DISSERTATION

Systemic antigen-reactive T cells as a marker for intestinal barrier function in critically ill patients

Systemische antigen-reaktive T-Zellen als Marker für die intestinale Barrierefunktion bei kritisch kranken Patienten

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 28.02.2025

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3. Abbreviations

AB	Antibody
AIDS	Acquired immune deficiency syndrome
APC	Allophycocyanin
ARDS	Acute respiratory distress syndrome
ARTE	Antigen-reactive T cell enrichment
BD	Becton Dickinson
B. longum	*Bifidobacterium longum*
BV	Brilliant Violet
C. albicans	*Candida albicans*
CCR	Chemokine receptor
CD	Cluster of differentiation
CI	Cell index
CKD	Chronic kidney disease
CLR	C-type lectin receptor
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CRC	Colorectal cancer
CrD	Crohn‘s disease
CRP	C-reactive protein
CTL	Cytotoxic T cell
CTRL	Control
Cy	Cyanine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate-buffered saline
ECMO	Extracorporeal membrane oxygenation
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EPEC	Enteropathogenic *Escherichia coli*
FACS	Fluorescence-activated cell sorting

FCS/FBS	Fetal calf serum / fetal bovine serum
FiO2	Fraction of inspired oxygen
FITC	Fluorescein-5-isothiocyanate
FOXP3	Forkhead box P3
FSC	Forward scatter
GCS	Glasgow coma scale
GIT	Gastrointestinal tract
GI	Gastrointestinal
GLP-2	Glucagon-like peptide-2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTPase	Guanosin triphosphate hydrolase
IBD	Inflammatory bowel disease
ICU	Intensive care unit
IEC	Intestinal epithelial cell
I-FABP	Intestinal-fatty acid-binding protein
IFN	Interferon
IG	Immunoglobulin
IL	Interleukin
K. pneumoniae	*Klebsiella pneumoniae*
K. oxytoca	*Klebsiella oxytoca*
MACS	Magnetic cell separation
MAP	Mean arterial pressure
MCI	Myocardial infarction
MHC	Major histocompatibility complex
MS	Mass spectrometry
NASH	Non-alcoholic steatohepatitis
NLR	NOD-like receptor
NK cell	Natural killer cell
NOD	Nucleotide-binding oligomerisation domain
ns	Not significant
PAMPs	Pathogen-associated molecular patterns

PaO2	Partial pressure of arterial oxygen
P. aeruginosa	*Pseudomonas aeruginosa*
PB	Permanent Blue
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein
P. mirabilis	*Proteus mirabilis*
PP	Polypropylene
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
S. agalactiae	*Streptococcus agalactiae*
SAPS	Simplified acute physiology score
SARS	Severe acute respiratory syndrome
S. aureus	*Staphylococcus aureus*
SEB	Staphylococcal enterotoxin B
S. gorondii	*Streptococcus gorondii*
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
SOFA	Sequential organ failure assessment
S. pneumoniae	*Streptococcus pneumoniae*
SSC	Sideward scatter
Tcm cell	T central memory cell
Tcon cell	T conventional cell
TCR	T cell receptor
Tem cell	T effector memory cell
TFh cell	Follicular T helper cell
Tg	Transglutaminase
TGF	Transforming growth factor
Th1 cell	T helper 1 cell

Th17 cell	T helper 17 cell
Th22 cell	T helper 22 cell
Th2 cell	T helper 2 cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg cell	Regulatory T cell
Trm cell	T tissue-resident memory cell
Tscm cell	Stem memory T cell
Tte cell	Terminal effector T cell
UC	Ulcerative colitis

4. Abstract

Patients receiving intensive care treatment often present with intestinal dysfunction, particularly impaired intestinal barrier function, independent of their underlying disease. Through various mechanisms, this leads to the development and maintenance of a systemic inflammatory response (SIRS/sepsis) with subsequent organ system failure and is associated with a poor prognosis. In the clinical context of intensive care treatment, direct detection and quantification of intestinal barrier dysfunction are not readily available. However, this would be a necessary prerequisite for the evaluation of therapeutic regimens. The aim of this study was to investigate the intestinal barrier function in ICU patients by phenotyping peripheral blood mononuclear cells (PBMCs). A total of 70 ICU patients were enrolled (30 patients with sepsis, 30 patients not meeting sepsis criteria, and 10 patients with Covid-19). In addition, 20 healthy volunteers without hospitalisation were included in the study. We hypothesised that an impaired intestinal barrier would lead to increased translocation of luminal antigens and increased priming of T cells. PBMCs were isolated and subsequently stimulated with different commensal antigens. Antigen-reactive T cell enrichment (ARTE) technology was used to enrich rare antigen-reactive T cells and characterise them by flow cytometry. Using the ARTE technology, we were able to show that patients with sepsis treated in the ICU had a significantly higher frequency of commensal antigen-reactive T cells in their peripheral blood, characterised by a proinflammatory cytokine profile, compared to patients without sepsis. This distinct signature found in the first cohort was confirmed by analysis of a second cohort of patients. In addition, the antigen-reactive T cell compartment, particularly in sepsis patients, was positive for the gut-homing integrin α4ß7, strongly suggesting a leaky gut. Thus, we were able to show that the analysis of PBMCs can be used to investigate the intestinal barrier function in intensive care patients.

5. Zusammenfassung

Patienten auf der Intensivstation weisen unabhängig von ihrer Grunderkrankung häufig Darmfunktionsstörungen auf, vor allem eine gestörte intestinale Barrierefunktion. Dies führt infolge unterschiedlicher Mechanismen zur Entwicklung und Aufrechterhaltung einer systemischen Entzündungsreaktion (SIRS/Sepsis) mit konsekutivem Versagen von Organsystemen und ist mit einer schlechten Prognose assoziiert. Der direkte Nachweis und die Quantifizierung der intestinalen Barrierefunktion ist in der klinischen Routine auf der Intensivstation nicht einfach möglich. Dies wäre jedoch notwendige Voraussetzung für die Etablierung und Evaluierung möglicher Therapieschemata.

Ziel der vorliegenden Arbeit war es, die intestinale Barrierefunktion mittels Analyse einer Blutprobe zu untersuchen und zwischen Patient:innen mit Sepsis und ohne Sepsis zu vergleichen.

Es wurden 30 Patient:innen mit Sepsis, 30 ohne Sepsis und 10 mit Covid-19 auf der Intensivstation rekrutiert. Ebenfalls wurden 20 gesunde, nicht hospitalisierte Proband:innen eingeschlossen. Aus einer Blutprobe erfolgte die Isolierung von peripheren mononukleären Zellen (PBMCs) und nachfolgend eine Stimulation der PMBCs mittels verschiedener kommensaler Antigenen. Mittels der *antigen-reactive T cell enrichment* (ARTE)-Technologie wurden die seltenen Antigen-reaktiven T-Zellen angereichert und mittels Durchflusszytometrie charakterisiert.

Mittels der ARTE-Technologie und durchflusszytometrischen Untersuchungen konnten wir zeigen, dass intensivstationär behandelte Patient:innen mit Sepsis im Vergleich zu Patient:innen ohne Sepsis nach eine signifikant höhere Frequenz Antigen-reaktiver T-Zellen im peripheren Blut aufwiesen, welche durch ein proinflammatorisches Zytokinprofil gekennzeichnet waren. Diese für Sepsispatient:innen spezifische Signatur konnte in einer zweiten Kohorte bestätigt werden. Darüber hinaus zeigte die für Sepsispatient:innen spezifische Antigen-reaktive T-Zell Population eine Positivität für den Darm Homing-Marker α4ß7, womit diese Zellen als suggestiv für das Vorliegen eines „leaky gut“ angesehen werden können. Zusammenfassend konnten wir eine Methode entwickeln, die anhand einer peripheren Blutprobe die Untersuchung und Charakterisierung der intestinalen Barriere bei intensivmedizinisch behandelten Patient:innen ermöglicht.

6. Introduction

6.1. The Intestinal Barrier

The gastrointestinal tract (GIT) represents one of the most significant interfaces between the host and the environment (1). As such, it is specialised for the dynamic interaction between intestinal cells and cells foreign to the body. The GIT harbours a complex and dynamic population of microorganisms, the gut microbiota, which plays a critical role in homeostasis and disease (2). The microbiome displays the totality of all microbial organisms (commensals, pathogens, viruses, and fungi), their structures, and their products. Due to potentially harmful pathogens, the GIT must act as a filter and barrier (3). The passage of essential nutrients and other vital commensals while neutralising potential pathogens is a hallmark feature of the GIT and is central to intestinal homeostasis (4). Given the existing crosstalk between microbiota, host epithelial cells, and immune cells in the underlying lamina propria, the epithelial lining of the GIT also exerts a role in immune regulation (1, 5).

Anatomically, the intestinal barrier is composed of a chemical barrier (mucus layer and lamina propria with immune cells) and a physical barrier (monolayer of intestinal epithelial cells (IECs)) (3). The monolayer of IECs plays a central role in the integrity of the intestinal barrier, allowing the passage of essential nutrients and ions while preventing pathogens from entering deeper layers of the GIT through phagocytosis (6). This occurs in close cooperation with immune system cells (e.g., dendritic cells, T cells, and B cells) (4). Below the monolayer of IECs, stem, goblet, tuft, Paneth, and enteroendocrine cells is the lamina propria, a layer of connective tissue where the microbiome and immune cells interact (Fig. 1; (7)). Table 1 summarises different elements of the intestinal barrier with its respective functions. Intestinal permeability is tightly regulated to avoid uncontrolled passage of antigens and possibly harmful microorganisms.

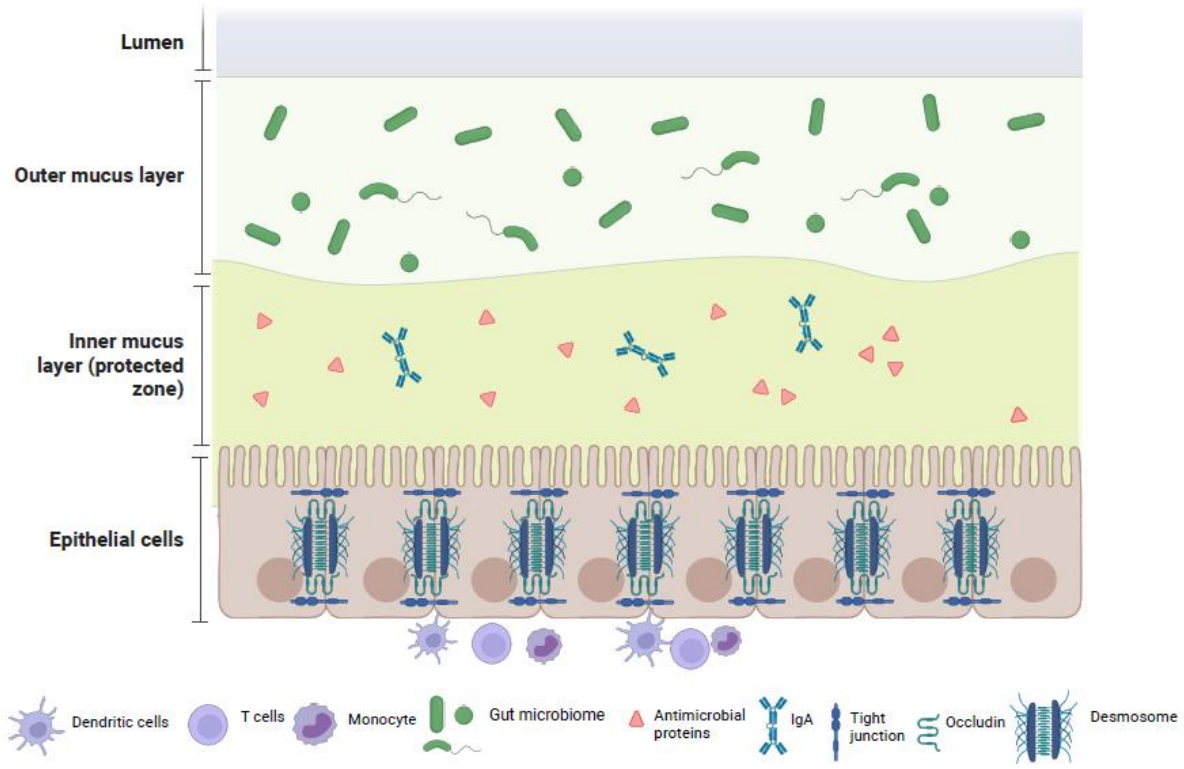

Figure 1: Structure of the Mucosal Barrier, adapted from "Structure of Mucosal Barrier" by BioRender.com (2022) Created with BioRender.com. Abbreviation: Ig – Immunoglobulin.

Cell types and elements of the intestinal barrier and their function	
Components	**Function**
Enterocytes	• Nutrient absorption and metabolisation • Maintenance of physical barrier through junctional protein complexes • Excretion of antimicrobial substances
Paneth cells	• Regulation of inflammation by producing antimicrobial peptides
Goblet cells	• Production and secretion of mucins
Tuft cells	• Regulation of Goblet cell-dependent mucin production
Mucus	• Physical (outer layer) and biochemical (inner layer) barrier
Enteroendocrine cells	• Hormone excretion (e.g., GLP-2)
M cells	• Capture and transport of antigens
Dendritic cells	• Stimulating intestinal repair
Innate lymphoid cells	• Immune response
Macrophages	• Immune defense
Commensal microbiota	• Colonisation resistance

Table 1: Cell types and Elements of the intestinal barrier and their function (adapted from Tab. 1 (8)). Abbreviation: GLP - Glucagon-like-peptide.

6.2. Sepsis

Sepsis and septic shock both describe a life-threatening condition in which organ dysfunction results from a dysregulated, exaggerated immune response to infection (8). Septic shock is characterised by persistent hypotension with elevated lactate and is associated with even higher mortality than sepsis (9). Systemic inflammatory response syndrome (SIRS) is a systemic inflammatory response of the immune system (10). The trigger may be infectious or non-infectious (e.g., trauma, ischemia, pulmonary embolism). The response is accompanied by changes in body temperature, heart rate, respiratory rate, and blood count (Fig. 2). SIRS is, therefore, non-specific, as it can be triggered by different factors (8).

The most common focus of infection in sepsis is the lungs, abdomen, and urinary tract. Typical pathogens are gram-positive organisms such as *Staphylococcus aureus* (*S. aureus*) (20%). The most common gram-negative isolates are *Pseudomonas aeruginosa* (*P. aeruginosa*) (20%) and *Escherichia coli* (*E. coli*) (16%) (11). Typical factors that increase the risk of mortality in sepsis patients include emergency surgery, trauma, transfer from the hospital, presence of chronic obstructive pulmonary disease, cancer, heart failure, immunosuppression, liver cirrhosis, prior mechanical ventilation or hemodialysis (12). The pathophysiology of sepsis is complex. Typical inflammatory biomarkers for sepsis include interleukin (IL)-1ß, IL-6, interferon (IFN)-γ, and Tumor necrosis factor (TNF)-α (13). However, the conventional view of a "cytokine storm" as the flashpoint for sepsis has been the subject of critical debate. Recent studies have shown that anti-inflammatory cytokines are also secreted in addition to pro-inflammatory cytokines (14). Furthermore, components of the cell wall of pathogens, known as pathogen-associated molecular patterns (PAMPs), induce endothelial damage. This leads to hyperpermeability, oedema formation and, ultimately, microcirculatory dysfunction. In addition, there is an increased release of nitric oxide, leading to vasodilatation and hypoperfusion (15). If sepsis is suspected to be present, therapy should be started immediately and not be delayed by diagnostic testing. Blood cultures should be obtained simultaneously as calculated antibiotic administration, and further focus-specific diagnostics should be initiated depending on the suspected site of infection. Imaging, such as a chest X-ray, should also be performed (16). Other therapeutic options include surgical or interventional focal decontamination, hydration, administration of

vasopressors and glucocorticoids, and vitamin supplementation or enteral nutrition, as appropriate. As organ failure progresses, organ replacement therapies such as invasive ventilation, hemodialysis or extracorporeal membrane oxygenation (ECMO) may become necessary (9).

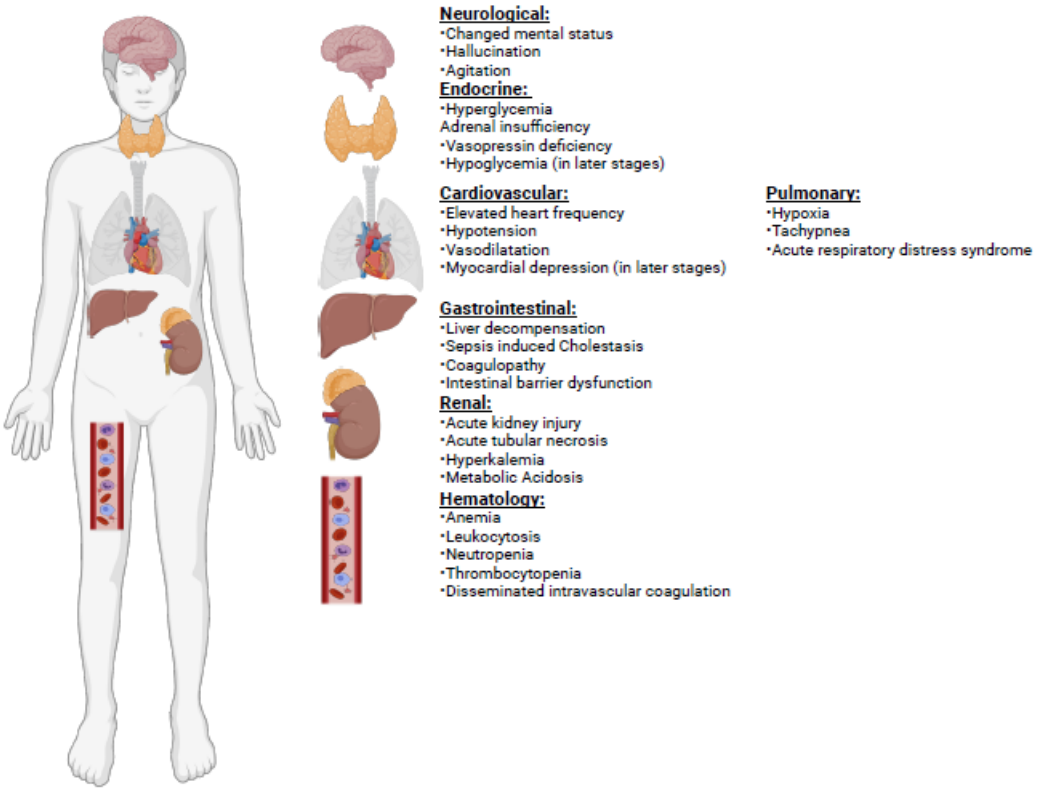

Figure 2: Pathophysiological changes in sepsis, created with BioRender.com.

6.3. Intestinal Barrier dysfunction in Sepsis

Critically ill patients treated in the intensive care unit (ICU) experience significant dysregulation of gastrointestinal (GI) function (17). GI dysfunction increases morbidity and mortality in these patients by exacerbating the underlying disease or causing further complications (18). GI dysfunction encompasses several aspects, including intestinal barrier dysfunction, mucosal integrity violation, microbial composition alterations, and motility and absorption disorders. Of particular relevance and severity are the characteristic disturbances in intestinal barrier function in patients with sepsis, where the intestinal proinflammatory response has been implicated in perpetuating the septic event (19). While it was initially hypothesised in the 1980s that structural changes in the intestinal epithelium caused by malnutrition and intestinal motility disorders could lead to

luminal bacteria migrating into the bloodstream and triggering a systemic inflammatory response/sepsis (translocation hypothesis), local immunologic processes are now increasingly being implicated in the development, maintenance and effects of intestinal barrier disorders (20, 21, 22). In addition to the typical clinical, laboratory, and radiologic signs of disease progression, intestinal barrier dysfunction may manifest as malabsorption, ileus, diarrhoea, haemorrhage or ischemia (20).

Although the functional status of the GIT plays a vital role in disease progression, few diagnostic tests are currently available to assess intestinal barrier function. Early and reliable diagnosis of barrier dysfunction and evaluation of therapeutic options in patients with sepsis are therefore lacking in clinical routine, representing a significant gap in the management of critically ill patients (23). A general clinical assessment and measurements of gastric residual volume and L-lactate are used to assess the intestinal barrier. In addition, citrulline and intestinal- fatty acid binding protein (I-FABP) levels can be determined as a sign of possible enterocyte damage or dysfunction (24). However, many confounding factors, such as red blood cell (RBC) transfusion and renal and hepatic dysfunction, can affect these diagnostic procedures. Therefore, these diagnostic tests are associated with inaccuracy and appear to be logistically infeasible. Early implementation of enteral nutrition in ICU patients as a treatment approach has been shown to stimulate early intestinal transit and prevent overgrowth of the intestinal microbiota (14, 25). This approach stems from expertise in pediatric Crohn's disease (CrD) patients, where immediate initiation of enteral nutrition led to remission and regeneration of the inflamed mucosa (26). Compared with corticosteroid therapy, enteral nutrition was superior in efficacy and side effect profile (27). Another protective effect of enteral nutrition has been demonstrated in laparoscopic colorectal surgery patients. In these patients, a high-fat enteral diet reduced intestinal inflammation (26, 28).

Taken together, GI dysfunction in ICU patients is thought to result from an evolved imbalance in the microbiome and intestinal epithelium homeostasis. This results in a loss of intestinal barrier function and the passage of pathogens across the mucosa into deeper tissue layers and the bloodstream and is associated with poor clinical outcomes for patients (29). Therefore, the gut can be considered a driver of disease in critically ill patients, which should be addressed in diagnostics and therapy, even if it does not display the focus of the initial disease.

6.4. Covid-19

Severe acute respiratory syndrome (SARS) coronavirus-2 is a novel coronavirus that belongs to the family *Coronaviridae* (30). Infection can lead to various manifestations, symptoms and morbidity (31). This depends on various parameters (e.g., individual genetics, age, previous diseases, lifestyle). Severe courses of Covid-19 are characterised by the destruction of lung epithelial cells, thrombosis, hypercoagulation, vascular leakage and ultimately, sepsis and septic shock (18). This results in acute respiratory distress syndrome (ARDS), which is associated with high mortality (32). The pathophysiology involves an exaggerated immune response, leading to a cytokine storm (33). Therapeutic priorities include optimisation of ventilation, antipyretic therapy, antivirals, antibiotics, steroids and immunomodulators, and in case of a severe ARDS, ECMO is a therapeutic option (18, 31, 32, 33).

6.5. SOFA and SAPS-II Score

The Sequential Organ Failure Assessment (SOFA; Tab. 2) score calculates the degree of organ dysfunction in the ICU. It is also used as a predictor of mortality (34). The SOFA score measures lung, liver, kidney, and central nervous system (CNS) function, platelet count, mean arterial pressure (MAP), and vasopressor requirements. An increase of ≥ 2 points is considered a positive SOFA score associated with acute organ dysfunction. The SOFA-Score is used to detect sepsis; a positive score is part of its definition (34, 35).

Respiratory System			**Points**
	PaO2/FiO2 (mmHg)	>400	0
		>400	1
		>300	2
		>200 with respiratory support	3
		<100 with respiratory support	4
CNS			
	GCS	15	0
		13-14	1
		10-12	2
		6-9	3
		<6	4
Cardiovascular system			
	MAP / administration of vasopressors required	MAP > 70 mmHg	0
		MAP < 70 mmHg	1
		Dopamine ≤ 5ug/kg/min OR dobutamine (any dose)	2
		Dopamine >5 ug/kg/min OR epinephrine ≤ 0.1 µg/kg/min OR norepinephrine ≤ 0.1 µg/kg/min	3
		Dopamine > 15 µh/kg/min OR epinephrine > 0.1 µg/kg/min OR norepinephrine > 0.1 µg/kg/min	4
Liver			
	Bilirubin (mg/dl) [µmol/L]	<1,2 [>20]	0
		1.2–1.9 [20–32]	1
		2.0–5.9 [33–101]	2
		6.0–11.9 [102–204]	3
		> 12.0 [> 204]	4
Coagulation			
	Platelets ×103/ml	>150	0
		<150	1
		<100	2
		<50	3
		20	4
Kidneys			
	Creatinine (mg/dl) [µmol/L]; urine output	< 1.2 [< 110]	0
		1.2–1.9 [110–170]	1
		2.0–3.4 [171–299]	2
		3.5–4.9 [300–440] (or urine output < 500 ml/day)	3
		> 5.0 [> 440]; urine output < 200 ml/day	4

Table 2: SOFA-Score, (adapted fromTab. 1 (35)). Abbreviations: CNS - Central nervous system; FiO2 - Fraction of inspired oxygen; GCS - Glasgow coma scale; MAP - Mean arterial pressure; mmHg - Millimetre of mercury; PaO2 - Partial oxygen pressure; SOFA - Sequential organ failure assessment.

The Simplified Acute Physiology Score-II (SAPS; Tab. 3) is another score used in intensive care medicine. It provides information about the severity of the patient's illness and is also used to calculate in-hospital mortality (36). It uses 12 physiological and 3 disease-related values (36, 37).

SAPS II	0 points	Abnormal value points				
Age (years)	<40	50-99 7 points	60-89 12 points	70-74 15 points	75-80 16 points	≥ 80 18 points
Heart rate (bpm)	70-119	40-69 2 points	120-159 4 points	≥ 160 7 points	< 40 11 points	
Systolic blood pressure (mmHg)	100-199	≥200 2 points	70-99 5 points	<70 13 points		
Body temperature (°C)	<39	≥39 3 points				
Only if on mechanical ventilation: PaO2/FiO2 (mmHg)		≥200 6 points	100-199 9 points	<100 11 points		
Urinary output (L/day)	≥1	0,5-0,9 4 points	<0,5 11 points			
Blood urea nitrogen (mmol/l)	<10	10-29,9 6 points	≥30 10 points			
White blood cells (/mm³)	1-19,9	≥20 3 points	<1,0 12 points			
Potassium (mmol/L)	3-4,9	<3 or ≥5 3 points				
Sodium (mmol/L)	125-144	≥145 1 point	<125 5 points			
Bicarbonate (mmol/L)	≥20	15-19 3 points	<15 6 points			
Bilirubin (µmol/L)	<68,4	68,4-102,5 4 points	≥102,6 9 points			
Glasgow Coma Scale	14-15	11-13 5 points	9-10 7 points	6-8 13 points	<6 26 points	
Chronic disease	none	Metastatic cancer 9 points	Haematological malignancy 10 points	AIDS 17 points		
Type of admission	Scheduled surgical	Medical 6 points	Unscheduled surgical 8 points			

Table 3: SAPS-II-Score (adapted from Tab.3 (39)). Abbreviations: AIDS - Acquired immunodeficiency; bpm - Beats per minute; FiO2 - Fraction of inspired oxygen; mmHg - millimetre of mercury; PaO2 - Partial oxygen pressure; SAPS - Simplified acute physiology score.

6.6. Immune responses - cell differentiation and cytokine production

The immune response to a pathogen involves both pro- and anti-inflammatory cells. The significant cells and cytokines involved in intestinal barrier function are presented below.

6.6.1. Conventional T cells

Lymphocytes that express an αß T cell receptor (TCR) and the co-receptor CD4 or CD8 are called conventional T cells (Tcon). Tcon cells are an essential component in directing immune responses against pathogens but also play a role in autoimmune responses or targeting of tumour cells (38). Tcon cells can be subdivided into subsets described below (39).

6.6.2. $CD4^+$ T cells

$CD4^+$ T cells can develop into different subsets of T helper (Th) cells, including Th1, Th2, Th9, Th17, Th22 and follicular T helper (TFh) cells, which are characterised by unique effector functions (40). Different types of Th cells are distinguished by their cytokine profile (41). First, there are type 1 T helper cells (Th1), which predominantly produce IFN-γ, IL-2 and TNF-α and type 2 T helper cells (Th2), which preferentially produce IL-4 (Tab. 4;(15). Other subpopulations include Th9, Th17, Th22 and TFh cells, which specifically produce IL-9, -17, -22 and -21(TFh), respectively (Fig. 3; (41, 42)). In addition, all Th cell subpopulations are capable of producing IL-2, IL-6, IL-10, IL-21, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) (43). One of the main functions of Th1 cells is the activation of macrophages through the production of IFN-γ and the proliferation of $CD8^+$ cytotoxic T cells. On the other hand, Th2 cells meditate reactions of the immune system against extracellular parasites (41). Likewise the play an important role in the pathogenesis of inflammatory asthmatic and allergic diseases (Tab. 4;(41)). Th9 cells are important in immune defense against worm diseases and developing autoimmune processes (44, 45). Th17 and Th22 cells are also important in immune defence function and are dysregulated in autoimmune diseases (46, 47). Through the production of IL-4, Th2 cells induce the conversion of B cell immunoglobulins (Ig) to IgG1 and IgE. By producing IL-5, Th2 cells recruit eosinophils (41). Figure 3 illustrates T cell activation and differentiation pathways.

	Th1 cells	Th2 cells
Cellular affiliates	Macrophages, $CD8^+$ T cells	B cells, eosinophils, mast cells
Cytokines	IFN-γ, TNF-α, IL-2	IL-4, IL-5, IL-6, IL-10
Function	• Proliferation of $CD8^+$ CTL • Cellular immune response • Support of lytic function of macrophages	• B cell stimulation • Auto-regulation • Pro-inflammatory inhibition

Table 4: Description of Th1- and Th2-cells, (adapted from Fig. 1 (49)). Abbreviations: CD – Cluster of differentiation; CTL – cytotoxic T lymphocyte; IFN – Interferon; IL – Interleukin; Th – T helper; TNF – Tumor necrosis factor.

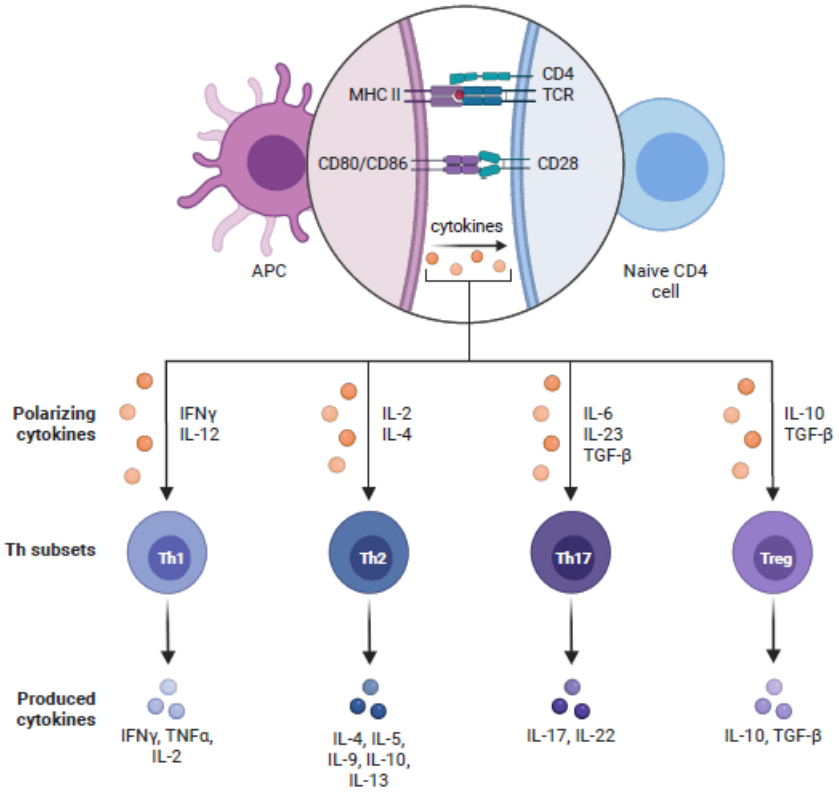

Figure 3: T-cell activation and differentiation, (adapted from Fig. 3 (48)), Created with BioRender.com. Abbreviations: APC – Antigen presenting cell; CD – Cluster of differentiation; IFN – Interferon; IL – Interleukin; MHC – Mayor histocompatibility complex; TCR – T-cell receptor

6.6.3. CD8$^+$ T cells

There are two major subpopulations distinguished within the CD8$^+$ T cell population.

T central memory cells (Tcm) are primarily responsible for enhancing the immune response (49). In contrast, T effector memory cells (Tem) are responsible for cytotoxicity against pathogens (50). In addition, small subsets exist, including T tissue-resident memory cells (Trm), that are involved in preventing barrier penetration of pathogens (51). Tcm cells secrete mainly IL-2 in addition to IFN-γ but have no direct cytolytic effect. In contrast, Tem cells preferentially produce IFN-γ and TNF-α but not IL-2 and are effector cells in terms of cytolytic activity using perforin granules (Tab. 5;(52, 53, 54)). In addition, Tcm and Tem cells differ concerning the expression of CCR7 and CD62L. CCR7 is involved in controlling the release and homing of lymphocytes and dendritic cells to various secondary lymphoid organs (i.e., lymph nodes, spleen) (55, 56). CD62L, also known as L-selectin, is a cell adhesion molecule and acts as a homing receptor for lymphocytes (57, 58). These are positive only in Tcm cells (59, 60). Moreover, CD137 can be expressed in both populations as a sign of antigen-driven activation to promote memory differentiation and effector function (61).

	CD154	**CD137**	**FoxP3**	**TNF-α**	**IFN-γ**	**IL-2**
CD8$^+$ T cell Subset						
Treg	-	+	+	-	-	-
Tte	+	-	-	+	+	-
Tcm	+	-	-	+	-	-
Tscm	+	-	-	-	-	+
CD4$^+$ T cell Subset						
Treg	-	+	+	-	-	-
Tcon (Th1)	+	-	-	+	+	+

Table 5: CD8$^+$ and CD4$^+$ T cell distinction, (adapted from Fig. 2 (41, 62)). Abbreviations: CD – Cluster of differentiation; FoxP3 – Forkhead box P3; IFN – Interferon; IL – Interleukin; Tcm – T central memory cell; Tcon – Conventional T cell; Th – T helper cell; TNF – Tumor necrosis factor; Treg – Regulatory T cell; Tscm – Stem memory T cell; Tte – Terminal effector T cell.

6.6.4. Regulatory T cells

Regulatory T cells (Treg cells) control the immune response to prevent pathological autoreactivity, such as autoimmune diseases (63). They are characterised within the $CD4^+$ T cell population by high expression of the transcription factor Forkhead Box P3 (FoxP3) and CD25 (40). Currently, measurement of FoxP3 expression is the gold standard for assessing the proportion of Treg cells in a population (64). The transcription factor FoxP3 plays a vital role in immune regulation (Tab. 5;(40)). CD137 is a member of the TNF family and provides a co-stimulatory signal that enhances the response of $CD4^+$ T cells and the cytotoxicity of $CD8^+$ T cells, as well as regulating Treg cells to counteract allergic inflammatory processes (40, 65). $CD4^+$ Treg cells express the highest levels of CD137 (40).

Overall, the measurement of $CD137^+CD154^-$ expression, as a sign of a universal Treg cell activation signature, proves to identify antigen-activated Treg cells in combination with FoxP3 (64).

6.6.5. Antigen-reactive T cells

For $CD4^+$ T cells, CD154 (CD40L), a co-stimulatory molecule, is a reliable functional marker for detecting antigen-reactive T cells and is upregulated within 4-12 hours after antigen stimulation (39). As a central mediator of T cell responses, CD154 is expressed by virtually all functionally activated $CD4^+$ T cells regardless of their differentiation status and by a subset of $CD8^+$ T cells (64). The interaction between CD154 and CD40 is critical for B cell activation and the development of high-affinity antibody responses (40). Most cells that produce at least one cytokine also express CD154 (39). This qualifies it for a marker for the most responding cells (40). Therefore, CD154 can be used as a marker for a general T cell response in terms of activation by antigens, independent of specific cytokine production (66). Another marker with sufficient specificity for antigen activation is CD137 (4-1BB). CD137 is expressed on antigen-activated $CD4^+$ and $CD8^+$ T cells, and γδ T cells approximately 16-24 hours after stimulation. In addition, CD137 is expressed on antigen-activated $CD4^+FoxP3^+$ Treg cells (39).

CD137 and CD154 are used to differentiate Treg from Th cells. After 6 hours of stimulation, only Treg cells express CD137, whereas only a small subpopulation of Treg

cells with unstable FoxP3 expression express CD154. Thus, $CD154^+$ $CD137^-$ cells can be identified as Th cells and $CD154^-$ $CD137^+$ cells as Treg cells (39, 67).

6.6.6. Cytokines

CD4+ and CD8+ memory T cells ubiquitously express TNF-α and IL-2 upon activation and are commonly used as markers of T cell effector responses (40). IL-2 is mainly produced by $CD4^+$ and $CD8^+$ T cells in lymphoid tissues and plays a critical role in T cell homeostasis, Th cell subset differentiation, and $CD8^+$ T-cell responses (40). IL-2 is frequently induced in the early stages of pro-inflammatory T cell activation, especially within the $CD4^+$ T cell compartment, where it is produced by less differentiated cells such as stem memory T (scm) and Tcm cells. It is essential for antigen-reactive T cells' proliferation, differentiation, and survival (52). TNF-α is also one of the early effector molecules activated T cells produce. Its main task is to enhance the pro-inflammatory status by mediating the lysis of pathogens, and increasing vascular permeability to enhance the outflow of fluids and the crossing of immune cells through cell barriers (68). This is essential for the recruitment of immune cells to the site of infection (68). Furthermore, TNF-α modulates the barrier function of epithelial cells (13). Terminal effector T cells (Tte), which are considered more differentiated T cells are unable to produce TNF-α (52). In addition, TNF-α is the most widely expressed cytokine produced by most activated $CD4^+$ T cells that arise under conditions favouring Th1 cell differentiation. T cells usually synthesise both IL-2 and TNF-α, regardless of whether IFN-γ is produced (68). $CD4^+$ T cells only producing IL-2 are able to do this for a longer time and eventually transform into IFN-γ-producing T cells (69).

IFN-γ production is mainly carried out by T cells at later stages of differentiation because it requires multiple rounds of production compared to IL-2 and TNF-α production (70). Tem and Tte cells in non-lymphoid tissue produce more IFN-γ than Tcm cells. This promotes a rapid response to reinfection. By activating macrophages, IFN-γ induces the differentiation and generation of different T-cell classes. It inhibits viral replication and stimulates natural killer (NK) cells' cytolytic activity. Thus, IFN-γ is essential in controlling the directed immune response to infection (52). Although IFN-γ plays a vital role in the defence against infection, particularly in immunological homeostasis, dysregulated expression is thought to contribute to the pathogenesis of various chronic diseases (e.g.,

inflammatory bowel disease, multiple sclerosis, systemic lupus erythematosus) (1). Thus, IFN-γ is involved in both acute and chronic diseases.

IL-17 is a pro-inflammatory cytokine produced by Th17 cells (71). It enhances the synthesis of other pro-inflammatory cytokines, such as IL-6, TNF-α and IL-8, in macrophages and endothelial cells (72). The differentiation of naïve $CD4^+$ T cells into Th17 cells is closely controlled by several modulators, such as tumour growth factor (TGF)-β, IL-1β and IL-6 (12).

IL-4 is classified as an anti-inflammatory cytokine. It is mainly produced by Th2 cells and primarily ensures adequate control of the immune response in infections, and dysregulation is found in autoimmune diseases. In addition, IL-4 stimulates B cells and provides the antibody isotype switching from IgM to IgG (15).

IL-10 is a central player in the anti-inflammatory cytokine system. Suppressing macrophage dendritic cells and producing pro-inflammatory cytokines regulates the immune response to pathogens to prevent an exaggerated harmful immune response (12, 15). Thus, it is essential in maintaining intestinal immune homeostasis (73).

6.7. Integrins

Integrins are transmembrane receptors on the cell surface responsible for the homing and adherence of leukocytes. After binding, integrins activate intracellular signalling pathways, contributing to immune defence (74, 75).

Integrin α4β7 is intestinal-specific and facilitates the passage of leukocytes across the blood-intestinal barrier, thus contributing to the homeostasis of the intestinal microbiome and the defence against mucosal pathogens (76).

Integrin α4β1 is found ubiquitously in the body and is not specific to the intestine (77).

6.8. The ARTE-Technique

The antigen-reactive-T cell enrichment- (ARTE) technique allows the direct measurement and quantification of rare antigen-reactive T cells by prior magnetic enrichment. After peripheral blood mononuclear cell (PBMC) isolation, cells are stimulated with a single antigen and express activation markers specific for $CD4^+$ T cells (CD154/CD137). The labelled cells can then be differentiated into Tcon ($CD154^+$) or Treg ($CD137^+$) cells and analysed for their frequency and effector functions by flow cytometry (Fig. 4 (78)).

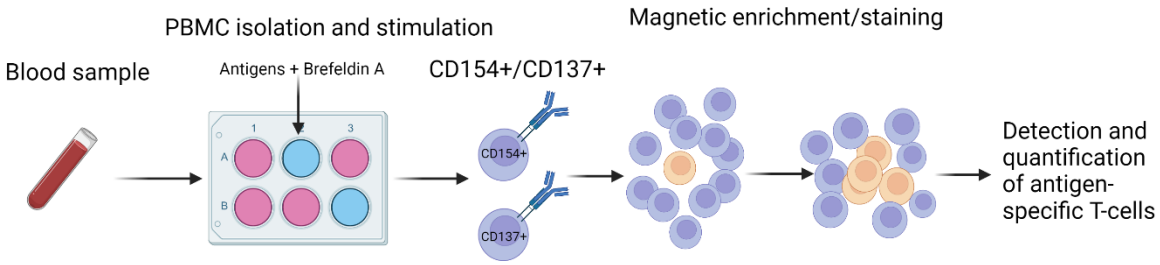

Figure 4: Overview of the ARTE-Technique workflow, created with BioRender.com. Abbreviations: CD – Cluster of differentiation; PBMC – Peripheral blood mononuclear cell.

6.9. Fluorescence Activated Cell Sorting

Flow cytometry involves staining patterns on or in cells with specific antibodies. Fluorescent dyes (fluorophores such as allophycocyanin (APC), peridinin chlorophyll protein (PerCP), possibly as a tandem fluorophore with cyanine (Cy) 5.5 or 7) are coupled to these antibodies (79). Each of these fluorophores has specific excitation and emission spectra. In the flow cytometer, it is possible to infer the antibody/fluorophore complexes bound to a given cell by using light sources of specific wavelengths and filters that pass only certain wavelengths of emitted light. This makes it possible to determine the expression of stained markers (79).

6.10. Hypothesis and aim of study

Impaired intestinal barrier function promotes and maintains the state of sepsis/SIRS. Similarly, impaired gut barrier function is associated with a poor prognosis in terms of mortality and morbidity. Therefore, early and rapid detection of a potential gut barrier dysfunction in ICU patients is inevitable and essential to better integrate this condition into the overall clinical context of critically ill patients. To date, a reliable measure of the gut barrier function is lacking in routine clinical practice (21, 22).

We hypothesise that sepsis patients treated in the ICU suffer from a leaky gut with consequent increased translocation of luminal antigens and increased priming of T cells. Therefore, we aimed to assess the integrity of the intestinal barrier by analysing PBMCs. Using the innovative ARTE technique, we identified rare antigen-reactive T cells in peripheral blood as potential markers of barrier dysfunctions. The functional analysis included a pro- and anti-inflammatory cytokines panel and the gut-homing marker α4ß7. This study investigated the integrity of the intestinal barrier in critically ill patients by a comprehensive analysis of the antigen-reactive T cell compartment, thereby characterising local immunological processes underlying intestinal barrier dysfunctions in these patients.

7. Method

7.1. Ethics Approval

The ethics approval was obtained from the Institutional Review Board of the Charité – Universitätsmedizin Berlin, Germany, with the study number EA4/070/20. All experiments were conducted following the Declaration of Helsinki.

7.2. Probands

We conducted a prospective observational study in a tertiary care medical ICU. A total of 70 patients requiring ICU care were enrolled: 30 with the diagnosis of sepsis or septic shock, 10 patients with Covid-19 and 30 without septic disease. The definition of sepsis was based on the Sepsis-3 Consensus Criteria. Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. In this context, organ dysfunction is present when there is an acute change of at least 2 points on the Sequential Organ Failure Assessment (SOFA) score. Septic shock is defined as sepsis with hypotension vasopressor requirements and lactic acidosis. The non-sepsis patients were ICU patients who did not meet the above sepsis criteria and required ICU care for other indications (e.g., neurological patients with stroke, epilepsy, neuromuscular diseases, myocardial infarction, pulmonary embolism). In addition, ICU patients' gut-specific proinflammatory immune response was compared with that of a healthy population. These 20 patients were not hospitalised at the time of enrollment.

Further, in the context of the Covid-19 pandemic, 10 patients with severe Covid-19 infection treated at the ICU were included in the study.

Exclusion criteria were inflammatory bowel disease, celiac disease, short bowel syndrome and patients with evidence of *Clostridioides difficile* infection. Patients with ostomy were also excluded.

Patient demographic and clinical data have been collected, including age, sex, diagnoses, intensive care scores (SAPS, SOFA), organ replacement procedures, enteral nutrition (type and amount used), gastric reflux and bowel emptying, catecholamine therapy, opiate therapy, microbial examination of blood cultures and antibiotic therapy.

7.3. Blood sampling and routine analysis

Written informed consent was obtained from all patients and healthy volunteers. If a patient had a legal representative, consent was obtained from the representative. Peripheral blood samples were obtained from healthy donors and ICU patients at the Charité – Universitätsmedizin Berlin, Department of Nephrology and Medical Intensive Care, Campus Benjamin Franklin. From ICU patients, 50 ml of blood was collected as part of routine blood sampling through an existing vascular access**.** Other laboratory tests routinely performed in the ICU that were relevant to the study included CRP, leukocytes, creatinine, urea, transaminases, bilirubin, procalcitonin, calprotectin, IgA, Tg antibodies and blood gas analysis. Blood sampling was performed on day 0 of the ICU stay. Blood was collected from healthy participants only for PBMC isolation.

7.4. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinised blood of participants by Ficoll gradient centrifugation. The separated PBMCs were washed once in PBS and once in wash buffer (Medium Mix). Cells were then counted using a Neubauer hemocytometer, adjusted to the desired cell density and resuspended in RPMI1640 (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 5% human AB serum (Sigma-Aldrich, St. Louis, MO, USA). 0,5-1x10^7 PBMCs were then cultured overnight at 36°C. PBMCs were isolated and subsequently stimulated with different commensal antigens. Using the antigen-reactive T cell enrichment (ARTE) technology, respective rare antigen-reactive T cell populations were enriched and characterised by flow cytometry.

7.5. Antigen-reactive T cell enrichment

Antigen-reactive T cells were identified and enriched by applying the ARTE technique. In the first step, PBMCs were stimulated for 6 hours with 1 µg/mL CD40 (Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence or absence of different antigens (SEB, *E. coli*, *C. albicans*, *B. longum;* see Fig. 5). During the last 2 hours, 1 µg/mL brefeldin A (Sigma Aldrich) was added. After 6 hours, cells were divided into original and ARTE samples for each antigen. The originals served as negative controls and were not intracellularly stained. Cells for ARTE analysis were indirectly labelled with anti-CD154 (both cohorts) and CD137 (only cohort 1) biotin antibodies followed by anti-biotin MicroBeads (CD154 & CD137 MicroBead kit, Miltenyi Biotec) and magnetically enriched with magnetic enrichment columns (MS column, Miltenyi Biotec). Surface staining was performed on the MS column. Samples were stained with either panel 1 (cohort 1) or panel 2 (cohort 2; see Tab. 6) (Panel 1: (ARTE-sample): Brilliant Violet 510™ anti-human CD4; beriglobin, Pe/Cy7 anti-human CD8; PBS, Panel 2: Brilliant Violet 510™ anti-human CD4; Pe/Cy7 ß1/CD29; beriglobin; PBS, all from BioLegend (Koblenz, Germany); VioBlue α4/CD49; PE ß7 all from Miltenyi Biotec). The negative fractions of MS columns were stored in a freezing medium at -80° Celsius. The enriched cell fraction (ARTE-sample) was fixed with eBioscience™, FoxP3 staining buffer (Thermo Fisher Scientific, Waltham, MA USA) and stained intracellularly (Panel 1: APC anti-human IFN-γ; APC/Cy7 anti-human IL-2; PerCP/Cy5.5 anti-human TNF-α; all from BioLegend; FITC anti- human CD154 from Miltenyi Biotec; Panel 2: PerCP/Cy5.5 anti-human IL-4; APC anti-human IL-10; APC/Cy7 anti-human IL-17a all from BioLegend; FITC anti- human CD154 from Miltenyi Biotec). The original samples were stained as well. (Panel 1: Brilliant Violet 510™ anti-human CD4; beriglobin, Pe/Cy7 anti-human CD8; PE CD137; PBS, all from BioLegend (Koblenz, Germany), FITC anti- human CD154 from Miltenyi Biotec; Panel 2: Brilliant Violet 510™ anti-human CD4; Pe/Cy7 ß1/CD29; beriglobin; PBS, all from BioLegend; VioBlue α4/CD49; PE ß7; FITC anti- human CD154 all from Miltenyi Biotec). Table 6 summarises concentrations of the respective antibody mixtures.

Figure 5: Schematic of the antigens used. Abbreviations: B. longum – Bifidobacterium longum; C. albicans – Candida albicans; E. coli – Escherichia coli; SEB – Staphylococcal enterotoxin B.

Panel 1			**Panel 2**		
Intracellular Mix	**Mixture**	**Volume**	**Intracellular Mix**	**Mixture**	**Volume**
IFN-γ APC	1:50	1 µL	IL-4 PerCp-Cy5	1:50	1 µL
TNF-a PerCP-Cy5	1:50	1 µL	IL-10 APC	1:50	1 µL
IL-2 APC-Cy7	1:400	0,125 µL	IL-17a APC/Cy7	1:50	1 µL
CD154 FITC	1:50	1 µL	CD154 FITC	1:50	1 µL
FoxP3 PB	1:40	1,25 µL	FixPerm		45 µL
FixPerm		45,625µL			
Surface Mix			**Surface Mix**		
CD4 BV510	1:40	1,25 µL	CD4 BV510	1:40	1,25 µL
CD8 PE-Cy7	1:20	2,5 µL	Alpha 4/CD49 VioBlue	1:50	1 µL
Beriglobin	1:50	1 µL	Beta7 PE	1:50	1 µL
PBS		45,25 µL	Beta 1/CD29 PE-Cy7	1:50	1 µL
			Beriglobin	1:50	1 µL
			PBS		44,75 µL
Original Mix			**Original Mix**		
CD4 BV510	1:40	1,25 µL	CD4 BV510	1:40	1,25 µL
CD8 PE-Cy7	1:20	2,5 µL	Alpha 4/CD49 VioBlue	1:50	1 µL
CD137 PE	1:40	1,25 µL	Beta7 PE	1:50	1 µL
CD154 FITC	1:50	1 µL	Beta 1/CD29 PE-Cy7	1:50	1 µL
Beriglobin	1:50	1 µL	CD154 FITC	1:50	1 µL
PBS		43 µL	Beriglobin	1:50	1 µL
			PBS		43,75 µL

Table 6: Antibody mixtures of Panel 1 and Panel 2. Abbreviations: APC – Allophycocyanin; BV – Brilliant Violet; CD – Cluster of differentiation; Cy – Cyanine; FITC – Fluorescein-5-isothiocyanat; FOXP3 – Forkhead box P3; IFN – Interferone; IL – Interleukin; PB – Permanent Blue; PBS – Phosphate buffered saline; PE – Phycoerythrin; PerCp – Peridinin-Chlorophyll-Protein; TNF – Tumor necrosis factor.

7.6. Flow cytometric cell analysis

Flow cytometry was analysed using the FACS Canto II instrument (BD Bioscience, Heidelberg, Germany). Data were analysed using FlowJo analysis software (Ashland, OR, USA).

7.7. Gating

Using FloJo, cells were first gated to FSC-H and -W and to SSC-H and -W. Subsequently, cells were gated to either $CD4^+$ or $CD8^+$ cells. The $CD4^+$ cells were further differentiated between $CD137^+$/$FoxP3^+$ (Treg) and $CD154^+$ cells (Tcon). For the $CD154^+$T-cell population, cytokine production was measured (IL-2, TNF-α, IFN-y).

$CD8^+$ cells were first divided into $CD137^+$(Treg) or $CD154^+$(Tmem/Teff) cells. Subsequently, $CD154^+$ cells were differentiated into Tte, Tscm, or Tcm based on their cytokine production (Figures 6 and 7; see Cell Differentiation and Cytokine Production section).

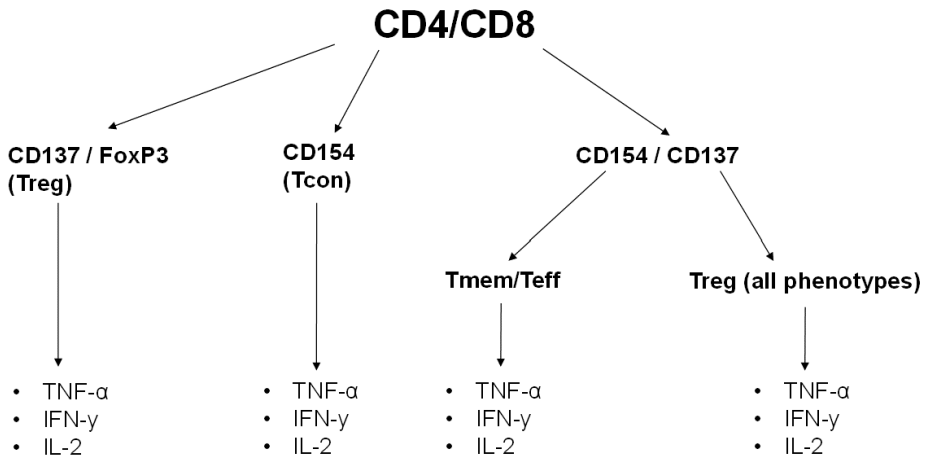

Figure 6: Gating strategy Panel 1. Abbreviations: CD – Cluster of differentiation; FOXP3 – Forkhead box P3; IFN – Interferone; IL – Interleukin; TNF – Tumor necrosis factor.

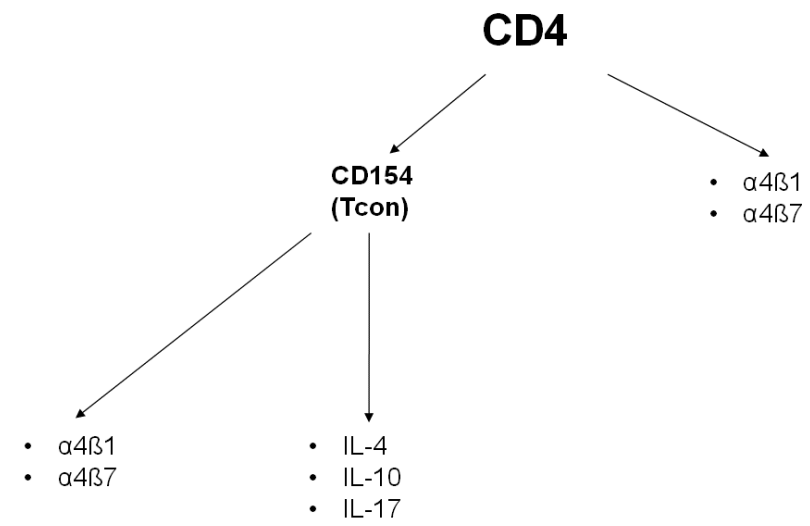

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Figure 7: Gating strategy Panel 2. Abbreviations: CD – Cluster of 34ifferentiation; IL – Interleukin.

7.8. Statistics

Statistical analysis was performed using Prism software (GraphPad Software). In order to obtain an overview of the patient characteristics of sepsis patients, using descriptive statistics, the median and standard deviation of age, sex, organ failure, SAPS II, SOFA score, origin of sepsis, microbiology, catecholamine therapy, leukocytes, CRP, creatinine, and lactate were determined for the measurement. The test for statistical significance was performed using an unpaired t-test in the case of normal distribution, otherwise using the Mann-Whitney-U-Test. When comparing more than 2 groups, the ANOVA (normal distribution) or Kruskal-Wallace test was used. Fisher's exact test was performed to statistically compare the two sepsis subpopulations related to mortality and the need for intubation and catecholamine therapy. Since this study was a pilot study, a correction for multiple comparisons (Bonferroni correction) was omitted. Statistical significance was assumed at $*p<0.05$, $**p<0.005$, $***p<0.001$. A counselling session was held at the Institute of Biometry of the Charité – Universitätsmedizin Berlin.

8. Materials

8.1. Plastics and commodities

Item	Company
6-Well-plate	Corning
PP Tubes 15mL, 50mL	Sarstedt
SepMate Tubes	StemCell
MS-Columns	Miltenyi
FACS-Tubes 5mL	Falcon
Serological pipettes 5mL, 10mL, 25 mL	Greiner bio-one
Pipette tips 20uL, 200 uL, 1000uL	Sarstedt
Vacutainer Lithium/Heparin Tubes	BD
Minisart® filter (0,45 µm)	Sartorius
Cryotubes	Greiner bio-one
Safe-Lock Tubes 1.5 mL	Eppendorf

Table 7: Plastics and commodities used. Abbreviations: MS – Mass spectrometry; PP – Polypropylene.

8.2. Equipment

Type of Equipment	Model	Company
Magnet-Stand	MACS® MultiStand	Miltenyi Biotec
Cell Separator	MiniMACS™ OctoMACS™	Miltenyi Biotec Miltenyi Biotec
Centrifuges	Centrifuge 5810R Multifuge 1 S-R	Eppendorf Heraeus
Flow cytometer	FACS Canto II	BD Biosciences
Incubator	HERAcell 150i	Heraeus
Pipette	Pipetman P10, 20, 200, 1000	Eppendorf
Pipettor	EasyPet	Eppendorf
Water bath	GN 1/3	VWR

Table 8: Equipment used.

8.3. Chemicals and reagents

Chemical / Reagent	Company
Ampuwa water	Fresenius
Brefeldin A	Sigma
DMSO	Sigma
PBS	Gibco
Ethanol	Roth
Fetal Bovine Serum (FBS)	Merck
Human Antibody Serum	Peprotech
Lymphoprep	StemCell
Penicillin / Streptomycin (10 000 U/ml / 10 000 µg/ml)	Merck
RPMI 1640 (1x)	Gibco

Table 9*:* Chemicals and reagents used. Abbreviations: DMSO – Dimethyl sulphoxide; FBS – fetal bovine serum; PBS – Phosphate buffered saline; RPMI – Roswell Park Memorial Institute medium.

8.4. Buffers and media

Medium	Composition
PBS-Buffer	DPBS (1x)
Medium-Mix	RPMI 1640 (1x) 1% Penicillin / Streptomycin
Medium-Mix/AB-Serum	Medium Mix 5% AB-Serum
MACS-Buffer	DPBS (1x) 10% MACS
Fix-Perm	Ampuwa 10% 10Xperm
Perm-Diluent	Perm-Diluent 25% Perm-Concentrate
Freezing Medium	DMSO 90% FCS

Table 10: Buffers and media used. Abbreviations: DPBS – Dulbecco's phosphate buffered saline; MACS – Magnetic cell separation; PBS – Phosphate buffered saline; RPMI – Roswell Park Memorial Institute medium.

8.5. Antibodies

Antibodies used for flow cytometry	**Company**
CD 154 MicroBead Kit	Miltenyi
CD 137 MicroBead Kit	Miltenyi
CD 154 FITC	Miltenyi
CD 137 PE	Biolegend
CD 4 BV510	Biolegend
INF-y APC	Biolegend
TNF-a PerCP-Cy5	Biolegend
IL-2 APC/Cy7	Biolegend
CD 8a PE-Cy7	Biolegend
FoxP3 Pacific Blue	Biolegend
Alpha 4/ CD49 VioBlue	Miltenyi
Beta 7 PE	Miltenyi
Beta 1/CD29 PE/Cy7	Biolegend
IL-4 PerCP-Cy5	Biolegend
IL-10 APC	Biolegend
IL-17a APC7Cy7	Biolegend
Beriglobin	CSL Behring GmbH
Antibodies used for stimulation	
CD40	Miltenyi

Table 11: Antibodies used. Abbreviations: APC - Allophycocyanin; BV - Brilliant Violet; CD - Cluster of differentiation; Cy - Cyanine; FITC - Fluorescein-5-isothiocyanat; FOXP3 - Forkhead box P3; IFN - Interferon; IL - Interleukin; PB - Permanent Blue; PBS - Phosphate buffered saline; PE - Phycoerythrin; PerCp - Peridinin-Chlorophyll-Protein; TNF - Tumor necrosis factor.

8.6. Kits

Kit	Company
BD Cytofix/Cytoperm™	BD Biosciences
CD4 MicroBeads, human	Miltenyi
FOXP3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent	eBioscience
CD 154 MicroBead Kit	Miltenyi
CD 137 MicroBead Kit	Miltenyi

Table 12: Kits used. Abbreviations: BD - Becton Dickinson; CD - Cluster of differentiation; FOXP3 - Forkhead box P3.

8.7. Antigens

Antigen	Company
SEB	Sigma/Aldrich
Escherichia coli	AG Heimesaat/Bereswill
Candida albicans	GREER laboratories
Bifidobacterium longum	AG Heimesaat/Bereswill
Brefeldin A	Sigma

Table 13: Antigens used. Abbreviations: B. longum - Bifidobacterium longum; C. albicans - Candida albicans; E. coli - Escherichia coli; SEB - Staphylococcal enterotoxin B.

8.8. Software

Software	Company
Excel 2016	Microsoft, Redmond, USA
FlowJo 10.1	Treestar, Ashland, USA
Powerpoint 2016	Microsoft, Redmond, USA
Prism 9 for Windows	GraphPad Software, La Jolla, USA
Word 2016	Microsoft, Redmond, USA
OneNote for Windows	Microsoft, Redmond, USA

Table 14: Software used.

9. Results

9.1. Study design

A total of 70 ICU patients (30 sepsis, 30 non-sepsis, 10 Covid-19) were included in the study. In addition, blood was collected from 20 healthy volunteers who were not hospitalised. Of all subjects enrolled in the study, the first cohort was assigned to the first antibody panel (20 sepsis, 20 non-sepsis, 10 Covid-19, 10 healthy controls). After analysing the results of this first cohort, we decided to extend the study with a second cohort to further investigate the systemic immune responses between sepsis and non-sepsis patients in more detail. Therefore, we collected blood samples from another 30 study participants (10 sepsis ICU patients, 10 non-sepsis ICU patients, and 10 healthy controls) and analysed them with the second antibody panel.

Demographics were matched between disease groups and healthy controls (Table 15). The median age in the sepsis- and Covid-19-subgroups was 72 years (± 10 and ± 12). In the non-sepsis and healthy control subgroups, the median age was 71 (± 11 and ± 9). In all subgroups, 60% of patients were female. None of the sepsis or non-sepsis group patients received therapy with corticosteroids or immunosuppressants. In the Covid group, all patients received corticosteroids. The mean SOFA score was significantly higher in sepsis and Covid-19 patients compared to non-sepsis patients (6.7 ± 3.4 and 9.0 ± 2.4 *vs.*1.5 ± 2.0; $p<0.001$). A similar pattern was found for the SAPS score between subgroups. As for the SOFA score, the sepsis and Covid-19 subgroups had significantly higher SAPS scores than non-sepsis ICU patients (52 ± 15 and 53 ± 8.5 *vs.* 35 ± 11*;* $p<0.05$). Neither SOFA nor SAPS scores significantly differed between the sepsis and the Covid-19 subgroups. In the sepsis subgroup, the most common site of involvement was the genitourinary tract, followed by the lungs. In the Covid-19 subgroup, no site other than the lungs was found. The main reasons for ICU admission in the non-sepsis subgroup were cardio-vascular and neurological diseases (e.g. stroke, intracerebral haemorrhage). Approximately one-third of the non-sepsis group presented with a urinary or pulmonary disease focus. The rates of organ failure (OF) and the need for catecholamine therapy differed significantly between the groups. During intensive care therapy, 65% of patients in the sepsis subgroup and 100% in the Covid-19 subgroup had an OF and received catecholamine vasopressor therapy. In contrast, 30% of patients in

the non-sepsis subgroup met the criteria for organ failure (all patients with OF had acute renal failure) and required catecholamine vasopressor therapy.

Microbiological examination of blood cultures revealed a predominantly Gram-negative pathogen spectrum as the primary pathogen in both the sepsis and Covid-19 subgroups, followed by Gram-positive cultures and fungal cultures. Two patients in the sepsis group had positive cultures, but the specific pathogen could not be identified. Because microbiological studies often revealed more than one pathogen per patient with positive blood cultures, Table 16 summarises the distribution of all pathogens detected per subgroup. Gram-negative species included *E. coli*, *Klebsiella pneumoniae (K. pneumoniae), Klebsiella oxytoca (K. oxytoca), P. aeruginosa* and *Proteus mirabilis (P. mirabilis)* (Table 16). Gram-positive species included *Streptococcus pneumoniae (S. pneumoniae)*, *Enterococcus faecalis (E. faecalis), S. aureus*, *Streptococcus agalactiae (S. agalactiae)* and *Streptococcus gorondii (S. gorondii)* (Table 16). Fungal cultures included the detection of *Aspergillus* and *C. albicans* (Table 16). No positive cultures were found in the non-sepsis subgroup.

Blood counts revealed significantly higher levels of leukocytes (16 ± 3 /nl and 19 ± 16 /nl vs. 9 ± 3/nl; $p<0.05$ and $p<0.01$) and CRP (168 ± 99 mg/dl and 174 ± 123 mg/dl vs. 72 ± 112 mg/dl; $p<0.01$ and $p<0.05$) within the sepsis- and Covid subgroups compared to patients in the non-sepsis subgroup (Table 15). In healthy controls, blood was collected for PBMC isolation only. No additional blood was collected for haematology or other clinical parameters. Lactate was analysed as an indicator of microcirculatory dysfunction. It was found to be significantly higher in the sepsis- and Covid-19-subgroups compared to the non-sepsis subgroup (22.5 mg/dl ± 22, 44 and 18.8 ± 5,63 *vs.* 9,8 mg/dl ± 4,56; $p<0.005$). Creatinine levels were also significantly higher in the sepsis and Covid-19 subgroups compared to the non-sepsis subgroup.

	Sepsis	Covid-19	Non-Sepsis	Healthy Control
n	20	10	20	10
Age (mean ± SD)	72 ± 10	72 ± 12	71 ± 11	71 ± 9
Female (%)	60	60	60	60
SOFA-Score (mean ± SD) (p-value)	6,7 ± 3,4 <0.001*	9 ± 2,36 <0.001*	1,5 ± 2	-
SAPS-Score (mean ± SD) (p-value)	52 ± 15 *< 0.05**	53 ± 8,5 *< 0.05**	35 ± 11	-
Origin of sepsis/Focus of disease				-
Abdominal (%)	5	0	0	-
Pulmonar (%)	40	100	20	-
Urinary (%)	55	0	10	-
CNS (%)	0	0	30	-
Cardiovascular (%)	0	0	40	-
Microbiological data (main pathogen)				-
Gram-negative (%)	50	40	0	-
Gram-positive (%)	25	20	0	-
Fungi (%)	15	30	0	-
Positive cultures (%)	10	0	0	-
Organ failure (%)	65	100	30	-
Catecholamine therapy (%)	65	100	30	-
Leukocytes /nl (mean ± SD) (p-value)	16 ± 3 *<0.05**	19 ± 16 *<0.05**	9 ± 3	-
CRP mg/dl (mean ± SD) (p-value)	168 ± 99 *<0.05**	174 ± 123 *<0.05**	72 ± 112	-
Creatinine mg/dl (mean ± SD) (p-value)	1,80 ± 0,94 *<0.05**	2,31 ± 1,45 *>0.05**	1,15 ± 1,14	-
Lactate mg/dl (mean ± SD) (p-value)	22,5 ± 22,44 *<0.05**	18,8 ± 5,63 *<0.05**	9,8 ± 4,56	-

Table 15: Summary of demographic and clinical characteristics of cohort 1. Abbreviations: CNS - Central nervous system; CRP - C-reactive protein; SAPS - Simplified acute physiology score; SD - Standard deviation; SOFA - Sequential organ failure assessment *compared to non-Sepsis.

	Gram-positive	Gram-negative	Fungi
n	*S. pneumoniae* Sepsis: 3 Covid: 2	*E. coli* Sepsis: 5 Covid: 1	*Aspergillus* Sepsis: 1 Covid: 3
n	*E. faecalis* Sepsis: 2 Covid: 1	*K. pneumoniae* Sepsis: 3 Covid: 3	*C. albicans* Sepsis: 2 Covid: 1
n	*S. aureus* Sepsis: 2 Covid:1	*P. aeruginosa* Sepsis: 3 Covid: 2	
n	Methicillin-resistant *S. aureus* Sepsis: 1 Covid:0	*K. oxytoca* Sepsis: 1 Covid: 0	
n	*S. agalactiae* Sepsis: 1 Covid: 0	*P. mirabilis* Sepsis: 1 Covid: 0	
n	*Streptococcus gorondii* Sepsis: 1 Covid: 0		

Table 16: Summary and distribution of all pathogens in the sepsis and Covid Population of cohort 1. Note: Table 16 only shows the primary pathogens. Most of the sepsis patients had evidence of multiple pathogens in blood cultures.

The second cohort included 10 ICU patients with sepsis and 10 ICU patients without sepsis, and non-hospitalized healthy volunteers, as in cohort 1. After Table 15, Table 17 shows the second cohort's main patient characteristics and demographics. The demographics were similar between the subgroups. As in cohort 1, there were significant differences in SOFA and SAPS scores between sepsis and non-sepsis subgroups (7.6 ± 3.6 *vs.* 0.7 ± 0.7; $p<0.001$ and 51.0 ± 17.0 *vs.* 26.0 ± 7.5; $p<0.001$). Similarly, the most common cause of sepsis was pulmonary and urinary tract infections, followed by abdominal infections (50%, 40% and 10%, respectively). As in cohort 1, the primary disease focus in the non-sepsis subgroup was the central nervous system and cardiovascular disease. The significantly higher incidence of organ failure and the need for catecholamine therapy in the sepsis group seen in cohort 1 was confirmed in cohort 2. Leukocyte counts were not significantly different between sepsis and non-sepsis patients as in cohort 1 (10 ± 5 *vs.* 8 ± 2; *n.s.*), CRP levels (189 ± 123 *vs.* 42 ± 60; $p<0.05$), lactate (26.1 ± 21.1 *vs.* 5.8 ± 2.0; $p<0.001$) and creatinine (2.9 ± 2.2 *vs.* 1.0 ± 0.6; $p<0.05$) were significantly higher in the sepsis- compared to the non-sepsis subgroup. Microbiological examination revealed an equal number of Gram-negative and Gram-positive bacteria in the sepsis group as the primary pathogens, followed by positive fungal cultures (Table 17). According to Table 16, Table 18 shows the distribution of species within cohort 2.

	Sepsis	Non-Sepsis	Healthy Control
n	10	10	10
Age (mean ± SD)	72 ± 12	69 ± 15	69 ± 9
Female (%)	60	60	60
SOFA-Score (mean ± SD) (p-value)	7,6 ± 3,6 *<0.001**	0,7 ± 0,7	-
SAPS-Score (mean ± SD) (p-value)	51 ± 17 *<0.001**	26 ± 7,5	-
Origin of sepsis/ Focus of disease			-
Abdominal (%)	10	0	-
Pulmonar (%)	50	10	-
Urinary (%)	40	0	-
CNS (%)	0	60	-
Cardio-vascular (%)	0	30	
Microbiological data (main pathogen)			-
Gram-negative (%)	40	0	-
Gram-positive (%)	40	0	-
Fungi (%)	10	0	-
Positive cultures (%)	10	0	-
Organ failure (%)	70	10	-
Catecholamine therapy (%)	60	0	-
Leukocytes /nl (mean ± SD) (p-value)	10 ± 5 *n.s.*	8 ± 2	-
CRP mg/dl (mean ± SD) (p-value)	189 ± 123 *<0.05**	42 ± 60	-
Creatinine mg/dl (mean ± SD) (p-value)	2,85 ± 2,15 <0.05*	1,03 ± 0,61	-
Lactate mg/dl (mean ± SD) (p-value)	26,1 ± 21,14 *<0.005**	5,8 ± 1,99	-

Table 17: Summary of demographic and clinical characteristics of cohort 2. Abbreviations: CNS - Central nervous system; CRP - C-reactive protein; SAPS - Simplified acute physiology score; SD - Standard deviation; SOFA - Sequential organ failure assessment. *compared to non-sepsis **main pathogen.

	Gram-positive	Gram-negative	Fungi
n	*S. pneumoniae* 2	*E. coli* 3	*Aspergillus* 1
n	*E. faecalis* 2	*K. pneumoniae* 2	*C. albicans* 1
n	*S. aureus* 2	*P. aeruginosa* 1	

Table 18: Summary and distribution of pathogens in the sepsis Population of cohort 2. Note: Table 18 only shows the primary pathogens. Most of the sepsis patients had evidence of multiple pathogens in blood cultures.

9.2. Analysis and functional characterisation of antigen-reactive T cells in cohort 1

As described in detail in the Methods section, antigen-reactive T cells were analysed using the ARTE technique. For detection and functional characterisation in cohort 1, PBMCs were stained with a panel of antibodies directed against lineage markers (CD4, CD8), activation markers (CD154, CD137), cytokines (TNF-α, IFN-γ, IL-2), and the integrin α4β7. Figure 8 shows the frequencies of $CD4^+$ T cells after stimulation with indicated antigens or control (without antigen). The frequencies were not significantly different between sepsis and non-sepsis patients (Fig. 8). non-sepsis patients showed the highest total CD4+ Th cell frequencies for all antigens and negative control.

Following stimulation with the negative control, *E. coli and C. albicans*, the frequencies in non-sepsis patients reached statistical significance compared to the Covid-19 subgroup (Fig. 8; neg. control *p<0.05; E. coli, C. albicans p<0.005).*

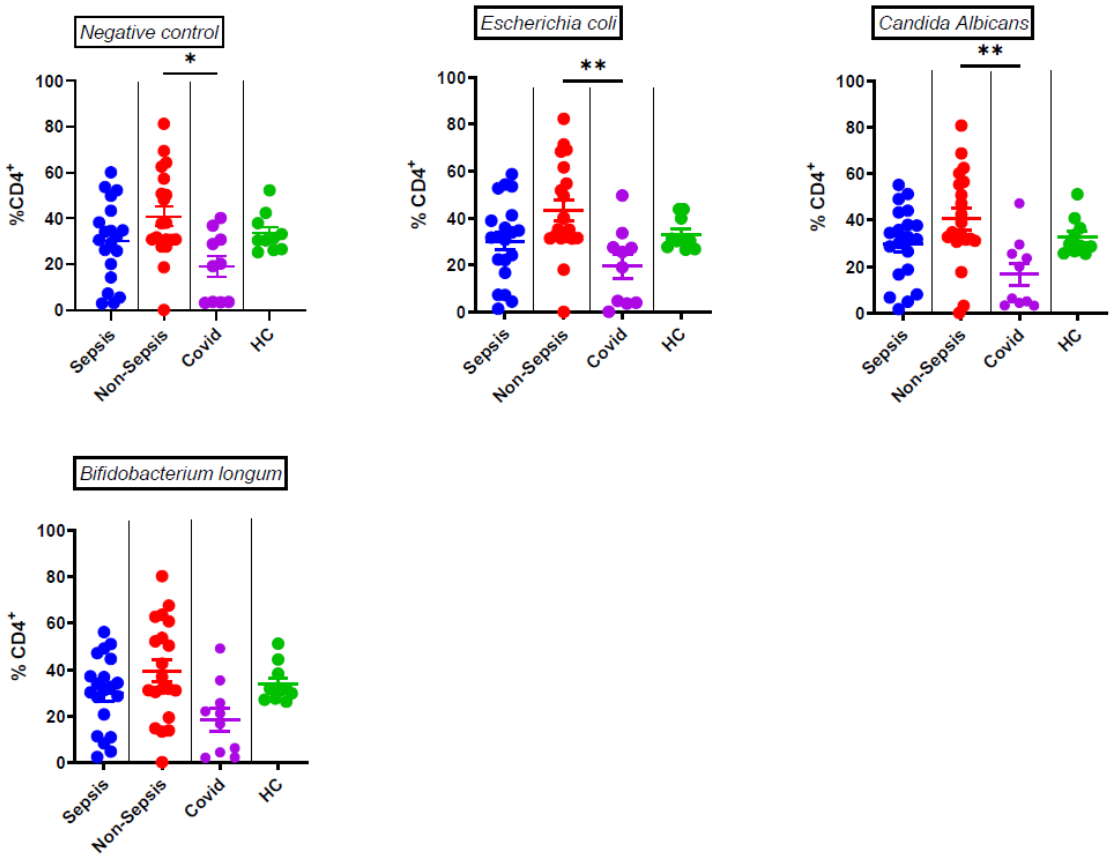

Figure 8: Frequencies of $CD4^+$ T cells Frequencies. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

When looking at absolute cell counts in the different antigen groups and controls, $CD4^+$ Th cells only showed a statistical difference between Covid and healthy control, with the higher numbers in healthy controls for all antigens analysed (Figure 9; all $p<0.005$). The Covid-19 subgroup also showed the lowest absolute CD4+ counts in the unstimulated control group following stimulation with respective antigens.

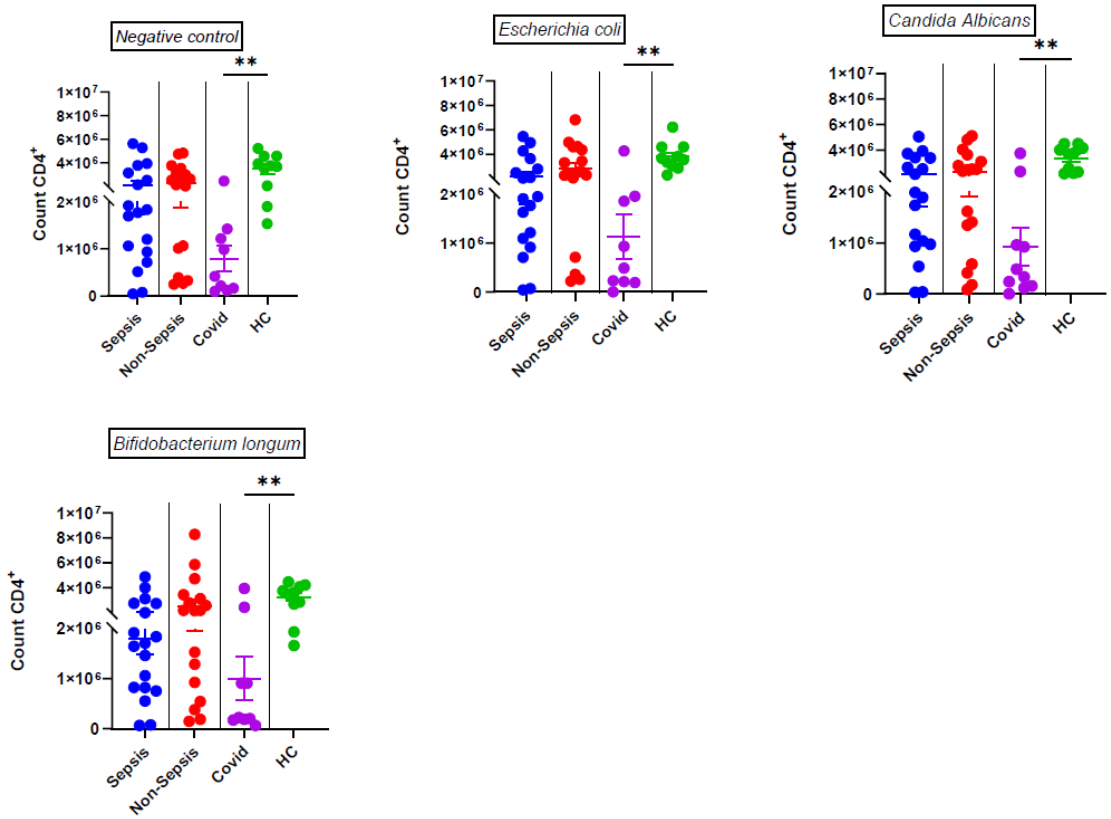

Figure 9: Cell counts of $CD4^+$ T cells, projected to total sample volume. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

Looking at the $CD8^+$ compartment, healthy controls had the highest $CD8^+$ frequencies and absolute cell counts among all patient subgroups studied, reaching statistical significance compared to all other subgroups (Fig. 10, 11). Tables 19 and 20 summarise the significance levels for cell frequencies and cell counts between the subgroups. Neither frequencies nor cell counts of the $CD8^+$ T cell compartment in sepsis patients showed significant differences compared to non-sepsis patients. As with $CD4^+$ T cells, the frequencies of $CD8^+$ T cells tended to be lower in patients with Covid-19, regardless of the antigen stimulation (Fig. 10,11).

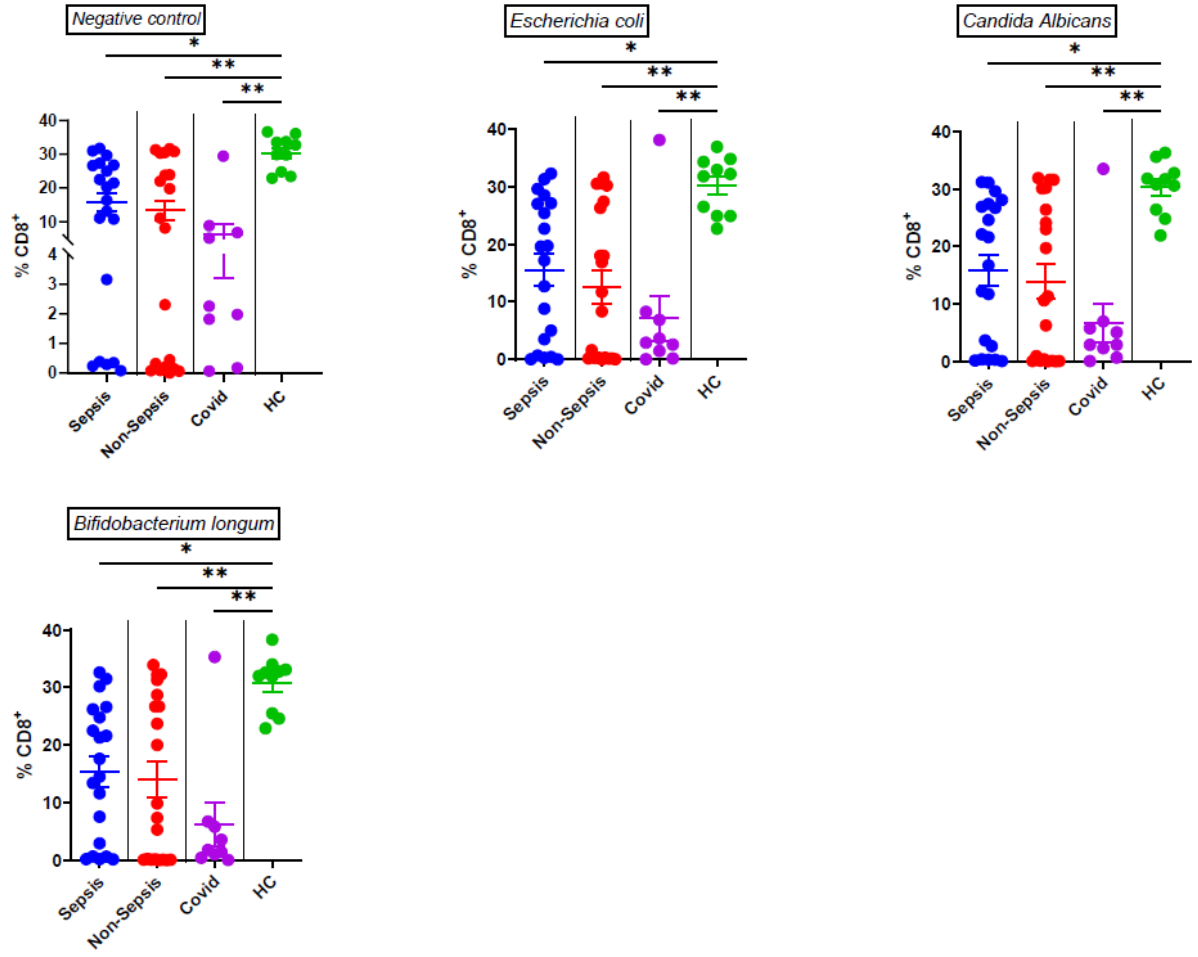

Figure 10: Frequencies of CD8⁺ T cells. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

	Control	***Escherichia coli***	***Candida albicans***	***Bifidobacterium longum***
Healthy control to sepsis (p-Value)	*<0.05*	*<0.05*	*<0.05*	*<0.05*
Healthy control to non-sepsis (p-Value)	*<0.005*	*<0.005*	*<0.005*	*<0.005*
Healthy control to Covid (p-Value)	*<0.005*	*<0.005*	*<0.005*	*<0.005*

Table 19: Levels of significance in frequencies of CD8⁺ T cells between subgroups.

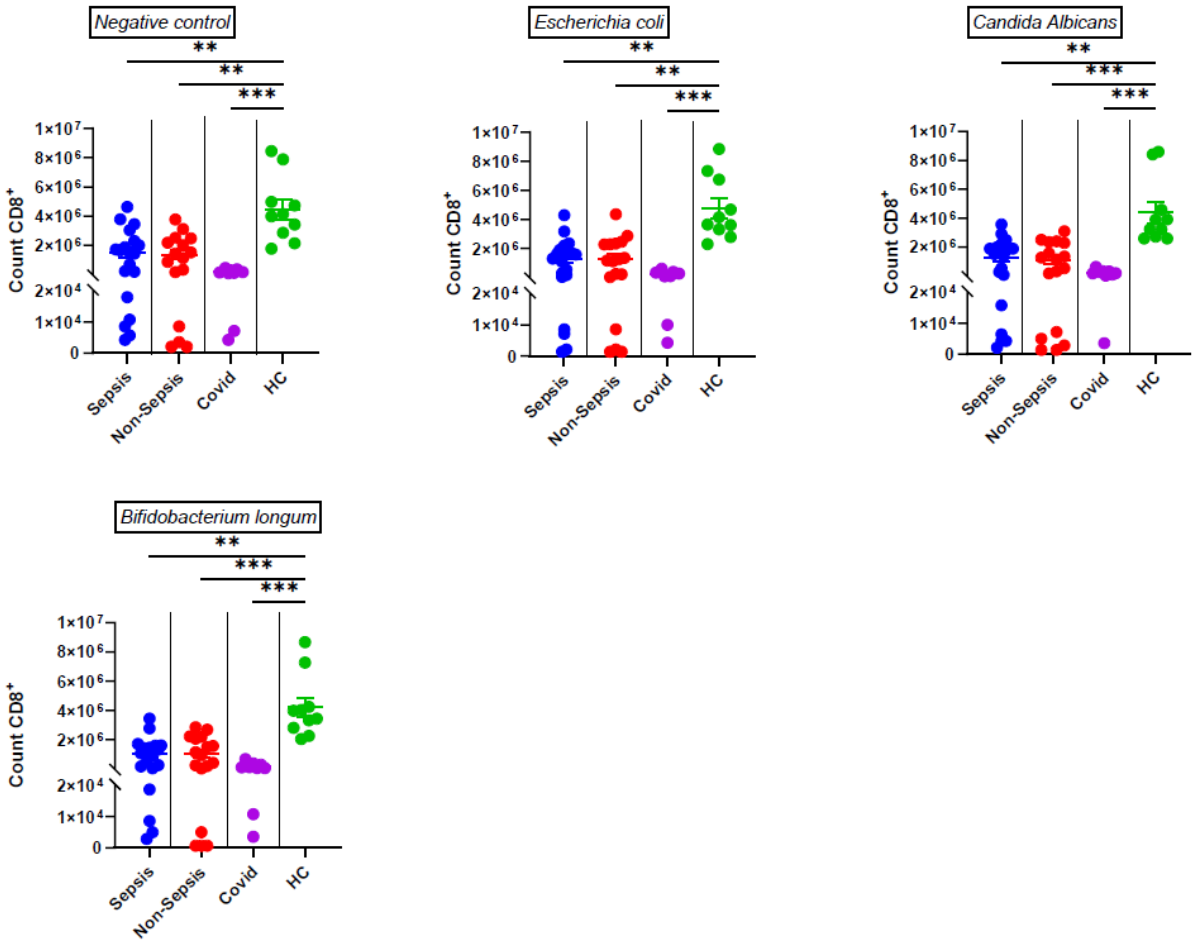

Figure 11: Cell counts of CD8⁺ T cells, projected to total sample volume. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

	Control	*Escherichia coli*	*Candida albicans*	*Bifidobacterium longum*
Healthy control to sepsis (p-Value)	*<0.005*	*<0.005*	*<0.005*	*<0.005*
Healthy control to non-sepsis (p-Value)	*<0.005*	*<0.005*	*<0.001*	*<0.001*
Healthy control to Covid (p-Value)	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Table 20: Levels of significance in counts of CD8⁺ T cells between subgroups.

9.2.1. Antigen-reactive T cells

CD154 is an activation marker used to detect antigen-reactive T cells. Combined with CD4, it allows the detection of antigen-reactive conventional T helper cells (Th cells). Figure 12 shows the frequencies of $CD4^+CD154^+$ in cohort 1 after stimulation with the indicated antigens and control (no antigen). Table 21 shows the significance levels for each antigen between the indicated groups. In cohort 1, sepsis patients had significantly higher frequencies of *E. coli*, *B. longum,* and *C. albicans*-reactive T cells in peripheral blood compared to non-sepsis patients (*p<0.001*, *p<0.005* and *p<0.001*, respectively). There was also statistical significance for *B. longum*- and *C. albicans*-reactive T cells compared to healthy controls (*p<0.001*). The frequencies of *E. coli*-reactive T cells were at a comparable level in sepsis- and Covid-19 patients. As in the sepsis subgroup, the frequencies of *E. coli*-reactive T cells were significantly higher in Covid-19 patients than in non-sepsis patients (*p<0.05;* Figure 12). *B. longum*-reactive T cells were statistically higher in the Covid-19 subgroup compared to healthy controls (*p<0.05*; Fig. 12).

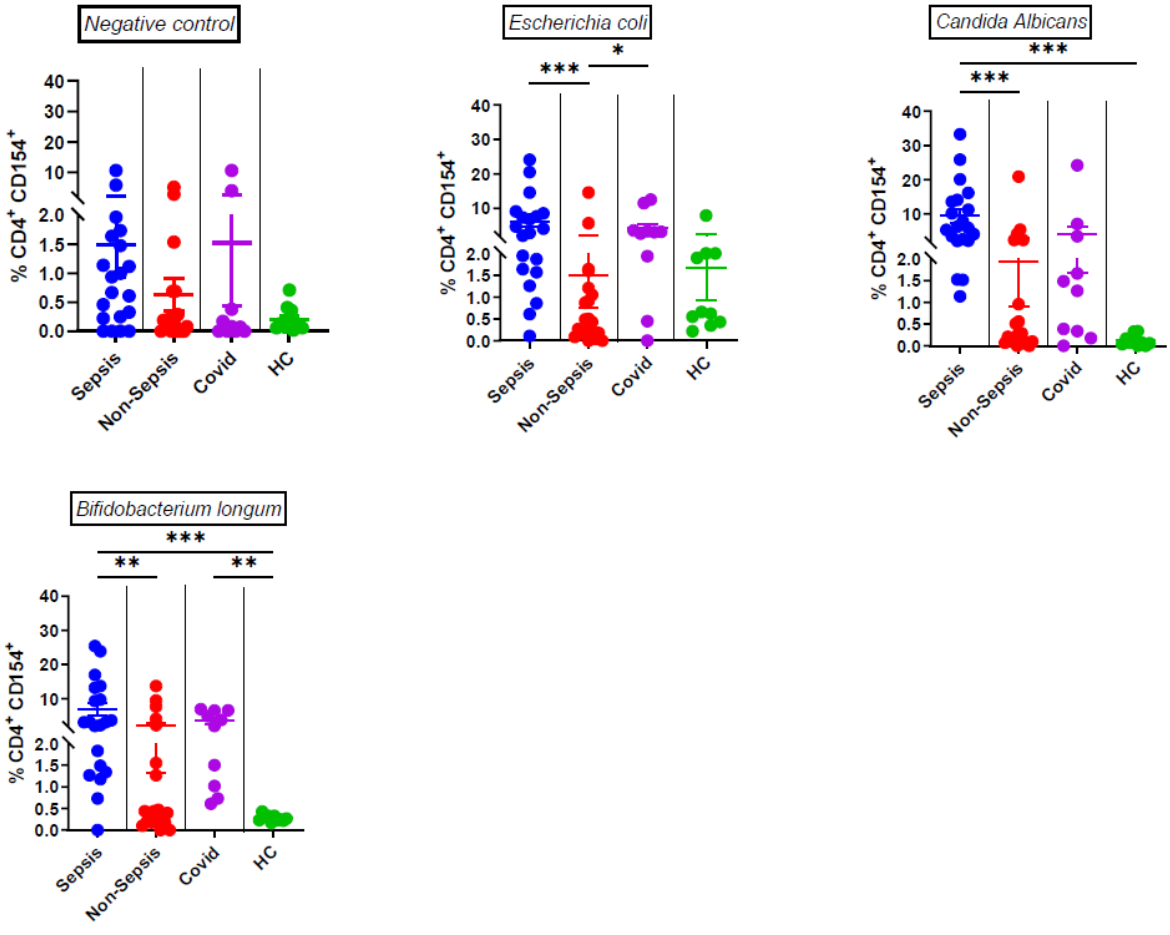

Figure 12: Frequencies of $CD4^+$ $CD154^+$ T cells. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

	Escherichia coli	*Candida albicans*	*Bifidobacterium longum*
Sepsis to non-sepsis (p-Value)	*<0.001*	*<0.001*	*<0.005*
Sepsis to healthy control (p-Value)	*n.s.*	*<0.001*	*<0.001*
Covid to non-sepsis (p-Value)	*<0.05*	*n.s.*	*n.s.*
Covid to healthy control (p-Value)	*n.s.*	*n.s.*	*<0.005*

Table 21: Levels of significance in the CD4⁺CD154⁺ T cell compartment.

Figure 13 displays an overview of the frequencies of CD4⁺CD154⁺ T cells in the sepsis population only. The antigen groups were compared to the control group (no antigen stimulation). There was a significant difference between all 3 antigen groups and the control group in terms of a higher frequency of antigen-reactive Th cells following stimulation with the respective antigen (Fig. 13; *E. coli p<0.005*; *C. albicans p<0.001*; *B. longum p<0.005*). This proves that the sepsis population did not have higher frequencies of antigen-reactive Th cells per se but that the higher frequencies that were detected were due to antigen stimulation.

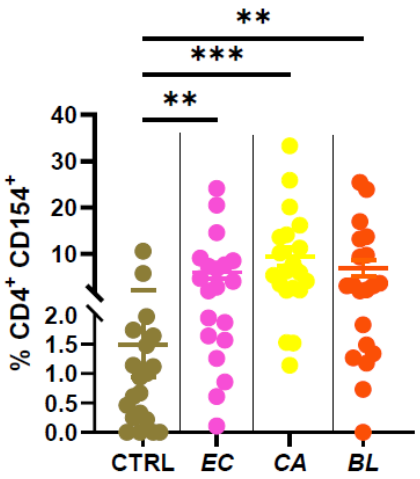

Figure 13: Frequencies of CD4⁺ CD154⁺ T cells in the sepsis-subgroup. Negative control compared to Antigens used for stimulation. Abbreviations: BL - Bifidobacterium longum; CA - Candida albicans; CD - Cluster of differentiation; CTRL - Negative control; EC - Escherichia coli.

9.2.2. Regulatory antigen-reactive T cells

CD137 displays an activation marker expressed on Treg cells and allows detection of the $CD137^+$ activated Treg cell compartment after antigen stimulation. The frequency of anti-inflammatory $CD4^+CD137^+$ T cells ($CD4^+$ Treg cells) showed no difference between the respective populations for all antigen stimulations (Fig. 14).

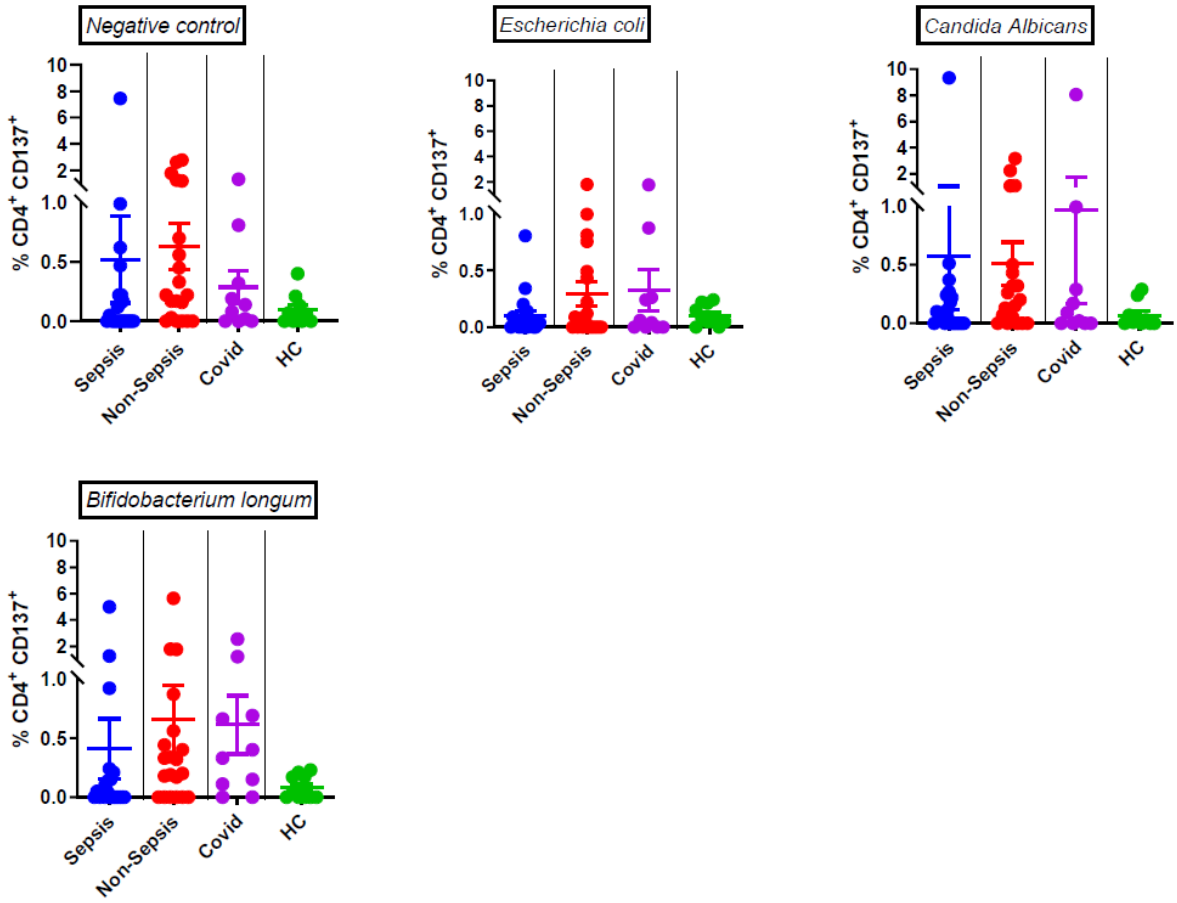

Figure 14: Frequencies of $CD4^+CD137^+$ T cells. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

9.2.3. Functional analysis of antigen-reactive conventional T cells

In cohort 1, IFN-γ, TNF-α, and IL-2 were used to investigate the functionality of the antigen-reactive conventional Th cell compartment. Figure 15 shows the frequencies of IFN-γ-producing antigen-reactive Tcon cells. Compared to non-sepsis patients and healthy controls, sepsis patients had significantly higher frequencies of proinflammatory IFN-γ producing *E. coli* (*p<0.001* and *p<0.05*), *C. albicans* (*p<0.001*, and *p<0.005*) and *B. longum* (*p<0.001* and *p<0.005*)-reactive T cells in peripheral blood (Fig. 15). The frequency of *C. albicans*-reactive T cells was also significantly higher in sepsis patients compared to Covid-19 patients (Fig. 15; *p<0.005*). *B. longum* (*p<0.001* and *p<0.005*)-reactive T cells in peripheral blood (Fig. 15).

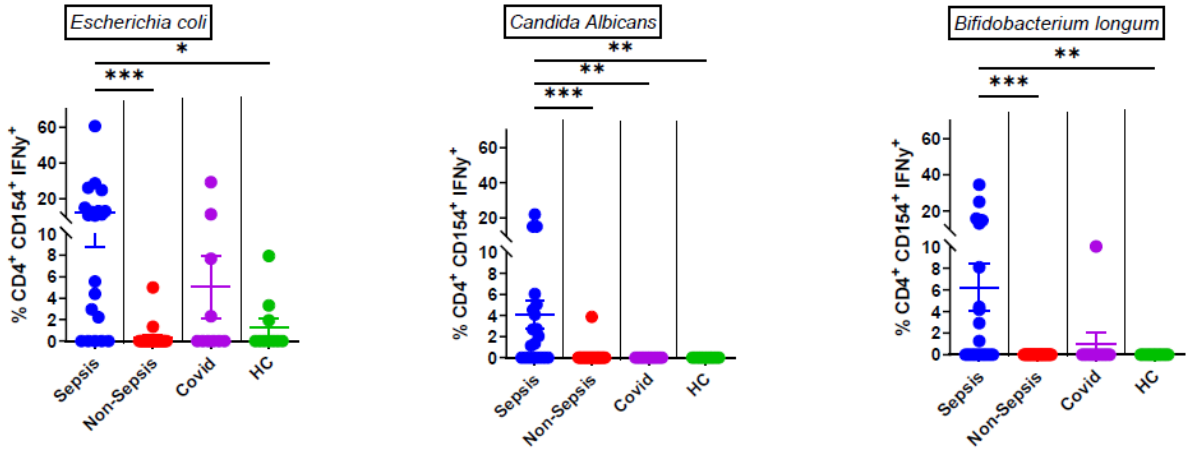

Figure 15: Frequencies of CD4⁺ CD154⁺ T cells producing IFN-y. Abbreviations: CD - Cluster of differentiation; IFN-y - Interferon y; HC - Healthy control.

These differences were also detectable when looking at the IL-2 production in CD4⁺ CD154⁺ T cells (*E. coli: p<0.001*, *p<0.005* and *p<0.005*; *C. albicans: p<0.001*, *p<0.001* and *p<0.001*; *B. longum: p<0.001* and *p<0.005*; Fig. 16).

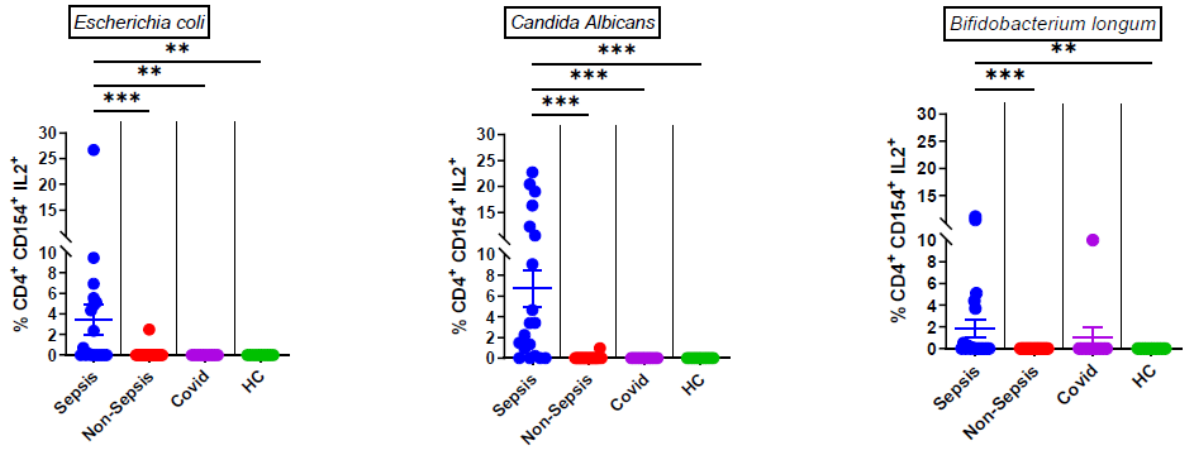

Figure 16: Frequencies of CD4⁺ CD154⁺ T cells producing IL-2. Abbreviations: CD - Cluster of differentiation; IL - Interleukin; HC - Healthy control.

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A similar result was obtained for the TNF-α production in CD4⁺ CD154⁺ T cells (*E. coli*: *p<0.001* and *p<0.005*; *C. albicans*: *p<0.001*, *p<0.001* and *p<0.001*; *B. longum*: *p<0.001* and *p<0.001*; Fig. 17). Table 22 summarises the levels of significance for all three cytokines produced by CD4⁺ CD154⁺ T cells between the sepsis group, the non-sepsis, healthy-control and Covid-19 group.

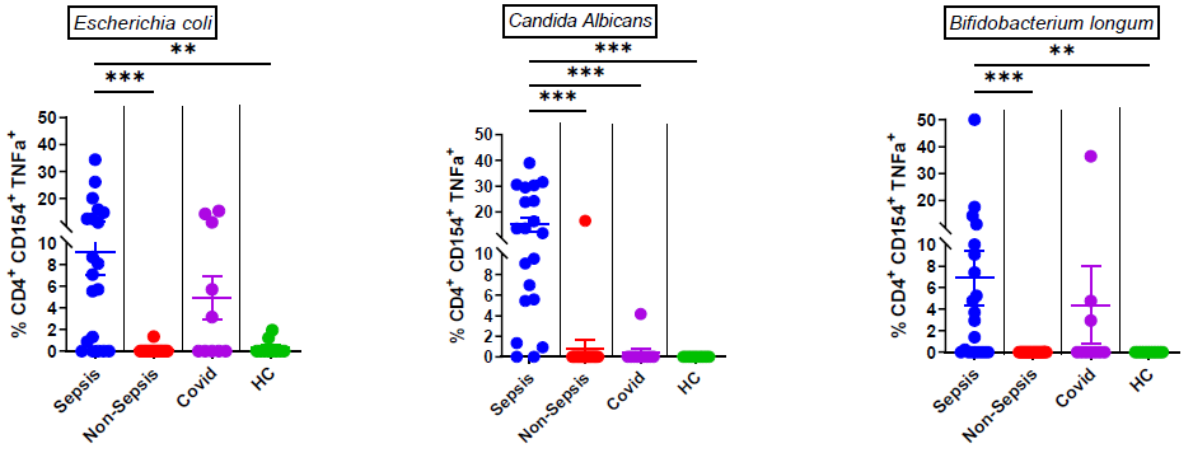

Figure 17: Frequencies of CD4⁺ CD154⁺ T cells producing TNF-α. Abbreviations: CD - Cluster of differentiation; HC - Healthy control; TNF-α - Tumor necrosis factor-alpha.

	Escherichia coli	***Candida albicans***	***Bifidobacterium longum***
IFN-γ Sepsis to non-sepsis (p-Value)	*<0.001*	*<0.001*	*<0.001*
IFN-γ Sepsis to healthy control (p-Value)	*<0.05*	*<0.001*	*<0.005*
IFN-γ Sepsis to Covid (p-Value)	*n.s.*	*<0.005*	*n.s.*
IL-2 Sepsis to non-sepsis (p-Value)	*<0.001*	*<0.001*	*<0.001*
IL-2 Sepsis to healthy control (p-Value)	*<0.005*	*<0.001*	*<0.005*
IL-2 Sepsis to Covid (p-Value)	*<0.05*	*<0.001*	*n.s.*
TNF-α Sepsis to non-sepsis (p-Value)	*<0.001*	*<0.001*	*<0.001*
TNF-α Sepsis to healthy control (p-Value)	*<0.005*	*<0.001*	*<0.001*
TNF-α Sepsis to Covid (p-Value)	*n.s.*	*<0.001*	*n.s.*

Table 22: Levels of significance for indicated Cytokine production in $CD4^+CD154^+$ T cell groups following indicated stimulation. Abbreviations: CD - Cluster of differentiation; IFN-y - Interferon y; IL - Interleukin; TNF-α - Tumor necrosis factor-alpha.

9.3. Analysis and functional characterisation of antigen-reactive T cells in cohort 2

We added another cohort to our study to more detail analyse the CD4+CD154+ T-cell compartment. This second cohort included sepsis ICU patients, non-sepsis ICU patients, and non-hospitalized healthy volunteers as controls (n=10 per group). PBMCs were stained with a panel of antibodies directed against lineage markers (CD4), activation markers (CD154), cytokines (IL-4, IL-10, IL-17), and integrins (α4β7). Figure 18 shows the frequencies of $CD4^+$ T cells. The frequencies were not significantly different between sepsis and non-sepsis patients. As in cohort 1, the frequencies of $CD4^+$ T cells after antigen stimulation and in the unstimulated group tended to be higher in the non-sepsis subgroup than in the sepsis group. For *C. albicans*- $CD4^+$ T cells, there was a significant difference between the sepsis and the healthy control subgroups, with healthy controls showing higher frequencies ($p<0.05$; Fig. 18).

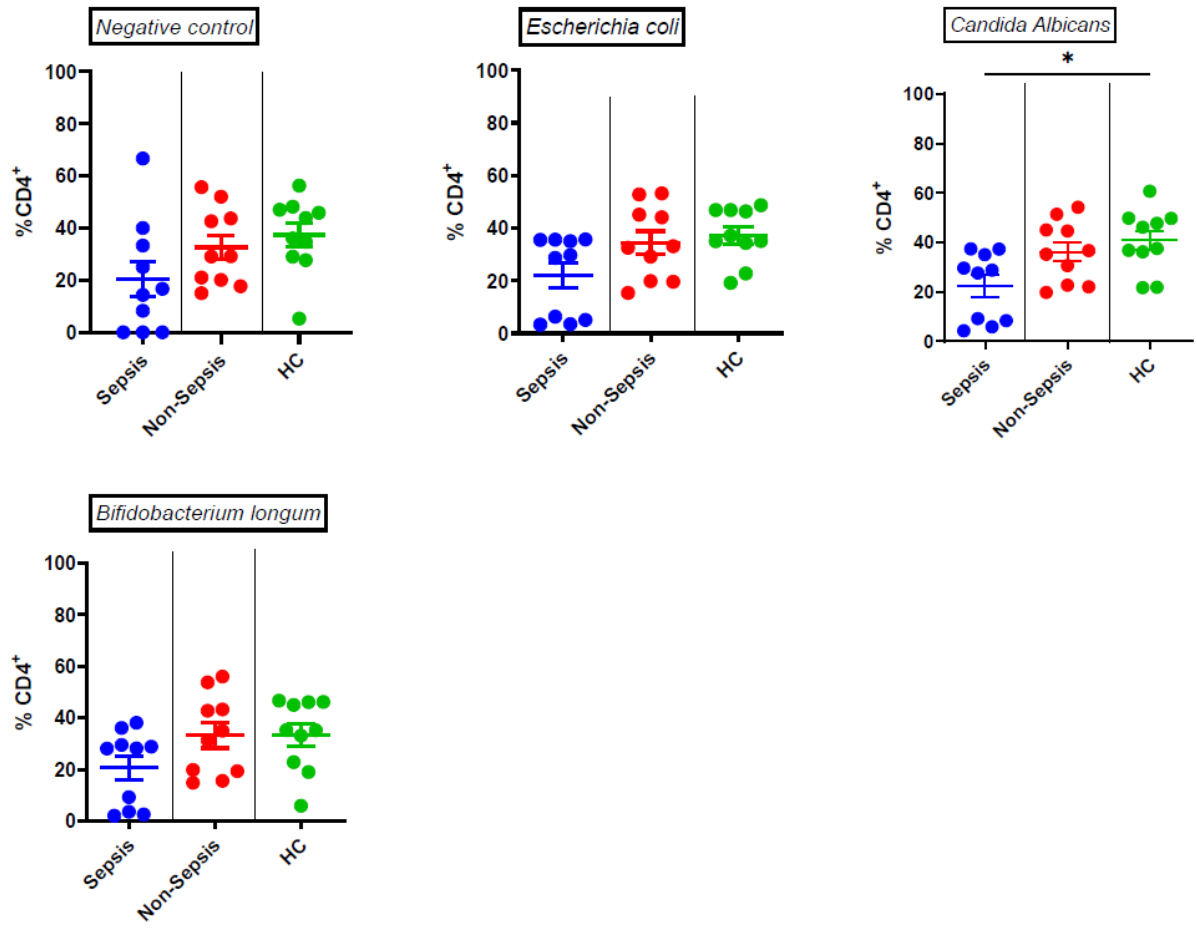

Figure 18: Frequencies of $CD4^+$ T cells in cohort 2. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

The statistically significant findings of the frequency analysis were not reflected in the absolute cell count (Fig. 19), where no significant difference was detected between all groups analysed.

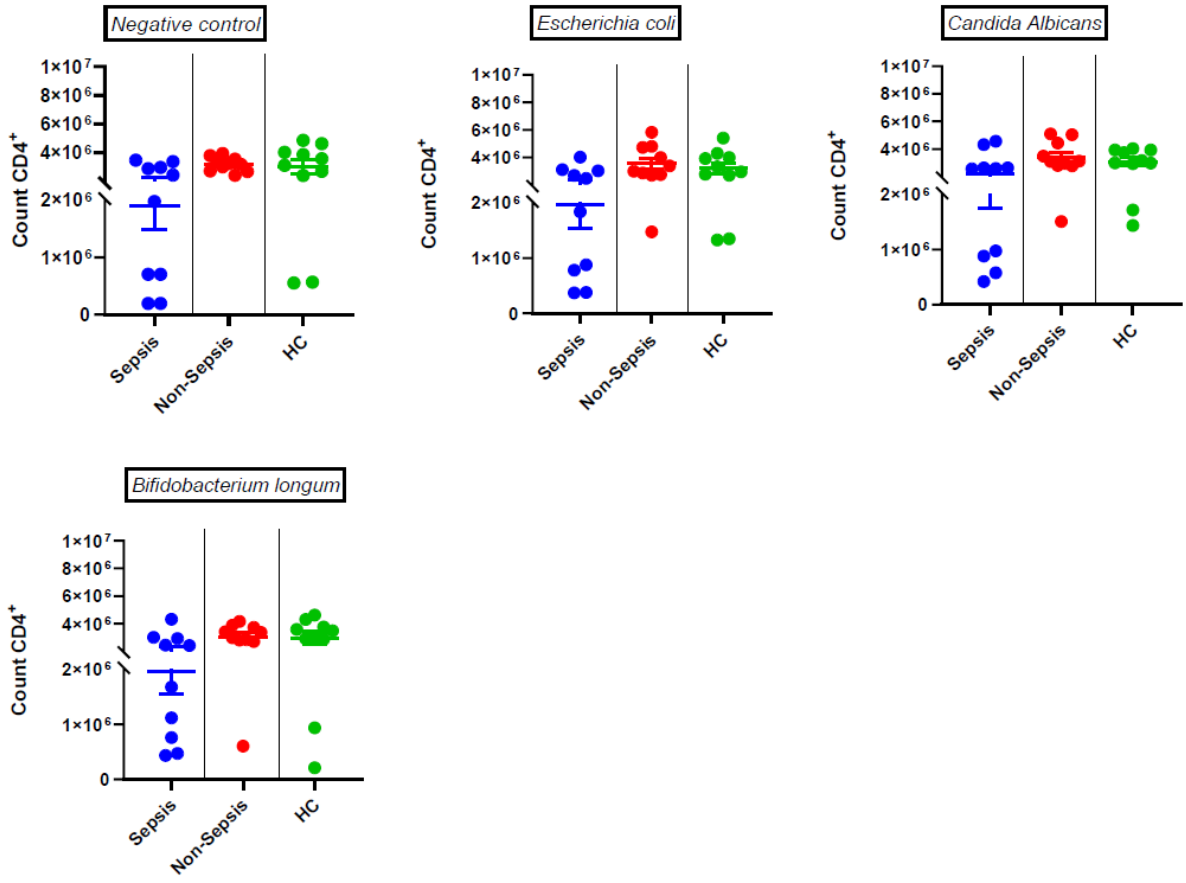

Figure 19: Cell counts of CD4⁺ T cells in cohort 2, projected to total sample volume. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

9.3.1. Antigen-reactive T cells

As in cohort 1, the frequencies of antigen-reactive CD4⁺ T cells were calculated from the total number of CD4⁺ cells obtained after enrichment. Figure 20 shows the frequencies of CD4⁺CD154⁺ in Cohort 2 after stimulation with the respective antigens and negative control. Analyses of the frequencies of CD4⁺CD154⁺ T cells in the second cohort confirmed the results of the first cohort. After stimulation with *E. coli, C. albicans,* and *B. longum*, sepsis patients had significantly higher frequencies of the respective antigen-reactive CD4⁺ T cells in peripheral blood compared to non-sepsis patients (all $p<0.05$; Fig. 20). The frequencies detected in sepsis patients were also significantly higher

compared to healthy controls (all *p<0.001*; Fig. 20). Table 23 summarises corresponding significance levels.

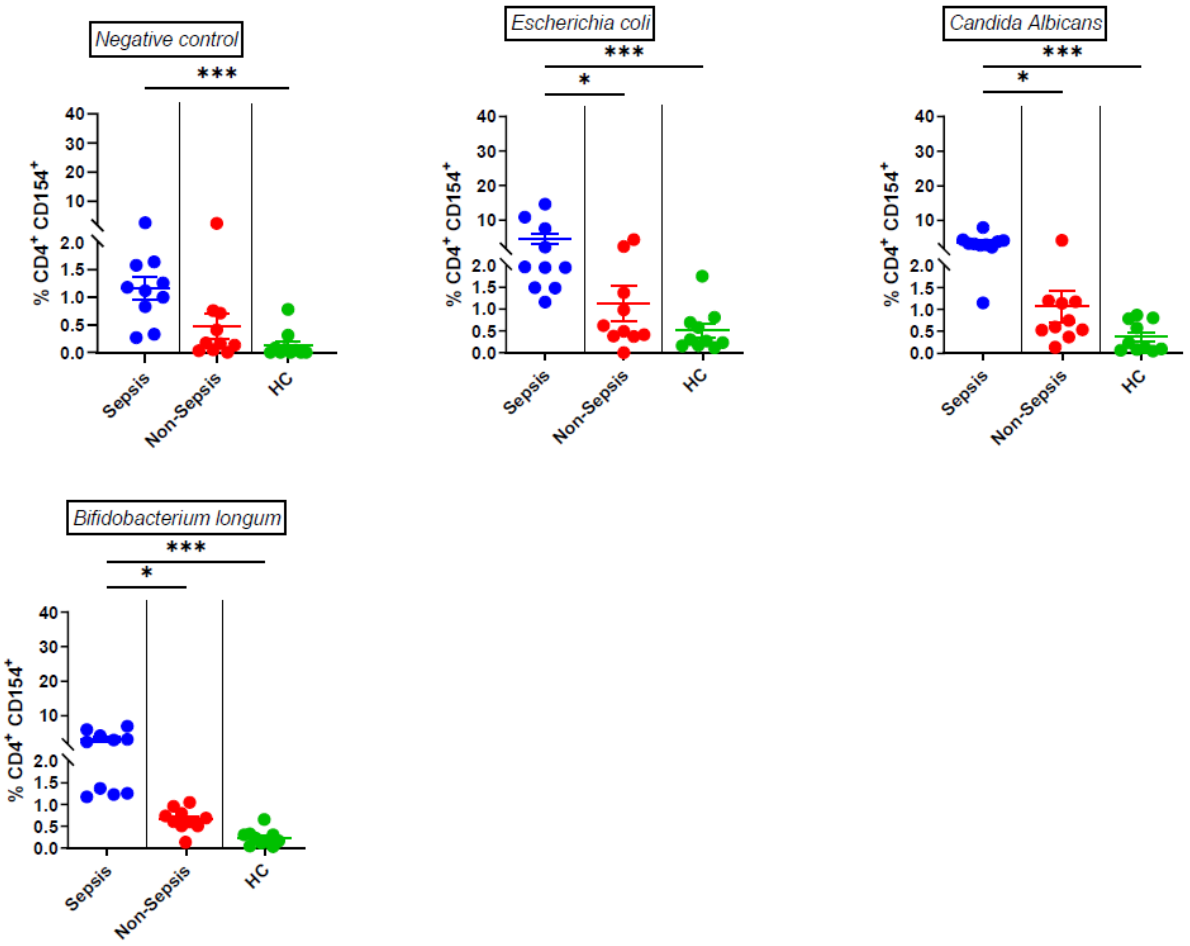

Figure 20: Frequencies of CD4⁺ CD154⁺ T cells in cohort 2. Abbreviations: CD - Cluster of differentiation; HC - Healthy control.

	Control	***Escherichia coli***	***Candida albicans***	***Bifidobacterium longum***
Sepsis to non-sepsis (p-Value)	*n.s.*	*<0.05*	*<0.05*	*<0.05*
Sepsis to healthy control (p-Value)	*<0.001*	*<0.001*	*<0.001*	*<0.001*

Table 23: Levels of significance in CD4⁺CD154⁺ T cells in Cohort 2.

As in cohort 1, stimulation with the respective antigens was responsible for the significant increase in cell frequencies in the sepsis subgroup. (*E. coli p<0.05*, *B. longum p<0.05* and *C. albicans* p<*0.001*; Fig. 21).

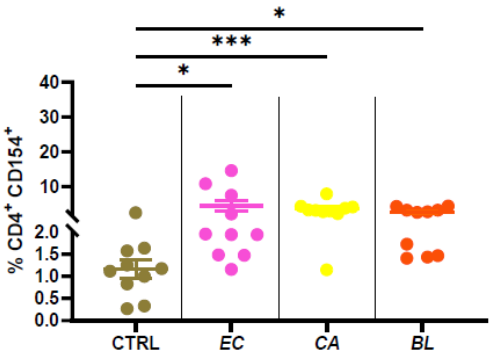

Figure 21: Frequencies of CD4⁺ CD154⁺ T cells in sepsis-patients in cohort 2, negative control compared to respective antigens. Abbreviations: BL - Bifidobacterium longum; CA - Candida albicans; CD - Cluster of differentiation; CTRL - Negative control; EC - Escherichia coli.

9.3.2. Intestinal target markers for antigen-reactive cells

The integrins studied in the second panel were an unspecific target marker (α4β1) and a general intestine target marker (α4β7). For all microbial antigens studied, sepsis patients showed significantly higher CD154+ α4β7+ T cell frequencies than non-sepsis patients and healthy controls (*E. coli*: *p<0.05* and *p<0.001*; *C. albicans: p<0.005* and *p<0.005*; *B. longum*: *p<0.005* and *p<0.001*; Fig. 22). Table 24 summarises the significance levels in the CD154⁺ α4β7⁺ T cell compartment in respective antigen groups.

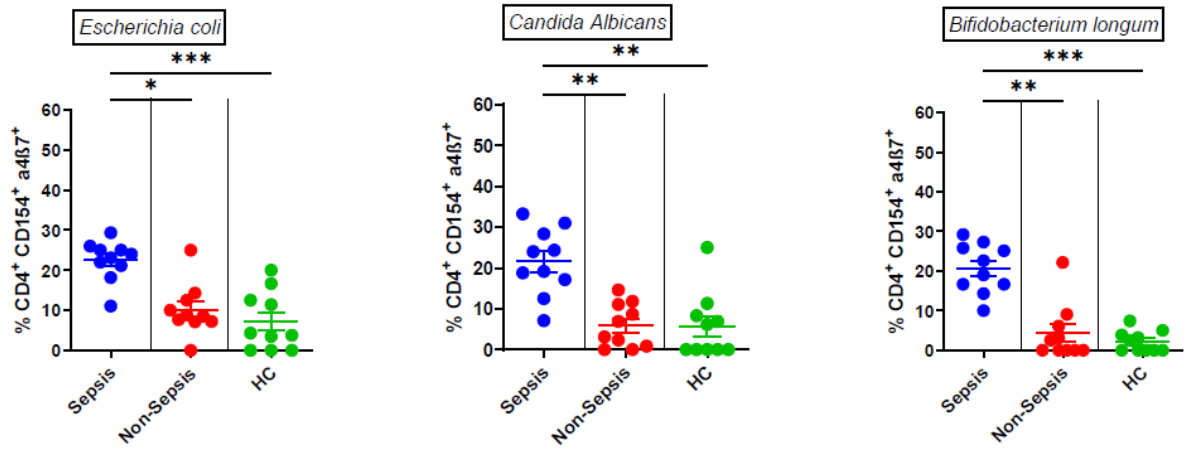

Figure 22: Frequencies of CD4⁺ CD154⁺ a4ß7⁺ T cells. Abbreviations: CD: Cluster of differentiation, HC: Healthy control.

	Escherichia coli	*Candida albicans*	*Bifidobacterium longum*
Sepsis to non-sepsis (p-Value)	*<0.05*	*<0.005*	*<0.005*
Sepsis to healthy control (p-Value)	*<0.001*	*<0.005*	*<0.001*

Table 24: Levels of significance in the CD4⁺CD154⁺ Th cell group for a4ß7⁺.

When looking at the frequencies of α4β1⁺ antigen-reactive CD4⁺ Th cells, for all antigens studied, sepsis and non-sepsis ICU patients showed no difference (not shown).

9.3.3. Functional analysis of antigen-reactive conventional T cells

In the second cohort, IL-4, IL-10, and IL-17 were used for functional analysis of the antigen-reactive T cell compartment. Figure 23 shows the frequencies of IL-4-producing antigen-reactive Tcon cells. Overall, only low levels of IL-4 were detected. The sepsis group had significantly higher frequencies of IL-4-producing antigen-reactive Th cells for all microbial antigens tested compared to the non-sepsis and healthy control groups (*E. coli*: *p<0.005* and *p<0.005*; *C. albicans: p<0.005* and *p<0.005*; *B. longum: p<0.05* and *p<0.05*; Tab. 12; Fig. 23).

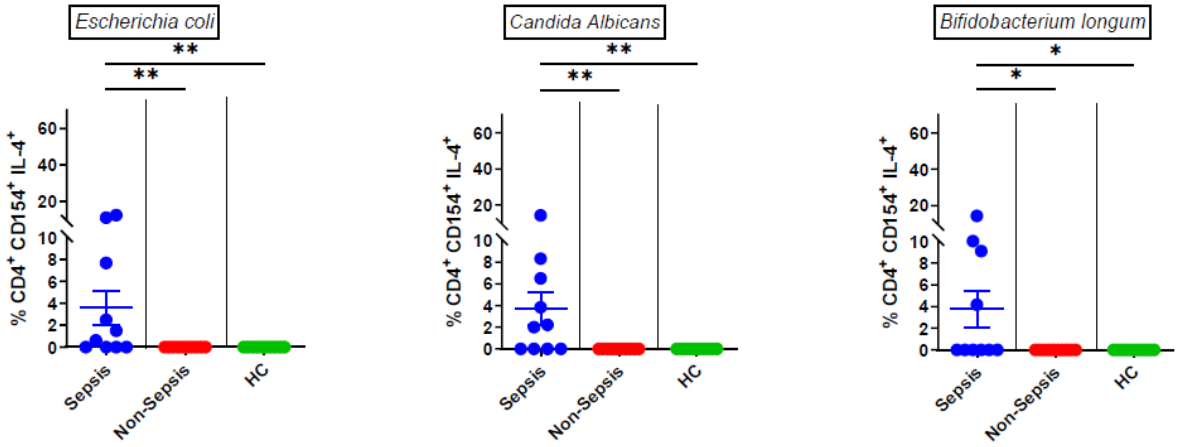

Figure 23: Frequencies of CD4⁺ CD154⁺ T cells producing IL-4. Abbreviations: CD - Cluster of differentiation; HC - Healthy control; IL - Interleukin.

Regarding IL-10, no significant differences could be detected between sepsis and non-sepsis patients and healthy controls. Only for *C. albicans* was a slightly higher frequency trend between sepsis and non-sepsis patients (Fig. 24).

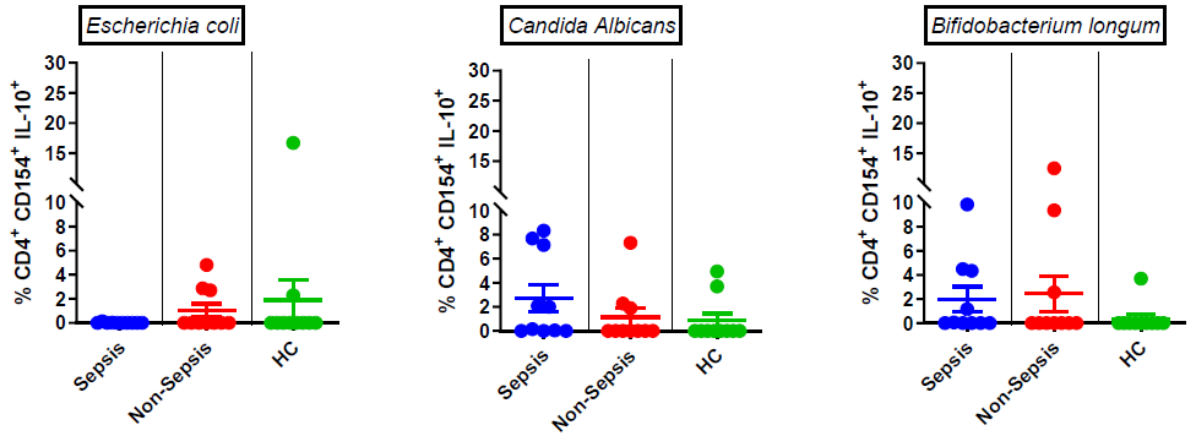

Figure 24: Frequencies of CD4⁺ CD154⁺ T cells producing IL-10. Abbreviations: CD - Cluster of differentiation; HC - Healthy control; IL - Interleukin.

Within cohort 2, we investigated IL-17 production as a proinflammatory cytokine. Figure 25 shows the frequencies of IL-17 positive antigen-reactive T cells. Sepsis patients showed significantly higher levels of *E. coli-, C. albicans*- and *B. longum*- reactive IL-17 producing CD4⁺ T cells compared to healthy controls (*E. coli: p<0.005*; *C. albicans: p<0.05*; *B. longum: p<0.05*; Fig. 25). Moreover, there was a statistically significant difference between non-sepsis patients and healthy control patients for *E. coli*- reactive CD4⁺ T cells (*p<0.05;* Fig. 25).

Table 25 illustrates the significance levels of the three cytokines investigated between the sepsis, non-sepsis, and healthy control groups.

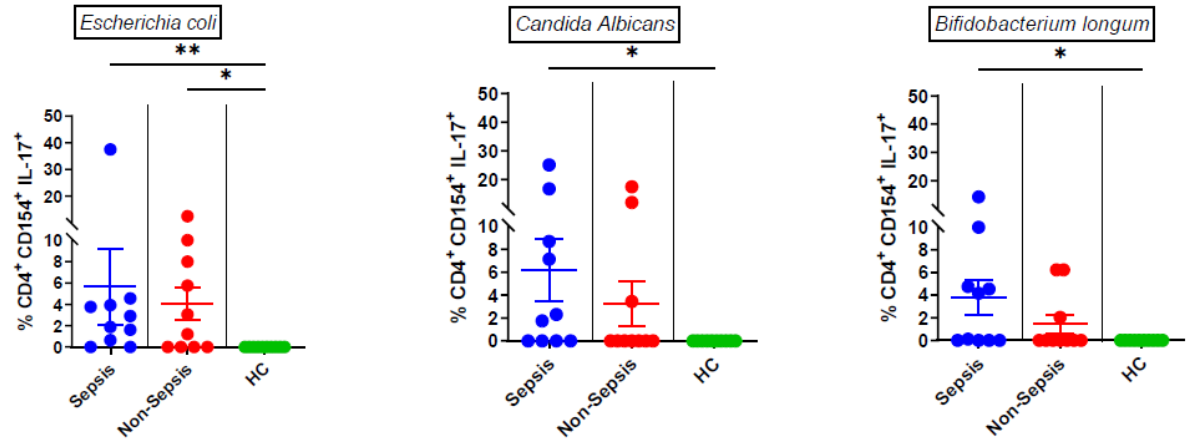

Figure 25: Frequencies of CD4⁺ CD154⁺ T cells producing IL-17. Abbreviations: CD - Cluster of differentiation; HC - Healthy control; IL - Interleukin.

	Escherichia coli	***Candida albicans***	***Bifidobacterium longum***
Il-4 **Sepsis to non-sepsis** (p-Value)	*<0.005*	*<0.005*	*<0.05*
IL-4 **Sepsis to healthy control** (p-Value)	*<0.005*	*<0.005*	*<0.05*
IL-10 **Sepsis to non-sepsis** (p-Value)	*n.s.*	*n.s.*	*n.s.*
IL-10 **Sepsis to healthy control** (p-Value)	*n.s.*	*n.s.*	*n.s.*
IL-17 **Sepsis to non-sepsis** (p-Value)	*<0.005*	*n.s.*	*n.s.*
IL-17 **Sepsis to healthy control** (p-Value)	*<0.05*	*<0.05*	*<0.05*

Table 25: Levels of significance for the IL-17 producing $CD4^+CD154^+$ T cell compartment. Abbreviations: CD - Cluster of differentiation; IL - Interleukin.

9.4. Subpopulations in the sepsis group and their different clinical outcome

A more detailed analysis of the clinical outcomes in the sepsis group showed a trend towards a higher frequency of $CD4^+CD154^+$ T cells for *B. longum-reactive cells* in the deceased sepsis patients compared to the sepsis patients who survived the hospital stay (Fig. 26). The same was observed for cytokine production (IFN-γ, TNF-α) but without reaching statistical significance (Fig. 26). Regarding clinical parameters, the group of deceased patients showed a slightly higher SOFA and SAPS score compared to the survivors but without reaching significant difference. Regarding infection parameters, only the lactate measurement showed a higher average value in the group of deceased patients. However, no statistically significant difference was found in the lactate measurement (Tab. 26).

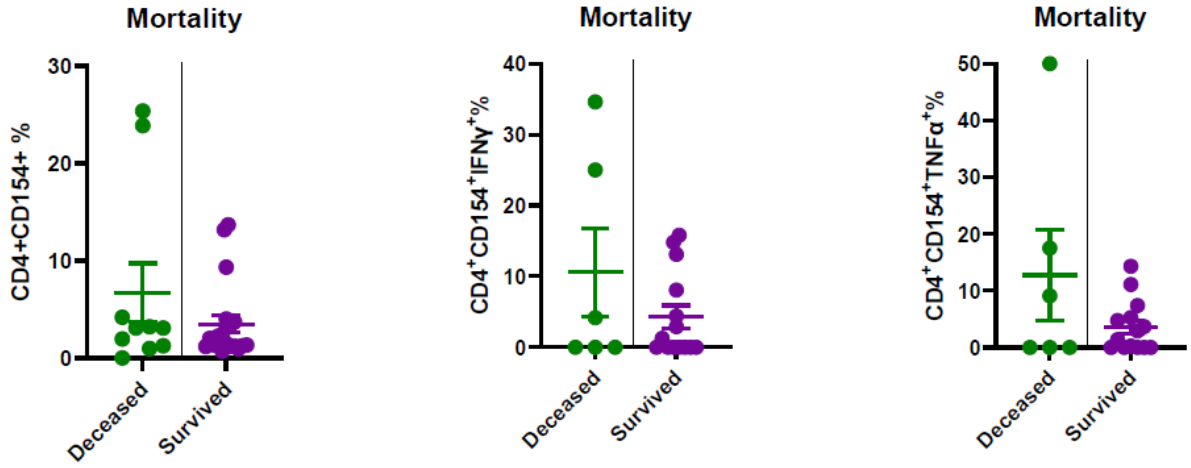

Figure 26: Comparison of the frequencies of $CD4^+$ $CD154^+$ and $CD4^+$ $CD154^+$ T cells producing IFN-γ and TNF-α after stimulation with B. longum between deceased and survivor patients in the sepsis-population. Abbreviations: CD - Cluster of differentiation.

	Deceased	**Survived**
Patients (n)	10	20
Ø SOFA Score	8.2	6.4
Ø SAPS-II Score	58.4	49.2
Ø CRP (mg/L)	149.2	187.5
Ø Leucozytes (/nL)	13.9	13.7
Ø Lactate (mg/dL)	30.4	20.85

Table 26: Characteristics of deceased and survivor patients in the sepsis group. Abbreviations: CRP - C-reactive protein; SAPS - Simplified acute physiology score; SOFA - Sequential Organ failure Assessment

Likewise, higher frequencies of $CD4^+CD154^+$ T cells and cytokine production (IFN-γ, TNF-α) were found in intubated patients after stimulation with *B. longum*, regardless of the clinical outcome (Fig. 27).

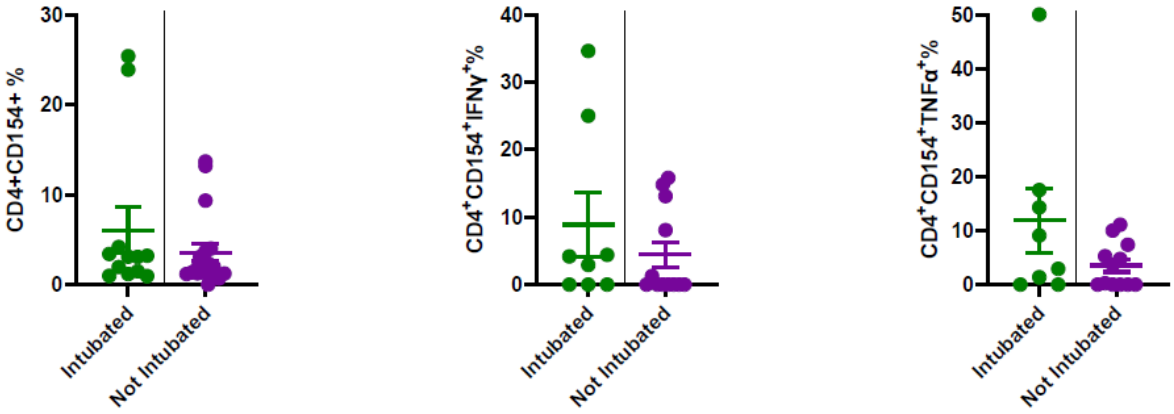

Figure 27: Comparison of frequencies of $CD4^+$ $CD154^+$ and $CD4^+$ $CD154^+$ T cells producing IFN-γ and TNF-α after stimulation with B. longum between intubated and not-intubated patients in sepsis-population. Abbreviations: CD - Cluster of differentiation.

10. Discussion

Sepsis and SIRS are life-threatening conditions. These conditions result in a disruption of the intestinal barrier. At the same time, an intestinal barrier dysfunction can maintain and even promote the pathological state of sepsis/SIRS (18). Therefore, gut barrier dysfunction is associated with increased mortality and a generally poorer outcome in patients diagnosed with sepsis/SIRS. In particular, in patients with sepsis who are admitted to intensive care, there is no established method in current clinical care to assess the function of the intestinal barrier. This would be necessary to evaluate potential therapeutic options. Dysfunction of the intestinal barrier leads to an increased translocation of luminal antigens and subsequent priming of immune cells. We therefore hypothesised that the analysis of systemic, circulating antigen-reactive T cells could provide information on the functionality of the intestinal barrier. We used different members of the commensal microbiota as antigens. Antigen-reactive T cells were analysed and functionally characterised using the ARTE technique.

Our cohort consisted of 90 patients. Seventy patients were admitted to the ICU. Of these, 30 patients were diagnosed with sepsis, 30 without sepsis, and 10 with Covid-19. Twenty patients were healthy controls who were not hospitalised. The most common site of sepsis was urological, followed by pulmonary and abdominal, reflecting the most common sites of sepsis diagnosed in the ICU (80, 81). In non-sepsis patients, the most common disease focus was CNS. The next most common were cardiovascular and pulmonary, also typical primary conditions in the ICU (82, 83). In setting up this pilot study, we started with a cohort of 60 patients (20 sepsis, 20 non-sepsis, 10 Covid-19 patients, all hospitalised at the ICU, and 10 healthy controls who were not hospitalised). Looking at the baseline characteristics and demographics of the first cohort, we saw a similar gender distribution (60%) and a similar mean age in all 4 groups. Therefore, all 4 groups were comparable, and we did not have to consider age or gender bias. We used the SOFA and SAPS scores to diagnose sepsis and calculate the patient's illness and in-hospital mortality. In clinical practice, the SOFA score is an integral part of the diagnostic bundle for sepsis. The SOFA score can detect organ dysfunction in sepsis (84, 85). The SAPS-2 score provides information on the severity of the patient's illness and mortality (36). As expected, the sepsis and Covid groups had higher expected mortality based on SOFA

and SAPS scores and significantly higher inflammatory parameters compared to the non-sepsis group. Elevated lactate in sepsis patients indicated microcirculatory dysfunction, which is also associated with poor prognosis (86), highlighting the severe illness of these patients. Other significantly different laboratory parameters between sepsis or Covid-19 patients and non-sepsis patients included creatinine, leukocytes and CRP, also highlighting the multisystemic nature of sepsis and Covid-19 infection, as well as the higher percentage of organ failure and need for catecholamine therapy in these patients.

The frequency and number of $CD4^+$ and $CD8^+$ T cells were not significantly different between sepsis and non-sepsis patients before T cell enrichment. In addition, after stimulation with the different antigens, there was no significant difference in the frequency and number of $CD4^+$ and $CD8^+$ T cells between sepsis and non-sepsis patients.

Compared to the other populations, Covid had the lowest $CD4^+$ T cell frequencies and counts. This supports the current state of research, which has shown that severe Covid infections in particular lead to blood lymphopenia with a significant reduction in $CD4^+$ and $CD8^+$ T cells (87).

Looking at the antigen-reactive $CD4^+$ $CD154^+$ T cell compartment, sepsis patients had significantly higher frequencies of *E. coli-, C. albicans*- and *B. longum*-reactive T cells in the peripheral blood than non-sepsis patients. The functional characterisation of these cells revealed their pro-inflammatory capacity. IFN-γ, IL-2, and TNF-α production were significantly increased in sepsis patients compared to all other subgroups, indicating an increase in immune cells. This follows from Alverdy's work showing that sepsis patients suffer from intestinal barrier disruption (88). These pro-inflammatory cytokines are evidence of an excessive immune response and a pro-inflammatory state in sepsis (13). With these findings, we were able to show evidence of excessive production of antigen-reactive T cells in response to a specific antigen in sepsis patients.

Looking at the frequencies of, e.g., *E. coli*-reactive T cells, it could be argued that within the first cohort, microbiological investigations revealed growth of *E. coli* in blood cultures of 4 patients in the sepsis subgroup and 1 patient in the Covid-19 subgroup. Therefore, it could not be excluded that the origin of *E. coli* in these patients was not intestinal. In the context of this controversy, we identified *B. longum* as a surrogate for intestinal translocation.

B. longum belongs to the Actinomycetes class and the family Bifidobacteria. It is a Gram-positive, catalase-negative, anaerobic bacterium. It is thought to be one of the earliest microorganisms to colonise the GI tract in neonates, and its presence is considered beneficial for the microbiome (89, 90, 91).

Since none of the sepsis patients had positive blood cultures for *B. longum*, the significantly higher frequency of *B. longum*-reactive T cells in the peripheral blood of sepsis patients (compared to non-sepsis patients and healthy controls) strongly suggested an increased translocation from the intestinal lumen.

When analysing the regulatory antigen reactive T cell compartment, we did not find any significant differences in frequencies between the sepsis and non-sepsis groups, nor in the cytokines studied. The timing of the blood collection in this study may explain this. PBMCs were isolated on day 1 after admission to the ICU. At this early stage, changes in the regulatory immune cell compartment are not expected (92, 93).

In particular, the analysis of frequencies and absolute cell counts before stimulation showed significantly higher values in the healthy subjects than in sepsis, non-sepsis, and Covid-19. These results are in line with the work of Hohlstein, who showed that critically ill patients had lower frequencies and cell counts of $CD8^+$ T cells compared to healthy subjects, which is associated with a worse prognosis (94).

The detection of this antigen-reactive signature in sepsis patients was further confirmed within our second cohort. Within this confirmatory cohort, we enrolled a further 30 patients. Ten patients with sepsis, 10 without sepsis, and 10 healthy subjects. The age and gender distribution was similar to cohort 1. The significantly higher SOFA and SAPS scores seen in sepsis patients were again reflective of the higher mortality in these patients. The demographic, laboratory, and clinical data showed that the two independent cohorts represented a similar patient population. Therefore, the results from cohort 2 were suitable for confirmation and further characterisation of the results from cohort 1.

With regard to the frequencies and cell counts of $CD4^+$ T cells, we confirmed our results from cohort 1. In cohort 2, there was no significant difference between the sepsis and non-sepsis populations before and after stimulation with the selected antigens.

The analysis of the antigen-reactive $CD4^+$ $CD154^+$ T cells confirmed the findings from cohort 1. For all antigens, *E. coli, C. albicans,* and *B. longum,* the frequency of antigen-reactive T cells was significantly higher in the sepsis population than in the non-sepsis population.

The cytokines analysed in cohort 2 were IL-4, -10 and -17. IL-4 and IL-10 are anti-inflammatory cytokines, whereas IL-17 is a pro-inflammatory cytokine (15). As IL-17 is a pro-inflammatory cytokine, mainly produced by Th17 cells and plays a role in the activation of other cytokines, we expected a difference in frequency between sepsis and non-sepsis based on the results of cohort 1 and our literature search (72). Although IL-17 levels were highest in sepsis patients, overall levels were low, and no significant differences were observed between sepsis and non-sepsis patients. Taken together, the proinflammatory cytokine profiles detected in cohort 1 suggest a Th1-driven immune response in sepsis. Our expectation, based on the results of cohort 1 and the literature, that levels of IL-4 and IL-10, which are mainly produced by Th2 cells (41), play a minor role as anti-inflammatory cytokines in sepsis, was confirmed (12). The significantly higher frequencies of $CD4^+CD154^+$ T cells producing IL-4 seen in sepsis patients could be interpreted as an early counterregulatory mechanism – however, given the overall low levels and the distribution within the sepsis subgroup, the differences did not seem biologically relevant.

Integrins are cell surface receptors that facilitate the trafficking and retention of leukocytes (74, 76). The receptor α4β7 is a binding site for lymphocytes to guide them from the blood into the gut. α4β7 is ubiquitously expressed in the intestine (95, 96). We included α4β7 in our second panel to demonstrate the priming of antigen reactive T cells in the gut. The results regarding the expression of the gut homing marker α4β7 were intriguing. Antigen-reactive T cells from sepsis patients expressed significantly higher levels of α4β7. Taken together, this strongly suggests that a leaky gut is the basis for increased priming of the T cell compartment after translocation.

In this study, we characterised a peripheral signature of antigen-reactive T cells, unique to ICU sepsis patients. We extended the functional characterisation by investigating this signature in a confirmatory second cohort. Our study showed that the immune response in gut barrier dysfunction is predominantly $CD4^+$ Th1 cell mediated. The increased frequency of proinflammatory antigen-reactive T cells and their positivity for a common

intestinal homing marker suggested an impaired intestinal barrier in ICU sepsis patients. Analysis of antigen-reactive T cells in peripheral blood may fill the gap in the diagnosis and characterisation of gut barrier function in routine clinical practice.

The analysis and interpretation of the results of the antigen-reactive T cells must be carried out in the context of the fact that in some cases the blood cultures show a positive bacterial count, especially for *E. coli*. In such cases, it is not clear whether the T-cell response to the antigen is caused by an intestinal barrier disorder or by bacteremia in the blood. The antigen used, *B. longum*, was not detected in blood cultures in any of the patients, making it suitable as a surrogate parameter for intestinal barrier dysfunction.

By characterising the population of *B. longum*-reactive T cells in peripheral blood, we identified these cells as a surrogate parameter for intestinal barrier leakage in sepsis patients treated in the ICU. This may provide a new and easily accessible method to gain insight into intestinal barrier function in critically ill patients on admission to the ICU. The frequency and proinflammatory capacity of this *B. longum*-reactive T-cell population are relevant to the clinical outcome in intensive care patients, as evidenced by mortality and the need for mechanical ventilation in all sepsis patients included in our study.

10.1. Covid-19

The Covid-19 patient population is critically ill based on laboratory parameters and scores (SOFA, SAPS). Based on this and the literature review, we expected a "cytokine storm" as a sign of a severe intestinal barrier dysfunction in our studies (33). Analysis of the Covid-19 subpopulation revealed that despite having antigen-reactive T cells comparable to sepsis patients, these patients had a lower positivity for proinflammatory cytokines. This could be explained by the fact that all patients with confirmed Covid-19 infection are immediately treated with dexamethasone on admission to the hospital (97). Other immunosuppressive drugs may be added as therapy progresses. Therefore, this study can only provide limited information on the gut-lung axis in Covid-19 infection. Future studies should address this issue.

10.2. Outlook

To our knowledge, this is the first study to identify and characterise a distinct antigen-reactive T cell compartment in the peripheral blood of ICU patients, with the potential to provide insight into gut barrier function at the time of ICU admission. Furthermore, the presence and functionality of a *B. longum*-reactive T-cell population appears to play a relevant role in the clinical outcome of sepsis patients. This provides an opportunity to expand the knowledge further in future studies. In this regard, future studies could address whether, e.g., early enteral nutrition could alter the pro-inflammatory capacity of these cells. Specific dietary components could also be investigated as potential mediators and therapeutic options.

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12. Declaration of authorship / Eidesstattliche Versicherung

„Ich, Markus, Müller, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Systemic antigen-reactive T cells as a marker for intestinal barrier function in critically ill patients (Systemische antigen-reaktive T-Zellen als Marker für die intestinale Barrierefunktion bei kritisch kranken Patienten)“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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13. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

14. Acknowledgements

Im Folgenden möchte ich mich bei allen Personen bedanken, die mich bei der Erstellung dieser Dissertation unterstützt haben.

Meiner Doktormutter Prof. Dr. Britta Siegmund danke ich für die Aufnahme in die Arbeitsgruppe in Berlin und die Bereitstellung des äußerst interessanten Themas. Mein Dank gilt ihrer Unterstützung, die mir während meiner Zeit als Doktorand entgegengebracht wurde.

Bei meiner Charité-internen Betreuerin Dr. Lea-Maxie Haag möchte ich mich für die sehr hilfsbereite Betreuung sowie für die großartige Unterstützung bei der Eröffnung des Promotionsverfahrens bedanken.

Ebenso danke ich den Gutachterinnen und Gutachtern dieser Arbeit.

Zudem bedanke ich mich bei allen Kolleginnen und Kollegen der AG Siegmund für die fachliche und persönliche Unterstützung während meiner Zeit im Labor. Yasmina Silke-Rodriguez und Dr. Rainer Glauben gilt besonderer Dank für ihre hervorragende vorausgegangene Arbeit an dem ARTE-Projekt. Mein Dank gebührt den Mitgliedern der AG Siegmund, insbesondere Dr. Jörn Ziegler für die Einführung und Unterstützung beim Zell Sorting und Inka Freise, die mir bei allen anfallenden Problemen zur Seite stand. Weiterhin danke ich meinen Kolleg:innen der AG Siegmund Max, Sonja ,Hsiang-Jung, Adrian, Sophiya, Julia und Marcell für die kritische Diskussion meiner Ergebnisse Weiterhin möchte ich mich bei den Kollegen der AG Heimesaat/Bereswill für die Bereitstellung des *B. longum*- und *E. coli*-Antigens bedanken.

Abschließend gilt mein besonderer Dank meinen Eltern Thomas und Ursula, die mich auf meinem bisherigen Weg immer in großem Vertrauen unterstützt und gefördert haben. Meiner Schwester Nicola, danke ich für die Unterstützung bei Fragen zur Statistik. Ebenso meiner Partnerin Helen, die mir während des gesamten Weges der Promotion jederzeit zur Seite stand.

15. Statistics certificate / Bescheinigung Statistik

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Bescheinigung

Hiermit bescheinige ich, dass *Herr Markus Müller* innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- *Termin 1: 07.01.2022*
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Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

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