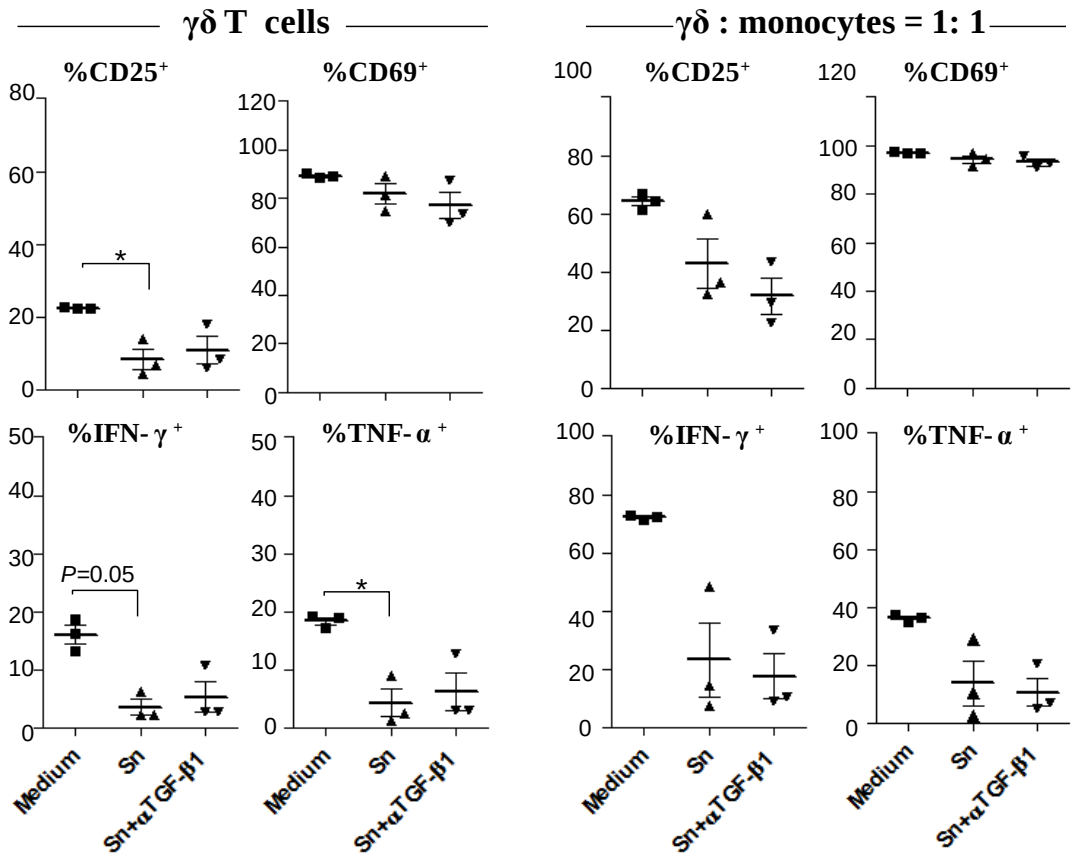

Figure 5.11 Inhibition of γδ T cell responses could not be reversed by anti-TGF-β1 antibody.
γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and in the absence (Medium) or presence of 50% HPMC supernatants (Sn), or in the presence of 10 ng/ml anti-TGF-β1 antibody. Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined using one blood donor and three different omental specimens. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

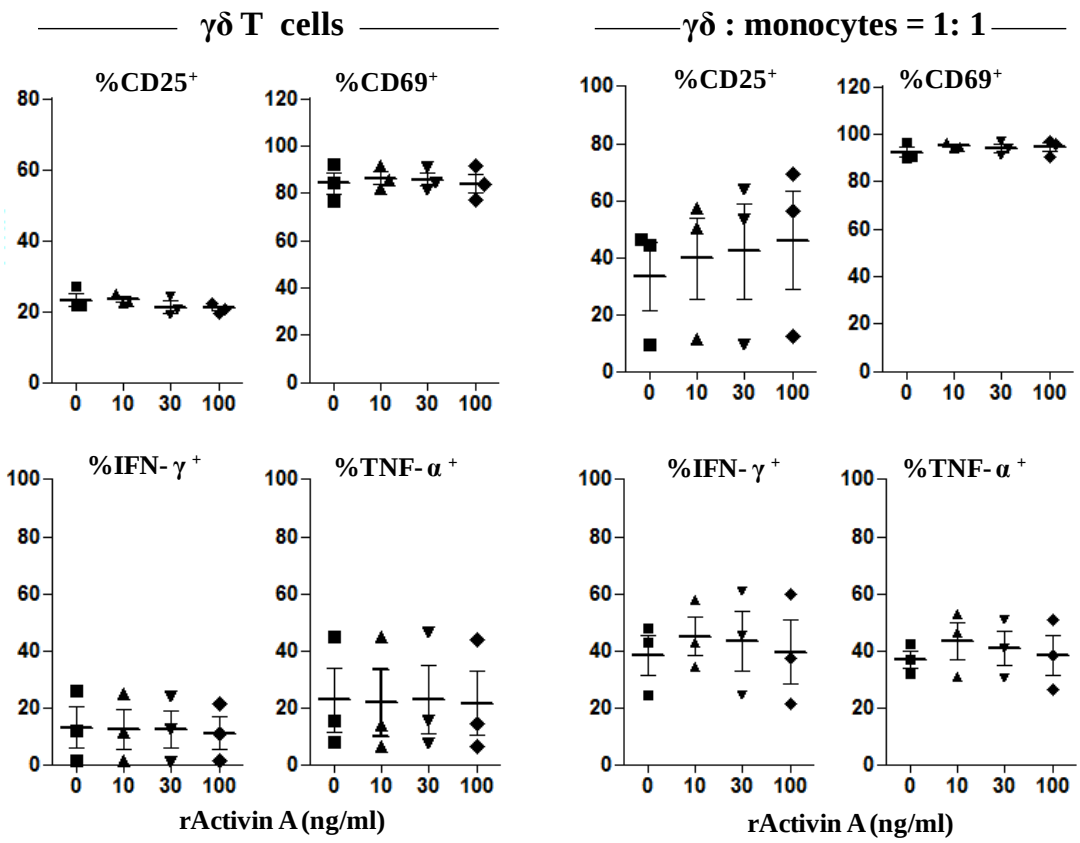

Figure 5.12 Recombinant activin-A could not inhibit γδ T cell responses.

γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and different concentrations of recombinant activin A (ng/ml). Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined in three independent experiments using three blood donors. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

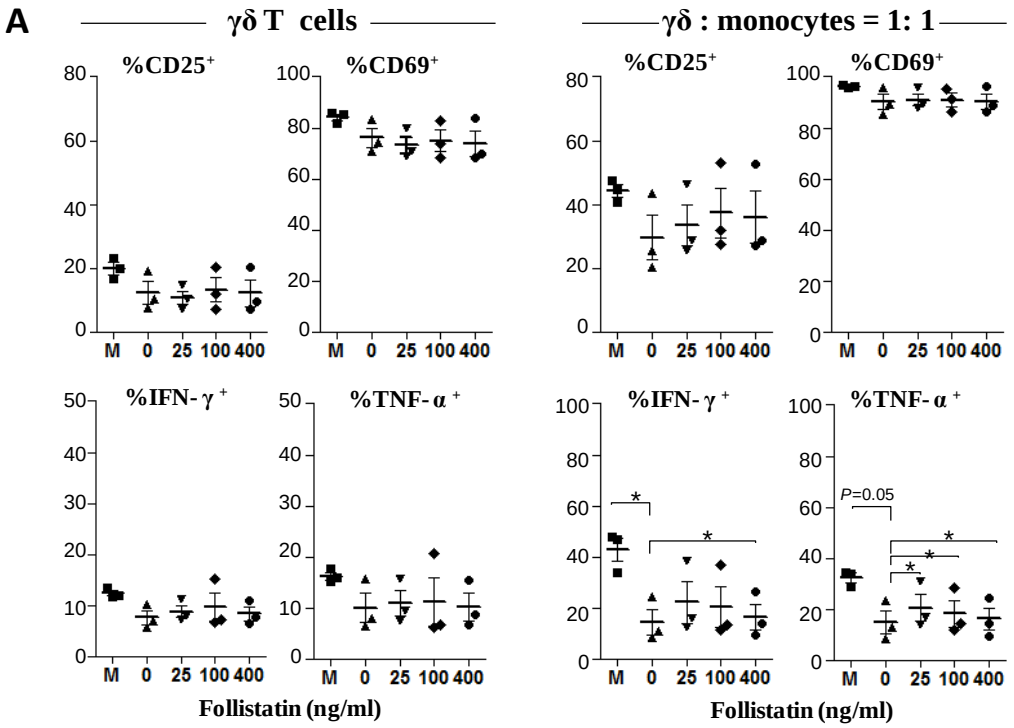

Figure 5.13 The effects of blocking suppression of HPMC supernatants on γδ T cell responses with follistatin.

(A) γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and in the absence (M) or presence of 50% HPMC supernatants, which were pre-treated with different concentrations of follistatin (ng/ml) for 30 minutes. Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined using one blood donor and three omental specimens. (B) γδ T cells ($V\gamma9^+$) were co-cultured with monocytes at a ratio of 1:1 for 4 days, in the presence of 100 nM HMB-PP and in the absence (M) or presence of 50% HPMC supernatants, which were pre-treated with different concentrations of follistatin (ng/ml) for 30 minutes. Data shown are proportions of divided CFSE-labeled $V\gamma9^+$ T cells, as determined using one blood donor and three omental specimens. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

5.3.6 IL-10 might not be involved in the HPMC-mediated inhibition of γδ T cell responses to HMB-PP.

Since it was shown that IL-10 could regulate γδ T-cell response to *M. tuberculosis* (193), we investigated whether IL-10 influenced the γδ T-cell response and proliferation in our in vitro model. Recombinant IL-10 did not inhibit γδ T-cell responses in the experiments without monocytes. In the presence of monocytes, the data about inhibition of the γδ T-cell responses and proliferation by IL-10 were inconsistent (Figure 5.14). The rescue effect of IL-10 receptor inhibitor is undetermined (Figure 5.15) due to inconsistent data.

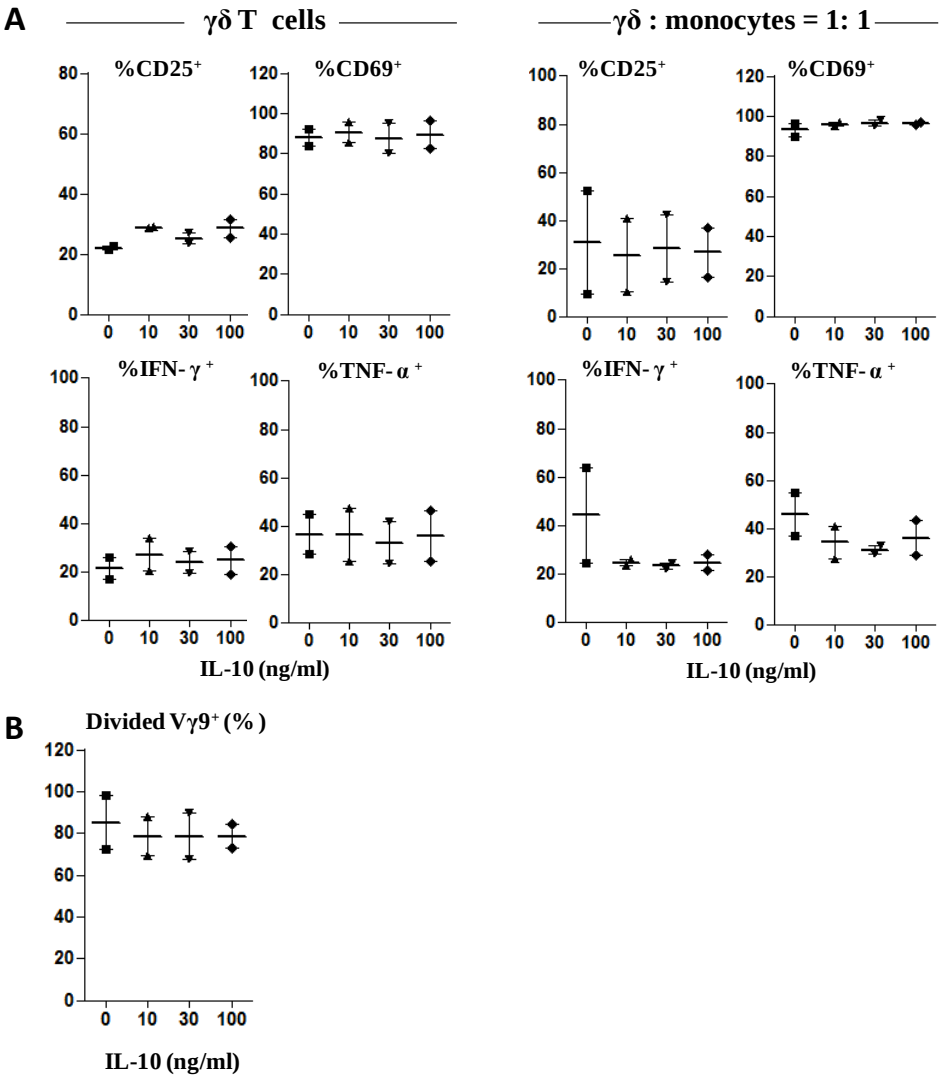

Figure 5.14 IL-10 could not inhibit γδ T cell responses and proliferation.

(A) γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and different concentrations of recombinant IL-10 (ng/ml). Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined in three independent experiments using two blood donors. (B) γδ T cells ($V\gamma9^+$) were co-cultured with monocytes at a ratio of 1:1 for 4 days, in the presence of 100 nM HMB-PP and different concentrations of recombinant IL-10 (ng/ml). Data shown are proportions of divided CFSE-labeled $V\gamma9^+$ T cells, as determined in two experiments using two blood donors. Error bars indicate SEM.

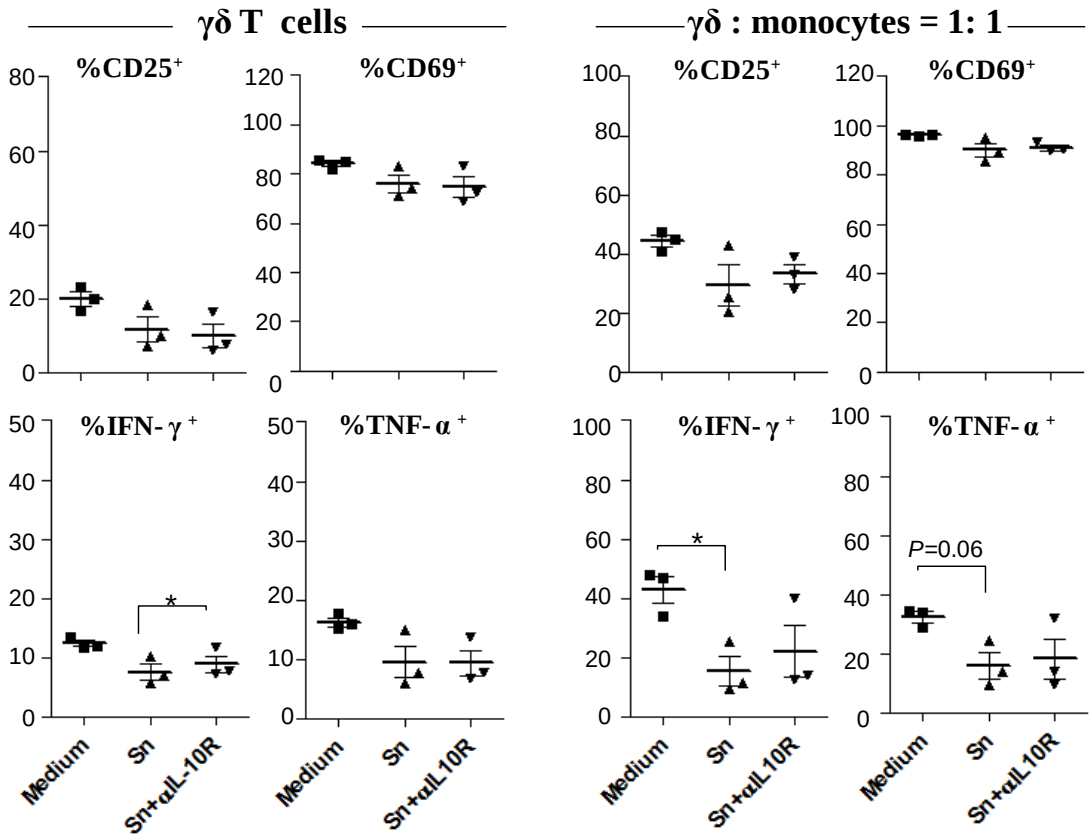

Figure 5.15 No consistent rescue effect of IL-10 receptor inhibitor.

γδ T cells ($V\gamma9^+$) were cultured alone (left) or co-cultured with monocytes (right) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and in the absence (M) or presence of 50% HPMC supernatants (Sn) or presence of 10 ng/ml anti-IL-10 receptor antibody (αIL-10R). Data shown are frequencies of $V\gamma9^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined using one blood donor and three different omental tissues. Statistical differences between groups were analyzed using the paired *t* test. Error bars indicate SEM. *, $P<0.05$.

5.3.6 The rescue effect of exosome-depletion for HPMC-mediated inhibition of γδ T-cell responses is undetermined.

Exosomes are nanometer-sized vesicles secreted by diverse cell types that play different roles according to their origin (194). For example, tumour exosomes express membrane-associated TGF-β1, which contributes to the anti-proliferative effects (195).

To evaluate whether exosomes play a role in the suppressive activity of mesothelial cells, we tested HPMC supernatants, which were subjected to serial centrifugation (2,000 g, 20 min, to remove dead cells; 10,000 g, 20 min, to remove cell debris; 100,000 g, 1 hour, to remove most exosomes (Optima-Max ultracentrifuge, Beckman Coulter); 200,000 g, 1 hour, to remove all exosomes (Optima-Max ultracentrifuge, Beckman Coulter). No consistent rescue of HPMC-mediated inhibition of γδ T-cell responses by exosome-depletion was achieved (Figure 5.16).

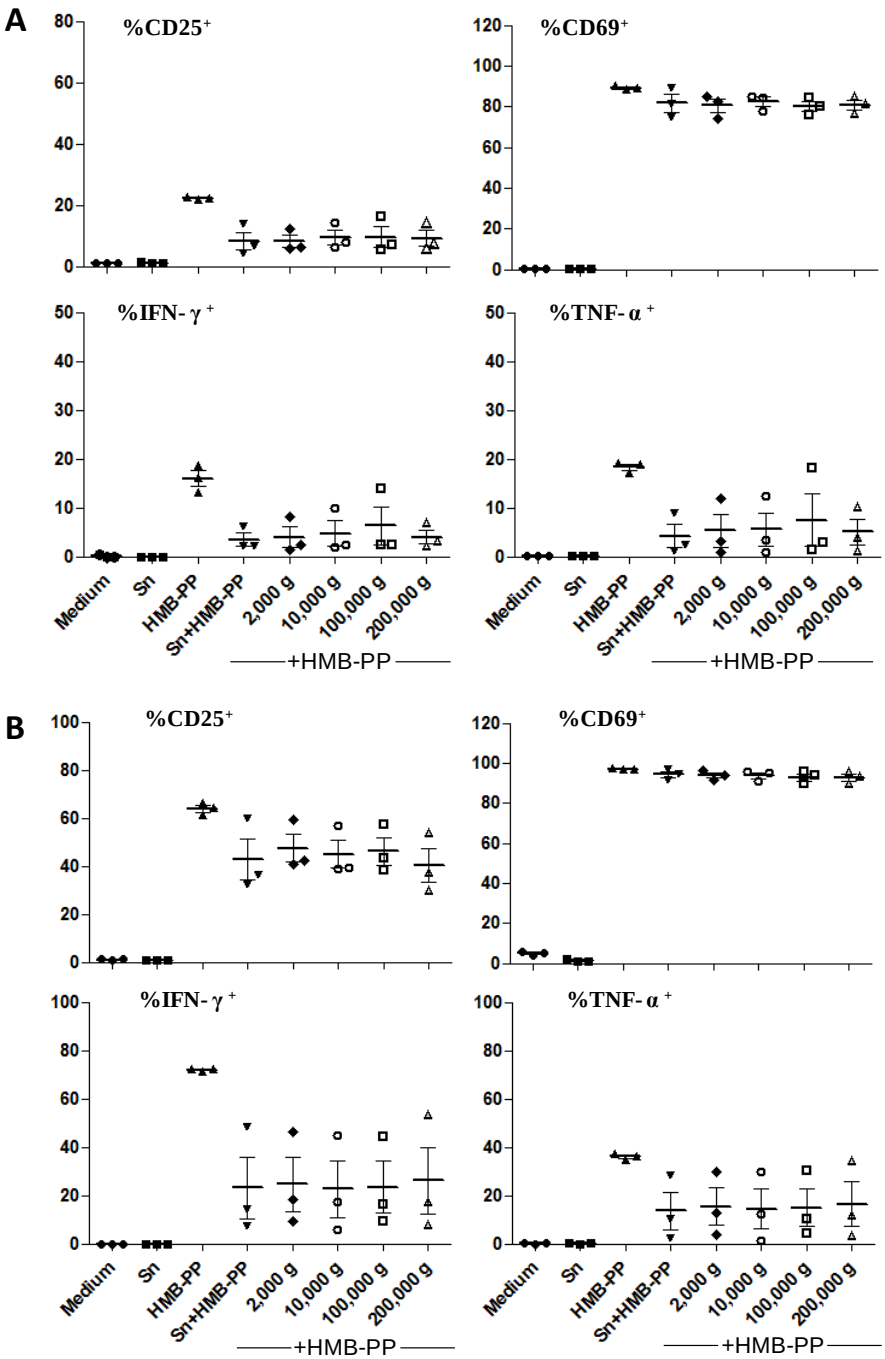

Figure 5.16 No consistent rescue by exosome depletion.

γδ T cells (Vγ9$^+$) were cultured alone (A) or co-cultured with monocytes (B) at a ratio of 1:1 for 20 hours, in the presence of 100 nM HMB-PP and in the absence (Medium) or presence of 50% HPMC supernatants (Sn) or in the presence of 50% HPMC supernatants pre-processed with centrifugation at different speeds. Data shown are frequencies of Vγ9$^+$ T cells expressing surface CD25 and CD69, and intracellular IFN-γ and TNF-α, as determined using one blood donor and three different omental tissues. Error bars indicate SEM.

5.4 Discussion

This study uncovered an unexpected regulation of γδ T-cell responses to HMB-PP by HPMC. Resting HPMC were potent suppressors of γδ T cell cytokine production and proliferation in the presence of HMB-PP. This inhibition was mediated by soluble factors released by primary HPMC and could be counteracted by SB-431542. Of note, recombinant TGF-β1 but not activin-A mimicked the HPMC-mediated inhibition of γδ T cell responses to HMB-PP.

HPMC actively participate in the control of inflammation in both the normal and inflamed peritoneal cavity (196). Unstimulated confluent HPMC were shown to constitutively release considerable amounts of TGF-β1 but only minimal amounts of TGF-β2 (197, 198). Most of TGF-β is released by HPMC in the latent form. The activation process may be the most important step for controlling TGF-β effects (198). It has been shown that HPMC produce dermatan sulfate proteoglycans, which may be involved in the control of the TGF-β activity in the peritoneum (199), suggesting that the mesothelium itself may play an important role in controlling bioavailability of secreted TGF-β.

The TGF-β superfamily is a large family of structurally related regulatory proteins in humans. The TGF-β superfamily ligands can be divided into distinct subgroups: the TGF-β subfamily, including TGF-β1-3, the activins, the bone morphogenetic proteins (BMPs) and nodal. These ligands bind to the constitutively active type II receptors that subsequently activate type I receptors (Figure 5.17) (200). There are seven type I receptors have been identified so far (201, 202). SB-431542 acts as a competitive ATP binding site kinase inhibitor and is a selective inhibitor for ALK-4, 5, and 7 (192). In this study, SB-431542 rescued the suppressive activity of HPMC, which suggested that TGF-β and/or activin A could play a role in the suppressive activity of HPMC.

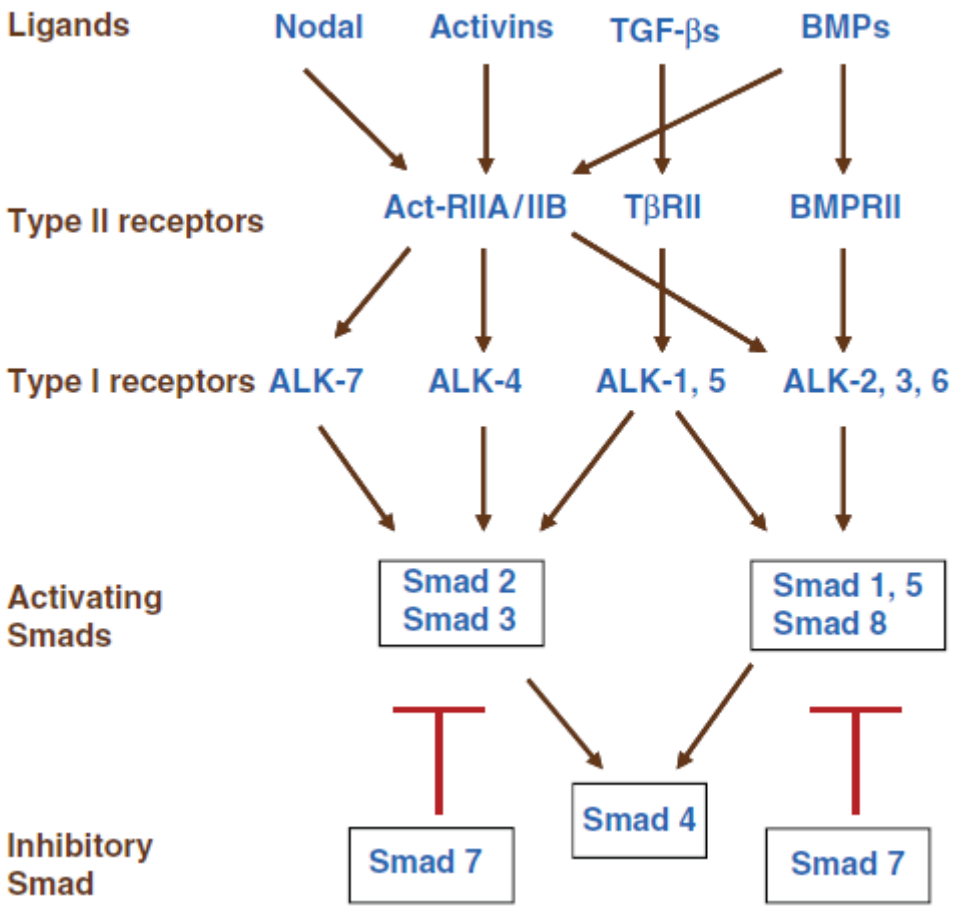

Figure 5. 17 TGF-β superfamily signalling pathways.

TGF-β superfamily ligands bind to the active type II receptors that subsequently phosphorylate type I receptors. Activation of type I receptors propagates the signal by phosphorylating intracellular receptor-regulated Smads. Smads associate with the common-mediated co-Smad 4 and translocate to the nucleus wherein they initiate gene transcription. Smad 7 is induced in response to TGF-β ligands and inhibits downstream signalling. ALK, activin-like kinase; BMP, bone morphogenetic protein. Taken from (200).

TGF-β is a multifunctional cytokine exerting diverse effects including the suppression of immune responses, stimulation of extracellular matrix formation, and modulation of cellular proliferation and differentiation (203). It has been shown to suppress the proliferation of bromohydrin pyrophosphate-activated γδ T cells in the presence of IL-2 (204) and to inhibit γδ T cell proliferation induced by *Mycobacterium tuberculosis*-pulsed monocytes (193). We showed above that recombinant TGF-β1 also suppressed the proliferation and cytokine production of HMB-PP-activated γδ T cells in the presence of monocytes.

Activin A is a pleiotropic cytokine that plays an important role in fundamental biological processes, such as development and tissue repair. An increasing body of evidence proposes that activin A exerts a crucial role in immune-mediated responses and associated diseases, with both pro- (205, 206) and anti-inflammatory effects (207, 208) depending on the cell type and the cytokine milieu (200). Activin A and its receptors are expressed by a wide range of immune cells (207, 209) and tissue cells, such as epithelial cells (in the mouse lung and intestine) and fibroblasts (200, 210, 211). The biological effects of activin A are controlled through interaction with its physiological antagonist, follistatin (212). Notably, follistatin can also inhibit certain functions of other TGF-β superfamily members, including TGF-β3 and the BMPs (213, 214). In this study, recombinant activin A did not inhibit γδ T cell response and proliferation nor did follistatin show any significant rescue effect. Previous studies have showed that stimulation of rat and mouse macrophages with recombinant activin A increased TNF-α, IL-1β and prostaglandin E2 release and enhanced their phagocytosis *in vivo* and *in vitro*, pointing to pro-inflammatory effects (205, 206). This might be the reason why γδ T-cell responses to HMB-PP were enhanced in experiments co-cultured with monocytes in the presence of activin A (Figure 5.12).

HPMC can dynamically participate in peritoneal homeostasis through release of pro- and anti-inflammatory mediators. Previous studies have identified a role of mesothelial cells in orchestrating peritoneal response during inflammation and infection (56, 215). They have been shown to secrete numerous pro-inflammatory cytokines and chemokines such as IL-6, IL-8 and MCP-1 on exposure to bacterial products and inflammatory cytokines (42-44). On the other hand, un-stimulated HPMC were showed to release anti-inflammatory cytokines such as TGF-β (198) and prostaglandins (196) and the secretion was enhanced when stimulated by IL-1β. Increased intraperitoneal secretion of TGF-β in PD-associated peritonitis (as shown in Chapter 4) may serve as a negative feedback loop inhibiting inflammation.

The data presented support the concept that HPMC are significantly incorporated in the cytokine network operating in peritoneal diseases. Resting HPMC are potent

suppressors of γδ T cell cytokine production and proliferation in the presence of HMB-PP. Additional studies are necessary to better define the precise functions of HPMC-derived TGF-β. Our results also propose that human $CD4^+$ and $CD8^+$ αβ T cells are similarly regulated by HPMC, suggesting a pivotal homeostatic function of HPMC in the regulation of peritoneal immunity.

Chapter 6

General Discussion and Future Work

6.1 General discussion

Over the last thirty years, PD has become an alternative to HD for the treatment of ESRD that is widespread around the world. Despite advances in treatment, PD-associated peritonitis and loss of peritoneal membrane function remain major causes for the transfer from PD to HD (13, 216). Continuing exposure of bio-incompatible PD solutions, glucose degradation products, and advanced glycation end-products cause peritoneal inflammation (30, 34), which leads to fibrosis, angiogenesis, progressive increased solute transport and ultimately ultrafiltration failure (56). In rare but serious cases, encapsulating peritoneal sclerosis may occur after chronic peritoneal inflammation (217). Peritoneal inflammation is characterized by increased vascular permeability, activation and expansion of peritoneal leukocytes, recruitment of infiltrating cells to the peritoneal cavity, release of pro- and anti-inflammatory mediators, and increased matrix protein synthesis and tissue remodelling (26). Chronic changes in the peritoneum with fibrosis develop after years of PD. The most noticeable changes are found in patients after severe and recurrent episodes of peritonitis (218). Peritonitis significantly contributes to peritoneal changes by inducing mesothelial damage, massive inflammatory response, and increased vascularisation of peritoneal tissue, leading to impaired membrane function (40). Given that peritonitis episodes may not only be the proximate cause of acute technique failure but also cause ultrafiltration failure and membrane-related problems at a later time, it is important to understand the peritoneal immune response, microbiology and clinical outcomes in these infections.

In the present thesis, my data demonstrate that leukocytes are recruited to the peritoneal cavity, starting with a rapid accumulation of $CD15^+$ neutrophils, which are later replaced by a population of mononuclear cells, including $CD14^+$ monocytes/macrophages and $CD3^+$ T cells. Of note, γδ T cells are also recruited to the peritoneal cavity in the early stage. I next examined the phenotypes and functional characteristics of T cell subsets, especially γδ T cells, and their correlation with clinical outcomes in PD-associated peritonitis patients. In comparison with circulating γδ T cells from healthy donors, peritoneal γδ T cells from acute peritonitis on day 1 are activated with higher expression of CD25 and CD69. In terms of memory subsets of γδ T cells, the frequencies of peritoneal

$CD45RA^{+}CD27^{+}$ γδ T cells are decreased and $CD45RA^{-}CD27^{-}$ γδ T cells are increased in peritonitis patients, compared with circulating γδ T cells from healthy donors. Expression of HLA-DR on peritoneal γδ T cells from peritonitis patients is also higher in comparison with circulating γδ T cells. In summary, peritoneal γδ T cells are activated and contribute to the inflammatory response on PD-associated peritonitis day 1. All together these data imply a significant role of Vγ9/Vδ2 T cells as early responders during acute peritonitis.

Vγ9/Vδ2 T cells are believed to play a beneficial role in bridging innate and adaptive immune responses in infection and inflammation (87, 89, 94). Stimulation of Vγ9/Vδ2 T cells at the site of infection is likely to amplify the local inflammatory response with important consequences for pathogen clearance and the development of microbe-specific immunity (113). However, their excessive activation in the peritoneal cavity of a vulnerable patient group may ultimately contribute both to irreversible damage on the peritoneal membrane and to detrimental outcomes. This conclusion is supported by my findings in patients with PD-associated peritonitis demonstrating that the capacity of the causative pathogen to produce HMB-PP and frequencies of peritoneal Vγ9/Vδ2 T cells on day 1 may predict the subsequent clinical outcome from infection.

My findings also highlight possible new avenues of therapeutic intervention in bacterial peritonitis. The antibiotic fosmidomycin and related compounds might be of particular interest in this respect. Fosmidomycin inhibits 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr), the first enzyme in the non-mevalonate pathway of isoprenoid biosynthesis, in which HMB-PP is a key intermediate (87, 219). Thus, fosmidomycin might serve two purposes in treating infections: by directly inhibiting an essential pathway in a broad range of pathogenic bacteria (220), and by abrogating HMB-PP driven hyper-inflammatory responses. Other ways of specifically manipulating excessive γδ T cell mediated responses may include the use of anti-γδ TCR antibodies or antagonistic compounds such as bromohydrin pyrophosphonate (221).

Given that each microorganism displays a distinct set of pathogen-associated

patterns and interacts with the human immune system in a unique and specific manner, my data provide evidence that the ensuing immunological signatures in acute PD-associated peritonitis can predict the nature of the causative pathogen and discriminate between culture-negative, $Gram^+$ and $Gram^-$ episodes. This research highlights the importance of combining humoral and cellular parameters to establish accurate 'immune fingerprints' in patients with a suspected infection. Particularly promising humoral and cellular parameters in the definition of disease-specific immune fingerprints include local levels of IL-1β, IL-10, IL-22, TNF-α and CXCL10, as well as the relative proportion of neutrophils and monocytes/macrophages among total peritoneal cells and the frequency of γδ T cells within the peritoneal T cell population.

Inflammation is essential to survival during infection and maintains tissue homeostasis under various noxious conditions (222). The effector mechanisms used to control infection include production of pro-inflammatory cytokines, chemokines and recruitment of inflammatory cells to the site of infection. Although these responses help to eliminate or slow the spread of the pathogen, if they are not tightly controlled, they can easily become detrimental when excessive and result in severe inflammation and collateral tissue damage. Inflammation and the immune response to pathogens are regulated by various host suppressor mechanisms, but the regulatory principles are still incompletely understood, in part due to the complexity of the inflammatory response and the multitude of components involved. One key mechanism is regulated by the production of anti-inflammatory cytokines by cells of the immune system in response to pathogen-derived products (222, 223).

HPMC play an important role in regulating the inflammatory response in the peritoneal cavity. During PD-associated peritonitis, they produce pro-inflammatory cytokines/chemokines, which contributing to the recruitment of leukocytes toward the site of infection. On the other hand, HPMC can release anti-inflammatory cytokines such as TGF-β (198) and prostaglandins (196) and the secretion was enhanced when stimulated by IL-1β. Increased intraperitoneal secretion of TGF-β in PD-associated peritonitis may serve as a negative feedback loop inhibiting inflammation. In the present study, I revealed a novel regulation of Vγ9/Vδ2 T cells

by HPMC and demonstrated that resting HPMC are potent suppressors of γδ T cell cytokine production and proliferation in the presence of HMB-PP, suggesting a pivotal homeostatic function of HPMC in the regulation of peritoneal immunity.

In conclusion, these findings improve our insight into the complex cellular interactions in acute PD peritonitis and peritoneal homeostasis, identify novel biomarkers of possible diagnostic and predictive value and highlight new avenues for therapeutic intervention.

6.2 Future work

The aim of my research was to understand the peritoneal immune response, microbiology and outcomes in PD-associated peritonitis. Despite the promising results obtained in this study, some of them are limited by the small sample size. Furthermore, the study of peritoneal immune responses was conducted in a single institution, the University Hospital of Wales, Cardiff, UK. Consequently, the results may not be directly extrapolated to other patient populations. In spite of these limitations, our findings have provided us with novel insights into the role of human peritoneal leukocytes, especially Vγ9/Vδ2 T cells, in PD-associated peritonitis. Our future aims are to investigate the following:

6.2.1 Predictive markers for clinical outcome in PD-associated peritonitis

In Chapter 3, my preliminary data imply that positive identification of the causative pathogen to produce HMB-PP and elevated frequencies of Vγ9/Vδ2 T cells might be negative predictors of clinical outcome. Due to the limited number of patient samples analyzed in the present study, we need to recruit more patient samples to improve the validity and accuracy of the results related to clinical outcome. External validation is also needed. Moreover, other clinically important outcomes such as the function of peritoneal membrane and the speed of responses (rapid vs. delayed) to antibiotics should be considered in future researches. Apart from confirming these findings, it is also important to concentrate on understanding why elevated frequencies of Vγ9/Vδ2 T cells are a negative predictor of clinical outcome.

6.2.2 Immune fingerprints for different bacteria species

The immune response to different bacteria differs greatly. My data in Chapter 4 suggest that the identification of pathogen-specific fingerprints is feasible. However, more patient samples are needed in order to reach sufficient statistical power. Moreover, the performance of diagnostic laboratories and their ability to positively identify bacteria by microbiological culture varies greatly between different centres, as well as the spectrum of micro-organisms patients become infected with (20, 169-171). Our findings will have to be confirmed in independent centres. Instead of merely identifying the immune fingerprints for different bacteria species, it is also important to find out possible underlying mechanisms in future studies.

6.2.3 Sequential measurement of acute peritonitis samples

Serial sampling of acute peritonitis samples (day 1-5) can reflect the dynamic aspects of PD-associated peritonitis. The serial samples can be examined for changes of leukocyte subpopulations and the function of these cells, thus providing superior information about the peritoneal immune defence mechanisms and outcome prediction.

6.2.4 The crosstalk between γδ T cells and mesothelial cells

In Chapter 5, my data show that resting HPMC are potent suppressors of γδ T cell cytokine production and proliferation in the presence of HMB-PP. The inhibition was likely mediated by HPMC-derived TGF-β. However, additional studies are necessary to better define the precise functions of HPMC-derived TGF-β. Moreover, HPMC were shown to release other anti-inflammatory cytokines such as prostaglandin E2 (196), which inhibit the cytokine production and cytotoxicity of Vγ9/Vδ2 T-cell lines (224). Prostaglandin E2 may also play a role in regulation of γδ T-cell responses to HMB-PP by HPMC. Last but not least, it will be interesting to evaluate whether γδ T cells can reciprocally affect the physiological function of HPMC.

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Appendix 1:
Peer reviewed publications arising from the study

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PLoS PATHOGENS

Human Neutrophil Clearance of Bacterial Pathogens Triggers Anti-Microbial γδ T Cell Responses in Early Infection

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Abstract

Human blood Vγ9/Vδ2 T cells, monocytes and neutrophils share a responsiveness toward inflammatory chemokines and are rapidly recruited to sites of infection. Studying their interaction *in vitro* and relating these findings to *in vivo* observations in patients may therefore provide crucial insight into inflammatory events. Our present data demonstrate that Vγ9/Vδ2 T cells provide potent survival signals resulting in neutrophil activation and the release of the neutrophil chemoattractant CXCL8 (IL-8). In turn, Vγ9/Vδ2 T cells readily respond to neutrophils harboring phagocytosed bacteria, as evidenced by expression of CD69, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. This response is dependent on the ability of these bacteria to produce the microbial metabolite (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), requires cell-cell contact of Vγ9/Vδ2 T cells with accessory monocytes through lymphocyte function-associated antigen-1 (LFA-1), and results in a TNF-α dependent proliferation of Vγ9/Vδ2 T cells. The antibiotic fosmidomycin, which targets the HMB-PP biosynthesis pathway, not only has a direct antibacterial effect on most HMB-PP producing bacteria but also possesses rapid anti-inflammatory properties by inhibiting γδ T cell responses *in vitro*. Patients with acute peritoneal-dialysis (PD)-associated bacterial peritonitis – characterized by an excessive influx of neutrophils and monocytes into the peritoneal cavity – show a selective activation of local Vγ9/Vδ2 T cells by HMB-PP producing but not by HMB-PP deficient bacterial pathogens. The γδ T cell-driven perpetuation of inflammatory responses during acute peritonitis is associated with elevated peritoneal levels of γδ T cells and TNF-α and detrimental clinical outcomes in infections caused by HMB-PP positive microorganisms. Taken together, our findings indicate a direct link between invading pathogens, neutrophils, monocytes and microbe-responsive γδ T cells in early infection and suggest novel diagnostic and therapeutic approaches.

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Introduction

The cellular immune system consists of an 'innate' arm of phagocytes and antigen-presenting cells, and an 'adaptive' arm of antigen-specific lymphocytes capable of developing immunological memory. Yet, there is increasing evidence of considerable crosstalk between the two [1]. Innate responses directly influence the shape and outcome of adaptive T cell responses, and *vice versa* specialized T cell subsets feedback to innate cells [2]. Among these interactions, the regulation of neutrophil-mediated inflammatory responses by Th17 cells has received enormous attention over the past few years [3], and with the emergence of novel T cell subsets additional networks are being proposed so that each polarized T cell eventually pairs with an innate counter player [4–7].

The necessity to integrate complex signals in order to mount the most effective defense is best illustrated by the existence of 'unconventional' T cells bridging the classical divide between innate and adaptive immunity, such as natural killer T cells, mucosal-associated invariant T cells, intestinal intraepithelial CD8αα$^+$ T cells and dendritic epidermal γδ T cells [8–14]. These often tissue-associated lymphocytes are characterised by restricted T cell receptor (TCR) repertoires that allow them to respond rapidly to a limited range of conserved structures. Unconventional T cells readily assume a plethora of effector functions, ranging

Author Summary

The immune system of all jawed vertebrates harbors three distinct lymphocyte populations – αβ T cells, γδ T cells and B cells – yet only higher primates including humans possess so-called Vγ9/Vδ2 T cells, an enigmatic γδ T cell subset that uniformly responds to the majority of bacterial pathogens. For reasons that are not understood, this responsiveness is absent in all other animals although they too are constantly exposed to a plethora of potentially harmful micro-organisms. We here investigated how Vγ9/Vδ2 T cells respond to live microbes by mimicking physiological conditions in acute disease. Our experiments demonstrate that Vγ9/Vδ2 T cells recognize a small common molecule released when invading bacteria become ingested and killed by other white blood cells. The stimulation of Vγ9/Vδ2 T cells at the site of infection amplifies the inflammatory response and has important consequences for pathogen clearance and the development of microbe-specific immunity. However, if triggered at the wrong time or the wrong place, this rapid reaction toward bacteria may also lead to inflammation-related damage. These findings improve our insight into the complex cellular interactions in early infection, identify novel biomarkers of diagnostic and predictive value and highlight new avenues for therapeutic intervention.

from sentinel tasks and targeted killing to engaging with keratinocytes, fibroblasts, phagocytes and antigen-presenting cells as well as other lymphocyte.

γδ T cells expressing a Vγ9/Vδ2 TCR – Vγ2/Vδ2 according to an alternative nomenclature – are only found in humans and higher primates and differ fundamentally from all other conventional and unconventional T cells [15]. Activated Vγ9/Vδ2 T cells produce a range of cytokines, kill infected and transformed target cells, regulate survival and differentiation of monocytes and maturation of dendritic cells, provide B cell help and present antigens to $CD4^+$ and $CD8^+$ T cells [11,12,16,17]. They expand considerably in many infections, at times to >50% of all circulating T cells within a few days [18], and respond selectively in a non-MHC restricted manner to the microbial metabolite (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) [19]. HMB-PP is an intermediate of the non-mevalonate pathway of isoprenoid biosynthesis that is present in many bacteria and in malaria parasites but not in humans [17–19]. The rapid and sensitive response of Vγ9/Vδ2 T cells to a broad range of pathogens evokes cardinal features of innate immunity. Indeed, HMB-PP fulfills Janeway's criteria for a 'pathogen-associated molecular pattern' in that it is (*i*) invariant among different species; (*ii*) a product of a pathway unique to micro-organisms; and (*iii*) essential in microbial physiology [17]. Yet, HMB-PP recognition is not mediated *via* germline-encoded pattern recognition receptors but involves the re-arranged Vγ9/Vδ2 TCR [20–22].

Bacteria that possess the non-mevalonate pathway and hence produce HMB-PP comprise some of the most detrimental human pathogens such as the causative agents of cholera, diphtheria, plague, tuberculosis and typhoid, but also numerous commensal and opportunistic species in the mucosal flora, skin and feces [19,23]. In all these micro-organisms, HMB-PP is an essential intracellular metabolite, and it is not clear whether and how it is released by invading bacteria and becomes visible to the immune system as soluble molecule. Indeed, earlier studies with mycobacteria suggested that uptake of whole bacteria by monocytes, macrophages, or DCs may be required for the recognition by Vγ9/Vδ2 T cells [24–27]. Neutrophils are the first immune cells infiltrating the site of infection and the main phagocytes responsible for early pathogen clearance, and growing evidence points toward a crucial role of γδ T cells in regulating neutrophil responses in mouse models of infection, hypersensitivity and autoimmunity [8,12]. Yet, the interplay between γδ T cells and neutrophils has not been addressed in detail [28,29]. Our present data demonstrate that Vγ9/Vδ2 T cells readily respond to neutrophils harboring phagocytosed bacteria, and that this response is strictly dependent on the ability of these bacteria to produce HMB-PP and cell-cell contact of Vγ9/Vδ2 T cells with accessory monocytes. The majority of circulating Vγ9/Vδ2 T cells shows migration properties similar to monocytes [30], suggesting that these two cell types are co-recruited to the site of inflammation and interact with each other at early stages of infection [17,31]. Our present findings thus indicate a direct link between invading pathogens, neutrophils, monocytes and microbe-responsive γδ T cells, and suggest novel diagnostic and therapeutic approaches in acute infection.

Results

Human γδ T cells induce neutrophil survival and activation

Neutrophils are short-lived phagocytes that undergo spontaneous apoptosis *in vitro* unless rescued by survival signals. We previously demonstrated that activated human Vγ9/Vδ2 T cells induce monocytes to survive and differentiate into inflammatory dendritic cells [31]. Here, HMB-PP stimulated Vγ9/Vδ2 T cells had a similar survival effect on autologous neutrophils and readily rescued them from undergoing apoptosis (Figure 1). This effect was selective and dependent on the number of Vγ9/Vδ2 T cells and the concentration of HMB-PP. An increase in neutrophil survival could already be observed at ratios of only 1 γδ T cell per 100 neutrophils and at HMB-PP concentrations as low as 0.1–1 nM. Activation of Vγ9/Vδ2 T cells in these cultures was confirmed by upregulation of CD69 and secretion of interferon (IFN)-γ (Figure S1 in Text S1). The low γδ T cell numbers and HMB-PP concentrations needed to promote neutrophil survival *in vitro* are likely to have physiologic relevance.

Activated neutrophils mobilize intracellular stores of CD11b to the cell surface and shed CD62L, thus enhancing their potential to undergo firm adhesions with endothelial cells and extravasate at the site of inflammation. In line with their anti-apoptotic effect on neutrophils, Vγ9/Vδ2 T cells induced upregulation of CD11b and loss of CD62L in surviving neutrophils in an HMB-PP dependent manner (Figure 2A). Importantly, synthetic HMB-PP alone did not have any activity on neutrophils in the absence of γδ T cells (Figure 1, Figure 2 and data not shown).

Rapid recruitment of neutrophils involves the chemotactic action of CXCL8 (IL-8) produced at the site of inflammation, and increased endothelial permeability mediated by tumor necrosis factor (TNF)-α. Analysis of the supernatants from the above experiments revealed that co-cultures of neutrophils and HMB-PP stimulated Vγ9/Vδ2 T cells produced considerable amounts of both CXCL8 and TNF-α, in a dose-dependent manner and at levels comparable to lipopolysaccharide (LPS) stimulated neutrophils (Figure 2B). Another cytokine implicated in neutrophil recruitment is IL-17, which in a number of infection models is readily produced by murine γδ T cells [8]. While activated Vγ9/Vδ2 T cells readily produce TNF-α, IFN-γ and granulocyte/macrophage colony-stimulating factor (GM-CSF) [31,32], we were unable to detect IL-17 in our co-cultures indicating that under the conditions tested human γδ T cells failed to secrete relevant levels of IL-17 (data not shown). This is reminiscent of recent findings

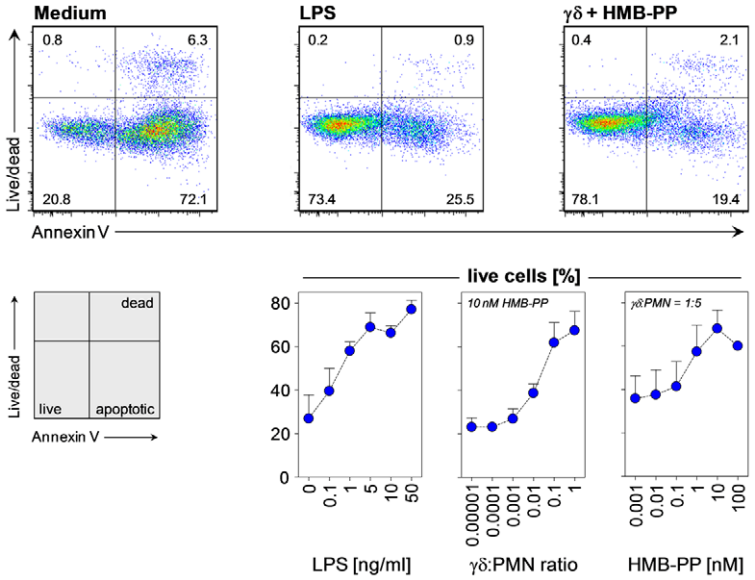

Figure 1. HMB-PP stimulated Vγ9/Vδ2 T cells induce neutrophil survival. Dose-dependent survival of neutrophils incubated with LPS, and of neutrophils (PMN) co-cultured with Vγ9/Vδ2 T cells at various ratios in the presence of HMB-PP at different concentrations. Neutrophils were analyzed after 20 hours in culture; dot plots depict representative annexin-V and fixable live/dead stainings for live $CD3^-$ $CD15^+$ neutrophils cultured under the indicated conditions. Data shown are mean percentages (%) + SEM of live cells from independent experiments using three different donors.
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that human αβ T cells including human Th17 cells modulate neutrophils (which lack the IL-17 receptor C chain) in an IL-17–independent manner through a combination of TNF-α, IFN-γ and GM-CSF [33]. In our cultures, blocking experiments demonstrated that TNF-α played a key role in the γδ T cell-mediated effect on neutrophils, as judged by a partial inhibition of neutrophil survival and a reduction of CD11b expression in the presence of soluble TNF-α receptor (sTNFR), while neutralizing antibodies against GM-CSF and IFN-γ had no significant effect (Figure 3).

Taken together, these data show that Vγ9/Vδ2 T cells become activated by soluble HMB-PP in the presence of autologous neutrophils and that they provide potent stimulatory signals inducing neutrophil survival and activation. The interaction of the two cell types leads to the rapid release of the pro-inflammatory mediators CXCL8 and TNF-α into the microenvironment, thereby potentially maintaining neutrophil influx at the site of infection.

Vγ9/Vδ2 T cells respond to neutrophils harboring phagocytosed bacteria

Under physiological conditions, invading pathogens are rapidly taken up by newly recruited neutrophils. We therefore tested whether Vγ9/Vδ2 T cells respond to neutrophils harboring phagocytosed bacteria in a similar manner as they respond to soluble HMB-PP. In order to do this, we set up triple cultures consisting of neutrophils, monocytes and Vγ9/Vδ2 T cells, mimicking physiological conditions at the site of infection.

Human neutrophils readily took up green fluorescent protein (GFP) expressing *Escherichia coli*, *Listeria innocua* and *Mycobacterium smegmatis*, with >95% of the neutrophils being GFP^+ within 30 min (Figure 4A and data not shown). Triple co-cultures of neutrophils harboring different strains of *M. smegmatis* with autologous Vγ9/Vδ2 T cells and monocytes led to rapid γδ T cell activation, as evidenced by upregulation of CD69 and expression of TNF-α and IFN-γ within 20 hours (Figure 4B and data not shown). Activation profiles were similar to those seen in control cultures with non-infected neutrophils in the presence of synthetic HMB-PP, demonstrating that Vγ9/Vδ2 T cells respond to bacterial degradation products released or presented by neutrophils. For the sake of clarity and simplicity all activation data in the following sections are shown as proportion of $CD69^+$ $TNF\text{-}\alpha^+$ γδ T cells in the cultures although the cells were always co-stained for IFN-γ as well. The proportion of $CD69^+$ $IFN\text{-}\gamma^+$ and $TNF\text{-}\alpha^+$ $IFN\text{-}\gamma^+$ γδ T cells followed essentially the same pattern throughout this study and led to the same conclusions.

The Vγ9/Vδ2 T cell response to phagocytosed bacteria depends on the ability of bacteria to produce HMB-PP

In order to investigate the correlation between the ability of bacteria to produce HMB-PP and their capacity to stimulate Vγ9/Vδ2 T cells, we designed experiments to distinguish a specific γδ T cell response to HMB-PP from a possible background stimulation by the plethora of other microbial compounds acting *via* pattern recognition receptors. Thus, we generated a *M. smegmatis* transfectant stably expressing a second copy of the gene encoding HMB-PP synthase (*gcpE*) and hence overproducing HMB-PP compared to the parental wildtype (wt) strain [34] (Figure S2 in Text S1). As a second bacterial model we utilized HMB-PP producing and HMB-PP deficient strains of the non-pathogenic Gram-positive bacterium *Listeria innocua* [35,36] (Figure S3 in Text S1).

Compared with *M. smegmatis* wt bacteria, higher levels of Vγ9/Vδ2 T cell activation were observed when using *M. smegmatis-gcpE*$^+$ (Figure 5). Furthermore, considerable Vγ9/Vδ2 T cell activation was seen with phagocytosed *L. innocua-gcpE*$^+$, a strain in which HMB-PP artificially accumulates, but not with the naturally HMB-PP deficient *L. innocua* wt strain that was >100× less potent (Figure 5). The double transfectant *L. innocua-gcpE*$^+$*lytB*$^+$, in which HMB-PP becomes converted into the downstream reaction products isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), resulted in no detectable Vγ9/Vδ2 T cell activation (data not shown). These data demonstrate that the

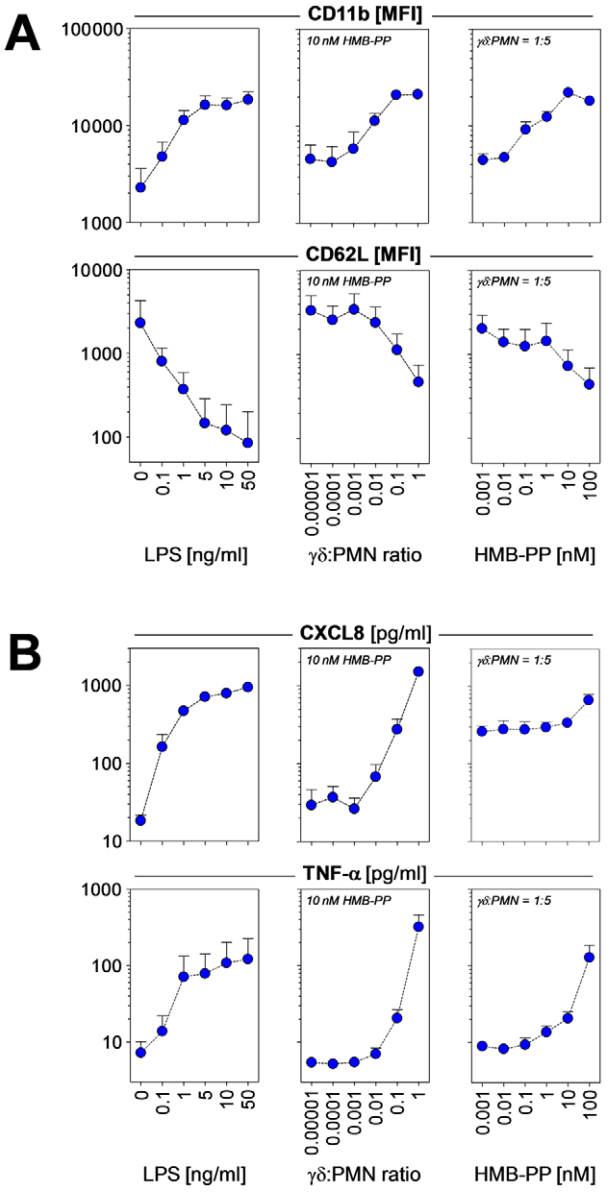

Figure 2. HMB-PP stimulated Vγ9/Vδ2 T cells induce neutrophil activation and production of pro-inflammatory mediators. (*A*) Dose-dependent up-regulation of CD11b and shedding of CD62L by neutrophils incubated with LPS as positive control, and of neutrophils co-cultured with Vγ9/Vδ2 T cells at various ratios in the presence of HMB-PP at different concentrations. Neutrophils were analyzed after 20 hours in culture; histograms depict representative CD11b and CD62L stainings for live $CD3^-$ $CD15^+$ neutrophils cultured under the indicated conditions. Data shown are mean fluorescence intensities (MFI) + SEM from independent experiments using three different donors. (*B*) Dose-dependent secretion of CXCL8 and TNF-α into the culture supernatant of neutrophils incubated with LPS as positive control, and of neutrophils co-cultured with Vγ9/Vδ2 T cells at various ratios in the presence of HMB-PP at different concentrations. Supernatants were analyzed after 20 hours by ELISA. Data shown are mean levels (pg/ml) + SEM from independent experiments using three different donors.
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response of Vγ9/Vδ2 T cells to neutrophils harboring phagocytosed bacteria depends on the ability of these bacteria to produce HMB-PP and suggest that phagocytosis and subsequent degradation of bacteria in neutrophils leads to either presentation of

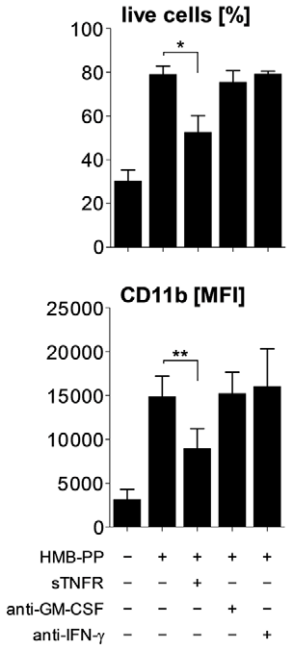

Figure 3. HMB-PP stimulated Vγ9/Vδ2 T cells induce neutrophil survival and activation through TNF-α. Neutrophils were co-cultured with Vγ9/Vδ2 T cells for 20 hours at a ratio of 5:1, in the absence or presence of 10 nM HMB-PP. Soluble cytokines were blocked by the addition of sTNFR, anti-GM-CSF or anti-IFN-γ. Data shown are mean values + SEM for neutrophil survival and expression of CD11b by live neutrophils, as determined in independent experiments using three different donors.
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HMB-PP on the cell surface or the release of soluble HMB-PP into the microenvironment.

Crosstalk with monocytes provides essential help for the Vγ9/Vδ2 T cell response to phagocytosed bacteria

Vγ9/Vδ2 T cells, monocytes and neutrophils share a responsiveness toward inflammatory chemokines and are the earliest leukocytes recruited to sites of infection. Vγ9/Vδ2 T cell responses *in vitro* are greatly facilitated by contact with monocytes as 'feeder cells', which most likely act by 'presenting' HMB-PP to Vγ9/Vδ2 T cells and by providing contact-dependent signals [17]. In support of our previous observation of a substantial HMB-PP dependent crosstalk between Vγ9/Vδ2 T cells and monocytes leading to optimum γδ T cell activation [31], the response of Vγ9/Vδ2 T cells to neutrophils harboring phagocytosed *L. innocua-gcpE*$^+$ was largely dependent on the presence of monocytes. Omission of monocytes from the co-cultures resulted in greatly reduced expression levels of CD69, TNF-α and IFN-γ, compared to triple co-cultures (Figure 6A and data not shown), suggesting that monocytes provide essential help for the recognition of bacteria by Vγ9/Vδ2 T cells and increase the sensitivity of the response especially at low HMB-PP concentrations.

We speculated that this accessory effect might have stemmed from contact-dependent interactions of monocytes with either neutrophils or γδ T cells and tested this hypothesis in transwell cultures where neutrophils harboring phagocytosed *L. innocua-gcpE*$^+$ in the lower chamber were separated from Vγ9/Vδ2 T cells in the upper chamber. As shown in Figure 6B, cell-cell contact between monocytes and Vγ9/Vδ2 T cells was crucial for the response to phagocytosed bacteria, while no contact was needed between Vγ9/Vδ2 T cells and neutrophils, and neither between

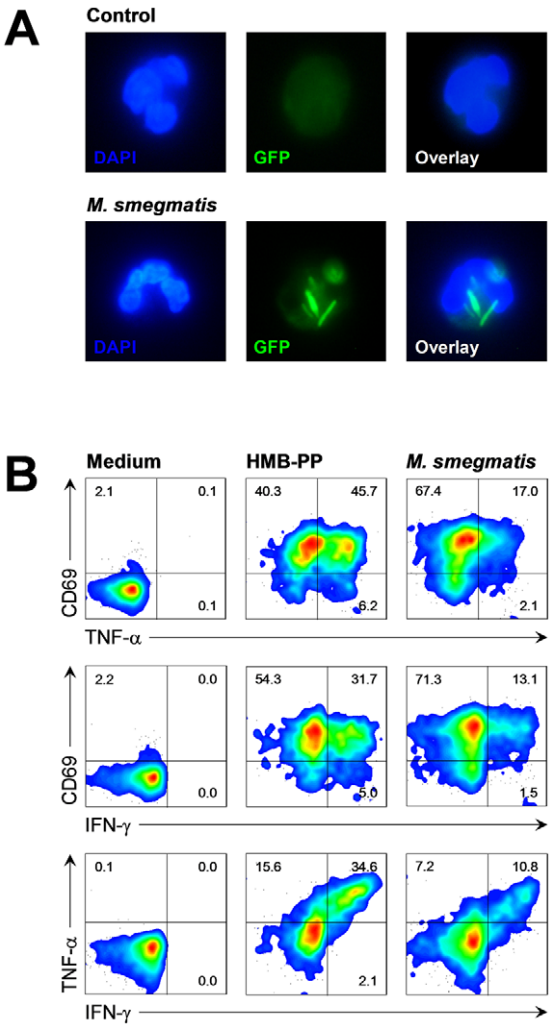

Figure 4. Vγ9/Vδ2 T cells respond to bacteria upon phagocytosis by neutrophils. (*A*) Resting neutrophils or neutrophils after phagocytosis of *M. smegmatis-gfp*$^{+}$ stably expressing GFP, at a multiplicity of infection (MOI) of 10. Cells were counter-stained with DAPI and imaged by fluorescence microscopy. Data shown are representative from independent experiments using two different donors. (*B*) Activation of Vγ9/Vδ2 T cells co-cultured for 20 hours with neutrophils in the absence (medium) or in the presence of 10 nM HMB-PP, or co-cultured with neutrophils harboring *M. smegmatis-gcpE*$^{+}$ overexpressing HMB-PP synthase. Data shown are representative from independent experiments using three different donors.
doi:10.1371/journal.ppat.1002040.g004

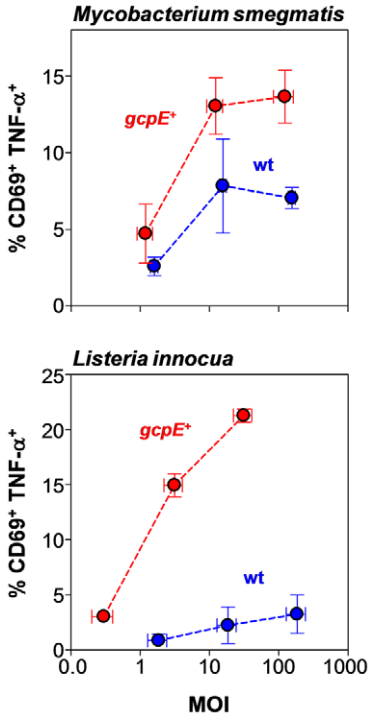

Figure 5. Vγ9/Vδ2 T cells show enhanced responses to phagocytosed bacteria that overproduce HMB-PP. Dose-dependent activation of Vγ9/Vδ2 T cells by neutrophils harboring genetically engineered transfectants of *M. smegmatis* (upper panel) or *L. innocua* (lower panel), in which HMB-PP accumulates intracellularly, compared to the parental wildtype strains (wt). Data shown are mean frequencies of CD69^{+} TNF-α^{+} Vγ9/Vδ2 T cells ± SEM after 20 hours in culture, as determined in independent experiments using three different donors. Error bars for MOIs depict mean values ± SEM for the true colony-forming unit (CFU) counts of the bacterial inocula used.
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monocytes and neutrophils. These data indicate that upon phagocytosis of HMB-PP^{+} bacteria, neutrophils release soluble factors that efficiently stimulate Vγ9/Vδ2 T cells, while monocytes provide important contact-dependent accessory signals.

Upon phagocytosis of HMB-PP^{+} bacteria neutrophils release HMB-PP into the culture supernatant

Since neutrophils harboring bacteria were able to stimulate Vγ9/Vδ2 T cells in a transwell system, we next examined whether cell-free culture supernatants derived from infected neutrophils stimulated Vγ9/Vδ2 T cells in a similar manner. Indeed, Vγ9/Vδ2 T cells readily responded to supernatants from neutrophils harboring *L. innocua-gcpE*$^{+}$ but not from neutrophils harboring *L. innocua* wt bacteria, as evidenced by expression of CD69, TNF-α and IFN-γ (Figure 6C and data not shown). Importantly, short-term pre-treatment of *L. innocua-gcpE*$^{+}$ supernatants with shrimp alkaline phosphatase abrogated the bioactivity on Vγ9/Vδ2 T cells completely (Figure 6C), evoking the known sensitivity of mycobacterial HMB-PP to dephosphorylation and the relative inactivity of the dephosphorylated products [37–39]. Control experiments confirmed that alkaline phosphatase affected the response of Vγ9/Vδ2 T cells to synthetic HMB-PP but not to the phosphatase-resistant diphosphonate analogue, HMB-PCP [40], demonstrating that the presence of alkaline phosphatase in the cultures had no inhibitory effect on the cells' ability to express CD69, TNF-α and IFN-γ (Figure 6D and data not shown). We conclude that upon phagocytosis of HMB-PP^{+} bacteria neutrophils release soluble HMB-PP into the microenvironment where it becomes accessible to monocytes and Vγ9/Vδ2 T cells.

The Vγ9/Vδ2 T cell response to phagocytosed bacteria is HMB-PP dependent but largely independent of other pathogen-associated molecular patterns

In order to assess the clinical relevance of our findings, we expanded our panel of bacteria by including clinical isolates of pathogens that are frequently associated with community- and hospital-acquired infections and pose serious threats to public health (Table S1 in Text S1). Of note, neutrophils harboring HMB-PP^{+} pathogens but not neutrophils harboring HMB-PP^{-} pathogens induced in Vγ9/Vδ2 T cells the co-expression of CD69, TNF-α and IFN-γ (Figure 7A and data not shown). This

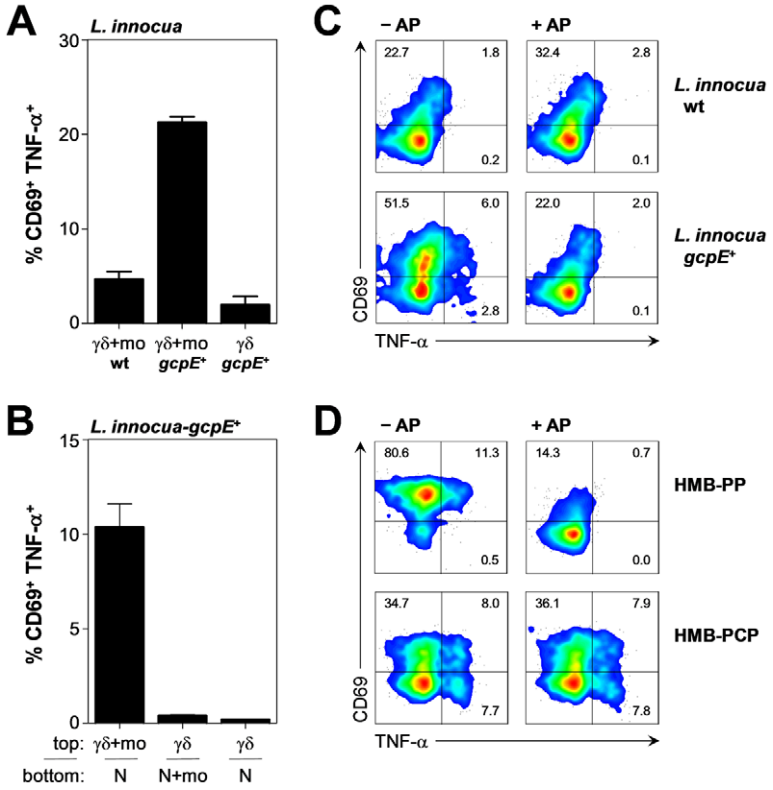

Figure 6. Vγ9/Vδ2 T cells respond, in a monocyte-dependent manner, to phosphatase-sensitive molecules released from phagocytosed bacteria. (*A*) Activation of Vγ9/Vδ2 T cells by neutrophils harboring *L. innocua* wt or *L. innocua-gcpE*$^+$ bacteria, in the presence (γδ-mo) or absence (γδ) of monocytes. Data shown are mean frequencies of CD69$^+$ TNF-α$^+$ Vγ9/Vδ2 T cells + SEM after 20 hours in culture, as determined in independent experiments using three different donors. (*B*) Activation of Vγ9/Vδ2 T cells in the top chamber of a transwell plate separated from neutrophils (N) harboring *L. innocua-gcpE*$^+$ in the bottom chamber, in the presence or absence of monocytes (mo). Data shown are mean frequencies of CD69$^+$ TNF-α$^+$ Vγ9/Vδ2 T cells + SEM after 20 hours in culture, as determined in independent experiments using three different donors. (*C*) Activation of Vγ9/Vδ2 T cells co-cultured for 20 hours with monocytes in the presence of supernatants from neutrophils harboring *L. innocua* wt or *L. innocua-gcpE*$^+$ bacteria that had been pretreated or not with alkaline phosphatase (AP). Data shown are representative from independent experiments using two different donors. (*D*) Activation of Vγ9/Vδ2 T cells co-cultured for 20 hours with monocytes in the presence of 1 nM HMB-PP or 100 μM HMB-PCP pretreated or not with alkaline phosphatase (AP). Data shown are representative from independent experiments using two different donors.

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response was largely independent of the presence of other pathogen-associated molecular patterns such as LPS as both Gram-negative (*Acinetobacter baumannii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*M. smegmatis*) capable of producing HMB-PP stimulated Vγ9/Vδ2 T cells equally. Direct addition of alkaline phosphatase to these co-cultures abrogated the HMB-PP dependent cytokine responses, confirming soluble HMB-PP as common Vγ9/Vδ2 T cell stimulator in these species (Figure 7A, Figure S4 in Text S1). The bioactivity of culture supernatants harvested after 5 hours from neutrophils harboring the above bacteria corresponded to levels of 0.1–10 nM HMB-PP, as titrated against a HMB-PP standard (data not shown). Residual levels of CD69 expression after phosphatase treatment may have been due to incomplete degradation of HMB-PP and to indirect stimulation of Vγ9/Vδ2 T cells by other microbial compounds such as LPS acting on neutrophils or monocytes [41,42]. In contrast to HMB-PP producing species, HMB-PP deficient Gram-negative (*Chryseobacterium indologenes*) and Gram-positive bacteria (*Enterococcus faecalis*, *L. innocua*, *Staphylococcus aureus*) did not elicit Vγ9/Vδ2 T cells responses above background as demonstrated by the complete lack of TNF-α (Figure 7A, Figure S4 in Text S1) and IFN-γ (data not shown). These findings illustrate the extraordinary specificity Vγ9/Vδ2 T cells for HMB-PP, even in the abundant presence of other microbial products and despite high levels of monocyte and/or neutrophil-derived mediators such as IL-1β, IL-6 and CXCL8 that were present in our triple co-cultures regardless of the HMB-PP status of the phagocytosed bacteria (data not shown).

Vγ9/Vδ2 T cells proliferate in response to phagocytosed bacteria in a TCR, LFA-1 and TNF-α dependent manner

γδ T cells expand rapidly in acute bacterial infections [18]. We therefore tested whether phagocytosed pathogens could induce expansion of 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled Vγ9/Vδ2 T cells. As shown in Figure 7B, Vγ9/Vδ2 T cells proliferated considerably in the presence of supernatants derived from neutrophils harboring HMB-PP$^+$ *Enterobacter cloacae* but not from neutrophils harboring HMB-PP$^-$ *Chryseobacterium indologenes* or *Staphylococcus aureus*. Similarly to the immediate up-regulation of CD69, TNF-α and IFN-γ, the proliferation of Vγ9/Vδ2 T cells in response to *Enterobacter cloacae* was HMB-PP dependent and could be abrogated by alkaline phosphatase. Expanding Vγ9/Vδ2 T cells also up-regulated the high affinity IL-2 receptor, CD25 (Figure 7B) and became responsive to exogenously added IL-2, which enhanced the proliferative response even further (data not shown). Blocking

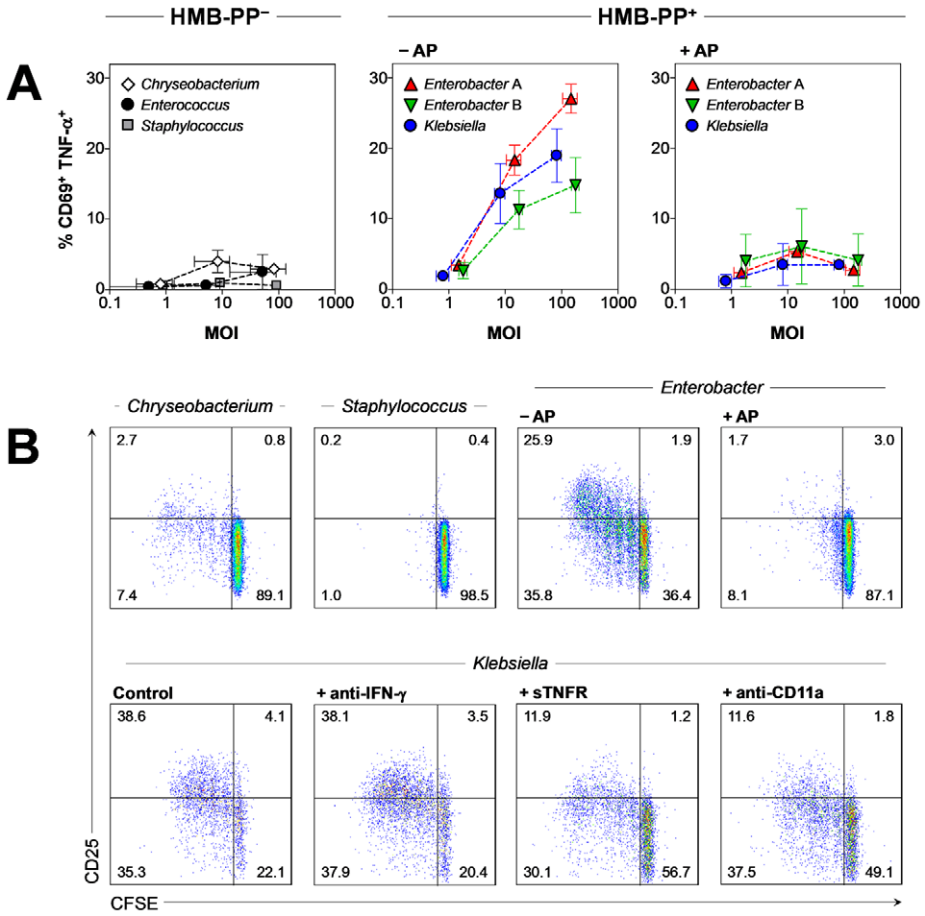

Figure 7. Vγ9/Vδ2 T cells respond to HMB-PP producing Gram$^+$ and Gram$^-$ bacteria but not to HMB-PP deficient bacteria. (*A*) Dose-dependent activation of Vγ9/Vδ2 T cells by neutrophils harboring clinical isolates of a range of different bacteria: Gram$^-$ HMB-PP$^+$, *Enterobacter cloacae* (two different isolates tested, A and B) and *Klebsiella pneumoniae*; Gram$^-$ HMB-PP$^-$, *Chryseobacterium indologenes*; and Gram$^+$ HMB-PP$^-$, *Enterococcus faecalis* and *Staphylococcus aureus*; in the presence or absence of alkaline phosphatase (AP). Data shown are mean frequencies of CD69$^+$ TNF-α$^+$ Vγ9/Vδ2 T cells ± SEM after 20 hours in culture, as determined in independent experiments using 3–5 donors. Error bars for MOIs depict mean values ± SEM for the true CFU counts of the bacterial inocula used. (*B*) Proliferation after 5 days in culture and CD25 expression of CFSE-labeled Vγ9/Vδ2 T cells in response to supernatants from neutrophils harboring clinical isolates of the indicated bacteria, in the presence or absence of alkaline phosphatase (AP), soluble TNF-α receptor (sTNFR) or blocking antibodies against IFN-γ or CD11a. Data shown are representative of independent experiments using cells from at least two different donors.
doi:10.1371/journal.ppat.1002040.g007

experiments demonstrated a crucial requirement of soluble and contact-dependent signals for optimum stimulation of Vγ9/Vδ2 T cells. TNF-α was recently implicated in Vγ9/Vδ2 T cell proliferation in response to IPP and IL-2 [43], and blocking of lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) efficiently disrupted cluster formation with monocytes [31]. Here, both Vγ9/Vδ2 T cell proliferation and CD25 up-regulation in response to supernatants derived from neutrophils harboring HMB-PP$^+$ *Klebsiella pneumoniae* (Figure 7B) or *Enterobacter cloacae* (data not shown) were readily inhibited by sTNFR and anti-CD11a antibodies but not by anti-IFN-γ antibodies. Finally, addition of anti-Vγ9 antibodies completely abrogated the Vγ9/Vδ2 T cell proliferation in response to HMB-PP (data not shown) and *Enterobacter cloacae* supernatants (Figure S7 in Text S1), confirming a requirement for the TCR [44]. Taken together, our findings demonstrate that Vγ9/Vδ2 T cells are rapidly activated by a broad range of HMB-PP producing pathogens, leading to TCR, LFA-1 and TNF-α dependent γδ T cell expansion.

Acute infections caused by HMB-PP producing bacteria are characterized by elevated numbers of activated γδ T cells

We next addressed whether the dichotomy between HMB-PP$^+$ and HMB-PP$^-$ pathogens in their potential to trigger γδ T cells *in vitro* is replicated under physiological conditions *in vivo*. As clinical correlate for HMB-PP$^+$ and HMB-PP$^-$ infections, we analyzed episodes of acute bacterial infections in peritoneal dialysis (PD) patients, in whom the peritoneal catheter affords continuous and non-invasive access to the inflammatory infiltrate (Table S2 in Text S1). PD-associated peritonitis is characterized by a considerable influx of neutrophils and monocytes into the peritoneal cavity [45,46], where the two cell types may become targets for local or infiltrating γδ T cells [31,47]. Here, in a total of 24 newly recruited patients examined on the first day of acute peritonitis (*i.e.* before administration of antibiotics), both the total number and the frequency of peritoneal Vγ9/Vδ2 T cells were elevated in HMB-PP$^+$ infections compared to HMB-PP$^-$ infections, suggesting increased recruitment and/or proliferation in response to

HMB-PP released by bacteria (Figure 8). Moreover, local activation was evidenced by higher percentages of Vγ9/Vδ2 T cells expressing CD69 in the HMB-PP$^+$ patient group. In contrast, we did not see any significant differences in the numbers and frequencies of peritoneal neutrophils, monocytes/macrophages and total CD3$^+$ T cells, regardless of the HMB-PP status of the causative pathogen (Figure S5 in Text S1). Similarly, while the proportion of Vγ9/Vδ2 T cells within peritoneal CD3$^+$ T cells was clearly elevated in HMB-PP$^+$ infections, CD4$^+$ and CD8$^+$ T cells showed no such bias (Figure S6 in Text S1).

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

As Medzhitov stated recently, "inflammation is beneficial in appropriate amounts but can easily become detrimental when excessive because of its tissue-damaging potential" [48]. PD patients constitute a particularly vulnerable group where inflammatory events can have profound consequences [49–51]. We speculated that local activation of γδ T cells may contribute to inflammation-related damage and tested whether the occurrence of clinical complications in PD patients depends on the capacity of the causative pathogen to produce HMB-PP. Our analysis of 26 patients treated at the University Hospital of Wales, Cardiff, UK, demonstrated that infections with HMB-PP$^+$ bacteria were associated with worse outcomes, evidenced as higher mortality rates and higher incidences of technique failure (*i.e.*, cessation of therapy due to catheter removal, transfer to hemodialysis or patient death), while HMB-PP$^-$ bacteria caused milder disease (Figure 9). Of note, we were able to validate this pattern in two larger and entirely independent cohorts treated in Australia (ANZDATA Registry) and at the University Hospital of North Staffordshire, Stoke-on-Trent, UK (Figure 9). In order to rule out that this pattern was not due to differences in Gram staining (and hence endotoxin-related), we divided the group of HMB-PP$^+$ bacteria further into Gram$^+$ and Gram$^-$ species. Our outcome analysis demonstrates that even within the Gram$^+$ group, bacteria capable of producing HMB-PP were associated with worse outcomes compared to HMB-PP$^-$ pathogens (Figure 9), suggesting that the HMB-PP producing capacity of the causative pathogen might be of predictive value for the clinical outcome from bacterial peritonitis (Table S3 in Text S1).

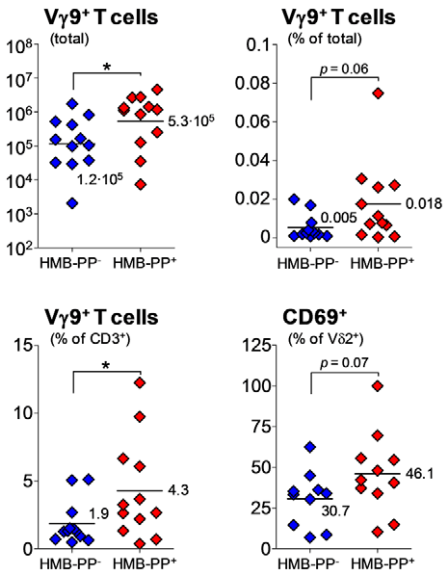

Figure 8. Vγ9/Vδ2 T cell numbers and CD69 expression are elevated in acute peritonitis caused by HMB-PP bacteria. Total numbers and frequencies of Vγ9/Vδ2 T cells (% of total cells and % of all CD3$^+$ T cells), and expression of CD69 on peritoneal Vγ9/Vδ2 T cells in patients with PD-associated peritonitis on day 1 (the day of hospital admission with a cloudy effluent, *i.e.* before commencement of antibiotic therapy), depending on whether or not the causative pathogen was capable of producing HMB-PP.
doi:10.1371/journal.ppat.1002040.g008

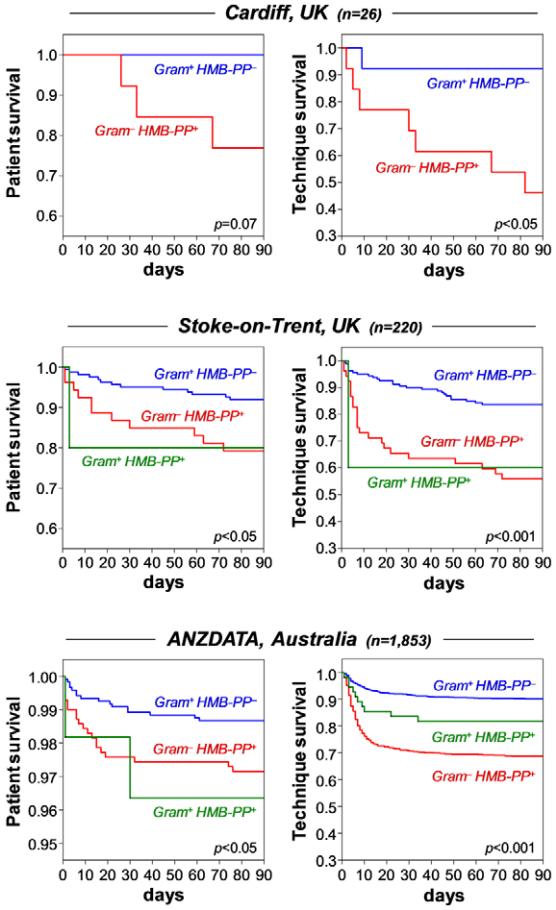

Figure 9. Episodes of peritonitis caused by HMB-PP producing bacteria are associated with poor clinical outcome. Cumulative patient survival (*left*) and cumulative technique survival (*right*) of patients with acute bacterial peritonitis, grouped into infections with Gram$^+$ HMB-PP$^-$ (blue), Gram$^+$ HMB-PP$^+$ (green) or Gram$^-$ HMB-PP$^+$ pathogens (red); episodes caused by Gram$^-$ HMB-PP$^-$ pathogens were not recorded in the patient databases. *Top*, PD patients admitted at the University Hospital of Wales, Cardiff, with acute peritonitis (day 1, *i.e.* first presentation with a cloudy bag). *Middle*, Australian PD patients from the ANZDATA registry with first-time peritonitis. *Bottom*, PD patients with first-time peritonitis treated at the University Hospital of North Staffordshire, Stoke-on-Trent, UK. Comparisons were made using log-rank tests.
doi:10.1371/journal.ppat.1002040.g009

Peritoneal γδ T cell frequencies and TNF-α levels might predict clinical outcome in peritonitis patients

In order to identify potentially useful diagnostic and prognostic biomarkers of inflammation severity and outcomes from bacterial infection, we measured a large number of immunological parameters. These analyses identified elevated frequencies of peritoneal Vγ9/Vδ2 T cells on day 1 as possible predictor of subsequent technique failure within three months after infection

Table 1. Identification of immune markers of possible predictive value for clinical outcome in 8 stable PD patients and 29 PD patients with acute peritonitis (means ± SD).

90th day technique failure	Stable patients	Survivors	Non-survivors
Vγ9$^+$ (% of CD3$^+$ T cells)	1.5±0.9	2.5±2.1	4.9±3.2*
TNF-α (pg/ml)	3.1±4.3	41.9±53.3	128.4±91.0*
90th day mortality			
HLA-DR$^+$ (% of Vδ2$^+$ T cells)	14.7±10.5	11.5±10.5	40.6±7.5***
TNF-α (pg/ml)	3.1±4.3	48.1±60.4	148.7±90.2*

Asterisks indicate significant differences between survivors and non-survivors.
doi:10.1371/journal.ppat.1002040.t001

(Table 1). Similarly, expression of the activation marker HLA-DR by peritoneal Vγ9/Vδ2 T cells on the day of admission was associated with increased mortality. No other parameters tested including the numbers and frequencies of neutrophils, monocytes or CD4$^+$ and CD8$^+$ T cells reached statistical significance (data not shown). Among soluble factors in peritoneal effluent, only elevated levels of TNF-α on day 1 indicated higher rates of technique failure and mortality (Table 1), while no such correlation was seen for other cytokines and chemokines, including GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, IL-17, IL-22, CXCL8, CXCL10 and sIL-6R (data not shown).

Pre-treatment of bacteria with fosmidomycin blocks HMB-PP production and abrogates γδ T cell responses

Our findings of a rapid γδ T cell response to neutrophil-engulfed HMB-PP producing pathogens and its potential detrimental consequence in episodes of acute peritonitis may not only be of diagnostic and predictive value for affected patients, they also highlight possible new avenues of therapeutic intervention in bacterial infections. HMB-PP is an intermediate of the non-mevalonate pathway of isoprenoid biosynthesis, in which the first enzymatic step catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr) can be inhibited by fosmidomycin (Figure S8A in Text S1), a natural antibiotic produced by *Streptomyces lavendulae* [52,53]. We therefore speculated that the effect of fosmidomycin pre-treatment of bacteria may serve a dual purpose in treating acute infections: by directly inhibiting an essential pathway in a broad range of pathogens and by abrogating HMB-PP driven inflammatory responses.

Tests with selected clinical isolates of common pathogens demonstrated that the majority of HMB-PP$^+$ bacteria (*Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) was susceptible to overnight treatment with fosmidomycin (with the exception of *Acinetobacter baumannii* as expected [54]), with a mean inhibitory concentration (MIC) of 1–32 μg/ml depending on the strain (Table S1 in Text S1). Of note, fosmidomycin also acted on multidrug-resistant strains including bacteria harboring the recently discovered 'New Delhi' metallo-β-lactamase 1 (NDM-1) [55,56] (Davey MS, Tyrrell JM *et al.*, submitted for publication). In contrast to the efficient killing of most HMB-PP$^+$ bacteria, the HMB-PP$^-$ bacteria *Chryseobacterium indologenes*, *Enterococcus faecalis* and *Staphylococcus aureus* were not affected by fosmidomycin (Table S1 in Text S1).

We next investigated the potential of short-term fosmidomycin treatment to affect γδ T cell activation by inhibiting the bacterial HMB-PP biosynthesis. Prior exposure of bacteria to fosmidomycin for 1 hour did not affect uptake by neutrophils as demonstrated using *Escherichia coli-gfp*$^+$ (Figure S8B in Text S1), and neither did it affect gross bacterial viability as confirmed by re-plating treated *Enterobacter cloacae* on antibiotic-free plates in order to overcome the competitive inhibition by fosmidomycin (Figure 10A). Yet, pre-incubation of *Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* with fosmidomycin for only 1 hour prior to phagocytosis by neutrophils clearly abrogated their capacity to stimulate Vγ9/Vδ2 T cells. This inhibitory effect on γδ T cell responses was evident both for activation of Vγ9/Vδ2 T cells in triple co-cultures with neutrophils harboring fosmidomycin-treated bacteria (Figure 10A and data not shown) as well as for activation (Figure S8B in Text S1) and proliferation (Figure 10B) of Vγ9/Vδ2 T cells in response to cell-free supernatants from neutrophils harboring fosmidomycin-treated bacteria. Together these results indicate that fosmidomycin not only has a direct antibacterial effect but also possesses immediate anti-inflammatory properties by inhibiting γδ T cell-driven responses (Figure 11), thus making the non-mevalonate pathway an attractive novel drug target for the treatment of acute infection.

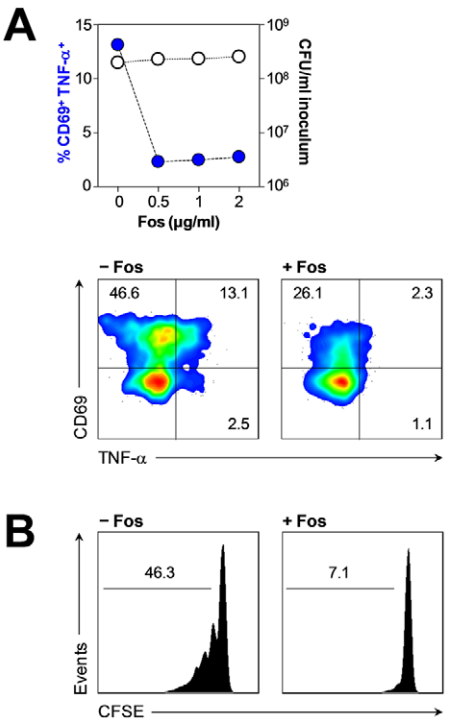

Figure 10. Abrogation of HMB-PP dependent γδ T cell responses by fosmidomycin. (*A*) Activation of Vγ9/Vδ2 T cells co-cultured for 20 hours with neutrophils harboring *Enterobacter cloacae* pre-treated or not for 1 hour with the indicated concentrations of fosmidomycin. The minimum inhibitory concentration (MIC) of fosmidomycin for this bacteria strain was 1 μg/ml, as determined by microbroth dilution (Table S1 in Text S1). Data shown are frequencies of CD69$^+$ TNF-α$^+$ Vγ9/Vδ2 T cells after 20 hours in culture, alongside CFU counts of the original bacterial inocula after 1 hour treatment with fosmidomycin, representative of independent experiments using cells from two different donors. FACS plots show typical Vγ9/Vδ2 T cell responses, representative of independent experiments using cells from three different donors. (*B*) Proliferation after 5 days in culture of CFSE-labeled Vγ9/Vδ2 T cells in response to supernatants from neutrophils harboring *Enterobacter cloacae* pre-treated or not for 1 hour with 0.25 μg/ml fosmidomycin. Data shown are representative of independent experiments using cells from two different donors.
doi:10.1371/journal.ppat.1002040.g010

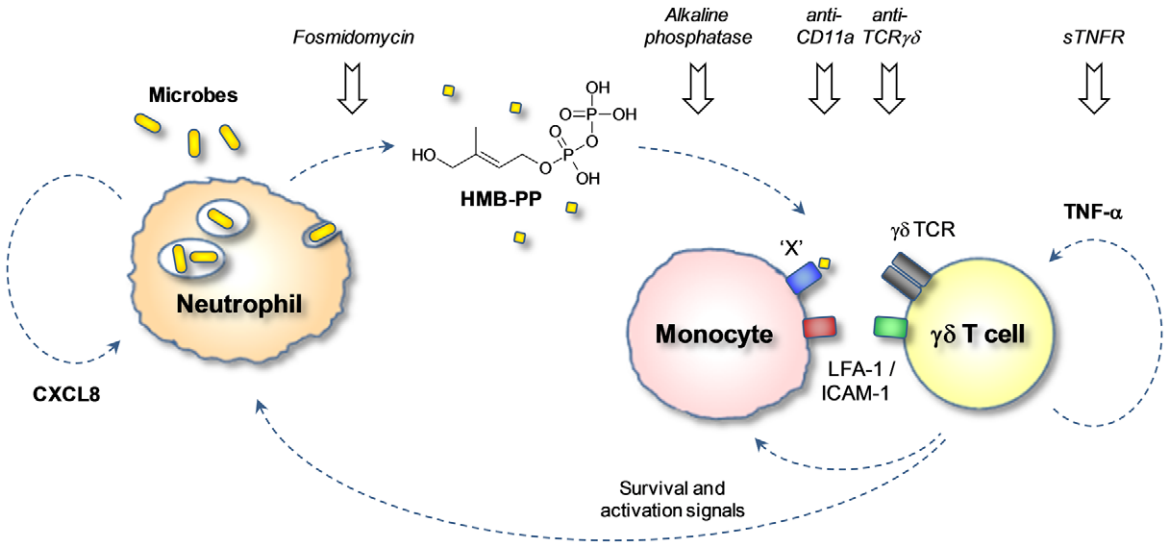

Figure 11. HMB-PP dependent interaction between γδ T cells, neutrophils and monocytes in acute microbial infection. Local secretion of inflammatory chemokines leads to extravasation of neutrophils, monocytes and γδ T cells toward the site of infection. Upon phagocytosis of invading microbes, neutrophils release traces of HMB-PP into the microenvironment where it becomes 'visible' to γδ T cells. γδ T cells recognize HMB-PP in the context of a yet unidentified presenting molecule 'X' and contact-dependent signals provided by monocytes. Crosstalk between the three different cell types triggers the production of pro-inflammatory cytokines such as TNF-α, which drives local γδ T cell expansion, and chemokines such as CXCL8, which recruits further neutrophils to the site of infection. Activated γδ T cells also provide survival and activation signals such as TNF-α for newly arriving neutrophils and monocytes. This γδ T cell-driven inflammatory reaction can be interrupted at various check-points as demonstrated in the present study.
doi:10.1371/journal.ppat.1002040.g011

Discussion

Despite its relevance in early infection, the immediate crosstalk of γδ T cells, monocytes and neutrophils in the presence of bacterial pathogens has not been addressed in detail. This is particularly the case in humans who possess a unique γδ T cell population uniformly targeting an invariant non-self-metabolite, HMB-PP. Previous reports already associated the activation of Vγ9/Vδ2 T cells with the production of HMB-PP by microbes. This link was mainly based on the observation that Vγ9/Vδ2 T cell levels are often elevated in the blood of patients infected with HMB-PP producing pathogens [18] and that bacterial extracts prepared from those species activate Vγ9/Vδ2 T cells *in vitro* much better than extracts prepared from HMB-PP deficient micro-organisms [19,35,57]. Other investigators have speculated that Vγ9/Vδ2 T cells respond *in vivo* toward infected host cells with dysregulated isoprenoid metabolism leading to accumulation of isopentenyl pyrophosphate (IPP) regardless of the presence or absence of HMB-PP [58]. Here we unequivocally demonstrate that Vγ9/Vδ2 T cells respond to live bacteria upon phagocytosis by neutrophils, that this response is strictly HMB-PP dependent, and that it is amplified by the presence of monocytes providing crucial accessory signals. While it has remained puzzling how the immune system actually 'sees' an intracellular metabolite that is unlikely to be secreted or released by live micro-organisms, our findings show that biologically relevant traces of HMB-PP escape into the microenvironment after phagocytosis of extracellular bacteria by neutrophils. These conditions are likely to occur during the acute stage of the infection when Vγ9/Vδ2 T cells and monocytes are co-recruited to the site of inflammation [17] where they encounter neutrophils engaged in clearing invading pathogens (Figure 11).

The present findings explain how HMB-PP may become released at the site of infection. However, the molecular mechanism of HMB-PP recognition by Vγ9/Vδ2 T cells remains poorly understood. Our observation that monocytes were required for Vγ9/Vδ2 T cell responses to phagocytosed bacteria offers important clues. Monocytes and monocyte-derived macrophages or DCs were shown before to provide accessory help and may constitute a pivotal trigger for Vγ9/Vδ2 T cell responses to different bacterial pathogens. In the case of direct infection of monocytic cells, HMB-PP derived from intracellular bacteria may reach the cell surface bound to a presenting molecule [24,25,27]. In the case of extracellular bacteria, monocytes may take up or bind soluble HMB-PP released by professional phagocytes and present it to Vγ9/Vδ2 T cells (Figure 11). The HMB-PP presenting pathway remains elusive but may involve cell surface F1-ATPase [59], together with tight cell-cell interactions via LFA-1/ICAM-1 [31,60], while it is independent of MHC class I, MHC class II, β_2-microglobulin or CD1 [61]. Of note, any chemical modification of the molecular structure of HMB-PP abrogates its bioactivity by several magnitudes, such that the closely related natural metabolites IPP and DMAPP are >10,000 times less active *in vitro* [39,40,62,63]. This is supported by our previous [35,64,65] and present demonstration that HMB-PP deficient bacteria (but which produce IPP and DMAPP) fail to stimulate cytokine production by Vγ9/Vδ2 T cells. Treatment with fosmidomycin or alkaline phosphatase abrogated the Vγ9/Vδ2 T cell responses to HMB-PP producing bacteria and emphasized the importance of HMB-PP for the induction of IFN-γ and TNF-α. However, fosmidomycin or alkaline phosphatase treated cultures as well as cultures involving HMB-PP deficient bacteria did show residual levels of CD69 expression, in line with a role for direct or indirect sensing of microbial TLR ligands [41,42,66] that is likely to amplify the overall response. In this respect it is intriguing that our present study identified a crucial role for TNF-α in supporting Vγ9/Vδ2 T cell proliferation, a cytokine which is readily produced not only by activated Vγ9/Vδ2 T cells themselves but also by neutrophils and monocytes exposed to

microbial compounds such as LPS. This is in stark contrast to other cytokines produced by innate immune cells such as IFN-α and IFN-β which may induce upregulation of CD69 on Vγ9/Vδ2 T cells but fail to co-stimulate Vγ9/Vδ2 T cell proliferation [32]. Taken together, we identified an inflammatory crosstalk of Vγ9/Vδ2 T cells, neutrophils and monocytes in the presence of HMB-PP producing bacteria that can be manipulated at various check-points: (*i*) the antibiotic fosmidomycin abrogates the microbial HMB-PP production and thus renders bacterial pathogens invisible for Vγ9/Vδ2 T cells; (*ii*) alkaline phosphatase degrades free HMB-PP released by neutrophils into the microenvironment; (*iii*) blocking antibodies against the TCR prevent the recognition of HMB-PP by Vγ9/Vδ2 T cells; (*iv*) blocking antibodies against CD11a disrupt the LFA-1/ICAM-1 dependent contact between γδ T cells and monocytes needed for γδ T cell stimulation; (*v*) and sTNFR neutralizes soluble TNF-α which is released by all three cell types in response to microbial ligands and acts as growth factor for Vγ9/Vδ2 T cells and survival factor for neutrophils (Figure 11).

How does the HMB-PP dependent crosstalk of Vγ9/Vδ2 T cells, monocytes and neutrophils *in vitro* translate into the situation *in vivo* in acutely infected patients? Studies in patients with systemic inflammatory response syndrome suggested a significant role for Vγ9/Vδ2 T cells as early responders after severe insult and identified a correlation between Vγ9/Vδ2 T cell activation and clinical scores [67]. Our own findings in patients with PD-related peritonitis support this notion and demonstrate that the capacity of the causative pathogen to produce HMB-PP and local infiltrates of activated Vγ9/Vδ2 T cells on day 1 are indicative of acute inflammatory responses and may predict the subsequent clinical outcome from infection. It is becoming increasingly clear that the nature of the infection is a major determinant of outcome, and future interventions may well have to focus on subgroups of patients with different forms of infection [68]. A careful re-analysis of peritonitis outcomes from validated registry data [69–76] confirms that HMB-PP$^+$ bacteria cause clinically more severe infection and emphasizes the need to pay more attention to detailed host-pathogen interactions. Bacterial infection remains a leading cause of morbidity and mortality worldwide, not the least due to the alarming spread of antibiotic-resistant pathogens that is posing an enormous challenge on clinical practice, public healthcare and biomedical research [55,56]. In most cases, antimicrobial treatment is largely empirical as microbiological culture results are typically not available for 2–3 days. Moreover, many times no organism can be identified, with rates of culture-negative infections occasionally reaching 50% [77]. In contrast, laboratory analyses of immune cells and/or soluble mediators may provide valuable information within a few hours and both aid diagnosis and refine treatment. In this respect, fosmidomycin or related HMB-PP inhibitors might constitute useful combination partners for antibiotic therapy especially in critical cases such as acute peritonitis or sepsis where the intervention has to commence before the nature of the causative pathogen is known, and where activated Vγ9/Vδ2 T cells may contribute to poor clinical outcome. Fosmidomycin targets an essential pathway in a broad range of pathogens [54,78,79] and simultaneously abrogates Vγ9/Vδ2 T cell responses [80,81]. Of note, the inhibitory effect of fosmidomycin on the Vγ9/Vδ2 T cell bioactivity was detectable after only 1 hour of treatment and well below its MIC, *i.e.* under conditions that would easily be achievable in patients. Other ways of specifically manipulating γδ T cell mediated responses may include the use of anti-γδ TCR antibodies or γδ TCR antagonists such as BrH-PCP [82]. Given the importance of TNF-α for γδ T cell proliferation and the association of peritoneal TNF-α levels with morbidity and mortality, one may also advocate the use of TNF-α blocking reagents for the treatment of acute PD-related peritonitis [83].

Taken together our experiments demonstrate that Vγ9/Vδ2 T cells recognize with HMB-PP a small common molecule released by the majority of invading bacteria when they become phagocytosed by neutrophils. Stimulation of Vγ9/Vδ2 T cells at the site of infection is likely to amplify the local inflammatory response with important consequences for pathogen clearance and the development of microbe-specific immunity. However, if triggered at the wrong time or the wrong place, this rapid reaction toward most bacteria may also lead to inflammation-related damage and detrimental clinical outcome. These findings improve our insight into the complex cellular interactions in early infection, identify novel biomarkers of possible diagnostic and predictive value and highlight new avenues for therapeutic intervention.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines (Bro Taf Health Authority, Wales). The study was approved by the South East Wales Local Ethics Committee under reference number 04WSE04/27. All patients provided written informed consent for the collection of samples and subsequent analysis.

Patients

The Cardiff study population included 39 adult patients who were receiving PD at the University Hospital of Wales, Cardiff, UK, and were admitted with acute peritonitis between September 2008 and October 2010 (Table S2 in Text S1). Eight stable patients with no infection in the previous 3 months were included in this study as non-infected controls. In addition, microbiological and survival data were obtained from all 739 adult patients who were receiving PD between 1987 and 2008 at the University Hospital of North Staffordshire, Stoke-on-Trent, UK; and from all 2,542 Australian adult patients from the Australia and New Zealand Dialysis Transplant (ANZDATA) Registry who were receiving PD between 2003 and 2008 (Table S2 in Text S1). Diagnosis of acute peritonitis was based on the presence of abdominal pain and cloudy peritoneal effluent with >100 WBC/mm^3. Infections were grouped into culture-positive and culture-negative episodes, according to the result of the microbiological analysis of the effluent. Bacteria species identified in culture-positive infections were grouped into HMB-PP$^+$ and HMB-PP$^-$, based on the presence or absence of HMB-PP in the microbial metabolism [17,19]. Endpoints of outcome analyses were 14th and 90th day mortality and technique failure (catheter removal, transfer to hemodialysis, and/or patient death). In order to rule out a history of previous antibiotic treatment as a potentially confounding factor, outcome studies were restricted to patients with first-episode peritonitis, excluding cases of fungal infection or unrecorded culture results.

Bacteria culture

Bacteria strains used in this study are listed in Table S1 in Text S1. *Mycobacterium smegmatis* strains were grown aerobically at 37°C in liquid Lemco medium (Oxoid) supplemented with 10 mg/ml peptone, 5 mg/ml NaCl and 0.25% Tween 80 (Sigma), or on solid Lemco plates with 15 mg/ml agar (Fisher). *Listeria innocua* strains were grown aerobically at 37°C in liquid brain heart infusion medium (Oxoid) or on agar plates. *Escherichia coli* laboratory strains and multi-drug resistant clinical isolates of *Acinetobacter baumannii*,

Chryseobacterium indologenes, *Enterobacter cloacae*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown in liquid LB broth and on solid Columbia blood agar (Oxoid). Where appropriate, antibiotics were added to the medium: *M. smegmatis-gcpE*$^{+}$, 100 μg/ml hygromycin B; *L. innocua-gcpE*$^{+}$, 7.5 μg/ml chloramphenicol; *E. coli-gfp*$^{+}$, 100 μg/ml ampicillin (all from Sigma). Bacterial susceptibilities to fosmidomycin were determined by microbroth dilution method, according to the Clinical and Laboratory Standards Institute guidelines [84]. A log 2 dilution series of 0.06 to 128 μg/ml allowed the identification of the minimal inhibitory concentration (MIC) where bacterial growth was absent. No defined break points have been acknowledged for fosmidomycin [85], therefore resistance was defined as concentrations >128 μg/ml.

Cell isolation

Peritoneal cells were harvested from chilled overnight dwell effluents [31,47]; cell-free supernatants were stored at −70°C. PBMC were isolated from peripheral blood of healthy volunteers using Lymphoprep (Axis-Shield). Vγ9^{+} T cells (>98%) were purified from PBMC using monoclonal antibodies (mAbs) against Vγ9-PE-Cy5 (Immu360; Beckman-Coulter) and anti-PE microbeads (Miltenyi). Monocytes (>98%) were purified using anti-CD14 microbeads (Miltenyi). Neutrophils (>95%) used for bacterial phagocytosis were isolated from peripheral blood using a Lymphoprep gradient followed by dextran sedimentation [86]. Remaining erythrocytes were lysed with ammonium chloride solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), and neutrophils were washed in HBSS without Mg^{2+} and Ca^{2+} (Sigma) and resuspended to a final cell concentration of 2×10^6/ml in HBSS with Mg^{2+} and Ca^{2+} (Sigma) supplemented with 10% human serum. Neutrophils (>98%) used in Vγ9/Vδ2 T cell co-culture experiments were isolated from peripheral blood by initial dextran sedimentation followed by centrifugation through discontinuous Percoll gradients [87]. The cell culture medium used throughout this study was RPMI-1640 with 2 mM L-glutamine, 1% sodium pyruvate, 50 μg/ml penicillin/streptomycin and 10% fetal calf serum (Invitrogen).

Phagocytosis of live bacteria

Single colonies were grown in culture broth for 18 hours, and bacteria were washed in PBS and resuspended in HBSS with Mg^{2+} and Ca^{2+} supplemented with 10% human serum. Freshly isolated neutrophils were incubated with bacteria at a multiplicity of infection (MOI) of 0.1–100 bacteria per neutrophil for 30–60 min at 37°C, with gentle shaking. In some experiments, bacteria were pre-treated with 0.5–25 μg/ml fosmidomycin for 1 hour prior to phagocytosis. Actual MOIs of all bacterial inocula used were determined by plating out serial dilutions on agar plates and expressed as colony forming units (CFU) per neutrophil. Non-phagocytosed bacteria were washed off three times. For microscopic analyses, neutrophils harboring GFP-expressing bacteria were washed, counterstained with DAPI (Sigma) and fixed in 2% paraformaldehyde. Images were acquired on a Nikon Eclipse 80i fluorescence microscope equipped with a Nikon DXM 1200F camera and processed with Adobe Photoshop. For the generation of cell-free supernatants, neutrophils pre-incubated with bacteria as described above were cultured for 5 hours in complete RPMI-1640. Supernatants were then harvested and cells removed by centrifugation at 12,000 *g* for 10 min. Samples were stored at −20°C and thawed a maximum of 5 times. For some experiments, neutrophil supernatants were treated with 0.015 U/μl shrimp alkaline phosphatase for 30 min at 37°C.

Triple co-cultures of neutrophils, monocytes and γδ T cells

Unless indicated otherwise, neutrophils were co-cultured for 20 hours in complete RPMI-1640 medium (further supplemented with 8 μg/ml colistin (Sigma) for assays involving multi-drug resistant clinical isolates) with autologous monocytes and γδ T cells at a ratio of 10 neutrophils and 1 monocyte per 1 γδ T cell (10:1:1), in the absence or presence of 0.015 U/μl shrimp alkaline phosphatase (Roche). Proliferation assays using γδ T cells that had been pre-labeled with CFSE (Molecular Probes) were incubated for 4–6 days. Controls included co-cultures in the absence or presence of 1–100 nM synthetic HMB-PP [40] or 1–100 ng/ml LPS from *Salmonella abortus equi* (Sigma). In transwell experiments, neutrophils were separated from γδ T cells by 0.4 μm pore polycarbonate membranes (Fisher Scientific). Cell-free supernatants derived from neutrophils after phagocytosis of bacteria were tested in monocyte-γδ T cell co-cultures (1:1) at a dilution of 1 in 3. Blocking reagents used were anti-IFN-γ (25718; R&D Systems); anti-CD11a (TS1/22) from Dr Ruggero Pardi (DIBIT-Scientific Institute San Raffaele, Milano, Italy); and sTNFR p75-IgG1 fusion protein (Enbrel; Amgen); alone or in combination at 10 μg/ml each. Anti-TCR-Vγ9 (Immu360; Beckman Coulter) was used at 1.25 μg/ml.

Flow cytometry

Cells were acquired on an eight-color FACSCanto II (BD Biosciences) and analyzed with FloJo 7.6 (TreeStar), using monoclonal antibodies against CD3 (UCHT1), CD15 (HI98), CD25 (M-A251), CD62L (DREG-56), CD69 (FN50), CD86 (2331) and HLA-DR (L243) from BD Biosciences; TCR-Vγ9 (Immu360) and CD40 (mAB89) from Beckman Coulter; and CD11b (ICRF44) and CD14 (61D3) from eBioscience; together with appropriate isotype controls. Cells of interest were gated based on their appearance in side scatter and forward scatter area/height, exclusion of live/dead staining (fixable Aqua; Invitrogen) and surface staining: CD3^{-} CD14^{-} CD15^{+} neutrophils, CD3^{-} CD14^{+} CD15^{-} monocytes, and CD3^{+} Vγ9^{+} γδ T cells. Apoptotic cells were identified using Annexin V (BD Biosciences). For detection of intracellular cytokines, 10 μg/ml brefeldin A (Sigma) was added to cultures 5 hours prior to harvesting. Surface-stained cells were labeled using the Fix & Perm kit (eBioscience) and monoclonal antibodies against IFN-γ (45.15; BD Biosciences) and TNF-α (188; Beckman Coulter).

Culture supernatants and effluent samples

Soluble cytokines in cell culture supernatants were detected using ELISA kits for IL-1β, IL-6 and IL-17 (R&D Systems); and IFN-γ, TNF-α and CXCL8 (BD Biosciences). All samples were measured in duplicate on a Dynex MRX II reader. Cell-free peritoneal effluents were analyzed on a SECTOR Imager 600 (Meso Scale Discovery) for TNF-α, GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, CXCL8 (IL-8) and soluble IL-6 receptor (sIL-6R). In addition, IL-17, IL-22 and CXCL10 in peritoneal effluents were measured in duplicate on a Dynex MRX II reader, using conventional kits (R&D Systems).

Statistical analysis

Data were analyzed using two-tailed Student's *t*-tests (GraphPad Prism 4.0), and considered significant as indicated in the figures and tables: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Cumulative survival curves as a function of time were generated using the Kaplan-Meier approach and compared using the log rank test (SPSS 16.0).

Supporting Information

Text S1 Supporting information.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: NT BM ME. Performed the experiments: MSD CYL. Analyzed the data: MSD CYL JAC BM ME. Contributed reagents/materials/analysis tools: GWR SH ACB MAT CGMG CH TP JDW SJD DWJ. Wrote the paper: BM ME.

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Text S1: Supporting Information

Table S1. Bacteria strains used in the present study.

Strain	Relevant feature	Gram	HMB-PP	Fosmidomycin MIC (μg/ml)
Escherichia coli	*[Clontech, Cambridge, UK]*			
E. coli wt	Parental laboratory strain BL21	−	+	*ND*
E. coli-gfp$^+$	BL21 expressing GFP (pAcGFP)	−	+	*ND*
Mycobacterium smegmatis	*[ref. 34]*			
M. smegmatis wt	Parental laboratory strain mc^{2}155	+	+	*ND*
M. smegmatis-gfp$^+$	mc^{2}155 expressing GFP (pFLAME7)	+	+	*ND*
M. smegmatis-gcpE$^+$	mc^{2}155 expressing GcpE from *M. tuberculosis*	+	+++	*ND*
Listeria innocua	*[refs. 35,36]*			
L. innocua wt	Parental laboratory strain APC	+	−	*ND*
L. innocua-gfp$^+$	APC expressing GFP	+	−	*ND*
L. innocua-gcpE$^+$	APC expressing GcpE from *Bacillus subtilis*	+	+++	*ND*
L. innocua-gcpE$^+$/*lytB*$^+$	APC expressing GcpE, LytB from *B. subtilis*	+	+	*ND*
Clinical isolates	*[this study]*			
Acinetobacter baumannii	Strain 48-694D	−	+	> 128
Chryseobacterium indologenes	Strain S281	−	−	> 128
Enterobacter cloacae 'A'	Strain 48-12346	−	+	1
Enterobacter cloacae 'B'	Strain 69-7329	−	+	1
Enterococcus faecalis	Strain IQA 7/09	+	−	> 128
Klebsiella pneumoniae	Strain WCH7	−	+	32
Pseudomonas aeruginosa	Strain 75-3755A	−	+	8
Staphylococcus aureus	Strain S288	+	−	> 128

ND, not determined

Table S2. Characteristics of all patients analyzed in the present study.

	Stable	***Acute peritonitis***		
	Cardiff (UK)	**Cardiff (UK)**	**Stoke-on-Trent (UK)**	**ANZDATA (Australia)**
Total number	8	39	385	2,424
Study period	2008-2010	2008-2010	1987-2008	2003-2008
Age (mean ± SD)	61.4 ± 19.5	66.3 ± 11.3	57.0 ± 16.0	58.4 ± 16.7
Women (%)	37.5	35.9	43.1	44.8
Days on PD (mean ± SD)	1088.5 ± 936.2	1106.1 ± 984.9	532.0 ± 581.9	738.7 ± 502.5
14^{th} day mortality (%)	−	2.6	3.6	1.2
14^{th} day technique failure (%)	−	12.5	9.6	13.4
90^{th} day mortality (%)	−	10.3	8.8	1.8
90^{th} day technique failure (%)	−	28.2	19.0	16.8
Culture-positive infections (%)	−	64.1	61.5	85.8
HMB-PP^{+} organisms among positively identified species (%)	−	50.0	26.4	38.6

Table S3. Odds ratios for risk of technique failure and mortality within 90 days after infection, depending on the causative pathogen.

90th day technique failure	**Stoke-on-Trent (UK)**	***p* value**	**ANZDATA (Australia)**	***p* value**	**ANZDATA + Stoke-on-Trent combined**	***p* value**
Reference: culture-negative	**1.0**		**1.0**		**1.0**	
HMB-PP$^-$	2.0	*	0.8	*n.s.*	1.0	*n.s.*
HMB-PP$^+$	7.4	***	3.4	***	4.1	***
Reference: HMB-PP$^-$	**1.0**		**1.0**		**1.0**	
Gram$^+$ HMB-PP$^+$	3.1	*n.s.*	2.0	0.066	2.1	*
Gram$^-$ HMB-PP$^+$	3.8	***	4.3	**	4.0	***
90th day mortality						
Reference: culture-negative	**1.0**		**1.0**		**1.0**	
HMB-PP$^-$	2.1	*n.s.*	2.3	*n.s.*	1.3	*n.s.*
HMB-PP$^+$	6.3	***	5.4	*	2.8	**
Reference: HMB-PP$^-$	**1.0**		**1.0**		**1.0**	
Gram$^+$ HMB-PP$^+$	2.9	*n.s.*	2.8	*n.s.*	2.4	*n.s.*
Gram$^-$ HMB-PP$^+$	3.0	*	2.4	**	2.2	**

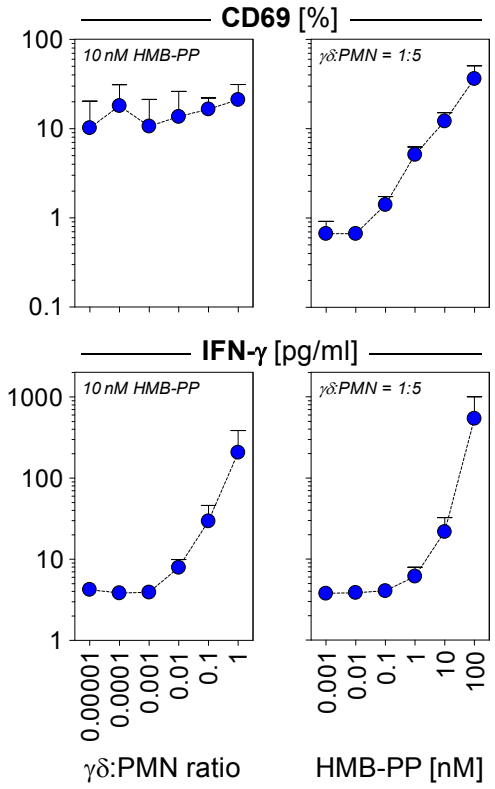

Figure S1. Activation of γδ T cells and dose-dependent secretion of IFN-γ in neutrophil-γδ T cell co-cultures. Expression of CD69 by $V\gamma9^+$ $CD3^+$ T cells was determined after 20 hours in culture by flow cytometry; supernatants were analyzed after 20 hours by ELISA. Data shown are mean values + SEM from independent experiments using three different donors.

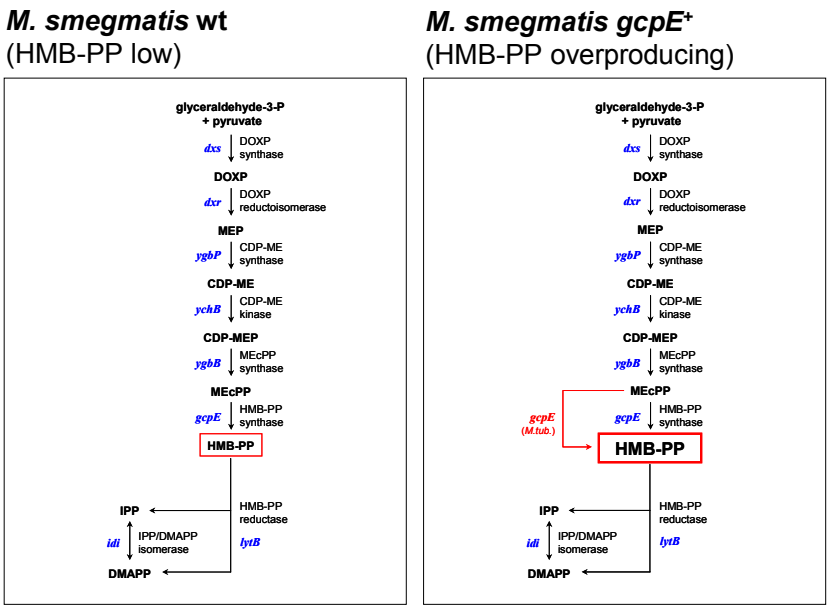

Figure S2. Isoprenoid biosynthesis in the *Mycobacterium* strains used in the present study. *M. smegmatis* possess a fully functional non-mevalonate pathway and hence is capable of producing HMB-PP. Transfection of *M. smegmatis* with an additional copy of *gcpE* from *M. tuberculosis* leads to a 2-3 fold accumulation of HMB-PP compared to the wildtype [35].

Abbreviations: CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP, isopentenyl pyrophosphate; MEcPP, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate.

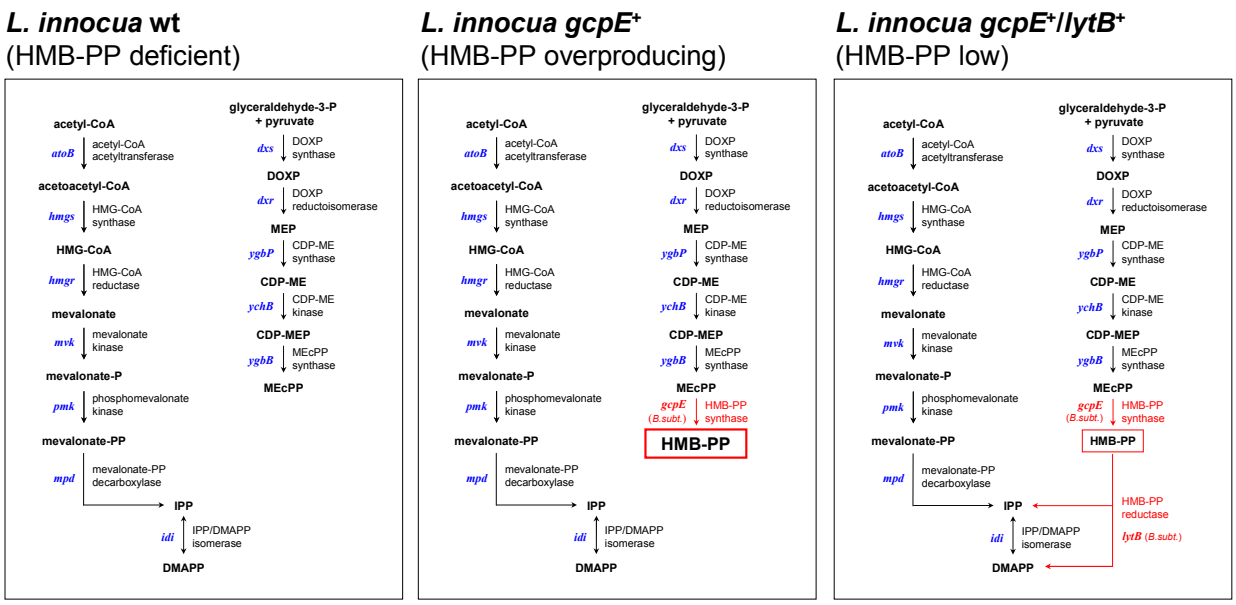

Figure S3. Isoprenoid biosynthesis in the *Listeria* strains used in the present study. *L. innocua* utilizes the mevalonate pathway (left) but also possesses a rudimentary non-mevalonate pathway (right) leading to the production of MEcPP but not of HMB-PP. Transfection of the wildtype strain with a copy of the *gcpE* (*ispG*) gene from *Bacillus subtilis* confers the ability to produce and accumulate HMB-PP; co-transfection of *L. innocua gcpE*$^{+}$ with the *lytB* (*ispH*) gene from *B. subtilis* rescues the non-mevalonate pathway in *L. innocua* and leads to intermediate HMB-PP levels due to reduction of HMB-PP into IPP and DMAPP [36,37].

Abbreviations: CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl pyrophosphate; MEcPP, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; P, phosphate.

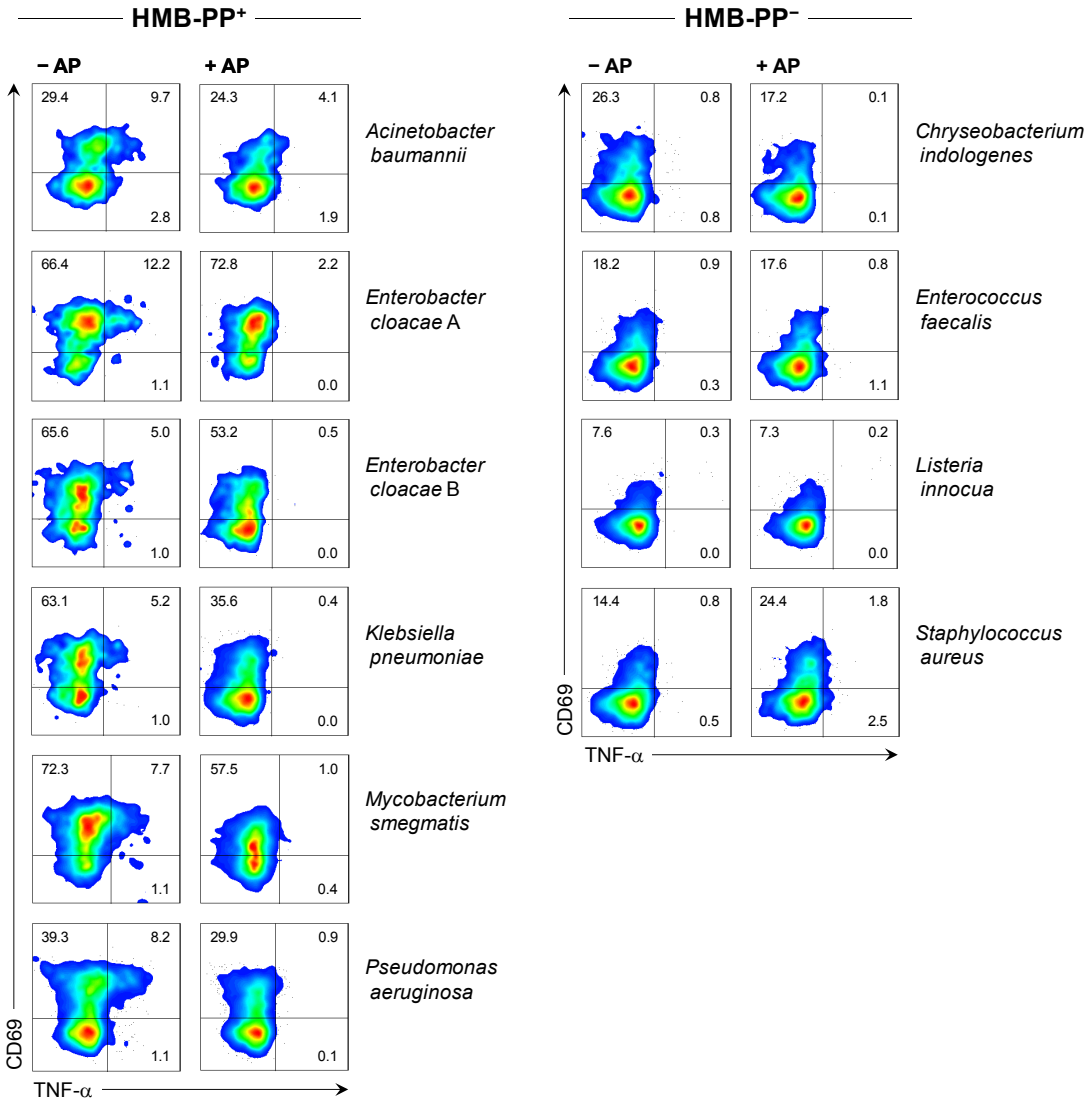

Figure S4. Vγ9/Vδ2 T cells respond to alkaline phosphatase-sensitive molecules released by phagocytosed HMB-PP producing bacteria. Activation of γδ T cells by neutrophils harboring a range of different bacteria: Gram$^-$ HMB-PP$^+$, *Acinetobacter baumannii*, *Enterobacter cloacae* (two different strains tested), *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*; Gram$^+$ HMB-PP$^+$, *Mycobacterium smegmatis*; Gram$^-$ HMB-PP$^-$, *Chryseobacterium indologenes*; and Gram$^+$ HMB-PP$^-$, *Enterococcus faecalis*, *Listeria innocua* and *Staphylococcus aureus*; in the presence or absence of alkaline phosphatase (AP). Data shown are representative from independent experiments using 2-5 different donors.

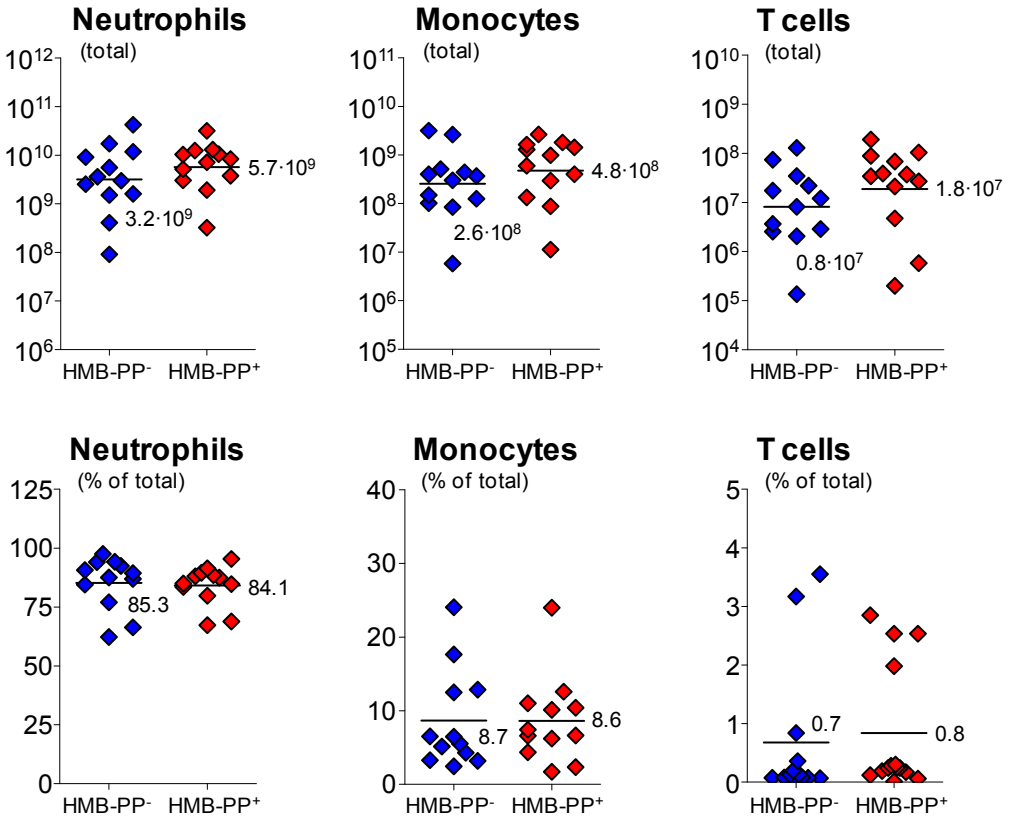

Figure S5. Neutrophil, monocytes and T cells in acute peritonitis are not influenced by the HMB-PP status of the causative pathogen. Total numbers and frequencies of peritoneal $CD15^+$ neutrophils, $CD14^+$ monocytes/macrophages and $CD3^+$ T cells in patients with PD-associated peritonitis on day 1 (the day of hospital admission with a cloudy effluent, *i.e*. before commencement of antibiotic therapy), depending on whether or not the causative pathogen was capable of producing HMB-PP.

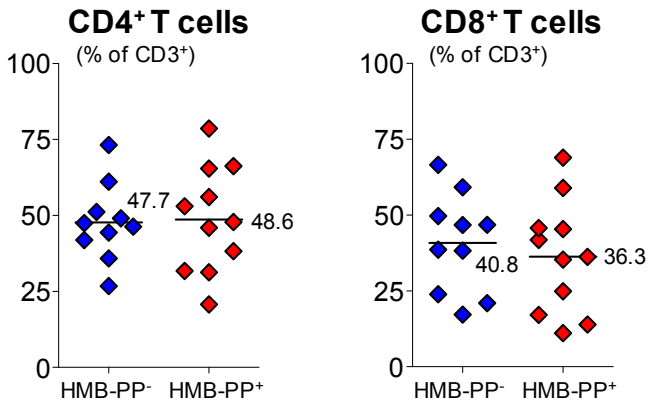

Figure S6. CD4$^+$ and CD8$^+$ T cells in acute peritonitis are not influenced by the HMB-PP status of the causative pathogen. Proportion of CD4$^+$ and CD8$^+$ T cells within all peritoneal CD3$^+$ T cells in patients with PD-associated peritonitis on day 1 (the day of hospital admission with a cloudy effluent, *i.e*. before commencement of antibiotic therapy), depending on whether or not the causative pathogen was capable of producing HMB-PP.

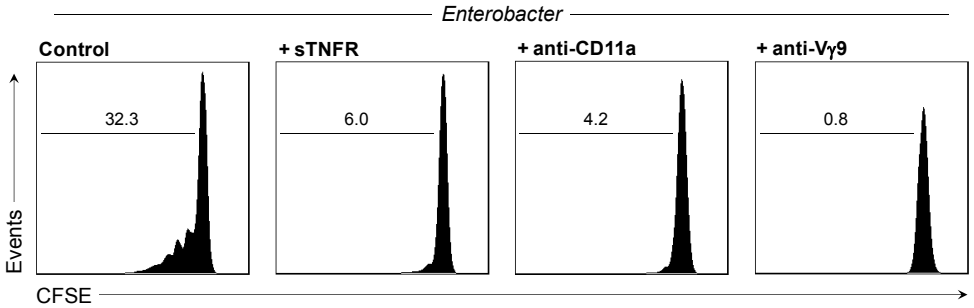

Figure S7. The Vγ9/Vδ2 T cells response to HMB-PP producing bacteria is TCR dependent. Proliferation after 5 days in culture of CFSE-labeled Vγ9/Vδ2 T cells in response to supernatants from neutrophils harboring *Enterobacter cloacae*, in the presence or absence of soluble TNF-α receptor (sTNFR) or antibodies against CD11a or TCR-Vγ9. Data shown are representative of independent experiments using cells from two different donors.

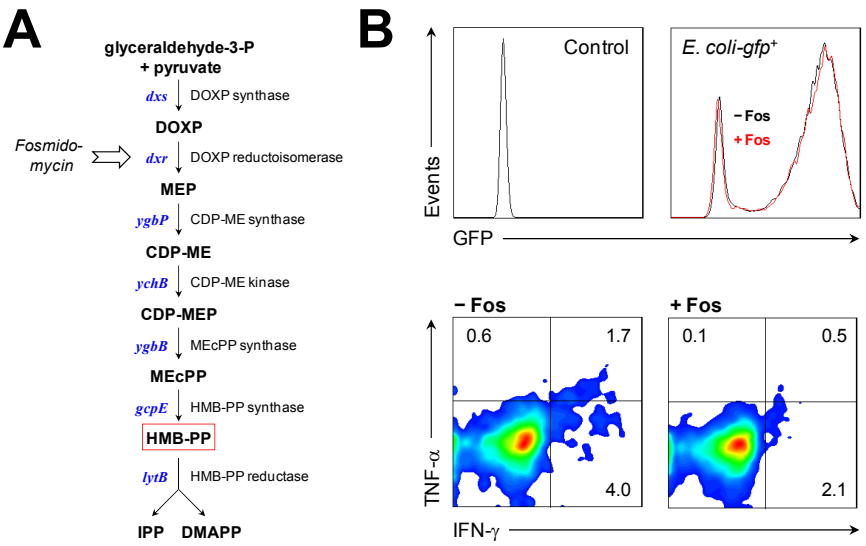

Figure S8. Fosmidomycin abrogates HMB-PP dependent γδ T cell responses to phagocytosed bacteria but does not affect bacterial uptake by neutrophils. (***A***) Overview of the non-mevalonate pathway of isoprenoid biosynthesis. The bacterial genes encoding the enzymes indicated are highlighted in blue. Fosmidomycin blocks HMB-PP production by inhibiting the second step of the pathway. DOXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; MEcPP, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate. (***B***) Phagocytosis by neutrophils of *E. coli-gfp*$^+$ that had been pre-treated or not for 1 hour with 50 μg/ml fosmidomycin (control, neutrophils only). GFP fluorescence was determined 1 hour later. Neutrophil supernatants were collected after 5 hours and added to γδ T cell-monocyte co-cultures for 20 hours. Data shown are representative of independent experiments using cells from two different donors.