Hypervirulent *Clostridioides difficile* induces a multifaceted response in human mucosal-associated invariant T cells

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Abstract

Clostridioides difficile infections (CDI) are the major cause of antibiotics-associated colitis with increasing prevalence in morbidity and mortality. Pathogenesis of *C. difficile*-associated colitis (CDAC) is linked to secreted *C. difficile* toxins. Severe CDAC is mostly caused by so-called hypervirulent *C. difficile* strains that are capable to produce the three *C. difficile* toxins TcdA, TcdB, and binary CDT. CDI is an age-associated infection and aged individuals show a substantial increase in CDI incidence. The recently identified mucosal-associated invariant T (MAIT) cells represent an abundant innate-like T cell population in the intestinal mucosa exhibiting anti-bacterial functions. MAIT cells are able to sense metabolites derived from bacterial riboflavin biosynthesis that are presented by the major histocompatibility complex class I-related protein MR1. *C. difficile* is considered to produce riboflavin, but the role of MAIT cells in CDI is largely unknown.

The first part of this thesis addressed the question whether MAIT cells from elderly individuals exhibit functional adaptions potentially contributing to their increased CDI susceptibility. A comparative quantitative proteome approach was used to analyze primary human MAIT cells in healthy young and aged individuals. MAIT cell proteomes of aged individuals did not indicate a global phenotypic switch. However, increased abundances of a few proteins in aged individuals could be detected including STING and BCL2 suggesting that with increasing age MAIT cells might exhibit reduced proliferative capacity. Moreover, donor variations for e.g. HLA components were found significantly higher in aged individuals, which together with reduced proliferative capacity might contribute to increased CDI susceptibility.

In the second part, it was investigated whether *C. difficile* activates human MAIT cells and whether this activation would occur MR1-dependent or -independent. Stimulation with toxin-free *C. difficile in vitro* indeed resulted in robust activation of primary human MAIT cells, which could be validated to depend on MR1 and partly on the cytokines IL-12 and IL-18. Activated MAIT cells were found to produce IFNγ that is known to play a protective role in CDI. However, *C. difficile* stimulated MAIT cell cytotoxicity, which is considered as anti-bacterial but might as well contribute to loss of barrier functions in CDI. Interestingly, cytotoxic MAIT cell response was found even more pronounced following stimulation with hypervirulent *C. difficile* strains. In summary, *C. difficile* provokes strong activation of primary human MAIT cells, which might directly contribute to the immunopathology in CDI.

The third part of this thesis focused on the specific contribution of *C. difficile* toxins TcdA, TcdB and binary CDT to the activation of human MAIT cells. All toxins are known to interfere with host GTPase signaling or actin polymerization following their internalization by different host receptors and were not studied in the context of MAIT cells before. Strikingly, this thesis revealed that TcdA and CDT, but not TcdB selectively

activated the MAIT cell subset in peripheral blood mononuclear cell culture, whereby TcdA induced superior cytotoxicity compared to CDT, and additionally significant IFNγ response. Furthermore, TcdA/ CDT stimulation was found to synergize with the MR1-dependent activation pathway. Eventually, toxin-mediated responses were demonstrated to be MR1-dependent even in the absence of bacterial metabolites. This MR1-dependency revealed a new and so far uncharacterized toxin-specific mechanism to trigger MAIT cell activation and suggests MR1 as a key regulator to modulate MAIT responses in CDI. Simultaneous stimulation with *C. difficile*, TcdA, and CDT in particular enhanced the proinflammatory IFNγ response of MAIT cells. Because IFNγ is known to play a protective role in CDI, increased IFNγ response might reflect a predominantly protective potential of MAIT cells in infections with hypervirulent *C. difficile* strains. In summary, in-depth characterization of *C. difficile*-induced MAIT cell response suggests a multifaceted role of MAIT cells that might be protective but as well detrimental in terms of immunopathology in CDI.

Zusammenfassung

Clostridioides difficile Infektionen (CDI) stellen die Hauptursache für Antibiotika-assoziierte Kolitis dar, die eine zunehmende Prävalenz an Morbidität und Mortalität aufweist. Die Pathogenese von *C. difficile*-assoziierter Kolitis (CDAK) wird mit den sekretierten *C. difficile*-Toxinen assoziiert. Schwere CDAK wird meist durch sogenannte hypervirulente *C. difficile*-Stämme verursacht, die in der Lage sind, drei *C. difficile*-Toxine zu produzieren: TcdA, TcdB und binäres CDT. CDI selbst ist eine altersassoziierte Infektion und ältere Menschen zeigen eine erhöhte Anfälligkeit für CDI. Die kürzlich identifizierten Mukosal-assoziierten invarianten T-(MAIT-)Zellen repräsentieren eine abundante T-Zellpopulation in der Darmschleimhaut, die Merkmale der angeborenen Immunität aufweist und antibakterielle Funktionen ausführen kann. MAIT-Zellen sind in der Lage, Metaboliten aus der bakteriellen Riboflavin-Biosynthese zu erkennen, die über das Haupthistokompatibilitätskomplex Klasse I-verwandte Protein MR1 präsentiert werden. *C. difficile* wird als aktiver Riboflavinproduzent betrachtet, jedoch ist die Rolle von MAIT-Zellen in CDI weitestgehend unbekannt.

Der erste Teil dieser Arbeit befasste sich mit der Frage, ob MAIT-Zellen von älteren Menschen funktionelle Anpassungen aufweisen, die möglicherweise zu ihrer erhöhten Anfälligkeit für CDI beitragen. Ein quantitativer Proteomansatz wurde verwendet, um primäre humane MAIT-Zellen von jungen und alten gesunden Spendern zu vergleichen. Dabei zeigten die MAIT-Zellproteome von ältere Spendern keine globalen phänotypischen Veränderungen. Allerdings konnte eine erhöhte Expression von einigen wenigen Proteinen, inklusive STING und BCL2, bei älteren Spendern nachgewiesen werden. Diese geben einen Hinweis darauf, dass MAIT-Zellen mit zunehmendem Alter eine verringerte Proliferationskapazität aufweisen könnten. Darüber hinaus wurden spenderabhängig signifikant höhere Expressionsstreuungen für z. Bsp. HLA-Komponenten bei älteren Spendern gefunden, was zusammen mit einer verminderten Proliferationskapazität zu einer erhöhten Anfälligkeit für CDI führen könnte.

Im zweiten Teil wurde untersucht, ob *C. difficile* in der Lage ist humane MAIT-Zellen zu aktivieren und ob diese Aktivierung MR1-abhängig oder -unabhängig erfolgen würde. *In vitro* Stimulation mit *C. difficile*, unter Ausschluss der Toxineffekte, führte zu einer robusten Aktivierung der primären humanen MAIT-Zellen, die nachweislich sowohl von MR1 als auch teilweise von den Zytokinen IL-12 und IL-18 abhängig war. Aktivierte MAIT-Zellen produzierten dabei IFNγ, dem eine protektive Funktion in der CDI zugewiesen wird. *C. difficile* induzierte jedoch auch die Zytotoxizität in MAIT-Zellen, die als antibakteriell angesehen wird, genauso gut aber auch zum Verlust von Barrierefunktionen in CDI beitragen kann. Interessanterweise war die zytotoxische MAIT-Zellantwort noch ausgeprägter nach der Stimulation mit hypervirulenten *C. difficile*-Stämmen. Zusammenfassend ist zu sagen, dass *C. difficile* eine starke Aktivierung von primären menschlichen

MAIT-Zellen induziert, die direkt zur Immunopathologie bei CDI beitragen könnte.
Der dritte Teil dieser Arbeit befasste sich mit dem Einfluss der *C. difficile*-Toxine TcdA, TcdB und binäres CDT auf die Aktivierung humaner MAIT-Zellen. Es ist bekannt, dass die Toxine nach ihrer Internalisierung durch verschiedene Wirtsrezeptoren die GTPase-Signalübertragung bzw. die Aktin-Polymerisation des Wirts stören und bisher nicht im Zusammenhang mit MAIT-Zellen untersucht wurden. In dieser Arbeit konnte gezeigt werden, dass TcdA und CDT, jedoch nicht TcdB, die MAIT-Zellpopulation innerhalb der mononukleären Zellen des peripheren Blutes selektiv aktivieren. Hierbei induzierte TcdA im Vergleich zu CDT eine höhere Zytotoxizität und zusätzlich eine signifikante IFNγ-Antwort. Darüber hinaus wurde festgestellt, dass die TcdA/ CDT-Stimulation synergistisch mit dem MR1-abhängigen Aktivierungsweg zusammenwirkt. Schließlich wurde gezeigt, dass die toxinvermittelten MAIT-Zellantworten auch in Abwesenheit von bakteriellen Metaboliten MR1-abhängig waren. Diese MR1-Abhängigkeit deckt einen neuen und bislang nicht charakterisierten toxinspezifischen Mechanismus zur MAIT-Zellaktivierung auf und legt MR1 als ausschlaggebenden Regulator zur Beeinflussung der MAIT-Zellantwort bei CDI nahe. Insbesondere die kombinierte Stimulation mit *C. difficile*, TcdA und CDT verstärkte die proinflammatorische IFNγ-Antwort der MAIT-Zellen. Da bekannt ist, dass IFNγ eine protektive Rolle bei CDI spielt, könnte eine erhöhte IFNγ-Antwort eine vorwiegend protektive Funktion der MAIT-Zellen bei Infektionen mit hypervirulentem *C. difficile* Stämme widerspiegeln. Zusammenfassend ist zu sagen, dass die detaillierte Charakterisierung der *C. difficile*-induzierten MAIT-Zellantwort auf eine vielfältige Rolle von MAIT-Zellen hindeutet, die zwar protektiv, aber hinsichtlich der Immunopathologie bei CDI auch schädlich sein könnte.

Supervision and contribution of student projects

Master student Jessica Scheibel "Characterizing the effect of *Clostridioides difficile* toxins on antibacterial mucosal-associated invariant T cells", Technische Universität Braunschweig, 2019.

Note: Jessica Scheibel has performed *C. difficile* toxin titration experiments and the experiments for basic characterization of the MAIT cell phenotype following stimulation with toxins, in the presence or absence of *C. difficile*, within the framework of the mentioned master thesis.

MD student Steffen Brauns "Characterization of gut-resident mucosal-associated invariant T cells in CDI patients", Otto-von-Guericke University, 2020 (in progress).

Contents

List of Figures

List of Tables

Abbreviations

% (v/v)	Volume percent
Ab	Antibody
ACK	Ammonium chloride potassium
APC	Allophycocyanin
APCs	Antigen presenting cells
bp	Base pairs
BV	Brilliant violet
CD	Cluster of Differentiation
CDAC	*C. difficile*-associated colitis
CDI	*C. difficile*
CDT	*C. difficile* transferase
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Desoxyribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FT	Fourier transform
HZI	Helmholtz Centre for Infection Research
IFN	Interferon
IL	Interleukin
MBL	Medical and Biological Laboratories
MHC	Major histocompatibility complex
MHH	Hannover Medical School
mRNA	Messenger ribonuleic acid
MSD	mean standard deviation
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanin7
PerCp-Cy5.5	Peridinin chlorophyll protein-cyanin5.5
PFA	Paraformaldehyde

pH	Potential of Hydrogen
PRR	Pattern recognition receptors
RBD	Receptor-binding domain
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
TCR	T cell receptor
TEAB	triethylammonium bicarbonate
Th1	T helper 1 cells
Th17	T helper 17 cells
TLR	Toll-like receptor
TMT	Tandem mass tagging
Treg	Regulatory T cell
unk.	Unkown
w/o	Without

Standard SI units and abbreviations of chemical elements were used. 'H_2O' always stands for purified, deionised water.

1 Introduction

1.1 *Clostridioides (Clostridium) difficile* infections

Discovered in 1935 as a commensal of healthy newborns (Hall Ivan C., 1935), the Gram-positive, spore-forming, strictly anaerobic gut bacterium was initially called *Bacillus difficilis* but later renamed to *Clostridium difficile (C. diffcile)*. Until the 1970s *C. difficile* was considered to be a part of the normal intestinal microbiota. When antibiotic treatment was introduced, the number of patients suffering from diarrhea and pseudomembranous colitis after clindamycin treatment increased (Tedesco et al., 1974). In the late 1970's *C. difficile* was identified as the causative pathogen for antibiotic-related pseudomembranous colitis (Bartlett et al., 1978). In 2016, *C. difficile* was reclassified as *Clostridioides difficile*, while maintaining the common name as *C. difficile* (Lawson et al., 2016). Because *C. difficile* is a strictly anaerobic bacterium, the vegetative cells are unable to survive in an aerobic environment. By producing aerotolerant dormant spores *C. difficile* is resistant against oxygen, but also against heat and commonly used ethanol-based disinfectants, which represents a drawback for preventing pathogen spreading in health care institutions (Dyer et al., 2019; Deakin et al., 2012; Rodriguez-Palacios and LeJeune, 2011). The spores are transmitted via the fecal-oral route into the patient's gastrointestinal tract and germinate in the intestine. The antibiotics-associated elimination of the intestinal microbiota that under normal conditions are competitors that prevent *C. difficile* outgrowth further supports the colonization and expansion of *C. difficile* in the intestine, which represents an important prerequisite for the manifestation of *C. difficile* infection (CDI).

Clinical symptoms of CDI can range from mild diarrhea to severe pseudomembranous colitis, toxic megacolon and even death (Chumbler et al., 2016; Olsen et al., 2015; Sayedy, 2010). In 2011, CDI reached highest clinical relevance as nosocomial infection and even replaced infection with methicillin-resistant *Staphylococcus aureus* (MRSA) (Miller et al., 2011). The economic burden for the German health-care system was determined as 464 million €/year (Reigadas Ramírez and Bouza, 2018). In 2012, the CDI incidence in Germany was estimated 83 cases per 100,000 people and it is still increasing (Stausberg, J., 2016). Mortality rate (within the follow-up period of 1 year) was determined as 13.5 % in inpatients with primary diagnosis of CDI and 7.1% in outpatients (Lübbert et al., 2016). Newly emerging, so-called hypervirulent *C. difficile* strains, are responsible for outbreaks and cause severe *C. difficile*-associated colitis (CDAC) with increased recurrence, morbidity, and mortality (He et al., 2013; Kuijper et al., 2008). Major risk factors to acquire CDI are long-term hospital stays, antibiotic therapy, immunosuppression and especially the age. For patients that are older than 65 the CDI incidence substantially increases and thus CDI is considered to be age-associated (Fig. 1, Lessa (2012)).

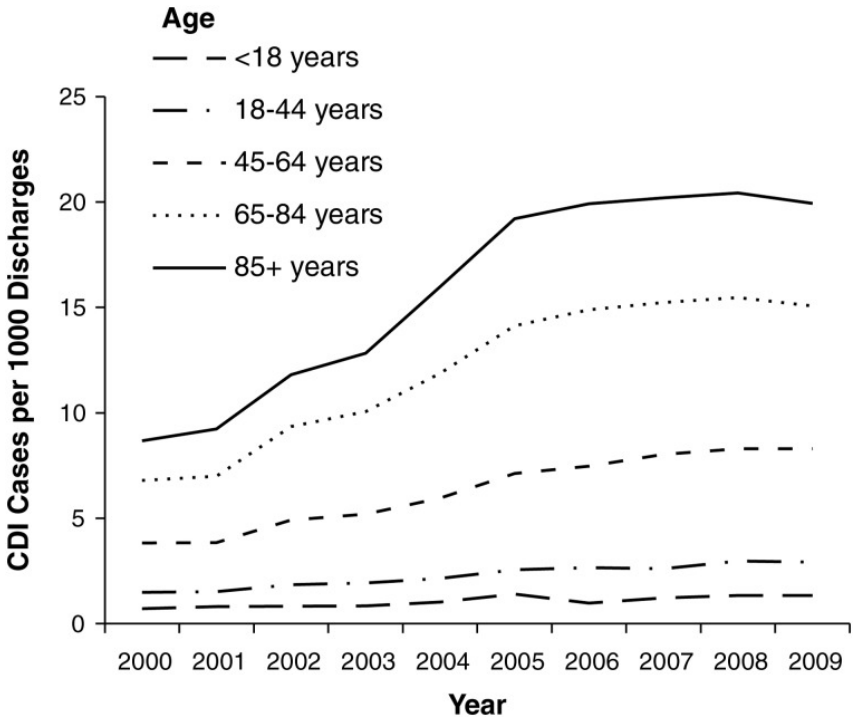

Figure 1: Increase of Age-associated CDI incidence in the years 2000 to 2009. Patient > 65 years of age show substantially increased CDI incidence. From Lessa (2012).

1.1.1 *C. difficile* pathogenicity

In general, pathogenesis is associated with *C. difficile* Toxin A (TcdA) and Toxin B (TcdB) which are secreted during acute CDI. Hypervirulent *C. difficile* strains are able to produce a third binary toxin, the *C. difficile* transferase (CDT). However, the role of CDT in CDI is still not fully understood (Cowardin et al., 2016a; Kasendra et al., 2014; Geric et al., 2006). The genes encoding TcdA and TcdB are organized and regulated in a chromosomal region of 19.6 kb that is called pathogenicity locus (PaLoc). However, TcdA and TcdB production is also regulated by environmental factors such as nutrient supply and temperature as well as antibiotic treatment (Karlsson et al., 2003; Yamakawa et al., 1996). *In vitro*, TcdA and TcdB are primarily produced in stationary growth phase of the bacteria (Hofmann et al., 2018). In contrast to TcdA and TcdB, the expression of CDT is organized in a separate 6.2 kb gene region, the Cdt locus (CdtLoc) (Carter et al., 2007; Perelle et al., 1997)).
In addition to the *C. difficile* classification as toxinotypes based on PaLoc and CdtLoc, information obtained from rRNA-based phylogenetic analyses is applied to discriminate different *C. difficile* ribotypes (RTs). This so-called ribotyping is used for individual finger printing of clinical isolates to better understand and control the spreading of *C. difficile*. Strains of ribotype RT084 do not produce any toxin and are prototypic non-toxigenic strains, which are found to be prevalent in symptomatic patients in sub-Saharan Africa (Janssen et al., 2016). The first fully sequenced and annotated *C. difficile* strain was a TcdA/B-toxigenic *C. difficile* strain of RT012 and its genome still serves as reference (Sebaihia et al., 2006). *C. difficile* strains of RT027 or RT023 are defined as hypervirulent

strains because they produce TcdA, TcdB, and CDT (Duerden et al., 2001). *C. difficile* strain of RT027 caused several outbreaks in the developed world with substantial morbidity and mortality (Arvand et al., 2014; He et al., 2013; Kuijper et al., 2008), whereas *C. difficile* strains of ribotype RT023 were responsible for recurrent CDI in Sweden (He et al., 2013).

1.1.2 *C. difficile* toxins

The two large *C. difficile* toxins, TcdA and TcdB, share a common domain structure with 44 % sequence identity and about 66 % sequence similarity with differences in their receptor-binding domains (RBD). Their common multi-domain structure is described as ABCD model (Jank and Aktories, 2008). The N-terminal, biologically active domain contains the glucosyltransferase domain. This glucosyltransferase modifies and thereby inactivates host cell's small Rho-family guanosine triphosphatases (GTPases) (Just et al., 1995a,b). The C-terminal domain contains combined repeated oligopeptides (CROPs) forming the RBD. The binary *C. difficile* transferase (CDT) consists of two components, CDTa and CDTb. CDTa has a two-domain structure: the N-terminal part serves as an adaptor that is involved in the interaction with the CDTb component and the C-terminal part of CDTa contains the ADP-ribosyltransferase activity, which inhibits host cell's actin polymerization. CDTb is responsible for receptor-binding, pore formation and toxin uptake (Hemmasi et al., 2015; Sundriyal et al., 2009).

Mechanisms of target cell intoxication

All *C. difficile* toxins enter their target cells via receptor-mediated endocytosis. In general, only a few *C. difficile* toxin-binding host receptors have been identified so far in humans. Effects of TcdB are considered to be more relevant for pathogenesis of infection than those of TcdA and CDT and so far more receptors have been discovered for TcdB than for TcdA and CDT. Chondroitin sulfate proteoglycan 4 (CSPG4) has been identified as the first binding receptor for TcdB which enables its internalization (Yuan et al., 2015). This proteoglycan is expressed on various progenitor cells such as mesenchymal stem cells, perivascular cells and others (Aktories et al., 2017). Moreover, Wnt receptor Frizzled (FZD) proteins are involved in TcdB binding and internalization (Chen et al., 2018; Tao et al., 2016). Another receptor that was reported to be involved in TcdB-mediated cytotoxicity is the protein poliovirus receptor–like 3 (PVRL3) which is also expressed on colonic epithelial cells (LaFrance et al., 2015). Recently, it was suggested that CSPG4, FZD 2, 7, and PVRL3 are non-internalizing binding receptors of TcdB and it was reported that LDL-receptor-related protein-1 (LRP1) serves as an internalizing co-receptor for TcdB (Schöttelndreier et al., 2018; Schorch et al., 2014). LRP1 binds to FZD receptors and acts as Wnt co-receptor (Zilberberg et al., 2004). TcdA binds to Lewis I, X, and Y glycan sequences that are expressed on human colonic epithetial cells (Na

et al., 2008). Moreover, TcdA binds to heat shock glycoprotein gp96 that is expressed on human colonocyte apical membranes and in the cytoplasm (Tucker and Wilkins, 1991; Clark et al., 1987). However, the pathophysiological relevant receptor of TcdA is still unknown. The host cell receptor for CDT is the lipolysis-stimulated lipoprotein receptor (LSR) (Papatheodoroua et al., 2011). LSR is highly expressed in the liver and in the gut (Mesli et al., 2004).

In order to elicit cytotoxic effects, *C. difficile* toxins bind to their specific receptors on the target cell and are internalized via endocytosis (Fig. 2). Following endosomal acidification, the biological active domain translocates into the cytosol (Florin and Thelestam, 1983; Henriques et al., 1987; Mitchell et al., 1987) and modifies the target molecule. Glucosyltransferase of TcdA and TcdB attaches glucose from the UDP-glucose onto Rho proteins (Di Bella et al., 2016). TcdA and TcdB have common glucosylation substrates of Rho protein family, including Rho A, B, and C, Rac, and Cdc42 (Just et al., 1995a,b). However, TcdA and TcdB modify also Rap 1, 2, TC10, Ral, Ras, TCL and RhoG but

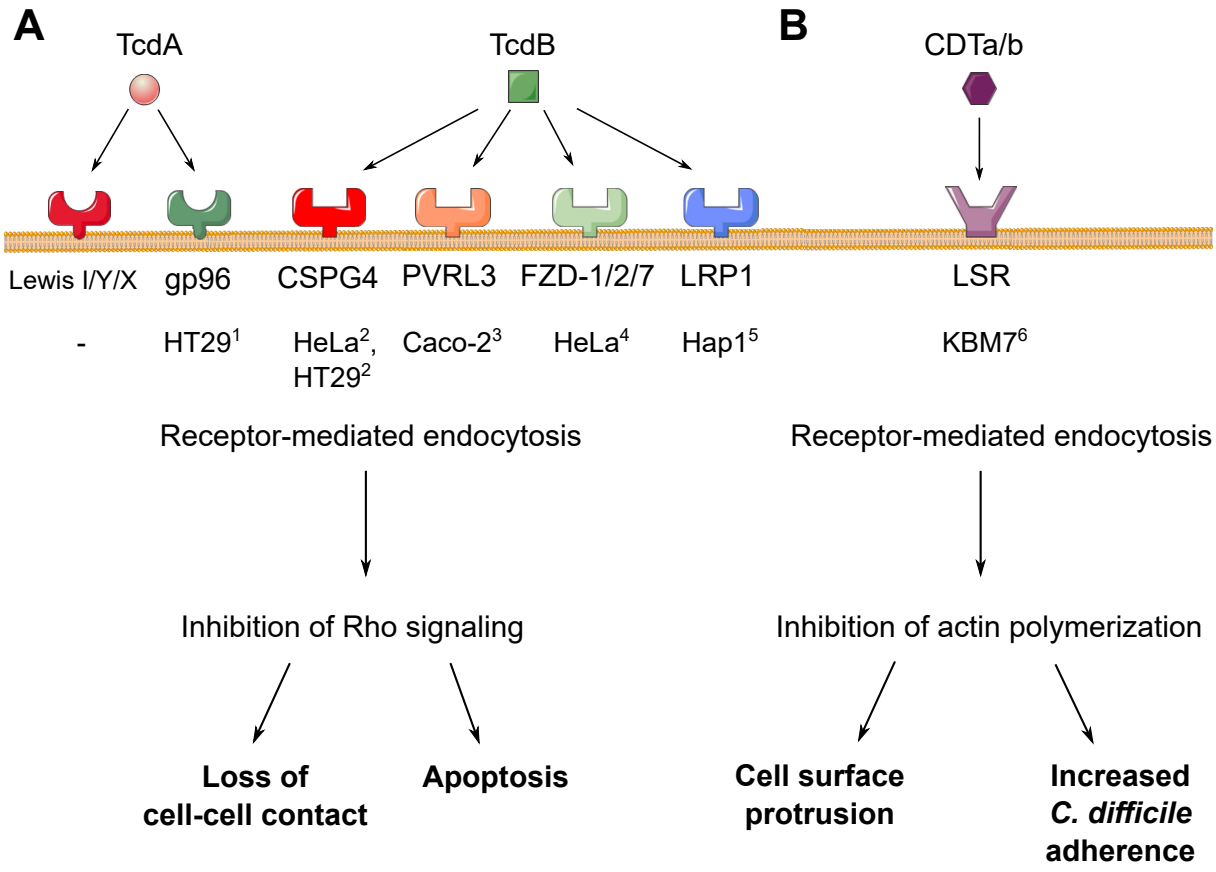

Figure 2: Target cell intoxication by *C. difficile* toxins. Receptors for toxin binding and uptake were identified in indicated cell types. Following receptor-mediated endocytosis toxins elicit their cytotoxic functions, which play a role in the context of immunological processes in CDI. A: TcdA and TcdB inhibit Rho signaling resulting in loss of cell-cell contact and apoptosis. B: CDT inhibits actin polymerization leading to cell surface protrusion and increased *C. difficile* adherence. [1]: Clark et al. (1987), [2]: Yuan et al. (2015), [3]: LaFrance et al. (2015), [4]: Tao et al. (2016), [5]: Schorch et al. (2014), [6]: Papatheodoroua et al. (2011).

show differences in substrate specificity (Genth et al., 2014; Zeiser et al., 2013). The Rho proteins regulate cytoskeleton and control processes such as phagocytosis, intracellular traffic, and migration. The glucosylation of Rho proteins by TcdA and TcdB blocks the interaction with their effectors and thereby inhibits Rho-dependent signaling (Sehr et al., 1998). Ultimately, this results in disruption of the actin-cytoskeleton, tight and adherent junctions, loss of cell-cell-contact, increased cell permeability, and apoptosis. Released CDTa ADP-ribosylates cellular actin and thereby inhibits actin polymerization (Gülke et al., 2001). ADP-ribosylated actin can block polymerization at the end of F-actin resulting in the depolymerization of the actin cytoskeleton. Ultimatively, this leads to formation of microtubules, cell surface protrusion, and enhanced *C. difficile* adherence (Schwan et al., 2014, 2009).

1.2 Immune responses in CDI

In general, the immune system can be divided into innate and adaptive immunity. The innate immune response is characterized by rapid, but unspecific responses towards a broad spectrum of pathogens. Among others, dendritic cells, macrophages, granulocytes, Natural killer (NK) cells, and mast cells belong to innate immunity. The adaptive immune response is relatively slow, but antigen-specific and thereby eliminates pathogens highly specifically. T cells and B cells belong to adaptive immunity. A selective number of these immune cells are capable to develop a memory phenotype, preventing reinfection with a specific pathogen and establishing long-lasting immunity. Recently, innate-like T cells have been discovered that blur the division lines between innate and adaptive immunity. This relatively new described innate-like T cell compartment consists of $\gamma\delta$ T cells, invariant Natural Killer (iNKT) cells, and mucosal-associated invariant T (MAIT) cells. These cells use innate receptors to quickly respond to a more unspecific stimulus (and thereby recognize broad classes of pathogens) and elicit an effector immune response comparable to cells belonging to the adaptive immune response.

1.2.1 Innate immunity in CDI

C. difficile toxins TcdA and TcdB cause breakdown of tight junctions and intestinal epithelial integrity to overcome the mucosal barrier and manifest *C. difficile* infection (CDI). Moreover, CDT induces cell surface protrusion and enhanced *C. difficile* adherence. This intoxication leads to an acute inflammatory response characterized by the expression of several proinflammatory cytokines and chemokines including IL-1β, IFNγ, IL-6, IL-12, IL-18, TNF-α, macrophage inflammatory protein 1 α (MIP-1α), and MIP-2 by activation of the inflammasome, NFκB, and MAP kinase pathways in epithelial cells and phagocytes (Cowardin et al., 2016a; Mahida et al., 1996). Following this, monocytes, dendritic cells, and macrophages are recruited and recognize *C. difficile* that have penetrated into the mu-

cosa. In particular, they recognize Pathogen-Associated Molecular Patterns (PAMPs) of *C. difficile* by their Pattern Recognition Receptors (PRR) and induce MyD88-mediated expression of neutrophil chemoattractant CXCL1 (Jarchum et al., 2012). This leads to transmigration of neutrophilic granulocytes from the peripheral blood into the tissue. Neutrophilic influx is a hallmark of CDI and beside their anti-bacterial function neutrophilic granulocytes have been described as major drivers of *C. difficile*-associated colitis (CDAC) (Bulusu et al., 2000; Burakoff et al., 1995; Kelly et al., 1994). In order to clear *C. difficile* and to reconstitute the intestinal epithelial barrier, neutrophils form extracellular traps (NET), which are composed of DNA structures and adherent primary and secondary granules with anti-bacterial activity (Khoruts and Sadowsky, 2016; Papayannopoulos and Zychlinsky, 2009; Brinkmann et al., 2004). These NETs are the building blocks of pseudomembranes, which are the hallmark clinical symptom for CDAC (summarized in Fig. 3). Intestinal mast cells also play an important role in *C. difficile* toxin-mediated proinflammatory responses by releasing histamines, which increase the permeability of the vascular endothelium (Meyer et al., 2007; Wershil et al., 1998). This leads to fluid loss into the intestinal lumen and watery diarrhea. Recently, eosinophilic granulocytes were identified to play a protective role in CDI, however, it is still unknown which beneficial function these cells display (Buonomo et al., 2016). Exposure to *C. diffi-*

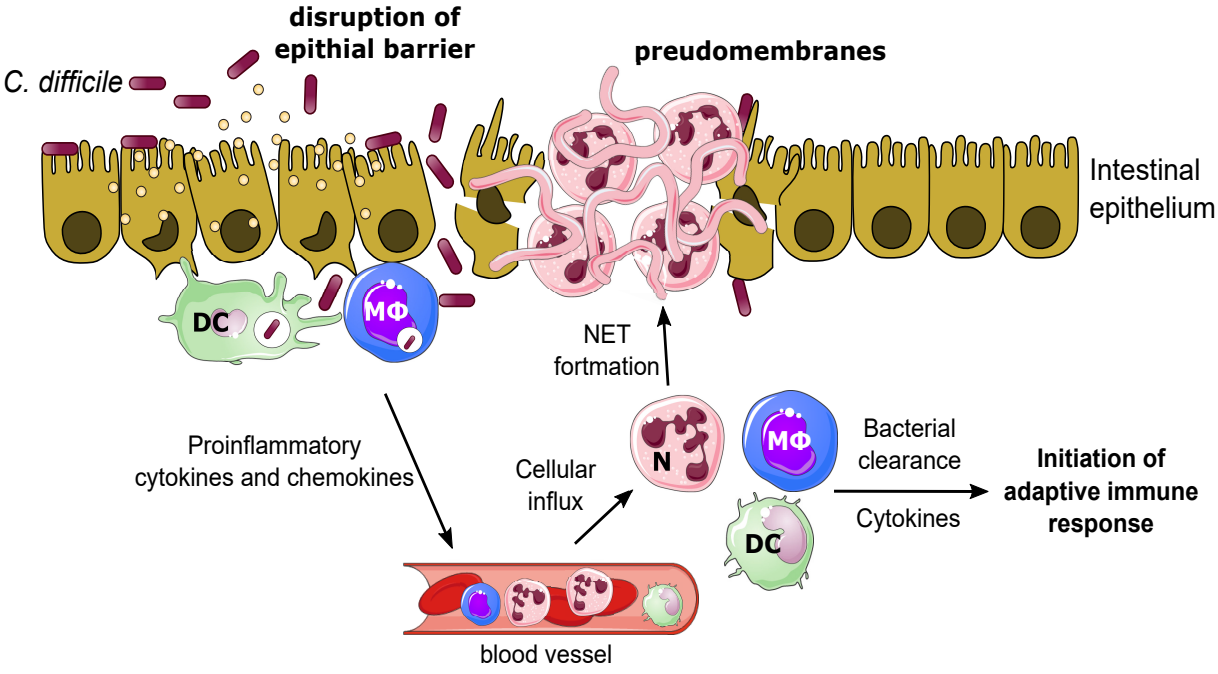

Figure 3: Innate immune response in *C. difficile* infection. *C. difficile* toxins disrupt the intestinal epithelial barrier and thereby facilitate *C. difficile* penetration into the mucosa. These two processes induce an innate immune response cascade characterized by the release of proinflammatory cytokines and chemokines that cause influx of dendritic cells (DC), macrophages (MΦ), and neutrophilic granulocytes (N) from peripheral blood vessels. N form neutrophil extracellular traps (NET) that build pseudomembranes at the epithelial barrier. DC and MΦ contribute to bacterial clearance and produce cytokines. Moreover, they present bacterial antigens to cells of the adaptive immune response.

cile TcdA leads to early loss of macrophages and eosinophils followed by T cell apoptosis (Mahida et al., 1998). Moreover, CDT induces apoptosis of eosinophils, which further prevents protective eosinophilia (Cowardin et al., 2016a).

1.2.2 Adaptive immunity in CDI

C. difficile toxins induce an acute and well-characterized innate immune response. Far less is known regarding adaptive immune responses in CDI, and so far, studies mainly focused on toxin-induced humoral immunity, while the investigation of T cell responses in CDI remains rudimentary (Rees and Steiner, 2018). *C. difficile* toxin-exposed B cells produce antibodies against TcdA and TcdB, which are associated with protection and decreased recurrence of CDI (Leav et al., 2010; Kyne et al., 2001). Moreover, detectable serum anti-TcdA and anti-TcdB antibody levels are associated with an asymptomatic *C. difficile* carrier state (Monaghan et al., 2013). However, B cells also produce antibodies against surface proteins of *C. difficile* but these serum antibodies do not prevent CDI. Activated B cells differentiate into professional antibody-producing plasma cells and few of them further into memory B cells. TcdA and TcdB-specific memory B cells are present in CDI patients contributing to protection, however, it is still under debate whether this protection is limited to antibody secretion (Devera et al., 2015; Monaghan et al., 2013). Although $CD4^+$ T cell signals are critical for B cell differentiation such as immunoglobulin class switching, affinity maturation, and memory establishment, the lack of $CD4^+$ T cells in murine CDI is associated with protection (Johnston et al., 2014). Recently, Saleh et al. (2019) identified a central role of $CD4^+$ T helper 17 (Th17) cells in CDI pathogenesis following colitis in mice. Here, adoptively transferred Th17 sufficiently increased CDI-associated mortality through elevated IL-17 production, which was suggested to play a role in *C. difficile*-associated epithelial cell damage and mortality (Nakagawa et al., 2016). There are only a few published studies in the context of CDI investigating the response of T cells that exhibit innate-like properties. One important finding is that T helper (Th) cells and invariant natural killer T (iNKT) cells and their follicular helper subsets (Tfh and iNKTfh cells) cooperatively provide B cell help to enhance antibody production against *C. difficile* TcdB, which ultimately improves humoral immunity in CDI (Rampuria et al., 2017). A recent study revealed that IL-17 producing $\gamma\delta$ T cells have a protective role in neonatal host defense in CDI (Chen et al., 2020). In mice deficient of IL-17 or $\gamma\delta$ T cells, the authors observed increased tissue inflammation and mortality following CDI. Neonatal mice, which possess naturally expanded IL-17 producing $\gamma\delta$ T cells, show resistance against CDI. Interestingly, Chen et al. (2020) have detected increased IL-17 and T cell receptor γ-chain expression in fecal extracts of children compared to adult. The authors stated that low $\gamma\delta$ T cell numbers in adults correlate with increased CDI susceptibility. However, additional studies are needed to understand the complex interplay between adaptive and innate-like immune responses and how this contributes to recovery

from CDI or immunopathology. Moreover, only very limited knowledge regarding the role of invariant mucosal-associated T (MAIT) cells in the pathogenesis of CDI does exist to date.

1.3 Human mucosal-associated invariant T (MAIT) cells

In 1993, a new T cell population with an invariant T cell receptor (TCR) α chain was described in the human blood (Porcelli et al., 1993). The same TCRα chain-bearing T cell population was also discovered in mice and was described to be restricted to monomorphic major histocompatibility complex class Ib (MHC-Ib)-related protein MR1 (Tilloy et al., 1999). Due to the fact that these T cells are enriched in mucosal tissues such as the gut, the name human mucosal-associated invariant T (MAIT) cells was coined (Treiner et al., 2003). MAIT cells are highly abundant in the intestinal lamina propria (up to 10 % of T cells) but also in the blood (up to 10 % of T cells) and the liver (45 % of T cells) (Gherardin et al., 2018a; Le Bourhis et al., 2010). Although MAIT cells are an abundant T cell population with a high TCR specificity, their role in immunity remained unclear for many years. In 2010, it has been demonstrated that MAIT respond to a broad range of bacteria and yeast (Le Bourhis et al., 2010; Gold et al., 2010). Since then, the number of studies highlighting the importance and uncovering the function of MAIT cells in different disease settings is steadily increasing. Initial investigations of the role of MAIT cells revealed that MAIT cells have a protective role in *Escherichia coli* (*E. coli*) infection *in vivo* (Le Bourhis et al., 2010). In addition to intestinal *E. coli* infection, MAIT cells play also a protective role in pulmonary infections caused by *Mycobacterium abscessus* and *Legionella longbeachae* (Wang et al., 2018; Le Bourhis et al., 2010). Interestingly, MAIT cells were initially reported to not respond to viruses *in vitro* however, MAIT cells are activated and display protective functions in *in vivo* pulmonary Influenza A virus infection (van Wilgenburg et al., 2018, 2016; Loh et al., 2016). There are further studies demonstrating MAIT cell activation in response to bacteria, viruses, and yeast however, the challenge is to clarify their either protective or detrimental function during infection (reviewed by Godfrey et al. (2019)).

1.3.1 Antigen specificity

MAIT cells are characterized by the expression of a semi-invariant $\alpha\beta$ T cell receptor (iTCR). This iTCR consists of an invariant TCRα chain paired with a limited repertoire of Vβ chains. Human MAIT cells express a Vα7.2-Jα33/12/20 (TRAV1-2/TRAJ33/12/20) TCRα chain together with Vβ2 or Vβ13 (TRBV20 or TRBV6) TCRβ chain (Lepore et al., 2014; Reantragoon et al., 2013; Tilloy et al., 1999; Porcelli et al., 1993). This iTCR restricts them to the monomorphic major histocompatibility complex class Ib (MHC-Ib)-related protein MR1, which is expressed on the surface and intracellularly by antigen presenting cells and epithelial cells (Moreira et al., 2017; Harriff et al., 2016; Dusseaux

et al., 2011; Le Bourhis et al., 2010).

MR1 presents metabolites that derive from the riboflavin biosynthesis pathway. Bacteria process guanosine triphosphate (GTP) to riboflavin, which is required for the production of Flavin-containing coenzymes. The metabolites that are able to bind and fold MR1 are derivatives of the precursor 5-amino-6-d-ribitylaminouracil (5-A-RU, Fig. 4, A). They are generated in a non-enzymatic reaction by converting 5-A-RU with glyoxal or methylglyoxal to 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), respectively (Fig. 4, B, Corbett et al. (2014); Kjer-Nielsen et al. (2012). 5-OP-RU and 5-OE-RU are the most potent MAIT cell-activating ligands but only stable when bound to MR1 (Schmaler et al., 2018; Corbett et al., 2014). When not bound to MR1, 5-OP-RU and 5-OE-RU are further processed to RL-6,7-DiMe which can also bind weakly to MR1 but shows less MAIT cell activating capacity (Schmaler et al., 2018). In contrast to riboflavin-derived metabolites, folic acid-derived metabolites have an inhibitory effect on MAIT cell activation (Schmaler

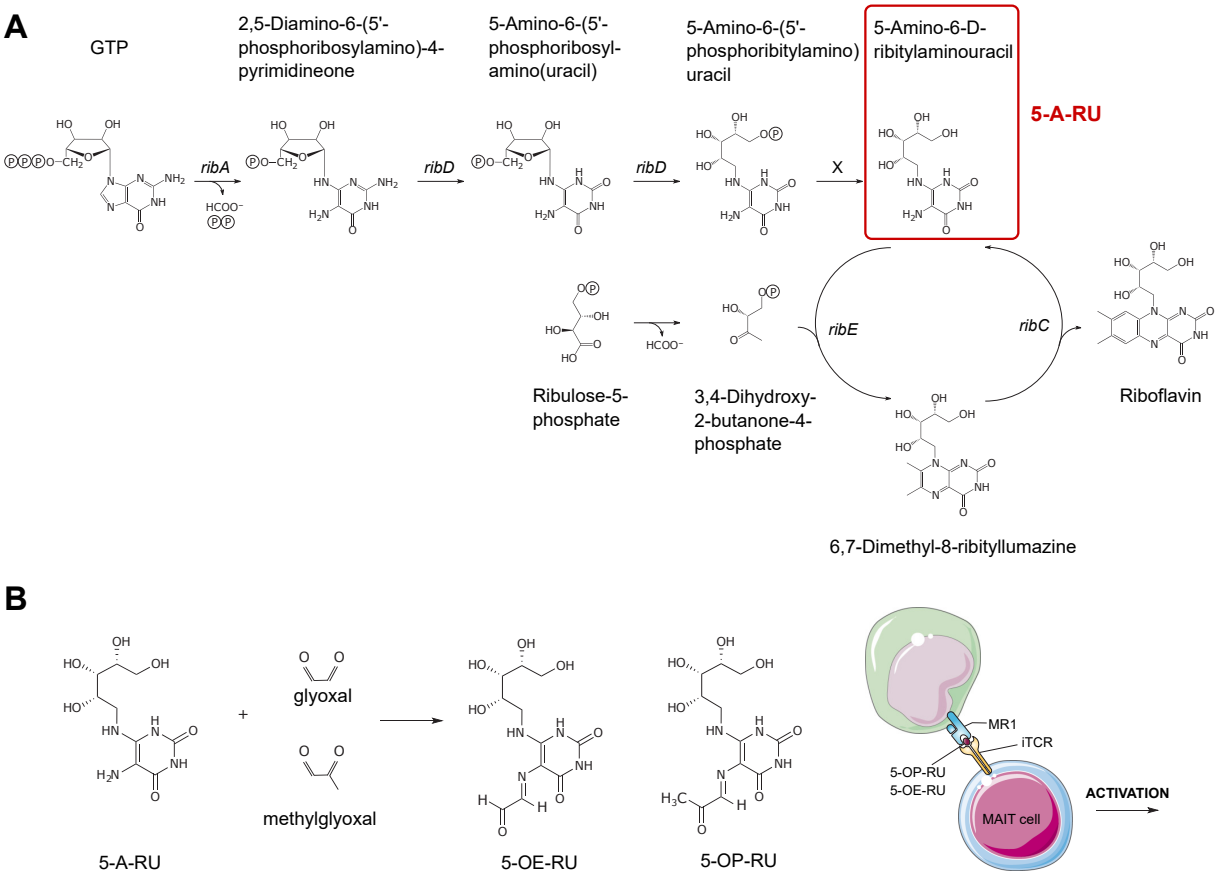

Figure 4: Riboflavin biosynthesis pathway in *C. difficile*. A: Guanosine triphosphate (GTP) is processed to riboflavin. Involved enzymes are mentioned above the arrows. Precursor molecule for MAIT cell-activating ligands (5-A-RU) is depicted in the red box. B: 5-A-RU is converted into 5-OE-RU and 5-OP-RU by non-enzymatic reaction of glyoxal and methylglyoxal, respectively. These adducts can bind to MR1 and activate MAIT cells via semi-invariant T cell receptor (iTCR) recognition. APC: Antigen presenting cell. Modified from Corbett et al. (2014).

et al., 2018). The number of known microbial but also non-microbial MR1-binding ligands and their capacity to modulate MAIT cell responses is still increasing (Keller et al., 2017; Lepore et al., 2017). Based on genomic data, *C. difficile* has been predicted to possess a functional riboflavin biosynthesis pathway (Janoir et al., 2013) and therefore Liuzzi et al. (2015) considered *C. difficile* as a potential MAIT cell-activating pathogen.

1.3.2 MAIT cell phenotype

Like conventional T cells, murine MAIT cells develop in the thymus where they are positively selected from MR1-expressing $CD8^+$ $CD4^+$ (DP) thymocytes (Seach et al., 2013), which might also hold true for human MAIT cells. The majority of human MAIT cells are $CD8\alpha\alpha^+$ (70 - 90 %), some are $CD4^-$ $CD8^-$ (DN) (10 - 20 %), and only a few of them are $CD4^+$ (Gherardin et al., 2018b; Reantragoon et al., 2013; Martin et al., 2009). MAIT cells exit the thymus with a naïve ($CD45RO^{lo}$ $CD45RA^+$ $CD27^+$) phenotype but quickly acquire an effector memory ($CD45RO^+$ $CD45RA^-$ $CCR7^-$ $CD62L^-$ $CD28^+$) phenotype in the periphery that allows immediate execution of their effector functions upon activation (Dusseaux et al., 2011). Peripheral MAIT cells expand in a B cell- and microbiota-dependent manner (Treiner et al., 2003). Adult MAIT cells universally express high levels of C-type lectin CD161 and CD26, which are both used in combination with the iTCR Vα7.2 chain to identify MAIT cells (Dusseaux et al., 2011; Billerbeck et al., 2010; Martin et al., 2009). To perform transmigration into target tissue, in particular liver and intestine, MAIT cells express tissue homing receptors for chemokines such as CCR5, CCR6, CCR9 and CXCR6 (Kurioka et al., 2017; Dusseaux et al., 2011).
MAIT cells possess the unique ability to secrete cytokines of type 1 and type 17 immunity. It is considered that this feature is associated to their transcription factor coexpression of promyelocytic leukemia zinc finger (PLZF), T-bet, CTL-associated transcription factor Eomesodermin (EOMES), and RORγt (Koay et al., 2016; Leeansyah et al., 2015). Due to its unique expression in innate-like T cells, PLZF is considered as a master transcription factor for innate-like functionality in MAIT cells (Le Bourhis et al., 2011). T-bet and EOMES drive IFNγ secretion and the expression of cytotoxic granules (granzymes and perforin) in $CD8^+$ T cells and EOMES expression increases as cells gain a memory-like phenotype (Knox et al., 2014; Pearce et al., 2003; Szabo et al., 2000). Retinoic acid-related orphan receptor γt (RORγt) drives type 17 immunity including IL-17 and IL-22 secretion and high CD161 expression (Maggi et al., 2010). However, only a very small proportion of blood human MAIT cells is capable of producing IL-17 and IL-22, whereas tissue-resident MAIT cells in the mouth or in the female genital tract mount substantial IL-17 and IL-22 responses following bacterial stimulation (Gibbs et al., 2017; Sobkowiak et al., 2019). The key cytokine for promotion and maintenance of type 17 immunity is IL-23, and MAIT cells are as well sensitive for this proinflammatory cytokine by expressing the IL-23 receptor (Wang et al., 2019; Billerbeck et al., 2010).

In addition to the iTCR, MAIT cells constitutively express interleukin (IL)-12, IL-15 and at high level IL-18 receptors (IL-12R, IL-15R, and IL-18R, Billerbeck et al. (2010). The expression of IL-12R, IL-15R and IL-18R enables a cytokine-mediated MAIT cell activation (Slichter et al., 2016; Ussher et al., 2014; Le Bourhis et al., 2010). Therefore, MAIT cells can be activated in a i) TCR-dependent, ii) cytokine-dependent or iii) TCR- and cytokine-dependent manner. Depending on the stimulus, blood MAIT cells can secrete IFNγ, tumor necrosis factor-α (TNF-α), pore-forming perforin and several granzymes (Gzm) such as GzmA, B, K, and M. Proteomic and flow cytometric phenotyping revealed that resting blood MAIT cells express already high level of GzmA, K, and M exhibiting a more pro-inflammatory than cytotoxic Gzm expression profile (Bulitta et al., 2018; Kurioka et al., 2015). Only GzmB is *de novo*-induced in MAIT cells following their activation (Kurioka et al., 2015).

In summary, human MAIT cells are a recently discovered innate-like T cell population that is highly abundant in the peripheral blood and exhibits tissue homing properties to transmigrate into target tissues such as the intestine. They are identified according to their expression of the iTCR Vα7.2 segment and high expression of CD161 or CD26. Due to their unique transcription factor coexpression, profile MAIT cells can execute cytotoxic type 1, type 17 and innate immune responses (summarized in Fig. 5).

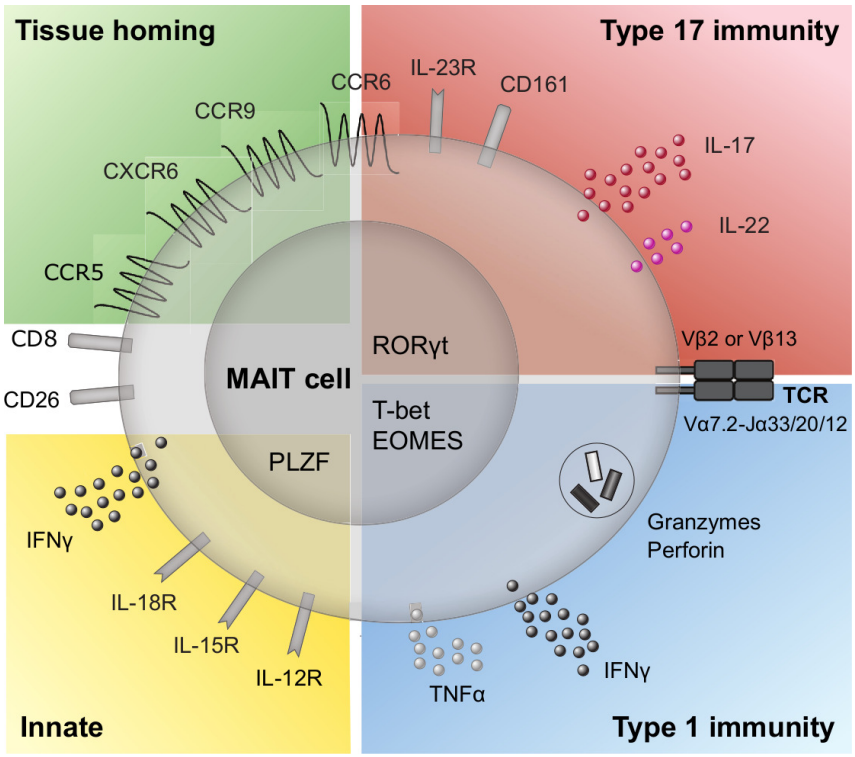

Figure 5: Transcription factors, cytokines and receptors of mature MAIT cells. MAIT cells coexpress promyelocytic leukemia zinc finger (PLZF), retinoic acid-related orphan receptor γt (RORγt), T-bet, and Eomesodermin (EOMES). This defines the MAIT cell phenotype and functions that can be characterized by four types: Innate-like, cytotoxic type 1, and type 17 immunity, and tissue homing. MAIT cells express the semi-invariant T cell receptor (iTCR) characterized by the Vα7.2 segment and most of them express the TCR co-receptor CD8. In combination with the iTCR, MAIT cells are identified by their high expression of CD161 and CD26. Modified from Provine and Klenerman (2020).

1.3.3 MAIT cell cytotoxicity

MAIT cells are activated in different disease settings, starting from microbial infection (bacteria and viruses) to inflammatory autoimmune disease and finally to malignancy (Yong et al., 2017; Shaler et al., 2017; Loh et al., 2016; Sundström et al., 2015; Willing et al., 2014; Cosgrove et al., 2013; Le Bourhis et al., 2010; Gold et al., 2010). In microbial infections they can sense riboflavin-producing bacteria via their invariant T cell receptor (iTCR) when bacterial metabolites are presented on the monomorphic MHC-Ib molecule MR1. However, they also respond to inflammatory or antimicrobial cytokines that are secreted by host cells during infections with bacteria not producing riboflavin and viruses, but also in autoimmune disease settings in which cognate MR1-antigen is missing. However, there is increasing evidence that ubiquitously expressed MR1 presents as well endogenous ligands that are involved in processes like MAIT cell homeostasis and tissue repair in the absence of inflammation (Hinks et al., 2019).

The hallmark of MAIT cell activation is their cytotoxic response via granzymes and perforin. While granzyme and perforin expression is increased following TCR and/or

cytokine stimulation, the targeted cell killing depends on MR1 (Leeansyah et al., 2015; Kurioka et al., 2015; Le Bourhis et al., 2013). In the so far best characterized bacterial MAIT cell stimulation model using the constitutive riboflavin-producer *Escherichia coli* (*E. coli*), MR1-expressing antigen presenting cells present riboflavin-derived metabolites (5-OP-RU and 5-OE-RU, mentioned in section 1.3) and secrete proinflammatory IL-12 and IL-18 (Fig. 6). MAIT cells recognize the MR1-antigen-complex by their iTCR and host cell-derived cytokines by their respective receptors (IL-12R and IL-18R). Early MAIT cell response is characterized by increased surface expression of the classical early activation marker CD69 (Dias et al., 2016). CD69 is known to regulate the secretion of IFNγ and *E. coli*-stimulated MAIT cells mount a substantial IFNγ response (Dias et al., 2016). Moreover, it was described that IL-12 and IL-18 also drive IFNγ production by MAIT cells. (Ussher et al., 2014). As mentioned above, already resting blood MAIT cells express high level of GzmA, K, and M (Bulitta et al., 2018; Kurioka et al., 2015). Following *E. coli*-mediated stimulation, MAIT cells rapidly degranulate as indicated by upregulation of surface CD107a expression within a few hours following stimulation. In particular, they rapidly release their granules which are initially armed with GzmA and K followed by induced GzmB and perforin expression to execute their full cytotoxic effector function

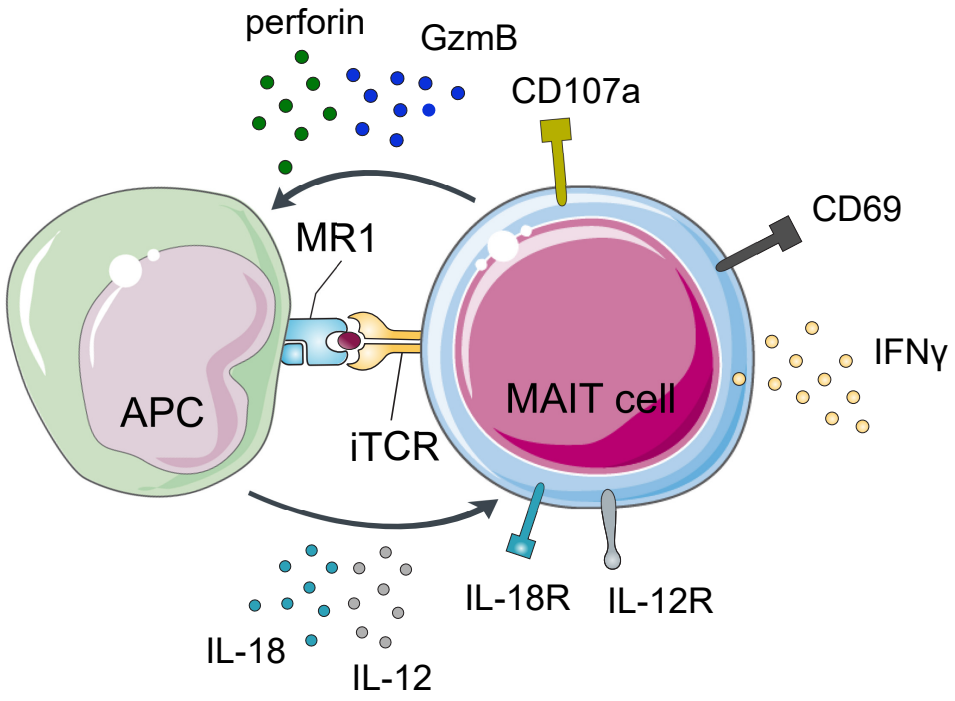

Figure 6: Cytotoxic MAIT cell response following *E. coli* stimulation. Antigen presenting cell (APC) presents *E. coli*-derived metabolites via MR1 and secretes proinflammatory cytokines IL-12 and IL-18. MAIT cells recognize MR1-ligand complex by their semi-invariant T cell receptor (iTCR) and sense the cytokines by their respective receptors (IL-12R and IL-18R). Activated MAIT cells increase CD69 surface expression and mount an IFNγ response. Following the release of granules initially armed with granzyme (Gzm) A and K, MAIT cells release granules armed with GzmB and perforin as indicated by increased CD107a surface expression.

(Leeansyah et al., 2015; Kurioka et al., 2015). By this, MAIT cells are able to identify and directly kill infected cells, thus contributing to rapid microbial clearance (Le Bourhis et al., 2010).

1.4 Aims of thesis

Clostridioides difficile infections (CDI) are the major cause of antibiotics-associated colitis with increasing prevalence in morbidity and mortality. Pathogenesis of *C. difficile*-associated colitis (CDAC) is linked to secreted *C. difficile* toxins. Severe CDAC is mostly caused by so-called hypervirulent *C. difficile* strains that are capable to produce the three *C. difficile* toxins TcdA, TcdB, and binary CDT. Following in-depth characterization of toxin-induced pathogenesis of CDAC, contribution of immunopathological processes are currently under investigation. The recently identified mucosal-associated invariant T (MAIT) cells represent an abundant innate-like T cell population in the intestinal mucosa exhibiting anti-bacterial functions. MAIT cells are able to sense metabolites derived from bacterial riboflavin biosynthesis pathway that are presented by the major histocompatibility complex class I-related protein MR1. Genomic data suggested that *C. difficile* possess a functional riboflavin biosynthesis and thus *C. difficile* was predicted to represent a potential target recognized by MAIT cells. In light of the fact that individuals with an age > 65 years show increased susceptibility to *C. difficile*, CDI is considered an age-associated infectious disease. Strikingly, MAIT cell frequency within the T cell compartment decreases with progressing age leading to the hypothesis that reduced MAIT cell numbers and/or their functional impairment in aged individuals might contribute to enhanced susceptibility to CDI in the elderly. Thus, the overall aim of this thesis was to gain first insight into the potential contribution of MAIT cells to the pathogenesis of CDI. In order to reach this goal, the following three questions have been addressed in the framework of this thesis:

1) Do MAIT cells from aged individuals exhibit functional adaptions that could potentially contribute to enhanced CDI susceptibility in the elderly?

2) Does *C. difficile* activate human MAIT cells and if so, does *C. difficile*-mediated MAIT cell activation occur MR1-dependent or -independent?

3) What is the contribution of *C. difficile* toxins TcdA, TcdB and binary CDT to the activation of human MAIT cells?

2 Material and Methods

2.1 Equipment and consumables

Table 2: Equipment

Unit type	Manufacturer	Model
Cell culture hood	Heraeus	Hera Safe KS 15
Cell sorter	BD Biosciences	BD FACSAria II SORP
Centrifuge	Heraeus	Heraeus Multifuge 1 S-R
CO_2-Incubator	New Brunswick Scientific™	Innova CO-170
CO_2-Incubator	Thermo Scientific Heraeus	Thermo Scientific BB15
ELISA plate reader	Tecan	Sunrise™
Exhaust pump	IBS Integra Biosciences	Vacu Safe
Flow Cytometer	BD Biosciences	BD LSR Fortessa™
	BD Biosciences	BD™ LSR II SORP
	Accuri Cytometers Inc.	BD Accuri C6 Flow Cytometer
HPLC system	Agilent Technologies	1260 Infinity
HPLC system	Thermo Scientific Dionex	UltiMate 3000
Mass spectrometer	Thermo Scientific	Orbitrap Fusion™ Tribrid™
	Thermo Scientific	LTQ Orbitrap Velos FT
Micro fraction collector	GE Healthcare	Frac-950™
Micro-LC system	GE Healthcare	Äkta Purifier™ UPC 10
Micro scales	Mettler Toledo	XS 204
Microscope	Nikon	Inverted microscope Ti-E
Precision scales	Sartorius	I 2000 D
Spectrophotometer	Thermo Scientific	NanoDrop® ND-1000
SpeedVac Concentrator	Christ	RVC 2-18 plus
Stainless steel emitter	Thermo Scientific	40mm, OD 1/32"
Thermomixer	Eppendorf	Thermomixer *comfort* 1.5 ml
Ultra centrifuge	Hitachi	Sorvall Discovery MS120SE
Vortex mixer	IKA® Vortex	VORTEX Genius 3
Water bath	Grant	SUB 14

Table 3: Consumables

Name	Type	Manufacturer
BD Falcon™ cell strainer	100 µm Nylon	BD Biosciences
C18 analytical column	Acclaim PepMAp RSLC	Thermo Scientific Dionex
C18 column	Zorbax 300	Agilent Technologies
C18 precolumn	Acclaim	Thermo Scientific Dionex
C18 separation column	Poroshell 120 EC	Agilent Technologies
FACS tubes	1.4 ml PP U-bottom	Thermo Scientific
Falcon™ tubes	15 ml, 50 ml	Thermo Fisher Scientific
Finntip™ pipette tips	200 µl	Thermo Scientific
Serologic pipettes	5 ml, 10 ml, 25 ml	Sarstedt AG & Co.
Cell culture flasks	50 ml, 100 ml	Thermo Fisher Scientific
Cell culture plates	96 - well plate U-bottom	TPP®
$Ni2^{+}$-NTA sepharose columns	Protino Ni-IDA columns	Macherey-Nagel
Nunc™ MicroWell™ plates	96 - well plate U-bottom	Thermo Fisher Scientific
TipOne® pipette tip	10 µl, 200 µl, 1000 µl	StarLab
ZEBA desalting columns	7.0 kDa	Pierce/Thermofischer

2.2 Software

Table 4: Software

Software	Version	Manufacturer
Chromeleon software	6.8	Thermo Scientific Dionex
Flow Jo	10.6.1	BD Biosciences
MS Office	2016	Microsoft
Proteome Discoverer	2.2	Thermo Scientific
Raw meat	2.1	VAST Scientific
R Studio	1.1.456	R Studio Inc.
XCalibur	3.0.63	Thermo Scientific

2.3 Chemicals

Table 5: Chemicals

Chemical	Article number	Manufacturer
BD FACS™ Clean	340345	BD Biosciences
BD FACS™ Flow	342003	BD Biosciences
BD FACS™ Rinse	340346	BD Biosciences
Brefeldin A	420601	BioLegend®

continued on the next page

Chemical	Article number	Manufacturer
CDTa	-	provided by Dr. R. Gerhard
CDTb	-	provided by Dr. R. Gerhard
cOmplete™ protease inhibitor	11697498001	Roche
EDTA	1001109897	Sigma-Aldrich
Fetal bovine serum gold	A15-751	PAA Laboratories
Ficoll® Paque PLUS gradient media	17144003	GE Healthcare
Fixable viability dye	L-34975	Invitrogen™
GT-dTcdA	-	provided by Dr. R. Gerhard
GT-dTcdB	-	provided by Dr. R. Gerhard
LIVE/DEAD™ fixable blue stain	L23105	Invitrogen
L-Glutamine	25030081	gibco®
Paraformaldehyde	252549-1L	Sigma-Aldrich
PBS tablets	18912-014	gibco®
Penicillin-Streptomycin	15070063	gibco®
peqGREEN	37.5000	peqLab/VWR
pGEX2T vector	27-1542-01	GE Healthcare
pHIS1522 vector	BMEG12	MoBiTec
pWH1520 vector	BMEG03	MoBiTec
RPMI 1640 medium	31980-022	Gibco/Life Technologies
Smart DNA ladder	MW-1700-10	Eurogentec
TcdA	-	provided by Dr. R. Gerhard
TcdB	-	provided by Dr. R. Gerhard
TMT labeling reagents	90110	Thermo Scientific
Trypan blue	15250-061	gibco®
Trypsin	V5111	Promega
UltraComp Beads™	01-1111-42	Invitrogen™

2.4 Kits

Table 6: Kits

Kit	Article number	Manufacturer
Annexin V Apoptosis Detection	640922	BioLegend
Bacillus megaterium expression system (WH320)	BMEG02	MoBiTec
BD Cytofix/Cytoperm™	554714	BD Biosciences
ELISA human IL-18	7620	MBL
ELISA MAX™ Standard IL-12p70	431701	BioLegend

continued on the next page

Kit	Article number	Manufacturer
ELISA MAX™ Deluxe IL-15	435104	BioLegend
QuikChange II Site-Directed Mutagenesis Kit	200524	Stratagene
RNeasy Mini	74104	Qiagen
Verso 1-Step RT-PCR Hot-Start kit	AB-1455/B	ThermoFisher

2.5 Buffers and Media

Table 7: Contents of buffers and media

Name	Contents
0.5 M EDTA	18.5 g Na_2-EDTA x $2H_2O$
	ad 100 ml distilled H_2O
	pH = 8
b-RP buffer	5 mM NH_4HCO_2 in ultrapure H_2O
PBS	PBS tablet resolved in H_2O
	pH = 7.4
ACK buffer	0.15 mM NH_4Cl
	10 mM $KHCO_3$ in 1 L H_2O
	0.1 mM 0.5 M EDTA
	(pH = 8)
FACS buffer	2 % (v/v) FBS
	2 mM EDTA in PBS
LB medium	10 g Bacto-Tryptone
	5 g Bacto-Yeast Extract
	7 g NaCL
	ad 1 l distilled H_20
Lysis buffer	50 mM TEAB in ultrapure H_2O
	1 % SDS
	1 x cOmplete™ protease inhibitor
	50 mM DTT
	250.000 U/ml benzonase
RPMI *complete*	10 % (v/v) FCS
	1 % (v/v) Pen/Strep
	2 nM L-glutamine
	ad 500 ml RPMI 1640

2.6 Antibodies

Table 8: Antibodies used for surface staining

Antibody	Conjugation	Clone	Dilution/conc.	Manufacturer
anti-CD107a	PerCP-Cy5.5	GK1.5	1:800	BioLegend®
anti-CD161	APC	DX12	1:25	BD Biosciences
anti-CD3	FITC	OKT3	1:50	BioLegend®
anti-CD3	BV605	OKT3	1:50	BioLegend®
anti-CD69	PE	FN50	1:50	BioLegend®
anti-CD16/CD32	-	-	1:50	Miltenyi Biotec
anti-IL-12 p35	-	B-T21	5 µg/ml	eBioscience
anti-IL-18	-	126-2H	5 µg/ml	MBL International
anti-MR1	-	26.5	20 µg/ml	BioLegend®
anti-Va7.2	PE-Cy7	3C10	1:80	BioLegend®

Table 9: Antibodies used for intracellular cytokine staining

antibody	conjugation	clone	dilution	manufacturer
anti-granzyme B	GB11	Pacific Blue	1:20	BioLegend®
anti-IFNγ	4S.B3	BV711	1:20	BioLegend®
anti-IFNγ	4S.B3	APC-Cy7	1:20	BioLegend®
anti-perforin	dG9	BV510	1:20	BioLegend®
anti-perforin	dG9	FITC	1:20	BioLegend®

2.7 Primers

Table 10: Primer used for RT-PCR

Primer	Sequence	(5' → 3')
ribD	Sense	5' - AATCAGTAAGTCTAGATGG - 3'
	Antisense	5' - CTGTCATTGAGAGTAGCACC - 3'
ribE	Sense	5' - CAGCCGATGTTATGATGGAG - 3'
	Antisense	5' - CTCCAACATTCTTTGTCAAGAG - 3'

2.8 Organisms

Table 11: *C. difficile* strains

Clade	Ribotype	TcdA	TcdB	CDT	DSM number
1	RT084	negative	negative	negative	DSM 28666
1	RT001	positive	positive	negative	DSM 29745

Continued on the next page

Clade	Ribotype	TcdA	TcdB	CDT	DSM number
1	RT012	positive	positive	negative	DSM 28645
2	RT027	positive	positive	positive	DSM 28196
3	RT023	positive	positive	positive	DSM 102859

Table 12: *C. difficile* toxins

Toxin	Other name	Source
Toxin A	TcdA	VPI10463
Toxin A D285/287N	GT-dTcdA	VPI10463
Toxin B	Tcd B	VPI10463
Toxin B D286/288N	GT-dTcdB	VPI10463
C. difficile transferase A	CDTa	R20291
C. difficile transferase B	CDTb	R20291

2.9 Bacteria cultures

Note: In cooperation with Dr. Meina Neumann-Schaal

***C. difficile* cultures.** *C. difficile* clinical isolates were provided by Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig). DSM 28196 (RT027), DSM 28666 (RT084), DSM 29745 (RT001) (depositor Dr. Uwe Groß), DSM 28645 (RT012) (depositor Dr. Ralf Gerhard), DSM 102859 (RT023) (depositor: Dr. Lutz von Müller) strains were cultured in riboflavin-free casamino acids containing medium (CDMM) under anaerobic conditions (Neumann-Schaal et al., 2015; Riedel et al., 2017). Cells were harvested at the mid-exponential phase (0.5 OD_{max}). Bacterial numbers were determined using a Neubauer improved counting chamber (C-Chip, NanoEnTek). Bacterial cell pellets were harvested by centrifugation (13.000 g, 10 min, 4 °C), fixed with 2 % paraformaldehyde (PFA) solution, washed three times with PBS, and stored at -80 °C. Prior PBMC stimulation, the bacterial cells were resuspended in PBS to a final concentration of 3 x 10^8 bacteria/ml.

2.10 Recombinant *C. difficile* toxins

Note: In cooperation with Dr. Ralf Gerhard

Expression and purification. Recombinant TcdA and TcdB as well as their glucosyltransferase deficient mutants were produced in the *Bacillus megaterium* expression system as described before (Burger et al., 2003). Coding sequences for strain VPI10463 TcdA and TcdB were cloned into a modified pWH1520 vector (TcdA, TcdA D285/287N) or into pHIS1522 vector (TcdB, TcdB D286/288N) to obtain C-terminal His6-tagged proteins. The glucosyltransferase deficient mutants (TcdA D285/287N, TcdB D286/288N)

were generated by site-directed mutagenesis kit according to supplied protocol. The *B. megaterium* strain WH320 was used for expression of proteins. In contrast to the large clostridial glucosyltransferases both components of the binary toxin CDT (CDTa and CDTb from *C. difficile* strain R20291 (DSM 27147)) were expressed in *E. coli* TG1. The coding sequence for mature CDTa, lacking the export sequence, was cloned into pQE30 vector for expression as N-terminally His6-tagged protein. Mature CDTb was expressed in *E. coli* as GST fusion protein using the pGEX2T vector as described earlier (Beer et al., 2018). GST-CDTb was purified via glutathione(GSH)-sepharose columns according to standard protocol. After elution from GSH-sepharose beads the GST-CDTb fusion protein was activated by limited digestion with trypsin (0.2 µg trypsin per µg GST-CDTb) for 30 min at room temperature. Thereby, the GST-tag as well as the N-terminal inhibitory part of CDTb was removed, resulting in activated CDTb. Incubation with trypsin was terminated by addition of 2 mM 4-(2-Aminoethyl)benzenesulfonylfluorid (AEBSF). All His6-tagged proteins were purified via $Ni2^{+}$-NTA sepharose columns. After buffer exchange via ZEBA desalting columns all toxins were stored in 20 mM Tris-HCl, pH7.2, and 50 mM NaCl at -80 °C. The specific concentration and purity of toxins was estimated by SDS gel electrophoresis.

2.11 Gene expression analysis of *C. difficile* riboflavin genes

RNA isolation. *C. difficile* RT084 and hypervirulent RT023 were cultured in riboflavin-free casamino acids containing medium (CDMM) under anaerobic conditions (Neumann-Schaal et al., 2015; Riedel et al., 2017). Bacteria were harvested at the mid-exponential phase (0.5 OD_{max}). For mechanical disintegration one spatula tip glass beads was added and vortexed. Additionally, bacteria were enzymatically digested with 15 mg/ml lysozyme for 30 min at RT. Bacterial RNA was isolated using Qiagen RNase easy minikit according to manufacturer's instructions. RNA was stored at -20 °C until use.

Reverse transcription and polymerase-chain-reaction (RT-PCR). Gene expression of *ribD* and *ribE gene* in *C. difficile* RT084 and hypervirulent RT023 was investigated using Verso 1-Step RT-PCR Hot-Start kit. *ribD* encodes for bifunctional diamino-hydroxy-phospho-ribosyl-amino-pyrimidine deaminase/ 5-amino-6- (5-phospho-ribosyl-amino) uracil reductase, which generates phosphorylated 5-A-RU, the precursor of the MR1-binding ligand 5-A-RU. 5-A-RU is converted by the *ribE* gene product lumazine synthase into 6,7-dimethyl-8-ribityllumazine (RL-6,7-diMe), which then can be processed to riboflavin. Sequences for *ribD* and *ribE* primers are shown in table 10. The reaction mastermix was used together with a LightCycler® 480 II device. For RT-PCR following cycle conditions were applied:

RT-PCR cycling protocol

Step	Temperature	Time	Cycles
cDNA synthesis	50 °C	15 min	1
Transciptase inactivation	95 °C	15 min	1
Denaturation	95 °C	20 s	41
Annealing	57 °C	30 s	
Extension	72 °C	60 s	
Final extension	72 °C	5 min	1

DNA gel electrophoresis. Gels were prepared with 1.5 % agarose in 1x TAE buffer. Suspension was boiled until agarose was completely solved. PeqGREEN was added to a final concentration of 40 % (v/v). PeqGREEN is a fluorescent dye that binds to desoxyribonucleic acids. The fluorescence of DNA is increased when excited with ultraviolet light. The boiled agarose solution was poured into the gel chamber and solidified after cooling at RT. The gel chamber was placed in the buffer tank and 1x TAE buffer was filled up to the mark. 1x DNA loading buffer was added to the samples. One lane with 10 µl Smart DNA ladder and the samples were loaded onto the gel. Electrophoresis was run at 150 V for approximately 30 min. For documentation, a picture of the gel was taken under UV light.

2.12 Quantification of bacterial riboflavin

Note: In cooperation with Dr. Meina Neumann-Schaal

For the determination of the riboflavin content 30 ml culture were harvested anaerobically in the exponential phase as described previously (Dannheim et al., 2017). Bacteria were collected by centrifugation (10 min, 10.000 g, 4 °C), washed with sterile, anaerobe PBS and the precipitated bacteria were immediately frozen in liquid nitrogen. Bacteria were resuspended in 100 µL 1 M NaOH, vigorously mixed (5 min, 2000 rpm), neutralized with 400 µL 1 M potassium phosphate buffer pH 6.0 and centrifuged (5 min, 17.000 g, 4 °C). The supernatant was sterile filtered and the riboflavin concentration was determined using a 1260 Infinity HPLC system equipped with a fluorescence detector and a Poroshell 120 EC - C18 separation column. The samples were measured at 35 °C with a flow rate of 1 ml min^{-1} according to Glinko et al. (2008), using a programmed gradient mobile phase A (25 mM NaH_2PO_4, pH 2.5) and mobile phase B (methanol) with the following modifications: 0 - 1 min, linear gradient from 1 % B to 12 % B; 1 min - 1.03 min, step from 12 % B to 30 % B; 1.03 - 10 min, isocratic at 30 % B; 10 - 15 min, linear gradient from 30 % B to 100 % B; 15 - 17 min, isocratic at 100 % B; 17 - 17.03 min, step from 100 % B to 1 % B; 17.03 - 20 min, isocratic at 1 % B (column equilibration). Riboflavin was detected by fluorescence detection (FLD) at 450 nm excitation and 530 nm emission according to Chen et al. (2009).

2.13 Human samples

Blood donations. This study was conducted in accordance with the rules of the Regional Ethics Committee of Lower Saxony, Germany and the declaration of Helsinki. Buffy coats from blood donations of healthy human volunteers, who provided informed consent, were obtained from the Institute for Clinical Transfusion Medicine, Klinikum Braunschweig, Germany. Blood donors' health was assessed prior to blood donation. This procedure also included standardized laboratory tests for infections with HIV1/2, HBV, HCV and Treponema pallidum (serology and/or nucleic acid testing) and hematological cell counts.

2.14 PBMC isolation and stimulation

Buffy Coats were produced from whole blood donations by using the Top & Bottom Extraction Bag System (Polymed Medical Devices). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll® Paque PLUS density gradient centrifugation. PBMCs were rested overnight in RPMI complete at 37 °C in a humid 7.5 % CO2 atmosphere. 5 x 10^5 PBMCs were either left untreated, stimulated with PFA-fixed bacteria at different multiplicities of infection (MOI; bacteria per PBMC cell) for 6, 12, 17, and 20 h at 37 °C or with 100 ng/ml recombinant *C. difficile* toxins for 20 h. For stimulation with CDT, 50 ng/ml CDTa and 50 ng/ml CDTb were mixed. If indicated, PBMCs were treated with blocking antibodies against MR1 and/or against IL-12 p35 or IL-18 or with recombinant IL-12 p70 and IL-18, 1 hour prior stimulation with PFA-fixed *C. difficile* bacteria and/or toxin.

2.15 Fluorescence-activated cell sorting (FACS) of MAIT cells and stimulation

PBMCs were isolated as described above. An aliquot was taken and cells were stained against CD3, Vα7.2, and CD161 for 15 min at 4 °C to identify suitable donors for MAIT cell sorting (see also table 8). MAIT cells were sorted as $CD3^+$ V$\alpha7.2^+$ $CD161^{++}$ lymphocytes following doublet exclusion using a FACSAria II flow cytometer and following settings: 70 PSI system pressure, 30 000 events/sec flow rate, 488 nm laser with 100 mWatt for FITC, PE-Cy5, and PE-Cy7, and 640 nm laser with 60 mWatt for APC, detection with bandpass filters for FITC 525/50, PE-Cy5 670/14, PE-Cy7 780/60, and APC 670/30. Cells scatter forward (FSC, forward scatter) and side light (SSC, side scatter) with unique characteristics defining cell size and granularity, which was used to gate on lymphocytes. Doublets show a disproportional high integrated area of signal intensity compared to the height of signal intensity when passing the detector. This mismatch is detectable with forward and side scatter light (FSC-A vs. FSC-H and SSC-A vs. SSC-H, respectively). After exclusion of doublets, $CD3^+$ lymphocytes were gated followed by gating on $CD161^{++}$ and $Va7.2^+$ MAIT cells. Sorted cells were washed with FACS buffer and

rested overnight in RPMI *complete* at 37 °C in a humid 7.5 % CO_2 atmosphere. 20.000 MAITs were either left untreated or stimulated with 100 ng/ml *C. difficile* toxins for 20 h at 37 °C.

2.16 Quantification of cytokines

0.5 x 10^6 PBMCs were either left untreated or stimulated in duplicates with fixed *C. difficile* bacteria at MOI 1 or with 100 ng/ml recombinant *C. difficile* toxins for 20 h at 37 °C in duplicates. Supernatants of respective samples were pooled and enzyme-linked immunosorbent assays were performed to detect human IL-12 using Human IL-12 (p70) ELISA MAX™ kit, human IL-15 using Human IL-15 ELISA MAX™ kit, and human IL-18 using Human IL-18 ELISA kit (see table 6).

2.17 Antibody staining

Extracellular staining. Stimulated PBMCs were centrifuged for 5 min at 1400 rpm and 4 °C and supernatant was discarded (from now called „washing step“, exceptions are indicated). To exclude dead cells, 1:1000 dilution of LIVE/ DEAD™ Fixable Blue Dead Cell Stain or 1:500 dilution of Fixable Viability Dye near-IR together with human anti-CD16/CD32 was prepared in PBS. Cell pellets were resuspended in 50 µL and, incubated in the dark for 15 min at 4 °C. Fixable viability dies passed through the membrane of apoptotic cells and stained their cell interiors. Vital cells were only touched on the surface. Purified anti-CD16/32 is directed against FcγIII/II receptor that is located on dendritic cells, macrophages or activated T cells. By binding this receptor non-specific bindings of fluorochrome-associated antibodies was prevented (also called Fc-block). After staining, cells were always washed once with 150 µL PBS. Cell pellets were resuspended in 50 µL surface marker staining solution (Table 8) and again incubated in the dark for 30 min at 4 °C. A washing step was performed with FACS buffer instead of PBS. If no intracellular staining followed, cells were fixed with paraformaldehyde (PFA). To this end, cell pellets were resuspended in 50 µL of 2 % (v/v) PFA solution and dark-incubated for 10 min at RT. FACS buffer washing step followed, cell pellets were resuspended in 80 µL FACS buffer and stored in the dark at 4 °C until measured.

Intracellular staining. Intracellular cytokines were stained according to cytokine staining panel (shown in Table 9). Prior to staining, cells were permeabilized with 100 µl BD Cytofix/Cytoperm™ fixation solution and incubated for 20 min at 4 °C. A washing step followed with Perm/Wash buffer, cell pellets were resuspended in 50 µL intracellular cytokine antibody mix in Perm/Wash buffer and dark-incubated for 30 min at 4 °C. After further two washing steps with Perm/Wash buffer, cell pellets were resuspended in 80 µL FACS and transferred into FACS tubes for subsequent analysis.

2.18 Flow cytometry

Data of stimulated PBMCs was acquired on BD™ LSRII SORP or BD LSRFortessa™ flow cytometer. For data mining BD FACSDiva v6.1.3 software was used. Using antibodies with different conjugated fluorochromes can cause detection overlap (known as „spillover“) and therefore lead to false-positive signals. To prevent these false-positive signals, single stained UltraComp Beads™ with each fluorochrome-conjugated antibody were used for compensation to eliminate false-positive signals in each used laser channel. Further, fluorescence minus one (FMO) stainings were prepared as follows: PBMCs were stained with all antibodies of staining panel except one, i.e. for six antibodies included in staining panel, six FMOs had to be prepared. FMOs were used to define the abscence of a given fluorochrome while at the same time integrating spillover signals from all other fluorocrome channels. Thus FMOs helped to define how a "negative staining" for a certain channel looks like. Evaluation was performed with FlowJo v10.6.1 Software.

2.19 Sample preparation for quantitative proteomics

Cell lysis, protein digestion, and TMT labeling. 1 x 10^6 sorted MAIT cells were lysed in 50 µl lysis buffer (see table 2.5) for 30 minutes at 37 °C. Lysis buffer contained 50 mM DTT for reduction of proteins. After incubation, 400 mM iodoacetamide was added for 30 minutes at 37 °C, for alkylation and thereby protection of cysteines. Proteins were purified and digested on carboxylated beads according to the single-pot solid phase enhanced sample preparation (SP3) protocol described by Hughes et al. (2019). For digestion trypsin reagent was added at a protease to protein ratio of 1:25 weight per weight and incubated at 37 °C overnight. Tryptic peptides dissociated from carboxylated beads, therefore samples were centrifuged at 13.000 rpm for 5 min, and peptide-containing supernatant was collected, while carboxylated beads were fixed on a magnet, and vacuum dried. Peptides were dissolved in 62,5 µl 50 mM TEAB buffer and labeled with 41 µl of 0.8 mg isobaric TMT reagent per sample for 1 h according to manufacturer's guidelines. Samples were vacuum dried and dissolved in 0.2 % trifluoroacetic acid/3 % acetonitrile. Labeling control with sample aliquots was performed by an LC-MS run and samples were combined to equal parts.

2.20 Peptide clean up and fractionation by basic reverse-phase chromatography

The combined TMT-labeled peptide samples were further subfractionated by basic reverse-phase (b-RP) chromatography to support representative and comprehensive protein identification by LC-MS/MS. Peptides were dissolved in b-RP buffer with 1 % ACN, fractionated on a Zorbax 300 C18 column connected to an Äkta Purifier micro-LC system, and separated at a flow rate of 1 ml/min with linear gradients from 0% to 35 % ACN in b-RP

buffer for 60 min, from 35 % to 50 % ACN in b-RP buffer for 15 min, and from 50 % to 80 % ACN in b-RP buffer for 10 min. Fractions were collected by a microfraction collector every minute. Peptide elution was monitored by an UV detector at 214 nm. Peptide-containing fractions were combined (e.g. fractions 2-9-12) and vacuum-dried, dissolved in 0.2 % trifluoroacetic acid/3 % acetonitrile, and analyzed by LC-MS/MS.

2.21 LC-MS/MS measurement, protein identification and quantification

LC-MS/MS analyses of purified and desalted peptides were performed on a Dionex UltiMate 3000 n-RSLC system connected to an Orbitrap Fusion™ Tribrid™ mass spectrometer. Peptides of each fraction were loaded onto a C18 precolumn (3 µm RP18 beads, 75 mm x 20 mm), washed for 3 min at a flow rate of 6 µl/min and separated on a C18 analytical column (3-µm, 75 mm x 25 cm) at a flow rate of 350 µl/min via a linear 120 min gradient from 97 % MS buffer A (0.1 % formic acid) to 25 % MS buffer B (0.1 % formic acid, 80 % acetonitrile), followed by a 15 min gradient from 25 % MS buffer B to 62 % MS buffer B. The LC system was operated with the Chromeleon software embedded in the Xcalibur software suite. The effluent was electro-sprayed by a stainless steel emitter. Using the Xcalibur software, the mass spectrometer was controlled and operated in the "top speed" mode, allowing the automatic selection of as many doubly and triply charged peptides as possible in a three-second time window, and the subsequent fragmentation of these peptides. During peptide sequencing, TMT reporter ions were generated and used for quantification of peptide intensities. All peptide intensities of one protein were used for cumulative quantification of the protein abundance. Peptide sequencing and quantification, based on MS/MS data files, was performed via Proteome Discoverer™ linked to a Mascot™ server using the SwissProt/Uniprot database. Following search parameters were used:

Table 13: Mascot search parameters

Parameter	**Setting**
Protein database	SwissProt/Uniprot
Species	Homo sapiens
Enzyme	Trypsin
Maximum missed cleavage site	1
Precursor mass tolerance	10 ppm
Fragment mass tolerance	0.05 Da

Continued on the next page

Parameter	Setting
Dynamic modifications	TMT10plex (K)
	TMT10plex (N-term)
	oxidation (M)
Static modifications	carbamidomethyl (C)
Mass precision	2 ppm

Table 14: Filters in Proteome Discoverer

Filter	Setting
Peptide confidence	high
Search engine rank	1
FDR	1 %

2.22 Statistics

Flow cytometry. Data were acquired on BD LSR-II SORP and BD LSR-Fortessa flow cytometers. Data analysis was then carried out by FlowJo (TreeStar, v10.6.1) and Prism (GraphPad Software, v7.0c and v8.0). Samples were always measured in duplicates and mean of median fluorescence intensity or percentage of parameter-positive events was used for further analysis. Graphics always show mean ± standard deviation (SD), if not otherwise indicated. To determine significant differences between donor-specific samples, p-values were determined by Wilcoxon matched-pairs signed rank test. Significant p-values were indicated (* for $p \leq 0.05$; ** for $p \leq 0.001$; *** for $p \leq 0.0001$).

Mass spectrometry. Data evaluation and determination of significantly regulated proteins was performed in collaboration with Prof. Dr. Frank Klawonn. For statistic evaluation, donor-specific reporter ion intensities were $\log_2$-transformed and normalized by median of all $\log_2$ reporter ion intensities. To identify regulated proteins, Welch's t-test was applied to compare the $\log_2$ reporter ion intensity means of the two groups for each protein. The difference of the two group $\log_2$ reporter ion intensity means was defined as $\log_2$ regulation factor ($\log_2$ RF). Proteins with uncorrected p-values < 0.05 based on Welch's t-test and with $|\log_2 \text{RF}| > 1$ were considered as regulated.

3 Results

3.1 MAIT cell protein expression shows higher donor variation in aged individuals

Incidence, morbidity, and mortality in CDI especially increases in patients that are older than 65 (Lucado et al., 2006) and thus CDI is considered to be age-related (Lessa, 2012). Interestingly, the frequency of MAIT cells within the T cell pool decreases with progressing age but only limited knowledge exists regarding a potential functional impairment of MAIT cells in the elderly (Chen et al., 2019; Walker et al., 2014). The leading hypothesis of this study was that reduced frequency and a potential functional impairment of MAIT cells might contribute to enhanced CDI susceptibility in the aged population.

To experimentally prove this hypothesis, the effects of age on MAIT cell inventory was investigated using an unbiased comparative MAIT cell proteome approach. To this end, MAIT cells were purified from blood of four young healthy donors between 19 and 21 years of age (Donor 1 - 4) and from four old healthy donors between 65 and 69 years (Donor 7 - 10). To do this, PBMCs were isolated and stained with primary antibodies against CD3, Vα7.2, and CD161 (see also table 8). Fluorescence-activated cell sorting (FACS, see section 2.15) was used to sort CD161^{++}Va7.2^{+}CD3^{+} lymphocytes (MAIT cells) following exclusion of doublets. The used separation strategy is shown in Fig. 7. Purified MAIT cells were lyzed, proteins were cleaned up and digested on carboxylated beads according to SP3 protocol (see also section 2.19). Tryptic peptides of each donor were labeled with one isobaric TMT label. In a 30 minute LC-MS/MS run, injecting 100 ng peptide of each donor sample, labeling efficiency was quantified by Proteome Discoverer and peptide amount was quantified correlating spectral area of the sample with spectral area of the standard with known peptide amount. Due to low peptide amounts, donor 5 and donor 6 had to be excluded from the analysis. 2.5 µg peptide of each of the remaining eight donors were combined to one sample, and fractionated by basic reverse-phase (b-RP)

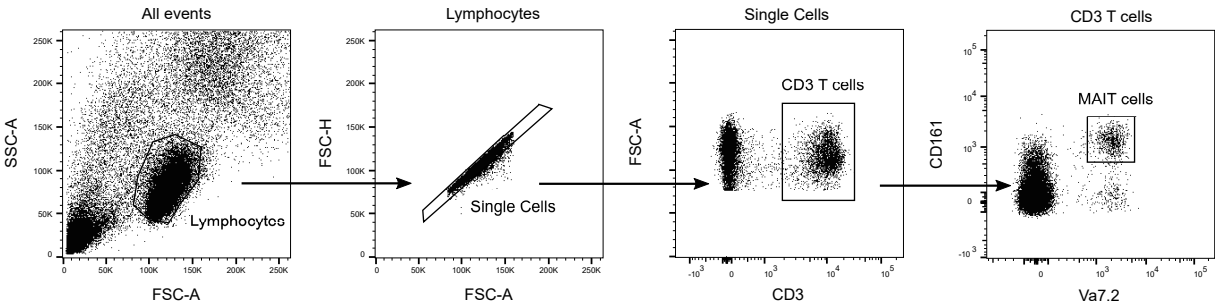

Figure 7: FACS strategy applied for the identification of human mucosal-associated invariant T (MAIT) cells. PBMCs were isolated from human blood. Live lymphocytes were discriminated by FSC-A and SSC-A. Doublets were discriminated by FSC-A and FCS-H. Cells were stained with antibodies specific for CD3, Vα7.2, and CD161 to separate CD161^{++}Va7.2^{+}CD3^{+} T (MAIT) cells by FACS.

chromatography to reduce sample complexity. After concatenation of peptide-containing fractions, 20 fractions were measured by LC-MS/MS (see also section 2.21).
The obtained MS and MS/MS spectra were annotated to peptides and proteins by Mascot and Proteome Discoverer software resulting in 6127 identified proteins in total. Of those, 5839 proteins could be quantified in all eight donors based on TMT reporter ions (see enclosed compact disk for complete list). For statistic evaluation, donor-specific reporter ion intensities were $\log_2$-transformed and normalized by median of all $\log_2$ reporter ion intensities. These ion reporter intensities were used for an unsupervised hierarchical clustering by Ward's method (Ward, 1963). Results summarized in Fig. 8 revealed that young and old individuals did not cluster according to their age (donor information in table 15). The first main cluster was formed by Donor 3 and Donor 9 that belong to the young or old group, respectively. All other donors clustered in the second main cluster. In particular, this indicates that MAIT cell proteomes do not exhibit global age-dependent alterations.

Table 15: Donor information

Donor	**Age**	**Sex**
1	19	female
2	20	male
3	21	female
4	21	male
7	69	male
8	68	male
9	66	male
10	65	female

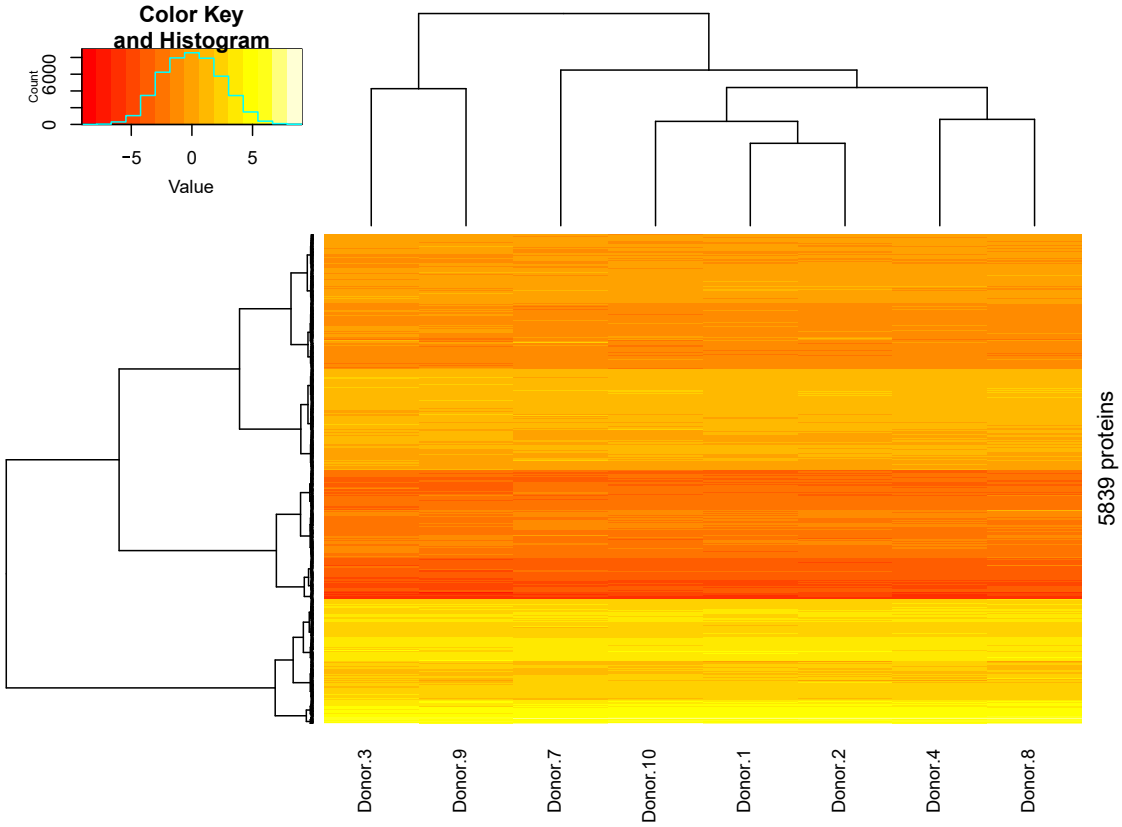

Figure 8: Hierarchical clustering of proteome data from MAIT cells obtained from young and old healthy donors. PBMCs were isolated from human blood of four young donors (Donor 1 - 4) and four old donors (Donor 7 - 10). MAIT cells were sorted and donor-specific MAIT cell proteins were identified by accurate LC-MS/MS. Heat map displays normalized $\log_2$ ion reporter intensities of all quantified proteins. Clustering was performed according to Ward's method (Ward, 1963).

To investigate whether individually regulated MAIT cell proteins show age-related differences, the mean of $\log_2$ reporter ion intensities for each protein per group (young or old individuals) was determined and the difference between old and young was defined as $\log_2$ regulation factor ($\log_2$RF). After calculation of the $\log_2$ RFs, a statistical approach was used to identify significantly regulated proteins in old individuals (see section 2.22). A protein was considered as significantly regulated if $|\log_2$ RF$|$ was higher than 1 and the probability (p-value) was less than 0.05. Differentially regulated MAIT cell proteins are displayed as red-bordered dots in the volcano plot (Fig. 9). These eight top-regulated proteins and their known T-cell specific functions are summarized in table 16. Upregulated MAIT cell proteins in the elderly are the Stimulator of interferon genes (STING), Methyl-CpG-binding protein 2 (MECP2), Apoptosis regulator Bcl-2 (BCL2), Activated RNA polymerase II transcriptional coactivator p15 (TCP4), and Hepatoma-derived growth factor-related protein 3 (HDGR3). Identified downregulated MAIT cell proteins in the elderly are Mucin-like protein 1 (MUCL1), α1-Antitrypsin (A1AT), and High mobility group nucleosome-binding domain-containing protein 3 (HMGN3).

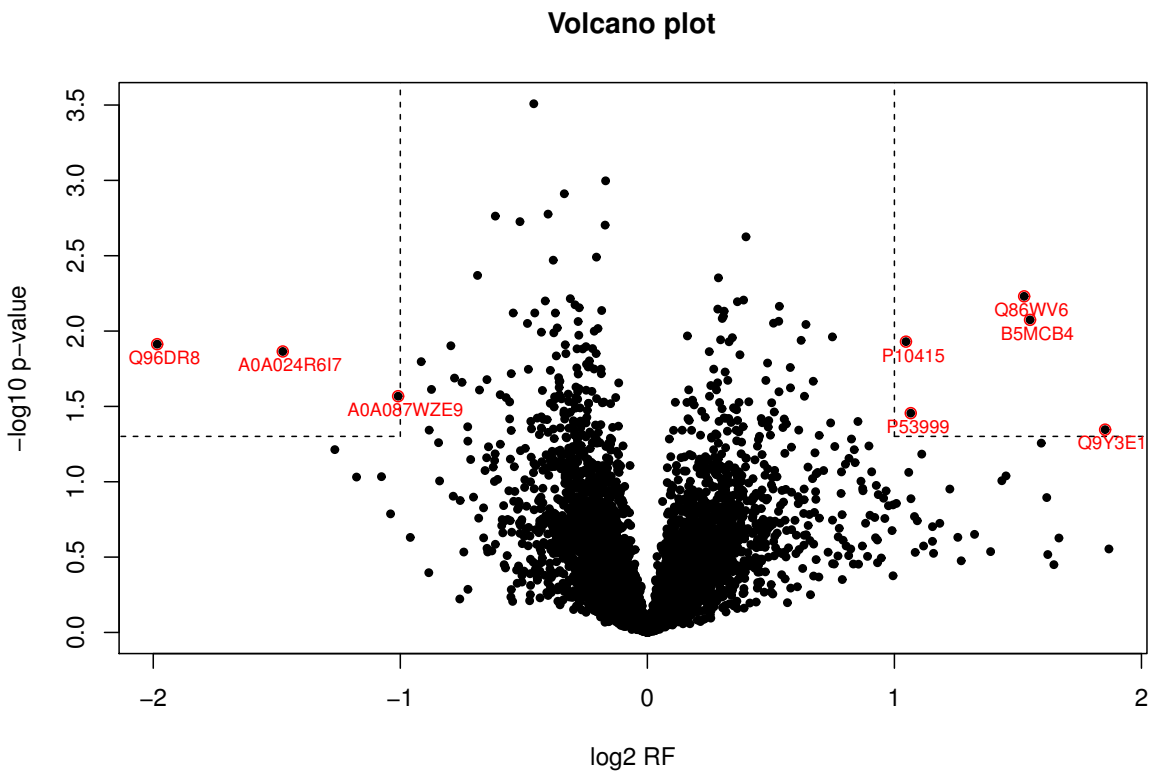

Figure 9: Volcano plot displaying MAIT cell proteins differentially expressed between old and young healthy individuals. PBMCs were isolated from human blood. MAIT cells were sorted by FACS and donor-specific MAIT cell proteins were identified by accurate LC-MS/MS. The vertical axis (y-axis) corresponds to the value of $\log_{10}$(p-value), and the horizontal axis (x-axis) displays the $\log_2$ RF of each protein. Proteins with -log10(p-value) $>$ 1.3 and $|\log_2 RF| > 1$ (dashed red line) are defined as regulated and depicted as red-bordered dots. Their corresponding Uniprot entries are indicated below.

Table 16: Differentially regulated MAIT cell proteins - old vs. young

			SD		
Uniprot	**Protein**	**$\log_2$RF**	**young**	**old**	**T cell-specific function**
Q86WV6	STING	1.53	0.42	0.56	Sensing of cytosolic DNA, stimulation of interferon genes (Larkin et al., 2017)
B5MCB4	MECP2	1.55	0.42	0.63	Epigenetic regulator critical for sustained Foxp3 expression in Tregs (Li et al., 2014)
P10415	BCL2	1.05	0.36	0.45	Inhibition of apoptosis (Cheng et al., 2004)

Continued on next page

			SD		
Uniprot	**Protein**	**log_2RF**	**young**	**old**	**T cell-specific function**
Q96DR8	MUCL1	-1.98	0.77	0.81	unk.; Analogue Mucin-protein 1 described as coinhibitory molecule (Mukherjee et al., 2005)
A0A024R6I7	A1AT	-1.48	0.67	0.44	unk.; described as protease inhibitor (Zhang et al., 2007; Petrache et al., 2006)
A0A087WZE9	HMGN3	-1.01	0.18	054	unk.; described as co-activator in tissue-specific gene expression (West et al., 2001)
P53999	TCP4	1.07	0.33	0.64	unk.,; described as coordinator of cellular responses after oxidative stress (Yu et al., 2016a)
Q9Y3E1	HDGR3	1.85	0.51	1.19	unk.; modulation of the neuronal cytoskeleton (El-Tahir et al., 2009)

Interestingly, mean standard deviation (MSD) of proteome data from young donors was determined as 0.364 (with confidence interval from 0.354 (2.5 %) to 0.375 (97.5 %)), whereas the MSD of proteome data from old donors was determined as 0.407 (with confidence interval from 0.395 (2.5 %) to 0.420 (97.5 %)). Standard deviations (SD) of differentially regulated MAIT cell proteins in the elderly are shown in table 17. This result indicates that donor variation of MAIT cell protein expression is higher in aged individuals compared to young individuals. Among the top ten MAIT cell proteins that show high expression variance in the elderly, determined as SD(young)-SD(old)-quotient (ΔSD), three human leukocyte antigen (HLA) proteins were identified that are involved in major histocompatibility complex class (MHC) antigen presentation (supplementary table 17). The log_2 reporter ion intensities of HLA-B(45), HLA-B(57), and DPB1 are shown in Fig. 10.

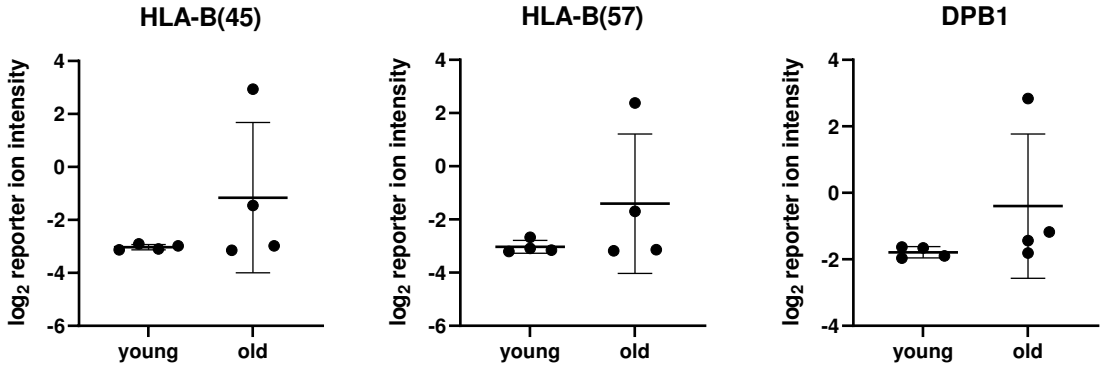

Figure 10: Expression variance of HLA proteins expressed on MAIT cells. PBMCs were isolated from human blood. MAIT cells were sorted by FACS and donor-specific MAIT cell proteins were identified by accurate LC-MS/MS. Donor-specific $\log_2$ reporter ion intensities of HLA-B(45), HLA-B(57), and DPB1 are shown. Mean ± SD.

In conclusion, apart from a small number of proteins that differed in abundance in MAIT cells from young and old individuals, no global age-related adaptions in the human MAIT cell proteome were observed that would explain impaired MAIT cell effector functions in the elderly. However, eight proteins were identified that are potentially regulated age-dependently. Interestingly, donor variation of protein expression in old individuals is higher than in young individuals. This might indicate that age-related factors such as co-morbidities and according medication may affect protein expression level in human MAIT cells.

3.2 Characterizing *C. difficile*-dependent MAIT cell responses

In the previous chapter it was demonstrated that age does not have global effects on MAIT cell proteome, suggesting that MAIT cell effector functions in the elderly are not impaired. In addition to age that is described as one of the major risk factors for enhanced susceptibility to CDI, the *C. difficile* ribotype causing the infection is as well relevant for the severity of infection. In the last decades, so-called hypervirulent *C. difficile* ribotypes causing severe *C. difficile*-associated colitis have been identified. Although MAIT cells represent an abundant T cell population residing in the gut and *C. difficile* was already predicted to be recognized by MAIT cells by Liuzzi et al. (2015) based on molecular characterization of the pathogen, the contribution of MAIT cells to immune response against *C. difficile* has not yet been investigated. To answer the question whether MAIT cells are responsive to *C. difficile*, it first has to be clarified whether *C. difficile* is a potential producer of MAIT cell-activating metabolites by investigating gene expression of riboflavin biosynthesis pathway.

3.2.1 *C. difficile* possesses an active riboflavin biosynthesis pathway

The production of bacterial riboflavin has been shown to be a crucial requirement for MR1-dependent MAIT cell activation (Kjer-Nielsen et al., 2012). Metabolites of this biosynthesis pathway can bind to the MHC-related protein, MR1, which is ubiquitously expressed in various mammalian tissues and cell types (Riegert et al., 1998; Hashimoto et al., 1995). This antigen-loaded MR1 complex interacts with the semi-invariant TCR on MAIT cells resulting in their activation. Genome analysis of *C. difficile* indicated the presence of a functional riboflavin pathway and thus suggested *C. difficile* to be capable to activate MAIT cells (Magnúsdóttir et al., 2015; Liuzzi et al., 2015).
However, expression of riboflavin pathway genes has not been experimentally investigated so far. Therefore, first of all the expression of riboflavin biosynthetic enzymes in two *C. difficile* strains grown in riboflavin-free medium was analyzed. The first investigated *C. difficile* strain with ribotype RT084 represents a prevalent non-toxigenic strain in hospitalized and non-hospitalized patients (Janssen et al., 2016). The second investigated *C. difficile* strain with RT023 represents a TcdA/B/CDT-toxigenic, hypervirulent strain causing diarrhea with high mortality rate in hospitalized patients (Shaw et al., 2019). *C. difficile* bacteria were harvested at mid-exponential phase (0.5 OD_{max}) and total bacterial RNA was isolated (see also section 2.11). Following reverse transcription, cDNA was visualized with DNA gel electrophoresis (shown in Fig. 11). Since *Escherichia coli* (*E .coli*) is a known constitutive producer of riboflavin (Vitreschak, 2002), *E. coli* strain BL21 grown in LB-medium was used as positive control. As negative control, a RT-PCR was performed without prior reverse transcription. Gene expression was analyzed for *ribD* that generates the MR1-binding ligand precursor 5-A-RU and additionally for *ribE* that, according to the KEGG pathway database, can influence 5-A-RU level as well.

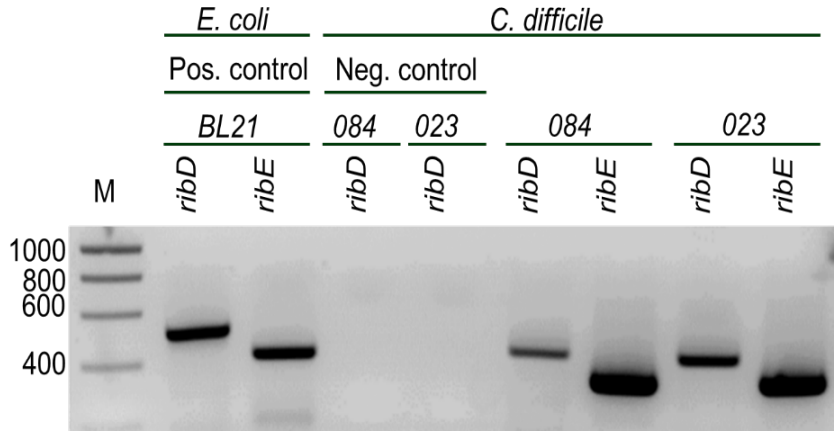

Figure 11: Functional riboflavin biosynthesis pathway in *C. difficile*. *C. difficile* strains of ribotype RT084 and RT023 were cultured in riboflavin-free CDMM medium under anaerobic conditions. Total RNA was isolated and RT-PCR was performed to analyze *ribD* and *ribE* gene expression. RT-PCR using a template without prior reverse transcription served as negative control. E. coli (BL21) was cultured in LB Medium overnight and isolated total RNA served as positive control for *ribD* and *ribE* gene expression. Smart DNA ladder was used as standard molecular-weight maker (M). Representative results from one of two independent experiments are shown. From Bernal et al. (2018).

As expected, in case of *E. coli* BL21 specific bands were detected at 500 bp for *ribD* and at 440 bp for *ribE* (shown in Fig. 11). The negative control did not show any contamination by remaining bacterial DNA. Indeed, substantial *ribD* and *ribE* expression was also observed on mRNA level in both *C. difficile* clinical isolates shown as specific bands at 450 bp for *ribD* and at 350 bp for *ribE*. These results reveal the active transcription of *ribD* and *ribE* genes indicating the expression of the corresponding enzymes. Consequently, an active riboflavin biosynthesis pathway in proliferating *C. difficile* was demonstrated, suggesting *C. difficile* to be competent to generate MAIT cell-activating ligands.

3.2.2 *C. difficile* induces activation and effector functions in primary human MAIT cells

Having demonstrated an active riboflavin biosynthesis pathway (section 3.2.1), it was examined whether *C. difficile* would be able to activate human MAIT cells *in vitro*. To this end, a hypervirulent strain with ribotype RT023 was selected and its ability to activate MAIT cells from healthy individuals was probed. As established for other bacterial species before (Dias et al., 2016), MAIT cell activation was tested by using PFA-fixed *C. difficile* that were applied to PBMCs from different donors at different MOIs (0.01, 0.1, and 1) for 20 h. Following stimulation cells were stained for the early activation marker CD69, for intracellular IFNγ, as well as for the lytic granule components perforin and granzyme B (GzmB). For flow cytometric analysis, cells were gated on live CD161^{++}Va7.2^{+}CD3^{+} T (MAIT) cells according to the gating strategy depicted in Fig. 12.

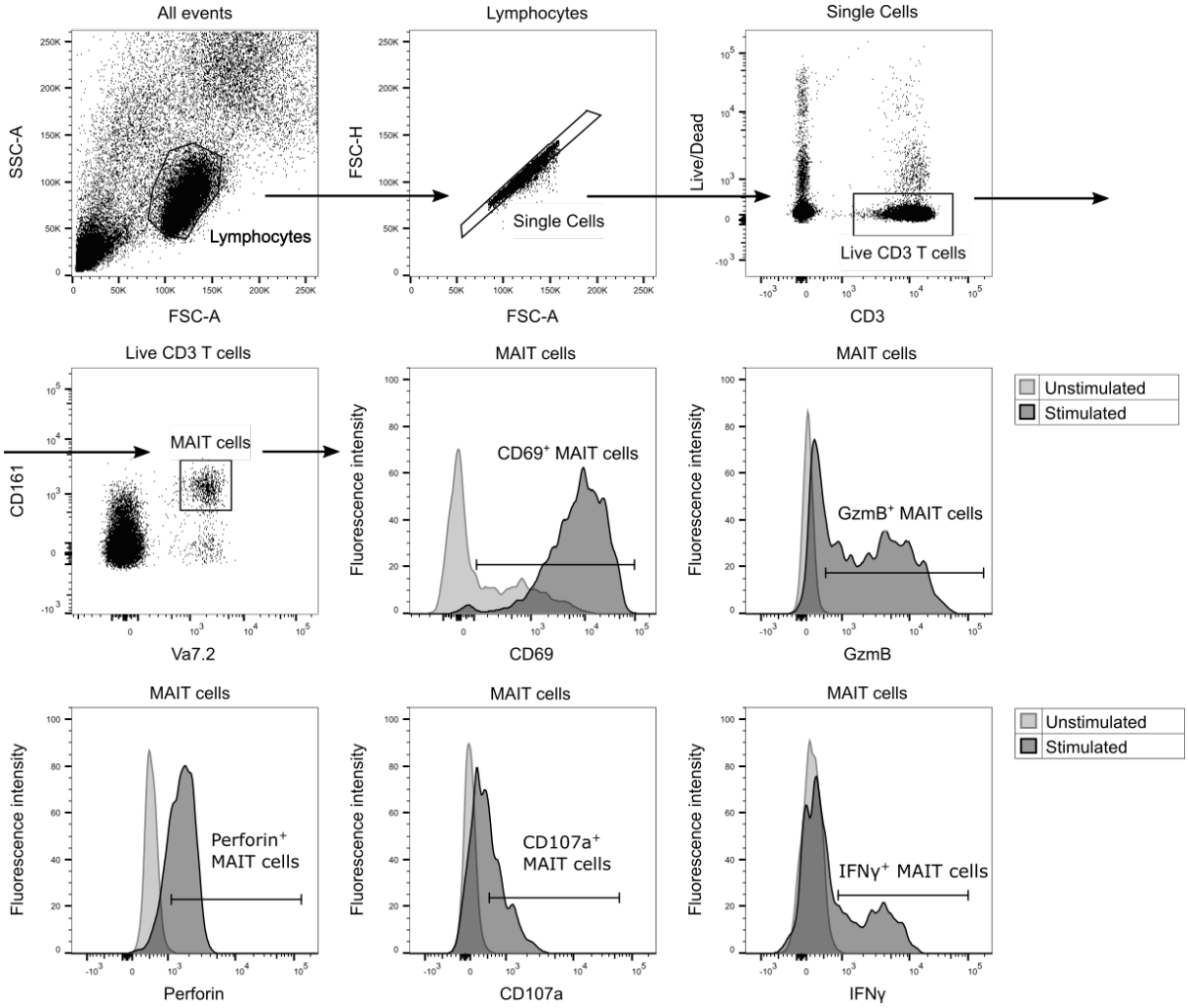

Figure 12: Gating strategy applied to identify human mucosa-associated invariant T (MAIT) cells. PBMCs were isolated from human blood; live cells were discriminated with Live/Dead dye. Cells were also stained with antibodies specific for CD3, Vα7.2, CD161, CD69, Interferon γ (IFNγ), perforin, granzyme B (GzmB), and CD107a followed by flow cytometric analysis.

Indeed, *C. difficile* RT023 caused marked MAIT cell activation already at low bacterial doses (MOI 0.01) as indicated by the significant increase of CD69 surface expression on stimulated compared to unstimulated MAIT cells (62.7 ± 18.4 % vs. 40.5 ± 14.3 %, Fig. 13 A), while 10-times higher bacterial numbers were required to induce significant IFNγ expression (2.1 ± 1.1 % vs. 1.1 ± 0.4 %, Fig. 13 B). Interestingly, as for CD69 induction, MOI of 0.01 was validated to be sufficient to trigger the cytotoxic effector phenotype of MAIT cells indicated by significant increase of perforin (67.7 ± 7.7 % vs. 47.0 ± 7.0 %) and granzyme B (GzmB, 7.3 ± 5.5 % vs. 5.1 ± 4.6 %) (Fig. 13, C and D). These data indicate that MAIT cell cytotoxicity and proinflammatory cytokine response triggered by *C. difficile* exhibit distinct antigenic activation thresholds. However, expression of all investigated markers showed a bacterial dose-dependent increase. At MOI 1 almost all MAIT cells were activated (89.0 ± 10.1 %) and enhanced expression of perforin (83.9 ± 7.9 %), GzmB (25.2 ± 20.2 %), and IFNγ (8.4 ± 7.0 %) was observed. In conclusion,

C. difficile is competent to induce MAIT cell effector functions including cytotoxicity mediated by GzmB and perforin and proinflammatory response via IFNγ.

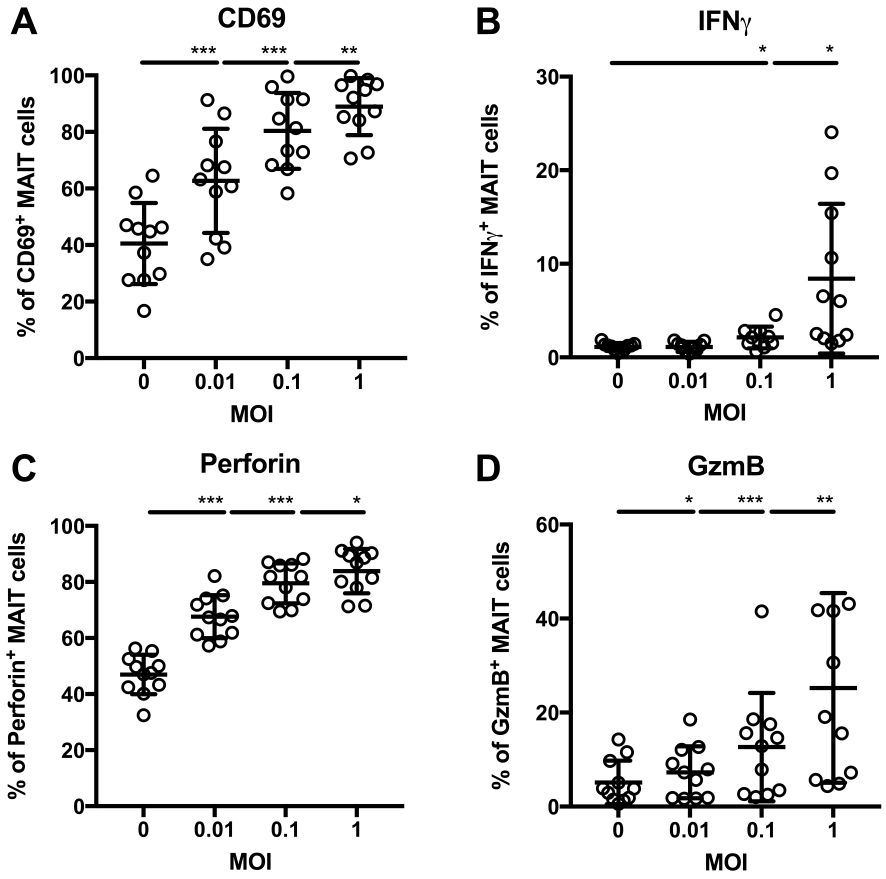

Figure 13: Dose-dependent activation of primary human MAIT cells following stimulation with *C. difficile* (ribotype RT023). PBMCs were isolated from healthy donors and stimulated with paraformaldehyde-fixed *C. difficile* isolate ribotype RT023 for 20 h followed by flow cytometric analyses of surface staining of CD69 (A) and intracellular staining of IFNγ (B), perforin (C) and granzyme B (GzmB, D). Mean percentages ± SD are shown. Cells were gated on CD161^{++}Va7.2^{+}CD3^{+} T cells (MAIT cells). Wilcoxon signed rank test for paired samples was used to detect significant differences and determine p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). From Bernal et al. (2018).

3.2.3 *C. difficile*-induced MAIT cell activation and effector functions depend on MR1 or IL-12/IL-18

In the previous section (3.2.2), it was shown that PFA-fixed hypervirulent *C. diffcile* (RT023) is capable to induce MAIT cell activation and effector responses. However, MAIT cells can be activated MR1/TCR-dependently or TCR-independently by the cytokines IL-12 and IL-18 (Ussher et al., 2014; Le Bourhis et al., 2010). To dissect the relevance of these two distinct stimulation conditions for *C. difficile*-mediated MAIT cell activation in more detail, PBMCs from healthy donors were stimulated *in vitro* with the hypervirulent *C. difficile* ribotype RT023 in the presence of MR1 and/or IL-12/IL-18 blocking antibodies

followed by analysis of MAIT cell responses after 20 h. MAIT cells were stimulated with *C. difficile* using a MOI of 1 that is able to induce robust and significant MAIT cell responses (Fig. 13).

Since IFNγ and GzmB expression is *de novo* induced in activated MAIT cells, the frequency of MAIT cells positive for these markers was measured (Fig. 14, B and D). For CD69 and perforin, which both showed basal expression levels already in unstimulated MAIT cells, changes in median of mean fluorescence intensity (MFI) were determined (Fig. 14, A and C). Representative dot plots obtained following gating on $CD3^+$ T cells are depicted on the left panel and cumulative expression data of $CD161^{++}V\alpha7.2^+CD3^+$ T cells (MAIT cells) are shown on the right panel. As shown before (Fig. 13), MAIT cells readily responded to *C. difficile* stimulation by significant upregulation of CD69 (3278.0 $\pm$ 2956.6 MFI vs. 100.2 $\pm$ 220.5 MFI), IFNγ (5.8 $\pm$ 5.9 % vs. 1.3 $\pm$ 0.9 %), perforin (1010.3 $\pm$ 158.8 MFI vs. 535.7 $\pm$ 100.4 MFI) and GzmB (24.5 $\pm$ 16.3 % vs. 4.3 $\pm$ 5.0 %) (Fig. 14, A - D).

CD69 induction was significantly (but not completely) blocked by anti-MR1 (1138.1 $\pm$ 1125.0 MFI), anti-IL-12/IL-18 (1175.6 $\pm$ 951.4 MFI) and a combination of both treatments (1455.0 $\pm$ 845.2 MFI) when compared to MAIT cells stimulated in the absence of MR1 and/or cytokine blocking antibodies, indicating that *C. difficile*-induced CD69 expression is both MR1- and cytokine-dependent (Fig. 14, A). The fact that combined treatment with anti-MR1 and anti-IL-12/IL-18 did not completely block the activation suggests that there are additional MAIT cell activating pathways that contribute to *C. difficile*-mediated MAIT cell activation.

Interestingly, IFNγ expression was blocked almost completely by anti-MR1 (1.7 $\pm$ 1.4 % vs. 5.8 $\pm$ 5.9 %), indicating that IFNγ induction by *C. difficile* requires cell-to-cell contact via MR1 on PBMCs and the TCR on MAIT cells. However, in contrast to CD69, perforin and GzmB, blockade of IL-12/IL-18 signaling alone did not significantly affect IFNγ production in *C. difficile*-stimulated MAIT cells (Fig. 14, B). To directly determine whether IL-12/IL-18 have an effect on IFNγ response of MAIT cells, PBMCs were stimulated with RT023 together with recombinant IL-12/IL-18. Indeed, addition of exogenous cytokines resulted in an enhanced IFNγ expression in MAIT cells (33.5 $\pm$ 20.2 % vs. RT023 alone: 3.0 $\pm$ 2.1 %) as shown in Fig. 15.

Regarding perforin, the blockade of MR1 or/and IL-12/IL-18 signaling resulted in a significant reduction of its expression (anti-MR1: 748.7 $\pm$ 127.7 MFI, anti-IL-12/IL-18: 829.4 $\pm$ 161.3 MFI, anti-MR1/IL-12/IL-18: 821 $\pm$ 151.7 MFI vs. RT023: 1010.3 $\pm$ 158.8 MFI) (Fig. 14, C). However, combined application of MR1 and IL-12/IL-18 blocking antibodies was not sufficient to completely inhibit perforin expression in *C. difficile*-stimulated MAIT cells, indicating that its expression is regulated also by other pathways. The induction of GzmB expression following stimulation by *C. difficile* was almost completely blocked by anti-MR1 (5.6 $\pm$ 5.1 % vs. RT023: 24.5 $\pm$ 16.3 %) and significantly reduced by anti-IL-12/IL-18 treatment (8.3 $\pm$ 6.3 %) as well as the combination of both treatments (6.8 $\pm$

6.9 %).

In conclusion, despite obvious donor-specific variations in the expression levels of the analyzed markers, it was shown that *C. difficile* activates effector functions in human MAIT cells in a MR1- and in part cytokine-dependent manner. Moreover, high MR1-dependency of IFNγ and GzmB expression following *C. difficile* stimulation indicates a cell contact-dependent MAIT cell response for these particular effector molecules.

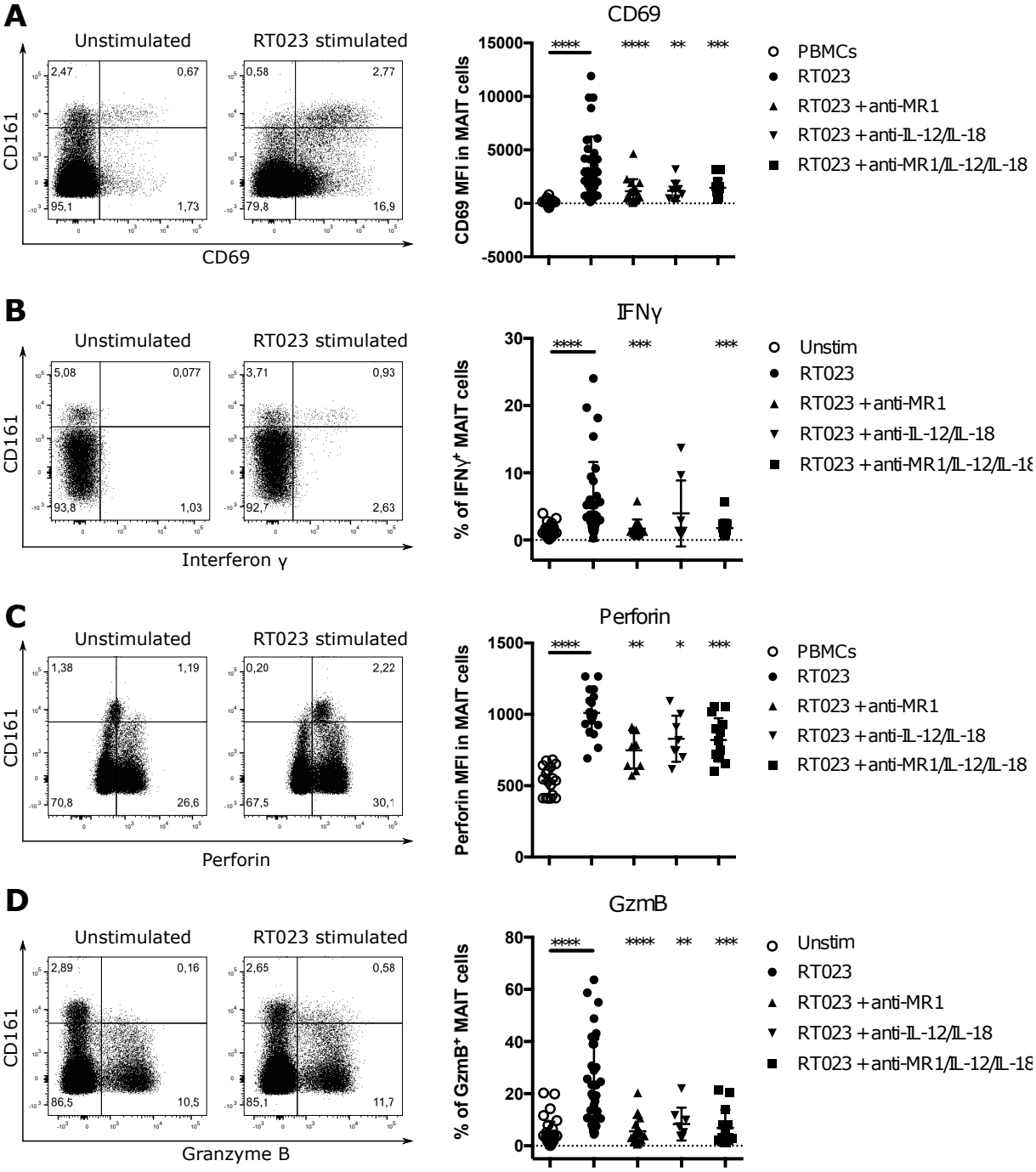

Figure 14: MR1- and cytokine-dependent activation of primary human MAIT cells following stimulation with *C. difficile* (ribotype RT023). PBMCs were isolated from healthy donors (A, B, and D: n = 8 - 36, for C: n = 8 - 14) and stimulated with *C. difficile* clinical isolate of ribotype RT023 at MOI 1 for 20 h followed by flow cytometric analyses of indicated parameters. Mean percentages ± SD are shown. Cells were gated on $CD161^{++}V\alpha7.2^{+}CD3^{+}$ T cells (MAIT cells). Wilcoxon signed rank test for paired samples was used to detect significant differences and determine p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Left: representative plots of $CD3^{+}$ T cells. Right: combined data from 12 independent experiments and 8 - 36 donors are shown. From Bernal et al. (2018).

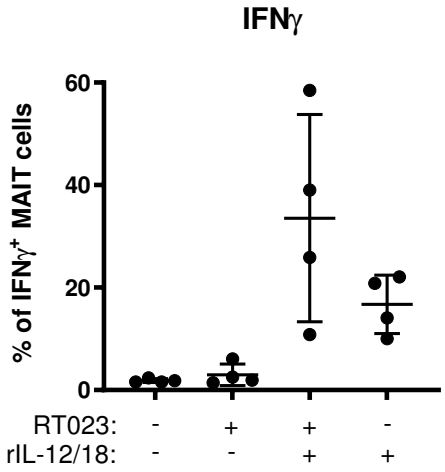

Figure 15: Effects of IL-12 and IL-18 on IFNγ response of primary human MAIT cells following stimulation with *C. difficile* (ribotype RT023). PBMCs were isolated from healthy donors (n=4) and stimulated with *C. difficile* strain of RT023 at MOI 1 for 20 h followed by flow cytometric analyses of IFNγ response. Mean percentages ± SD are shown. Cells were gated on CD161^{++}Vα7.2^{+}CD3^{+} T cells (MAIT cells). From Bernal et al. (2018).

Finally, the kinetics of *C. difficile*-induced MAIT cell responses has been probed by investigating CD69, IFNγ, perforin, and GzmB expression in response to *C. difficile* with ribotype RT023 at 6, 12, 17, and 20 h (Fig. 16). Additionally, the surface level of CD107a expression was examined to monitor potential degranulation of lytic granules. Interestingly, significant upregulation of CD69 (4244.25 ± 1015.8 MFI) compared to unstimulated (unstim) cells (227.7 ± 53.8 MFI) was observed at 12 h with a peak at 17 h post *C. difficile* stimulation (12758.3 ± 2797.7 MFI vs. 385.2 ± 73.0 MFI; Fig. 16 A, C, D). Similar kinetics were observed for perforin (985.8 ± 94.7 MFI vs. unstim: 418.3 ± 32.6 MFI), and GzmB (13.9 ± 3.0 % vs. unstim. 1.8 ± 0.5 %) at 12 h with a peak at 17 h (GzmB 36.7 ± 7.2 % vs. unstim. 1.9 ± 0.6 % and perforin 1658.5 ± 177.3 MFI vs. 785,125 ± 124.2 MFI). At 17 h post stimulation, the highest increase in IFNγ expression (11.4 ± 3.9 % vs. unstim. 1.6 ± 0.9 %) was detected, which however showed relatively strong donor variation (Fig. 16, B). Interestingly, at 17 h post *C. difficile* stimulation, enhanced surface expression of CD107a (884.9 ± 175.0 MFI vs. 352.8 ± 67.9 MFI) was observed indicating cytotoxic activity of MAIT cells in response to *C. difficile* (Fig. 16, E). In general, time course experiments confirmed CD69, IFNγ, perforin, and GzmB expression to be dependent on MR1 as described before for the 20 h stimulation experiments (Fig. 14), and an additional contribution of IL-12/IL-18 became apparent for CD69 (e.g. at 17 h, anti-MR1/IL-12/IL-18: 2392.7 ± 330.8 MFI vs. anti-MR1: 4948.8 ± 1048.4 MFI) and GzmB (e.g. at 17 h, anti-MR1/IL-12/IL-18: 5.7 ± 1.4 % vs. anti-MR1: 15.3 ± 4.4 %) shown in Fig. 16, A and D. In contrast, IFNγ and perforin induction on MAIT cells were found to be mainly MR1-dependent at all investigated time points, since additional block-

ade of IL-12/IL-18 showed only limited effects. In conclusion, *C. difficile*-induced MAIT cell response was detectable earliest at 12 h following stimulation with a peak response at 17 h. Expression dynamics of the effector molecules IFNγ, GzmB and perforin were found to be largely MR1-dependent while MAIT cell degranulation appears to involve additional IL-12/IL-18 cytokine signaling.

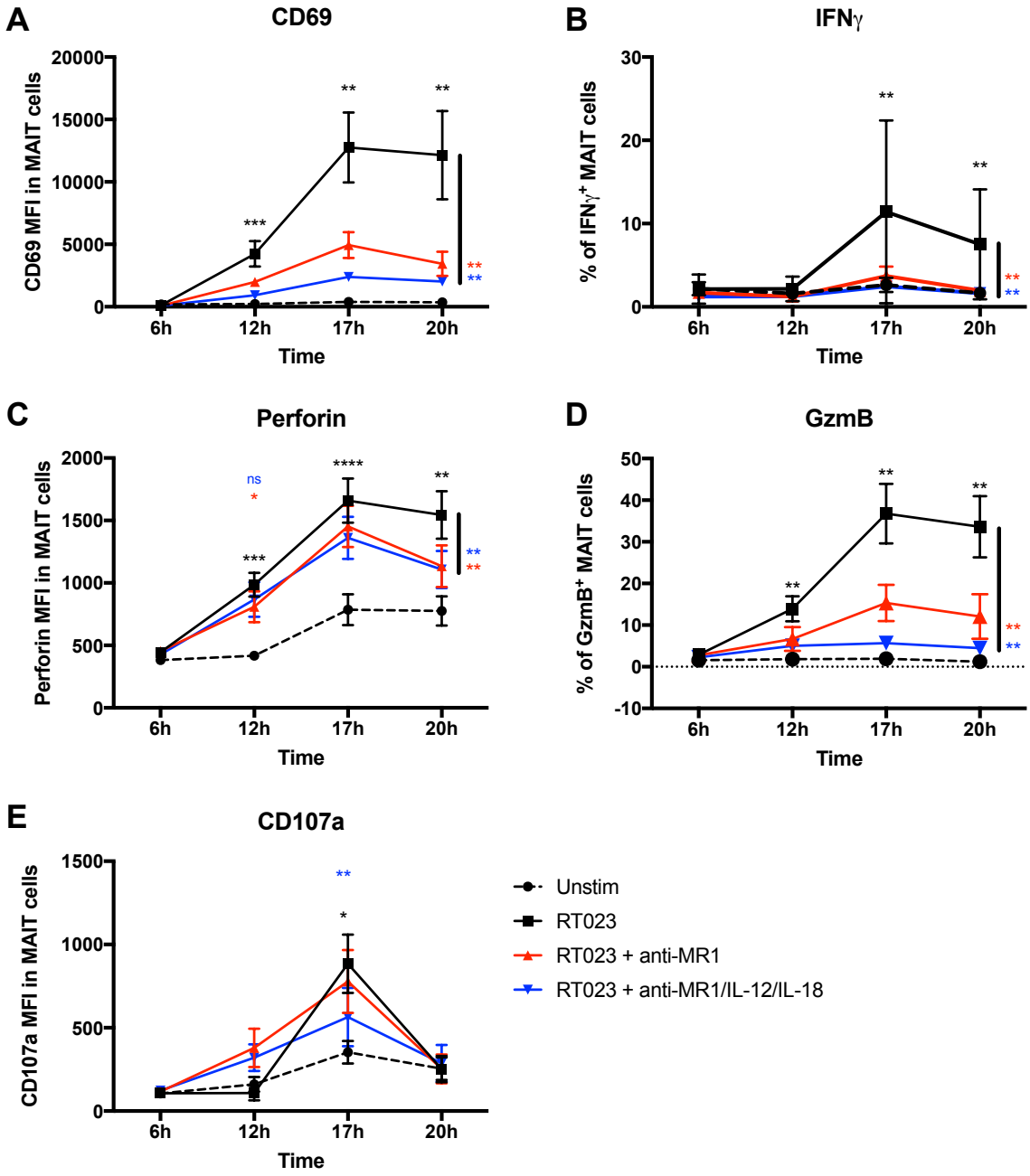

Figure 16: Kinetics of parameter expression of primary human MAIT cells following stimulation with *C. difficile* (ribotype RT023). PBMCs were isolated from healthy donors and stimulated with *C. difficile* of ribotype RT023 at MOI 1 for indicated time periods followed by flow cytometric analyses of surface staining of CD69 (A), CD107a (E) and intracellular staining of IFNγ (B), perforin (C), and granzyme B (GzmB, D). Means ± SEM are shown. Cells were gated on $CD161^{++}V\alpha7.2^{+}CD3^{+}$ T cells (MAIT cells). Black asterisks (*) indicate significant differences of marker expression in stimulated MAIT cells compared to the unstimulated controls. Blue and red asterisks indicate significant differences of marker expression in stimulated MAIT cells compared to antibody treated samples. 8 - 12 donors per group were tested, by Wilcoxon signed rank test for paired samples and determine p-values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Combined data from five independent experiments are shown. From Bernal et al. (2018).

3.2.4 Hypervirulent *C. difficile* strains provoke strongest MAIT cell activation and effector function

Next to their antibacterial function MAIT cell activation can result in exaggerated release of proinflammatory mediators such as IFNγ, GM-CSF, and TNF-α and thus may contribute to excessive immunopathology at the site of infection (Shaler et al., 2017). The toxins of hypervirulent ribotypes are considered to be the causative agents for detrimental courses of CDAC (Chumbler et al., 2016; Pothoulakis, 1996). Therefore, it was next tested whether these strains would be particularly active in riboflavin synthesis and whether this might be the basis for an extraordinary strong MAIT cell activation.

To this end, riboflavin levels were quantified by fluorescence detection in five different *C. difficile* clinical isolates (described in section 2.12). These included a non-toxigenic strain (ribotype RT084), two TcdA/B-toxigenic strains (ribotypes RT012 and RT001) as well as two hypervirulent TcdA/B/CDT-toxigenic strains (ribotypes RT023 and RT027). Since it is known that riboflavin biosynthesis is highly regulated in *C. difficile* and *C. difficile* toxins are not detectable in the exponential growth phase (Hofmann et al., 2018), bacteria were harvested at exponential growth phase for quantification of riboflavin and fixed bacteria from the same culture to analyze MAIT cell response without toxin-dependent effects. Indeed, strain-dependent differences with respect to riboflavin level were observed (Fig. 17, A). Surprisingly, highest riboflavin content was not detected in the hypervirulent strains (ribotypes RT023 and RT027) but in the non-toxigenic strain (RT084: 10.1 $\pm$ 0.6 ng/mg dry weight). The second highest riboflavin level was determined in TcdA/B toxigenic strain with RT012 with 7.5 $\pm$ 0.1 ng/mg dry weight. The hypervirulent strains with RT023 and RT027 showed intermediate riboflavin levels with 6.0 $\pm$ 0.5 ng/mg dry weight and 6.3 $\pm$ 0.4 ng/mg dry weight, respectively. The lowest riboflavin level was found in TcdA/B-toxigenic strain with RT001 with 4.2 $\pm$ 0.3 ng/mg dry weight. In particular, one hypothesis was that the riboflavin level correlate with the magnitude of MAIT cell responses and thus could serve as an indicator for the contribution of MAIT cells to the inflammatory response during CDAC.

Interestingly, despite containing highest riboflavin level, the non-toxigenic strain (ribotype RT084) did not induce MAIT cell activation (CD69) but cytotoxic effector function (GzmB: 3.4 $\pm$ 2.1 % vs. unstim. 0.9 $\pm$ 0.5 %) (Fig. 17, B and C). Moreover, TcdA/B toxigenic *C. diffcile* RT012 did as well only induce GzmB expression (6.6 $\pm$ 3.7 % vs. unstim. 0.9 $\pm$ 0.5 %) which was slightly higher than that observed in RT084-stimulated MAIT cells. The second investigated TcdA/B-toxigenic *C. difficile* strain RT001 was capable to induce both MAIT cell activation (CD69: 65.6 $\pm$ 25.0 % vs. unstim. 42.6 $\pm$ 11.7 %) and cytotoxicity (GzmB: 6.1 $\pm$ 4.6 % vs. unstim. 0.9 $\pm$ 0.5 %), however, cytotoxic response was similar to RT012. Strikingly, strongest CD69 and GzmB induction was observed in MAIT cells stimulated with the hypervirulent *C. difficile* that only contained an intermediate riboflavin level (CD69 73.1 $\pm$ 22.4 % RT023 and 85.2 $\pm$ 13.5 % RT027, GzmB: 13.1

± 9.6 % RT023 and 14.6 ± 9.3 % RT027). Consequently, bacterial riboflavin concentration did not correlate with the observed strength of MAIT cell responses. This indicates that the measurable riboflavin content does not necessarily reflect the concentration of MAIT cell-activating ligands (riboflavin intermediates) being present in *C. difficile*.
Differences in MAIT cell activation may be caused by different antigen concentrations but also by different cytokine concentrations. Thus, we hypothesized that strain-dependent differences in cytokine-induced activation might also contribute to enhanced MAIT cell activation following stimulation with hypervirulent *C. difficile* (ribotype RT023 and RT027). To investigate this, the concentrations of MAIT cell-activating cytokines IL-12 and IL-18 were quantified in the supernatant of PBMCs stimulated with fixed *C. difficile* for 20 h by ELISA (described in section 2.16). IL-12 and IL-18 were not detectable in the supernatant of unstimulated PBMCs (17, D and E). Interestingly, *C. difficile* RT084 induced only low concentration of IL-12 (10.3 ± 9.3 pg/ml) but no IL-18, whereas *C. difficile* RT001 induced no IL-12 but the highest concentration of IL-18 (79.8 ± 73.7 pg/ml) among tested isolates. *C. difficile* RT001, which induced significant MAIT cell activation and cytotoxicity, caused only moderate secretion of IL-12 (18.3 ± 11.5 pg/ml) and IL-18 (24.7 ± 12.6 pg/ml). Surprisingly, hypervirulent *C. difficile* (ribotype RT023 and RT027) induced a higher IL-12 secretion by antigen presenting cells than less virulent strains (RT023: 146.3 ± 104.5 pg/ml and RT027: 94.8 ± 114.3 pg/ml). However, IL-18 secretion was moderate or even low in hypervirulent *C. difficile* (RT023: 38.0 ± 9.7 pg/ml and RT027: 5.7 ± 1.3 pg/ml), suggesting that hypervirulent strains induce an IL-12 specific cytokine pattern in antigen presenting cells.

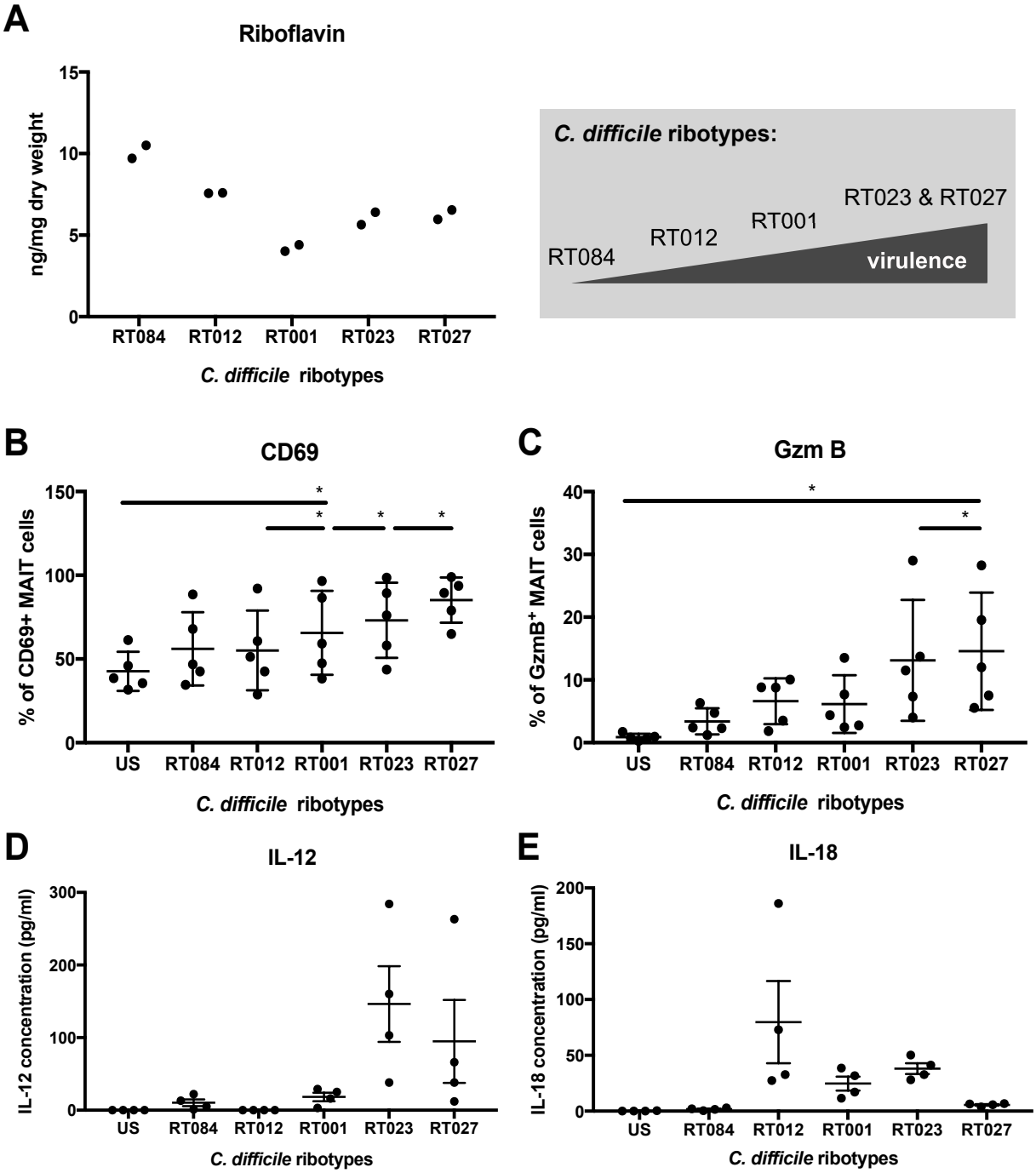

Figure 17: MAIT cell activation by *C. difficile* clinical isolates that differ in riboflavin metabolism. Riboflavin content of *C. difficile* clinical isolates was measured by fluorescence detection and ng/mg dry weight ± SD is shown (A). Data of technical replicates from two cultivations are shown. PBMCs were isolated from healthy donors and stimulated with *C. difficile* clinical isolates of the ribotypes RT084, RT012, RT001, RT023 and RT027 at MOI 1 for 20 h followed by flow cytometric analyses of activation marker CD69 (B) and intracellular GzmB (C). Means ± SD are shown. Cells were gated on $CD161^{++}Va7.2^{+}CD3^{+}$ T cells (MAIT cells). Wilcoxon signed rank test for paired samples was used to detect significant differences and determine p-values (*$p < 0.05$). Combined data from two independent experiments using five donors are shown. IL-12 (D) and IL-18 (E) were quantified in PBMC supernatant. Means ± SD are shown. Data from one experiment and four donors are shown. From Bernal et al. (2018).

Summarizing this chapter, proliferating *C. difficile* were demonstrated to possess an active riboflavin biosynthesis pathway, suggesting *C. difficile* to be generally competent to produce MAIT cell-activating ligands. Indeed, data collected in framework of this study demonstrated that *C. difficile* does induce MAIT cell effector functions including cytotoxic responses by GzmB and perforin and proinflammatory response via IFNγ. Despite obvious donor-specific variations in the expression levels of the analyzed markers, it was shown that *C. difficile* activates effector functions in human MAIT cells in a MR1- and in part cytokine-dependent manner. Moreover, high MR1-dependency of IFNγ and GzmB expression indicates a cell contact-dependent targeted MAIT cell response. Investigating the kinetics, *C. difficile*-induced MAIT cell response was detectable earliest at 12 h following stimulation with a peak response at 17 h. Expression dynamics of the effector molecules IFNγ, GzmB and perforin were found to be largely MR1-dependent while MAIT cell degranulation appears to involve additional IL-12/IL-18 cytokine signaling. *C. difficile* clinical isolates included in this survey differed in riboflavin production, induced different cytokine patterns in antigen presenting cells, and showed different capacity to induce MAIT cell activation and cytotoxicity. Interestingly, hypervirulent *C. difficile* produced only an intermediate riboflavin level but induced an IL-12-specific cytokine pattern and superior MAIT cell responses, suggesting a role of MAIT cells as drivers of inflammation and immunopathology in CDAC.

3.3 *C. difficile* toxins modulate MAIT cell response

In the previous chapter, MAIT cells were suggested as new players in CDI. It has been shown for the first time that *C. difficile* induces MAIT cell effector phenotype including cytotoxicity and proinflammatory response. Moreover, hypervirulent *C. difficile* ribotypes showed superior MAIT cell activation compared to less virulent ribotypes (Bernal et al., 2018). The chosen experimental approach so far excluded *C. difficile* toxin-dependent effects on MAIT cells due to the specific preparation and PFA fixation of *C. difficile* bacteria that were used for *in vitro* stimulation assays (section 2.9). However, *C. difficile* toxins TcdA, TcdB, and binary toxin CDT are characteristic for hypervirulent *C. difficile* ribotypes causing severe CDAC and therefore knowledge of their specific effects on MAIT cells would be of utmost importance as well. For this purpose, the previously established *in vitro* MAIT cell stimulation system was adapted to the stimulation of PBMCs from healthy individuals with purified *C. difficile* toxins in the presence or absence of fixed *C. difficile* RT023 bacteria. With this approach, toxin-induced MAIT cell apoptosis and effector phenotype can be investigated. To experimentally prove whether toxin-specific effects do depend on their enzymatic activity, glucosyltransferase-deficient TcdA and TcdB have been generated, and examined whether these mutated toxins are still be capable to induce MAIT cell activation. To understand the underlying mechanisms of toxin-specific MAIT cell activation, prototypic MAIT cell activation pathways like MR1 antigen presentation and cytokine signaling were perturbed and differences in MAIT cell response were assessed. By combined stimulation with hypervirulent *C. difficile* RT023 bacteria and their toxins, synergistic effects on MAIT cell response were investigated.

3.3.1 *C. difficile* toxins TcdA and CDT activate MAIT cells

Hypervirulent *C. difficile* ribotypes produce TcdA, TcdB, and binary toxin CDT which are described as causative agents for the clinical spectrum of severe CDAC (Chumbler et al., 2016; Pothoulakis, 1996). So far, there are no experimental data available regarding the potential effects of *C. difficile* toxins on MAIT cell activation and effector phenotype.
To experimentally address this, *ex vivo* isolated MAIT cells were stimulated with hypervirulent *C. difficile*-derived toxins TcdA, TcdB, and binary toxin CDT. TcdA, TcdB, glucosyltransferase-deficient TcdA (GT-dTcdA), glucosyltransferase-deficient TcdB (GT-dTcdB), and CDT were generated and provided by Dr. Ralf Gerhard (as described in section 2.10), and their capacity to activate MAIT cells from healthy individuals was examined. PBMCs were stimulated with TcdA, GT-dTcdA, TcdB, GT-dTcdB, and CDT at different concentrations ranging from 25 ng/ml to 150 ng/ml for 20 h followed by flow cytometric assessment of CD69 expression on MAIT cells (gating strategy according to Figure 12). To visualize differences in strength of activation, median fluorescence intensities (MFI) are shown (Fig. 18). Surprisingly, TcdA and CDT induced significant MAIT cell activation already at low concentrations (25 ng/ml) as indicated by the sig-

nificant increase of CD69 surface expression (Figure 18, A and E). Higher concentrations of TcdA and CDT did not further enhance CD69 expression in MAIT cells, suggesting that already low doses resembling physiological concentrations are sufficient to trigger a full MAIT cell activation. TcdA-induced CD69 expression was even seven-times higher than CDT-induced CD69 expression (e.g. at 25 ng/ml: 37,663.9 ± 37,733.2 MFI and 5435.4 ± 4842.2 MFI compared to unstim. 914.9 ± 573.4 MFI). Glucosyltransferase activity of *C. difficile* toxins was shown to be essential for target cell intoxication (Yang et al., 2015; Sehr et al., 1998; Hofmann et al., 1997) and stimulation with GT-dTcdA did not induce CD69 expression in MAIT cells (Fig. 18, B), demonstrating that at least for TcdA, MAIT cell activation also depends on glucosyltransferase activity. Unexpectedly, TcdB and GT-dTcdB stimulation did not induce CD69 expression in MAIT cells (Fig 18, C and D), although TcdB has been described to be at least 100-fold more potent than TcdA in its ability to induce cell rounding and apoptotic cell death in various cell types (Chaves-Olarte et al., 1997; Donta et al., 1982).

Since MAIT cells are part of the T cell compartment, it was analyzed whether non-MAIT cells were activated following stimulation with either 100 ng/ml TcdA or CDT for 20 h. Because notable CD69 expression in non-MAIT cells is only induced upon activation, the frequencies of CD69-positive cells were determined. Indeed, CD69 expression of non-MAIT cells was significantly increased following stimulation with TcdA and CDT (Figure 19, B). However, after stimulation with TcdA a much lower frequency of $CD69^+$ non-MAIT cells (31.8 ± 10.4 % vs. unstim. 7.1 ± 2.5 %) than $CD69^+$ MAIT cells (90.9 ± 7.0 % vs. unstim. 50.5 ± 17.8 %) was observed (Fig. 19 A and B). Stimulation with GT-dTcdA did also not activate non-MAIT cells, suggesting that activation of non-MAIT cells also depends on glucosyltransferase activity. Regarding CDT, 18.9 ± 11.4 % of non-MAIT cells were $CD69^+$ following stimulation compared to 7.1 ± 2.5 % $CD69^+$ unstimulated non-MAIT cells. TcdA and CDT showed similar capacities to induce significantly CD69 expression in MAIT cells and non-MAIT cells, however, MFI revealed that TcdA-induced CD69 expression intensity on MAIT cells was 4 times higher than CDT-induced CD69 expression intensity (27948.4 ± 29482.0 % vs. 6975.4 ± 5539.6 MFI, data not shown).

In conclusion, only TcdA and CDT, but not TcdB induced MAIT cell-specific activation. Already low concentrations of TcdA and CDT led to a full MAIT cell activation. However, TcdA-induced CD69 expression was four to seven times higher than CDT-induced expression, suggesting that *C. difficile* toxin TcdA is far more competent in activating MAIT cells than CDT.

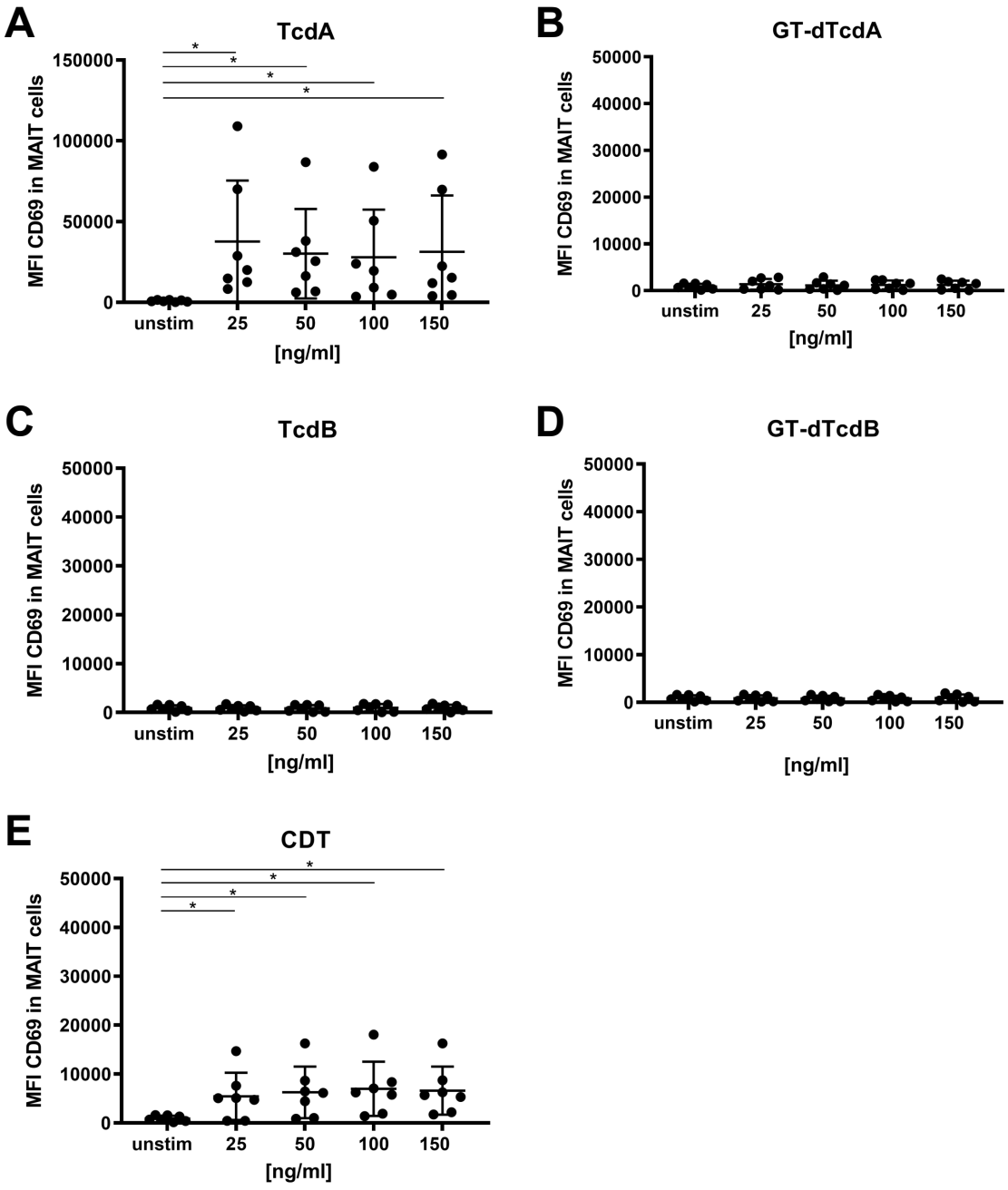

Figure 18: MAIT cell activation following stimulation with *C. difficile* toxins. PBMCs were isolated from healthy donors and stimulated with 25-150 ng/ml of *C. difficile* toxins TcdA (A), GT-dTcdA (B), TcdB (C), and CDT (D) for 20 h followed by flow cytometric analysis of CD69 expression. Cells were gated on $CD161^{++}Va7.2^{+}CD3^{+}$ T cells (MAIT cells). Horizontal lines indicate mean fluorescence intensity ± SD. Stars indicate significant differences determined by Wilcoxon matched-pairs signed rank test: *$p<0.05$. Combined data from five independent experiments with seven donors are shown.

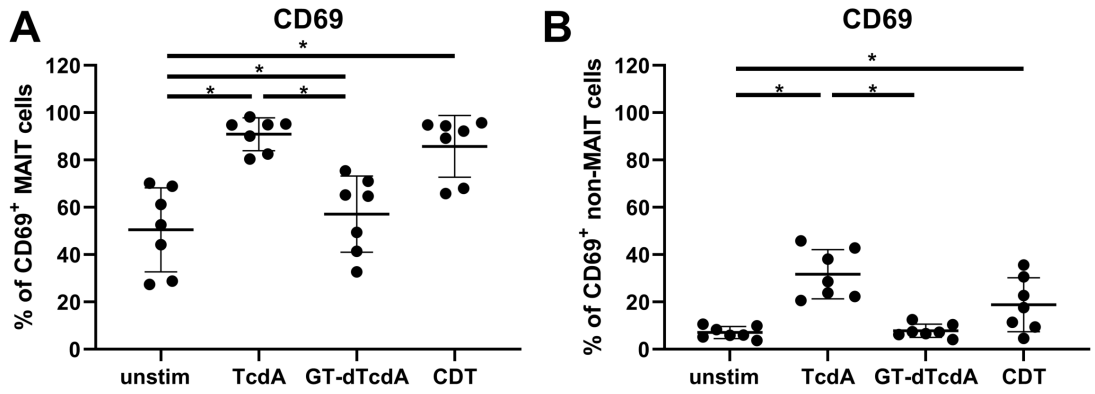

Figure 19: CD69 expression by MAIT cells and non-MAIT cells following stimulation with *C. difficile* toxins. PBMCs were isolated from healthy donors and stimulated with 100 ng/ml of *C. difficile* toxins for 20 h followed by flow cytometric analyses of CD69 surface expression. Cells were gated on $CD161^{++}Va7.2^{+}CD3^{+}$ T cells (MAIT cells). Non-MAIT cells include $CD161^{+/-}$ $V\alpha7.2^{-}$ and $CD161^{-}$ $V\alpha7.2^{+}$ CD3 T cells. Horizontal lines indicate mean fluorescence intensity ± SD. Asterisks indicate significant differences determined by Wilcoxon matched-pairs signed rank test: *$p<0.05$. Combined data from five independent experiments with seven donors are shown.

3.3.2 TcdA provokes stronger MAIT cell effector response than CDT

Upon activation, MAIT cells gain effector functions including cytotoxic response and secretion of cytokines (Le Bourhis et al., 2013). So far, the effects of *C. difficile* toxins TcdA and CDT on MAIT cell response were not investigated.

To next elucidate MAIT cell function following TcdA- or CDT-induced activation, PBMCs from healthy individuals were stimulated with 100 ng/ml TcdA, GT-dTcdA, and CDT for 20 h followed by assessing apoptosis (by 7-AAD and annexin V) and intracellular cytokine expression by flow cytometry. Interestingly, neither TcdA nor GT-dTcdA and CDT induced apoptosis in MAIT cells, suggesting that MAIT cells are not sensitive for intoxication by TcdA and CDT (Fig. 20, A). As shown before (Fig. 18), MAIT cells readily responded to TcdA and CDT stimulation as indicated by significant upregulation of CD69 (Figure 20, B). Since granzyme B and IFNγ expression are known to be induced upon activation, the frequency of MAIT cells positive for these markers was measured (Figure 20, C and F). For perforin and CD107a, which were already expressed at basal levels in unstimulated MAIT cells, changes in median fluorescence intensity (MFI) were determined (Figure 20, D and E). As shown in Fig. 20, C and D, TcdA induced a strong cytotoxic response in MAIT cells indicated by a significant increase of granzyme B (GzmB, 67.0 ± 27.1 % vs. unstim. 3.9 ± 1.5 %) and perforin expression (1001.3 ± 571.7 % vs. unstim. 387.6 ± 248.0 %). TcdA-induced cytotoxic MAIT cell response was significantly higher than CDT-induced cytotoxic response (GzmB: 67.0 ± 27.1 % vs. 10.5 ± 4.6 %) and depended on glucosyltransferase activity because stimulation with GT-dTcdA did

not induce GzmB and perforin expression. TcdA and CDT showed no differences in their capacity to induce significant MAIT cell degranulation as measured by CD107a surface expression (265.7 ± 56.2 MFI vs. 281.7 ± 89.4 MFI) (Fig. 20, E). As expected, GT-dTcdA did not induce MAIT cell degranulation, suggesting that glucosyltransferase activity of

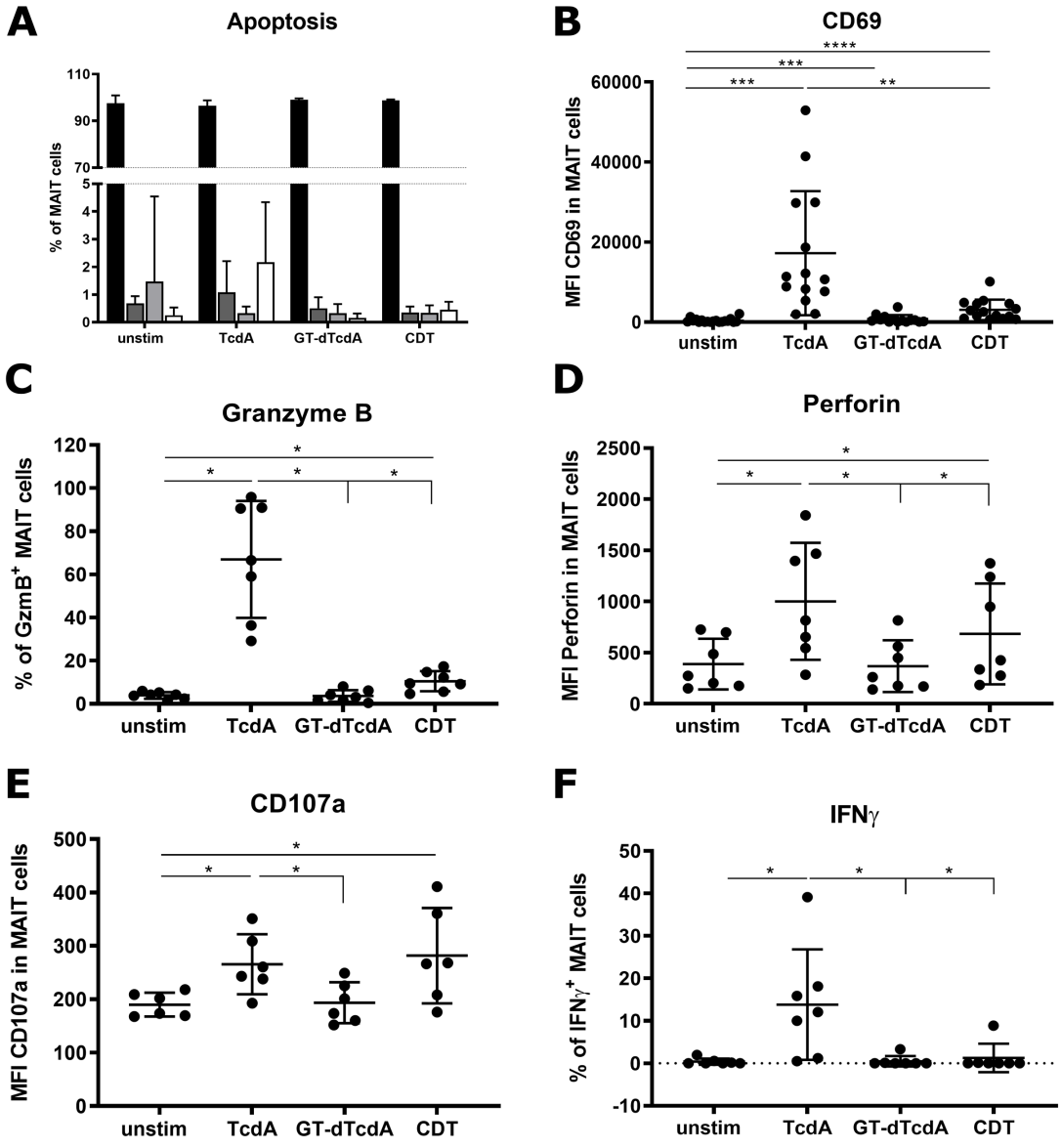

Figure 20: Effector phenotype of primary human MAIT cells following stimulation with *C. difficile* toxins. PBMCs were isolated from healthy donors and stimulated with 100 ng/ml *C. difficile* toxins for 20 h followed by flow cytometric analysis of apoptosis (A), granzyme B (B), CD69 (C), perforin (D), CD107a (E) and IFNγ expression (F). Cells were gated on CD161^{++}Va7.2^{+}CD3^{+} T cells (MAIT cells). A: Live (black), early apoptotic (dark grey), late apoptotic (light grey), and dead (white) MAIT cells are depicted. Horizontal lines indicate mean ± SD. Asterisks indicate significant differences determined by Wilcoxon matched-pairs signed rank test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Combined data from nine independent experiments with 6 - 19 donors are shown.

TcdA is essential for stimulation of MAIT cell cytotoxicity.
Interestingly, TcdA but not CDT was capable to significantly induce IFNγ expression (13.8 ± 13.0 % vs. unstim. 0.4 ± 0.7 %), suggesting that TcdA triggers different MAIT cell activating mechanisms compared to CDT (Figure 20, F). In conclusion, TcdA and CDT induced cytotoxic MAIT cell response but not apoptosis. TcdA induced a more pronounced MAIT cell effector phenotype than CDT and additionally a proinflammatory cytokine response.

3.3.3 TcdA-induced MAIT cell activation depends on IL-18, IL-12, and MR1

In the previous section, it was demonstrated that *C. difficile* toxin TcdA induced a distinct MAIT cell effector phenotype. To elucidate the underlying mechanisms, it was next investigated whether *C. difficile* toxins have a direct or indirect effect on MAIT cells. Although it is known that MAIT cells can be activated in a TCR- and cytokine-dependent manner (Lamichhane et al., 2019), there is so far no evidence that MAIT cells are targets for TcdA and CDT intoxication.
$CD161^{++}Va7.2^{+}CD3^{+}$ MAIT cells from healthy individuals were sorted followed by stimulation with TcdA, GT-dTcdA, and CDT, and subsequent analysis of CD69 expression via flow cytometry (Fig. 21, A). Surprisingly, direct stimulation of MAIT cells with *C. difficile* toxins TcdA and CDT did not lead to induction of CD69 expression, suggesting that the toxin-induced CD69 expression on MAIT cells that was found previously using an assay system based on PBMC culture (Fig. 18 - 20) was an indirect effect that requires accessory cells present in the culture. As shown before in PBMC culture (Fig. 20, A), viability of sorted MAIT cells was also not altered following toxin stimulation (data not shown), suggesting that MAIT cells do not express toxin-binding receptors and thereby are not sensitive for toxic effects.
However, MAIT cells could also respond to cytokines secreted by other intoxicated cells and *C. difficile* toxin TcdA was shown to induce the expression of several cytokines including MAIT cell-activating IL-12 in PBMCs (Yu et al., 2017). Therefore, concentrations of known MAIT cell-activating cytokines IL-12, IL-15, and IL-18 were determined in the supernatant of TcdA- and CDT-stimulated PBMCs. Interestingly, no IL-15 was detectable, suggesting that IL-15 is not required for *C. difficile* toxin-induced MAIT cell response (data not shown). The concentration of IL-18 was significantly increased following stimulation of PBMCs with TcdA (43.6 ± 41.9 pg/ml) and CDT (34.5 ± 23.9 pg/ml) compared to unstimulated PBMCs (6.7 ± 4.8 pg/ml) with higher donor variability observed for TcdA (Fig. 21, B). Interestingly, only TcdA was capable to induce IL-12 secretion in PBMCs (19.9 ± 24.4 pg/ml vs. unstim. 0.1 ± 0.3 pg/ml).
To confirm IL-12 and IL-18 dependency of MAIT cell responses following TcdA and CDT stimulation, blocking antibodies against IL-12 or IL-18 were added 1 h prior to stimulation. Alternatively, either recombinant IL-18 (rIL-18) or IL-12 (rIL-12) was supplemented

at the beginning of stimulation (Fig. 21, C). After 20 h, MAIT cell expression of CD69, Granzyme B (GzmB), perforin, CD107a, and IFNγ was assessed by flow cytometry. Regarding CD69 (Fig. 21, C upper panel), again a significant increase of CD69 expression was observed following stimulation with TcdA and CDT (9647.1 ± 7145.7 MFI and 2042.9 ± 1429.6 vs. unstim. 150.0 ± 199.0). TcdA-induced CD69 expression could significantly (but not completely) be blocked by anti-IL-18 (5979.9 ± 5066.6 MFI) and by anti-IL-12 (6170.5 ± 5351.7 MFI). Addition of rIL-18 and rIL-12 resulted in enhanced CD69 expression of TcdA-stimulated MAIT cells (rIL-18: 13399.5 ± 7276.9 MFI and rIL-12: 14267.6 ± 7601.9 MFI). As for TcdA, CDT-induced CD69 expression was as well significantly blocked by anti-IL-18 (1916.7 ± 1631.0 MFI) and enhanced by rIL-18 (5794.7 ± 4445.1 MFI). Since CDT did not induce IL-12 secretion (Fig. 21, B), it was only examined whether rIL-12 supplementation would enhance MAIT cell response. However, no increase of CD69 expression was observed (Fig. 21, C), indicating that CDT-induced MAIT response does not depend on IL-12.

As expected, TcdA and CDT significantly increased GzmB expression in MAIT cells (67.6 ± 17.3 % and 8.9 ± 11.1 % vs. unstim. 1.4 ± 1.8 %) as shown in Fig. 21 C, upper panel. TcdA-induced GzmB expression could significantly be blocked by anti-IL-18 (43.7 ± 17.9 %) and by anti-IL-12 (37.3 ± 13.3 %). Accordingly, the addition of rIL-18 and rIL-12 resulted in enhanced GzmB expression in TcdA-stimulated MAIT cells (rIL-18: 78.7 ± 13.8 % and rIL-12: 74.5 ± 12.3 %). In the same line, CDT-induced GzmB expression was as well significantly blocked by anti-IL-18 (2.3 ± 1.0 %) and enhanced by rIL-18 (15.1 ± 14.0 %). Again, addition of rIL-12 did not result in increased GzmB expression, indicating that also CDT-induced MAIT cell cytotoxicity does not depend on IL-12.

Surface expression of the degranulation marker CD107a was significantly increased on MAIT cells following stimulation with TcdA (384.6 ± 164.9 MFI) and CDT (203.5 ± 45.3 MFI) compared to unstimulated MAIT cells (130.5 ± 21.4 MFI). TcdA-induced degranulation and thus CD107a surface expression could significantly be blocked by anti-IL-18 (280.1 ± 131.1 MFI) and by anti-IL-12 (305.6 ± 132.8 MFI). The addition of rIL-18 and rIL-12 resulted in enhanced CD107a expression in TcdA-stimulated MAIT cells (rIL-18: 459.1 ± 170.8 MFI and rIL-12: 465.5 ± 190.2). CDT-induced CD107a expression could also be significantly blocked by anti-IL-18 (187.3 ± 40.2 MFI) and enhanced by rIL-18 (300.2 ± 110.2 MFI). As expected from the previous results, addition of rIL-12 did not result in increased CD107a expression.

Perforin expression was significantly increased following stimulation with TcdA and CDT compared to unstimulated MAIT cells (1192.5 ± 192.2 MFI and 701.5 ± 89.5 MFI vs. unstim. 516.8 ± 64.0 MFI). TcdA-induced perforin expression could significantly be blocked by anti-IL-12 (1061.0 ± 156.4 MFI) but was not enhanced by rIL-12 or rIL-18 supplementation. CDT-induced perforin expression was not altered by anti-IL-18, rIL-18, or rIL-12, suggesting that CDT-dependent perforin expression in MAIT cells is regulated by other pathways.

As expected, TcdA but not CDT induced IFNγ expression (22.2 $\pm$ 13.8 % vs. unstim. 1.0 $\pm$ 0.8 %) as depicted in Fig. 21 C, lower panel. TcdA-induced IFNγ expression in MAIT cells was blocked almost completely by anti-IL-12 or anti-IL-18 (3.5 $\pm$ 3.9 % and 6.9 $\pm$ 3.7 %) and stimulation with rIL-12 or rIL-18 resulted in an even more pronounced IFNγ expression (44.0 $\pm$ 17.4 % and 35.0 $\pm$ 19.7 %). Interestingly, the addition of rIL-18 induced significant IFNγ expression in CDT-stimulated MAIT cells.

To additionally investigate whether the MR1 antigen presentation pathway is involved in toxin-induced MAIT cell activation, an antibody against MR1 was added 1 h prior to stimulation (Fig. 21, C). Unexpectedly, TcdA- and CDT-induced MAIT cell activation (CD69) and effector functions (IFNγ, GzmB, perforin, CD107a) could be blocked almost completely by anti-MR1 treatment, suggesting that MR1-mediated antigen presentation is crucial for toxin-induced MAIT cell effector function even in the absence of bacterial metabolites.

In conclusion, toxin-induced MAIT cell activation requires accessory cells contained in PBMC cultures. TcdA-stimu-lated PBMCs produce IL-12 and IL-18, whereas CDT-stimulated PBMCs produce IL-18. MAIT cell activation and effector function that are indicated by enhanced CD69, GzmB, CD107a, and TcdA-induced IFNγ expression depend on IL-12 and IL-18 produced by accessory cells. In addition to cytokine-dependent stimulation, MR1-mediated antigen presentation contributes to toxin-dependent MAIT cell activation, cytotoxicity, and proinflammatory response, suggesting that next to cytokines cell contact between intoxicated antigen presenting cells and MAIT cells is required for a robust MAIT cell response.

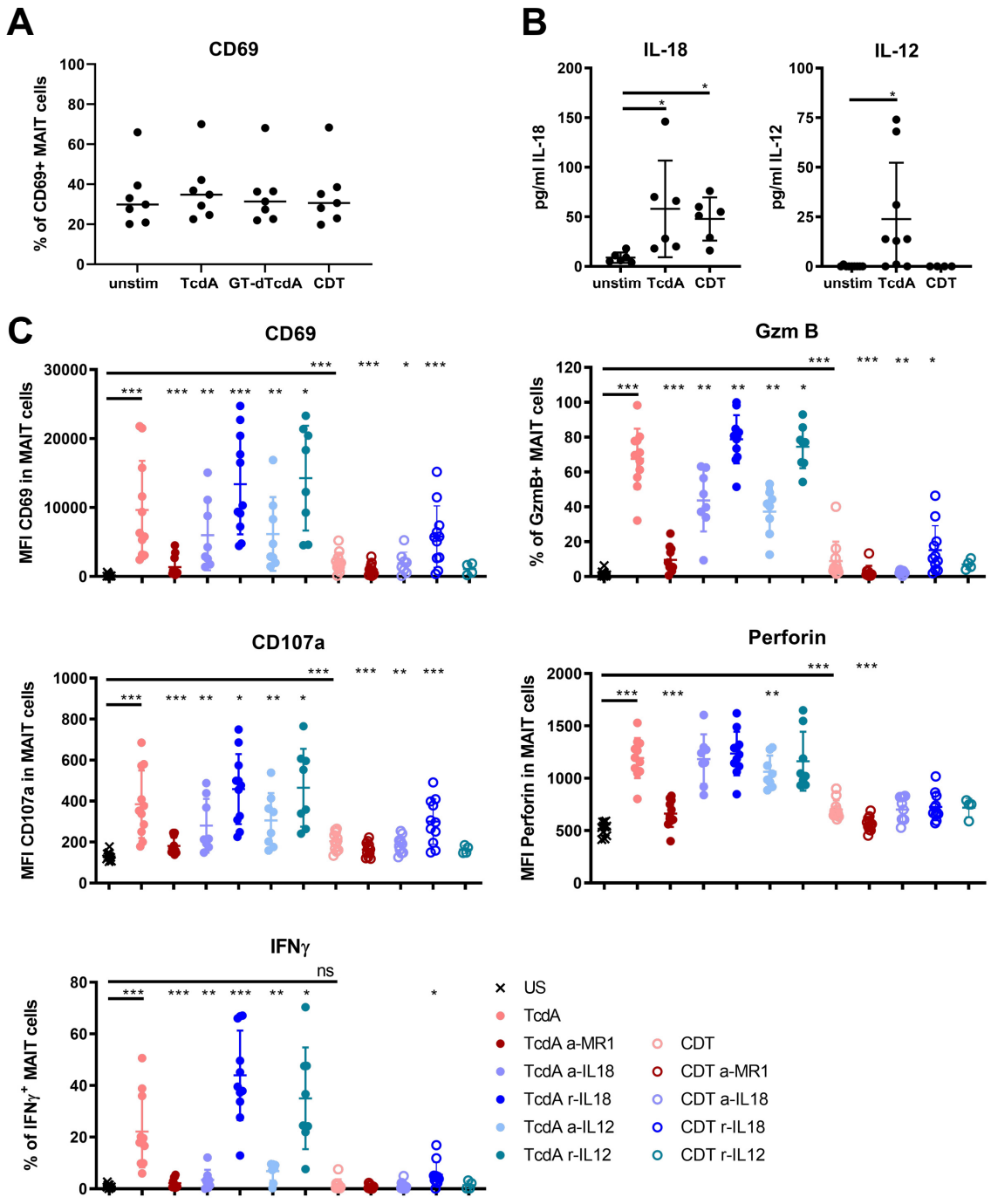

Figure 21: IL-18, IL-12, and MR1-dependent activation of MAIT cells following stimulation with *C. difficile* toxins. PBMCs were isolated from healthy donors and CD161^{++}Va7.2^{+}CD3^{+} T cells (MAIT cells) were sorted by FACS. MAIT cells were stimulated with 100 ng/ml of indicated *C. difficile* toxins for 20 h followed by flow cytometric analyses of extracellular CD69 expression (A). PBMCs were isolated and stimulated with 100 ng/ml *C. difficile* toxins and/or with *C. difficile* clinical isolate of ribotype RT023 at MOI 1 for 20 h followed by quantification of IL-18 and IL-12p70 (B) in the PBMC supernatant or flow cytometric analysis of CD69, CD107a, intracellular granzyme B, perforin, and IFNγ (C). If indicated, PBMCs were treated with anti-IL-18, anti-IL-12 or anti-MR1 for 1 h before stimulation or with rIL-12 or rIL-18 at the beginning of stimulation. Unstim: unstimulated. Horizontal lines indicate mean ± SD. Asterisks indicate significant differences determined by Wilcoxon matched-pairs signed rank test: *p<0.05, **p<0.01, ***p<0.001. Combined data from three independent experiments with 4 - 11 donors are shown.

3.3.4 Simultaneous stimulation with hypervirulent *C. difficile* and TcdA enhances proinflammatory MAIT cell responses

In section 3.2.4 it was demonstrated that hypervirulent *C. difficile* ribotypes, even in the absence of toxins, trigger the strongest cytotoxic MAIT cell response compared to less virulent ribotypes (Bernal et al., 2018). Additionally, it was demonstrated that *C. difficile* toxins TcdA and CDT also indirectly induce MAIT cell effector functions in a cytokine- and an MR1-dependent manner (section 3.3.3).

Based on that, it was next analyzed whether simultaneous stimulation of MAIT cells with the hypervirulent *C. difficile* ribotype RT023 together with TcdA and/or CDT would have a synergistic effect on MAIT cell activation and effector function. To this end, PBMCs from healthy donors were stimulated with *C. difficile* RT023 at MOI 1 and/or with 100 ng/ml TcdA and/or CDT for 20 h followed by flow cytometric analysis of CD69, CD107a, GzmB, perforin, and IFNγ expression. As depicted in Fig. 22, A, TcdA exhibits the highest capacity to activate MAIT cells as indicated by the most pronounced increase in CD69 expression (8793.8 ± 5810.7 MFI) compared to CDT (4400.9 ± 3481.6 MFI) and *C. difficile* RT023 (4845.1 ± 5239.6 MFI). CD69 expression was further increased following combined stimulation with *C. difficile* RT023 and TcdA (13288.3 ± 10532.5 MFI) and strikingly, activation was additionally strengthened when CDT was added as a third stimulus (22344.4 ± 12778.3 MFI).

With regard to the expression of cytotoxic molecules, 43.7 ± 20.0 % MAIT cells were GzmB$^+$ following stimulation with TcdA compared to 6.4 ± 5.5 % GzmB$^+$ MAIT cells following stimulation with CDT and 18.1 ± 15.4 % GzmB$^+$ MAIT cells following stimulation with *C. difficile* RT023 (Fig. 22, B). TcdA-induced GzmB expression was only slightly but significantly increased following combined stimulation with RT023 (48.3 ± 19.9 %). However, addition of CDT did not further enhance GzmB expression. TcdA-induced perforin expression (1446.6 ± 580.3 %) was significantly higher than CDT-induced (1068.9 ± 346.6 %) and *C. difficile* RT023-induced (1091 ± 384.1 %) perforin expression in MAIT cells. TcdA-induced perforin expression was not further increased by combined stimulation with RT023 and/or CDT (Fig. 22, C). CDT-induced degranulation indicated by CD107a surface expression on MAIT cells was significantly higher than TcdA-induced degranulation (297 ± 113.4 MFI vs. 223.1 ± 91.8 MFI) but was not increased following combined stimulation with TcdA and *C. difficile* RT023.

IFNγ expression in TcdA-stimulated MAIT cells (12.5 ± 5.5 %) was higher than in *C. difficile* RT023-stimulated MAIT cells (3.6 ± 4.4 %) and, as expected, CDT did not induce IFNγ expression (Fig. 22, E). Interestingly, TcdA-induced IFNγ expression was significantly enhanced following combined stimulation with TcdA, CDT, and/or *C. difficile* RT023 (TcdA and RT023: 20.5 ± 9.1 % and TcdA, CDT, and RT023: 26.6 ± 11.8 %).

In conclusion, despite obvious donor-specific variations in the expression levels of the analyzed markers, it was shown that simultaneous stimulation with TcdA, CDT, and

hypervirulent *C. difficile* significantly enhances MAIT cell activation (CD69) and proinflammatory response (IFNγ).

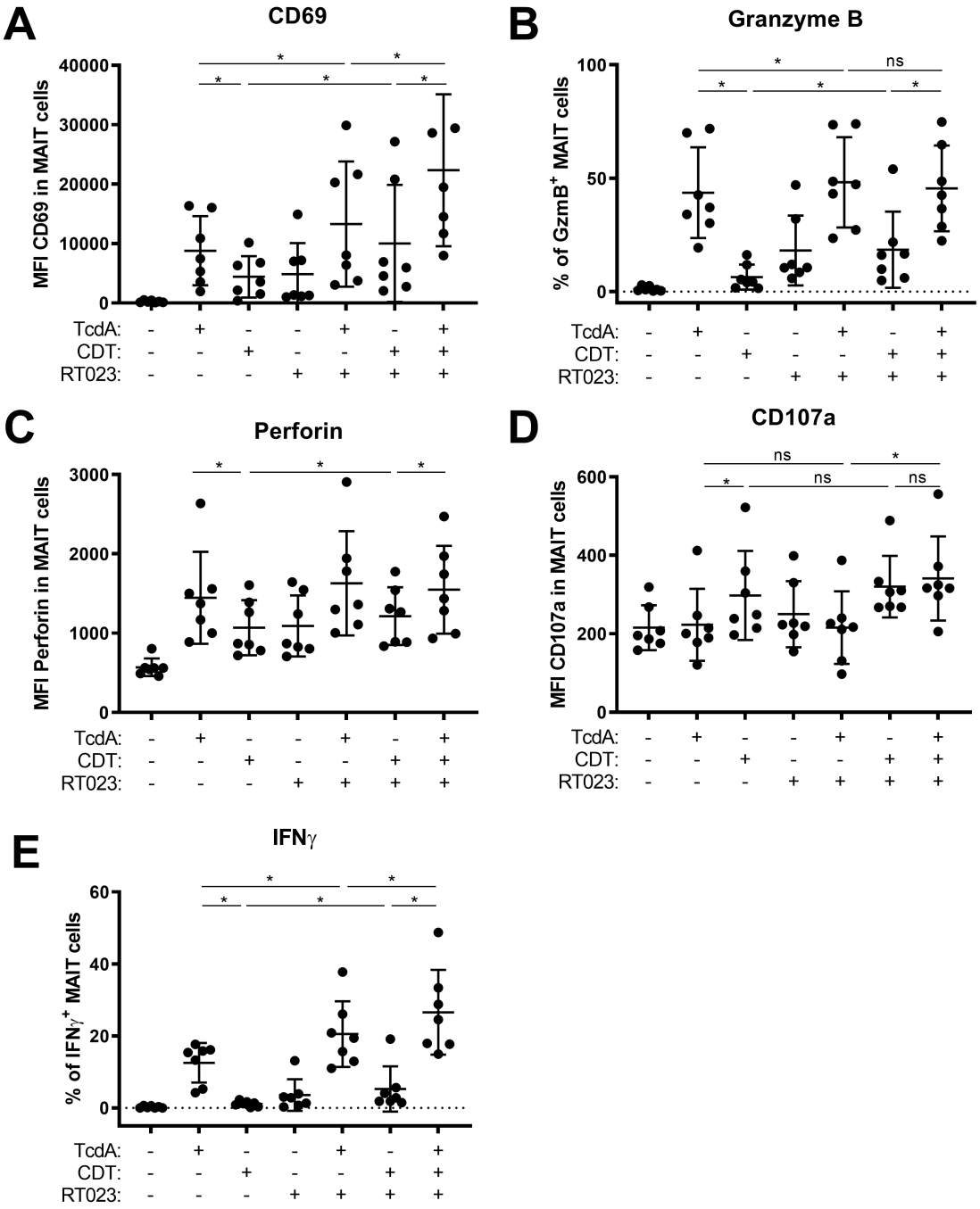

Figure 22: MAIT cell effector phenotype following simultaneous stimulation with hypervirulent *C. difficile* isolate RT023, TcdA and/or CDT. PBMCs were isolated from healthy donors and stimulated with *C. difficile* isolate of ribotype RT023 at MOI 1 and/or 100 ng/ml of indicated *C. difficile* toxins for 20 h followed by flow cytometric analyses of CD69 (A) and CD107a (D) surface expression as well as intracellular granzyme B (B), perforin (C), and IFNγ (E) expression. Cells were gated on CD161^{++}Va7.2^{+}CD3^{+} T cells (MAIT cells). Horizontal lines indicate mean fluorescence intensity ± SD. Asterisks indicate significant differences determined by Wilcoxon matched-pairs signed rank test: *$p<0.05$. For clarity, some asterisks that show additional significant differences are not shown. Combined data of three independent experiments with seven donors are shown.

3.3.5 MAIT cell activation by hypervirulent *C. difficile* is markedly reduced by MR1 blockade

As described above *C. difficile* toxin TcdA induces a strong MAIT cell activation and cytotoxicity and enhances proinflammatory MAIT cell response when administered together with CDT and hypervirulent *C. difficile* RT023 (section 3.3.4). Moreover, it was demonstrated that *C. difficile* RT023 and toxin-induced MAIT cell effector functions require in addition to IL-12 and IL-18, MR1-dependent MAIT cell activation (section 3.2.3 and 3.3.3).

Based on these findings it was next investigated to which extent combined stimulation with *C. difficile* RT023 and its toxins can be inhibited by blocking MR1. To this end, anti-MR1 antibody was added 1 h prior to PBMC stimulation with *C. difficile* ribotype RT023 in the presence or absence of both 100 ng/ml TcdA and 100 ng/ml CDT. After 20 h, CD69 expression on MAIT cells was assessed by flow cytometry (Figure 23). As expected, *C. difficile* RT023 induced significant increase of CD69 expression (8928.9 ± 8231.8 MFI vs. unstim. 1111.2 ± 642.2 MFI) that could be blocked by anti-MR1 (1888.1 ± 735.0 MFI). The combined stimulation with *C. difficile* RT023, TcdA and CDT resulted in 4-times higher CD69 expression with high donor variability (32392.6 ± 35830.7 MFI vs. RT023: 8928.9 ± 8231.8 MFI). Surprisingly, pronounced MAIT cell activation could significantly be blocked by anti-MR1 treatment (5138.6 ± 4396.6 MFI) which was in the range of *C. difficile* RT023-induced CD69 expression. These results underscore the requirement

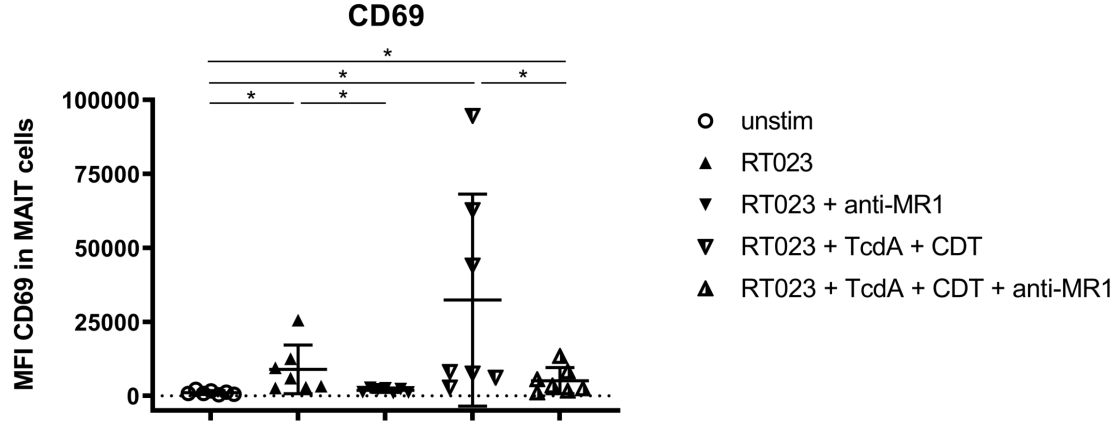

Figure 23: MR1-dependent activation of MAIT cells following stimulation with *C. difficile* RT023 and/or TcdA and CDT. PBMCs were isolated from healthy donors and stimulated with *C. difficile* isolate of ribotype RT023 at MOI 1 in the presence or absence of 100 ng/ml of toxin TcdA and CDT for 20 h followed by flow cytometric analysis of CD69 expression. If indicated MR1-blocking antibody was added 1 h before stimulation. Cells were gated on $CD161^{++}Va7.2^{+}CD3^{+}$ T cells (MAIT cells). Horizontal lines indicate mean fluorescence intensity ± SD. Asterisks indicate significant differences determined by Wilcoxon matched-pairs signed rank test: *$p<0.05$. Combined data from two independent experiments with seven donors are shown.

of MR1 for toxin-induced MAIT cell activation and demonstrate that hypervirulent *C. difficile* toxins TcdA and CDT have the capacity to induce superior MAIT cell activation in individual donors.

To summarize data presented within this chapter, only TcdA and CDT, but not TcdB induced MAIT cell-specific activation. Already low concentrations of TcdA and CDT were sufficient for full MAIT cell activation. Moreover, TcdA and CDT induced cytotoxic MAIT cell responses without stimulating apoptosis. TcdA induced a more pronounced MAIT cell effector phenotype than CDT and additionally a proinflammatory response. The observed toxin-induced MAIT cell activation requires accessory cells. TcdA-stimulated PBMCs produce IL-12 and IL-18, while in contrast CDT-stimulated PBMCs only produce IL-18 but no IL-12. Indeed, induction of MAIT cell effector function by *C. difficile* toxins was found to be cytokine-dependent. In addition to cytokine-dependent mechanisms, MR1 is involved in toxin-dependent MAIT cell activation, suggesting that cell contact between intoxicated cells and MAIT cells is required for robust MAIT cell responses. Simultaneous stimulation with TcdA, CDT, and hypervirulent *C. difficile* significantly enhanced proinflammatory MAIT cell responses, suggesting that MAIT cells would develop a distinct proinflammatory phenotype during *in vivo* infection with hypervirulent *C. difficile*.

4 Discussion

4.1 How to discover MAIT cell function in CDI?

Clostridioides difficile infections (CDI) are the major cause of antibiotic-associated colitis and an emerging threat for hospitalized patients. Beside long-term hospital stays, administration of antibiotics and immune suppressive drugs are important risk factors (Lessa, 2012). Notably, especially patients older than 65 years are predisposed to CDI (Jump, 2013). Severe courses of this disease are associated with the secretion of *C. difficile* toxins during acute CDI. The toxins induce intestinal epithelial cell damage, increased mucosal permeability, and cause an acute host inflammatory response characterized by the expression of inflammatory cytokines, tissue damage, hemorrhaging, and infiltration of neutrophilic granulocytes (Savidge et al., 2003; Pothoulakis, 2000; Rocha et al., 1997). Beside their anti-bacterial function, neutrophilic granulocytes have been described as major drivers of *C. difficile*-associated colitis (Bulusu et al., 2000; Burakoff et al., 1995; Kelly et al., 1994).

Investigating the contribution of adaptive immune responses, Abt and colleagues have recently demonstrated that recovery from acute CDI is independent of adaptive T cell responses (Abt et al., 2015). However, they also have demonstrated that *C. difficile*-induced activation of innate lymphoid cells (ILCs) resulted in protection against acute CDI in mice. Here they mentioned especially IFNγ-producing ILC1, which are believed to enhance bacterial clearance and to prevent systemic spreading of the pathogen. Like ILC1, MAIT cells represent another highly abundant (innate-like) T cell population that resides in the intestinal mucosa. The MAIT cell population in humans is known for its antigen specificity to riboflavin-derived metabolites and anti-bacterial function, but also for its rapid activation-induced secretion of inflammatory cytokines such as IFNγ, TNFα, and IL-17. Although other innate-like T cells have been demonstrated to enhance protection against CDI by increasing humoral immunity (Rampuria et al., 2017), only very limited information regarding the role of MAIT cell responses during CDI does exist, despite obvious similarities to ILC1. In mice, MAIT cells are less abundant compared to human and there is only limited access to suitable mouse strains to study MAIT cell-specific function in CDI. The MR1$^{-/-}$ mouse strain lacks MAIT cells, however, the fact that MR1 is completely missing affects the gut microbiota resulting in resistance against *C. difficile* colonization following antibiotic treatment (Smith et al., 2019). Moreover, transgenic mouse strains that overexpress MAIT cells (due to overexpression of invariant TCRα chain (Vα19-Jα33)) exhibit dysregulated T cell development characterized by significantly lower conventional T cell numbers in several peripheral tissues and a higher proportion of double-negative T cells (Garner et al., 2018). Based on these facts, many studies investigate MAIT cell function in the human system by analyzing immune responses to bacterial and viral pathogens using blood-derived primary MAIT cells.

Because CDI induces strong inflammation at the intestinal mucosa that is associated with elevated cytokine level in patient blood (Yu et al., 2017), it was assumed that MAIT cells following their cytokine-mediated activation might contribute to the immune response in age-related CDI (Lessa, 2012). Moreover, genomic data suggest the existence of a functional riboflavin pathway in *C. difficile* and therefore *C. difficile* has already been predicted as MAIT cell-activating pathogen (Liuzzi et al., 2015; Janoir et al., 2013). Interestingly, MAIT cell frequency decreases with progressing age but a potential functional impairment of these cells has not been described (Walker et al., 2014). We speculated that less frequent and functionally impaired MAIT cells might contribute to CDI susceptibility in the elderly population. To address this issue, age-dependent effects on the MAIT cell proteome were investigated by a comparative study of *ex vivo* isolated MAIT cells from healthy young and old individuals. Next, *C. difficile* bacteria- and toxin-induced MAIT cell responses were investigated in an *in vitro* model system. In this newly established model, blood-derived PBMCs were stimulated with fixed *C. difficile* bacteria and/or *C. difficile* toxins under different conditions followed by flow cytometric assessment of MAIT cell phenotype and/or quantification of MAIT cell activating cytokines. Consequently, this study provides new insights into *C. difficile*-specific mechanisms involved in MAIT cell activation but also reveals potential (immune modulatory) roles of MAIT cells during CDI.

4.2 Proteome analysis of MAIT cells from aged individuals revealed higher variation of protein expression

The first part of this thesis revealed that the global MAIT cell proteome was not affected by age. However, eight proteins were identified to be potentially regulated age-dependently (see table 16). The first differentially regulated protein candidate Stimulator of interferon genes (STING) was upregulated in the elderly ($\log_2$ RF = 1.53). Interestingly, STING is usually described in innate immune cells as sensor for cytosolic nucleic acids derived from endogenous or exogenous DNA derived from cell damage, viral, or some bacterial infections (Cai et al., 2014; Sauer et al., 2011; Woodward et al., 2010; Ishikawa et al., 2009). Following STING activation, innate immune response is induced characterized by increased type I interferon (IFN-I) production and subsequent enhanced expression of of interferon stimulating genes (ISG) (Ishikawa et al., 2009). Recently, Larkin et al. (2017) showed for the first time that STING activation also induces IFN-I as well as cell death in conventional T cells. It was demonstrated that following STING activation in conventional T cells their IL-2 expression level decreases while at the same time the T cell apoptosis was induced, suggesting that virus-infected T cells are impaired in proliferation and prone to cell death. One might speculate that MAIT cells of the elderly, which showed increased expression of STING, are less proliferative than MAIT cells from young individuals following STING activation.

Interstingly, Larkin et al. (2017) also described that B-cell lymphoma 2 (BCL2) protein expression was decreased following STING activation, supporting the observed proapoptotic T cell phenotype. In the present study, BCL2 expression was found slightly upregulated in MAIT cells of aged individuals. BCL2 is a multifunctional protein regulating cell cycle and cell death (Huang et al., 1997). Due to antiapoptotic features, BCL2 is considered to promote oncogenesis but due to its antiproliferative feature it is proposed to suppress tumorigenesis (de La Coste et al., 1999; Öfner et al., 1995; Tsujimoto et al., 1985). Moreover, BCL2-dependent inhibition of T cell proliferation was demonstrated to be age-dependent (Cheng et al., 2004). In accordance with our findings, Larkin et al. (2017) described elevated BCL2 expression levels in conventional T cells isolated from aged mice. It is tempting to speculate that basal expression of BCL2 is increased in MAIT cells of aged indivduals to compensate the potential effects caused by increased STING expression or that MAIT cells of elderly are less proliferative due to higher BCL2 expression.
Methyl-CpG-binding protein 2 (MECP2) is a nuclear protein that binds methylated DNA to regulate gene expression (Chahrour et al., 2008; Lewis et al., 1992). Li et al. (2014) defined MECP2 as a critical regulator for sustained Foxp3 expression in regulatory T cells (Tregs) in inflammatory conditions. MECP2 binds to a non-coding region of the *foxp3* gene and simulates its expression. Thus, MECP2 counteracts inflammation-induced epigenetic silencing of *foxp3* maintaining immune homeostasis and tolerance (Sakaguchi et al., 2010; Dudda et al., 2008). Moreover, Yang et al. (2012) have demonstrated that T helper cell subset 1 (T_H1)-specific IFNγ response is impaired in children with *MECP2* duplication syndrome, suggesting a negative effect of MECP2 on IFNγ expression. In contrast to this and our finding that MAIT cells from aged individuals exhibit elevated MECP2 levels, Chen et al. (2019) have recently shown that TCR- and cytokine-stimulated MAIT cells in aged indivduals express higher levels of IFNγ than activated MAIT cells in young individuals. In case of CDI, higher IFNγ expression of MAIT cells in the blood would be beneficial because high IFNγ levels in CDI patient blood are associated with low disease severity (Yu et al., 2017). However, age-dependent evaluation of *C. difficile*-stimulated MAIT cell IFNγ response from this study did not reveal any difference of MAIT cell response between young and aged individuals (data not shown).
The most downregulated protein in MAIT cells from aged individuals was mucin-like protein 1 (MUCL1, $\log_2$ RF = -1.98). MUCL1 was initially described to be specifically expressed in normal and cancerous human breast tissue (Miksicek et al., 2002; Colpitts et al., 2002). Later its activating function in proliferation of breast cancer cells was documented (Conley et al., 2016). Very recently, Navarro-Barriuso et al. (2019) defined MUCL1 as a biomarker for vitamin D3-induced tolerogenic dendritic cells in Multiple Sclerosis patients. However, so far there are no studies investigating the function of MUCL1 in conventional T cells and MAIT cells. Interestingly, MUCL1 shows structural analogy with mucin proteins and the mucin-family member MUC1 was identified as a new coinhibitory receptor molecule on T cells (Mukherjee et al., 2005). Speculating that

MUCL1 might have coinhibitory functions on MAIT cells as well, decreased MUCL1 levels in aged individuals might contribute to an hyperresponsive MAIT cell phenotype.
Interestingly, three HLA proteins were identified among the top ten protein candidates with highest differential expression levels. HLA-B(45) and HLA-B(57) are major histocompatibility complex class I (MHC I) molecules, and DPB1 is an MHC II molecule. MHC molecules are expressed on conventional T cells as well and are involved in T cell-mediated self or non-self presentation (Hillen et al., 2008; Pichler and Wyss-Coray, 1994). On the one hand, antigen presenting T cells can induce cytotoxic responses in T cells, on the other hand they can induce expression of inhibitory signals in T cells which in turn induce anergy in antigen presenting T cell (Taams et al., 1999). Speculating that MAIT cells can serve as antigen presenting cells as well, differential expression levels of HLA proteins on MAIT cells in aged individuals might indicate an altered homeostasis of these processes. MAIT cells in aged individuals with higher HLA protein expression could be more efficient in inducing T cell cytotoxicity, which in turn could result in enhanced killing of MAIT cells contributing to decreased MAIT cell numbers in aged individuals and thus increased susceptibility to CDI.

In conclusion, unbiased proteome profiling revealed that MAIT cells of aged individuals do not show functional adaptations at the global protein level. However, due to their increased expression of antiproliferative STING and BCL2, MAIT cells from aged individ-

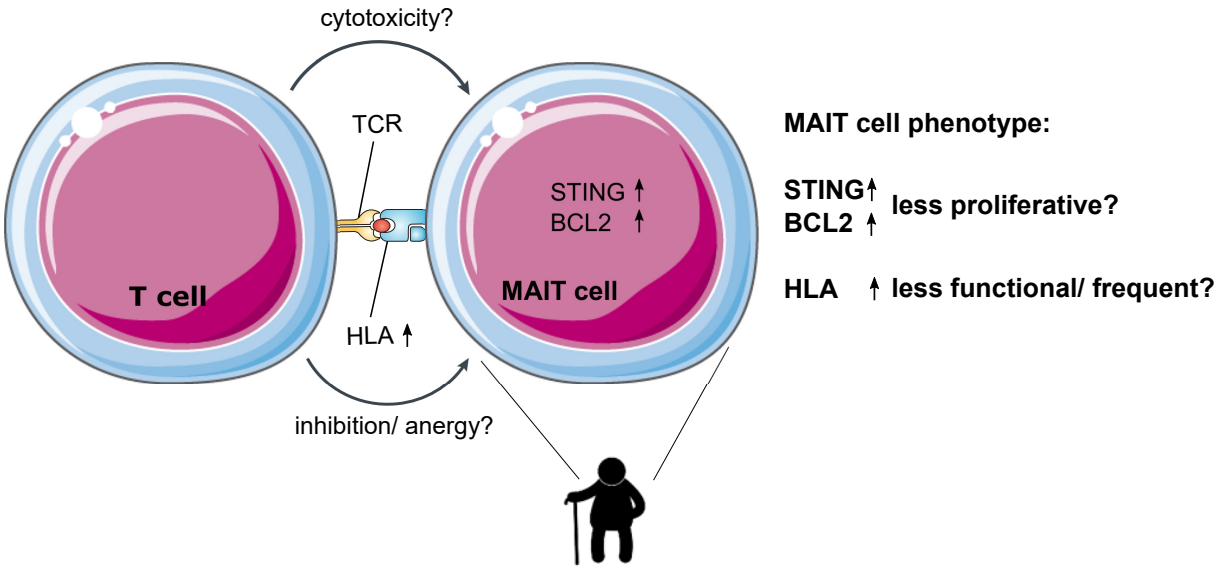

Figure 24: Proposed model for age-related functional adaptations of MAIT cells from aged individuals. Proteome study of MAIT cells from young and aged individuals revealed increased expression of antiproliferative STING and BCL2. Due to this, MAIT cells could exhibit a less proliferative phenotype in aged individuals. Moreover, differential expression levels of HLA proteins in MAIT cells were identified. Increased HLA expression in aged individuals could lead to T-cell dependent induction of MAIT cell anergy or death contributing to the reduced numbers and moreover functionally impaired MAIT cells in aged individuals.

uals could exhibit impaired proliferation. Decreased frequency and impaired proliferative capacity of MAIT cells in aged individuals might contribute to increased susceptibility to CDI. However, further studies are needed to decipher in more detail the functional role of proteins found to be differentially expressed in MAIT cells from young and aged individuals.

4.3 *C. difficile*-induced MAIT cell response - protective or detrimental?

In the second part of this thesis, human MAIT cells were identified as a new player in CDI. *C. difficile* was shown to possess a functional riboflavin biosynthesis pathway and the capacity to MR1-dependently activate MAIT cells. However, *C. difficile* was demonstrated before to express as well a riboflavin transporter encoded by the *ribZ* gene (Gutiérrez-Preciado et al., 2015). This riboflavin transporter enables *C. difficile* to take up external riboflavin produced by other bacteria present in the gut (García-Angulo, 2017). In case riboflavin is externally available, bacteria would stop their own riboflavin biosynthesis and thus *C. difficile* would, under physiological conditions in the gut, not produce 5-A-RU, the precursor of MAIT cell-activating ligands 5-OP-RU and 5-OE-RU. In this case, *C. difficile* could escape MR1-dependent MAIT cell recognition in early onset of CDI. However, to date there are no published data available regarding the dynamics of riboflavin transporter expression in *C. difficile*. In the present approach, *C. difficile* was cultured in riboflavin-free medium to investigate whether endogenously produced MAIT cell-activating ligands would induce MAIT cells activation. Interestingly, a ten-times higher MOI was required to induce MAIT cell activation comparable to that induced following *E. coli* BL21 stimulation (Supplementary Fig. 27) indicating that antigen concentration differs between bacteria. This is corroborated by the fact that *C. difficile* riboflavin biosynthesis is tightly regulated and not as efficient as observed for the constitutive producer *E. coli* (Vitreschak, 2002).

Earliest *C. difficile*-induced MAIT cell activation was observed after 12 h with a peak response after 17 h. Most problably processing and presentation of MR1-binding ligands by antigen presenting cells to MAIT cells requires a certain period of time, which however might differ between living and fixed bacteria (Kurioka et al., 2015). After antigen recognition, expression of activation marker CD69 and cytotoxic effector molecules (perforin, GzmB) was increased, followed by a predominantly cytokine-dependent degranulation (CD107a, Fig. 16). At the same time, *C. difficile*-activated MAIT cells produced the proinflammatory cytokine IFNγ. In a murine CDI model, IFNγ was identified to be protective during the acute phase of the infection (Abt et al., 2015) and the present study provides first evidence that in addition to murine ILC1, human MAIT cells are able to mount an IFNγ response in CDI. IFNγ is proposed to strengthen the immunological barrier in the intestine and thus to play a beneficial role in controlling acute CDI (Abt

et al., 2015). In this scenario, human MAIT cells might play a protective rather than a detrimental role in CDI similar to murine ILC1, whereby their IFNγ response seemed to be mostly dependent on antigen presentation. Based on the observation that IFNγ could not significantly be inhibited by IL-12/IL-18 blockade, *C. difficile*-induced IFNγ expression in MAIT cells was found to be mostly MR1-dependent. This suggests the existence of a pathogen-specific mechanism in MAIT cell activation. However, IL-12/IL-18 has an impact on IFNγ expression, since IL-12/IL-18 alone primes IFNγ expression in MAIT cells (Ussher et al., 2014) and an additional MR1/TCR stimulation results in more pronounced IFNγ expression in individual donors (Fig. 15). This finding is in accordance with other *in vitro* studies that revealed *E. coli*-induced IFNγ expression in MAIT cells to be co-dependent on MR1/TCR- and IL-12/IL-18 signaling after 20 h stimulation (Ussher et al., 2014). Data from this and earlier time points indicate that *C. difficile*-induced IFNγ expression is mostly MR1-dependent, suggesting that the cell contact with antigen-presenting cells is essential for IFNγ responses in the acute phase of infection.
In contrast to IFNγ, expression of CD69 and the cytolytic effector molecules perforin and GzmB required MR1 and IL-12/IL-18 signaling and potentially other MAIT cell co-stimulating factors or cytokines (e.g. IL-23) that can support the induction of effector function (Wang et al., 2019). Like IFNγ, expression of these markers peaked at 17 h, which is similar to the kinetics of cytolytic molecules expressed in *E. coli*-stimulated MAIT cells (Kurioka et al., 2015). However, cytotoxicity was always more pronounced than IFNγ response, suggesting that a major function of *C. difficile*-stimulated MAIT cells might be targeted lysis of infected cells and prevention of bacterial spread. Although the expression of effector molecules in *C. difficile*-stimulated MAIT cells already indicates arming of lytic granules and adaptation of their prototypic granzyme expression pattern including GzmB, GzmA and GzmK (Bulitta et al., 2018; Kurioka et al., 2015), we additionally examined potential effects on MAIT cell degranulation. Indeed, CD107a surface expression also peaked at 17 h post stimulation with *C. difficile* indicating the release of lytic granules by this time. Therefore, granzymes together with perforin are most probably present at elevated levels intracellularly in *C. difficile*-activated MAIT cells as well as extracellularly. In the present study, the peak of *C. difficile*-induced degranulation was MR1-independent and could only be prevented by stimulation in the presence of both MR1 and IL-12/IL-18 blocking antibodies. Therefore, one might speculate that MAIT cells, once activated MR1/TCR-dependently by *C. difficile*, might be able to release lytic granules with MAIT effector molecules in a cytokine-dependent, but MR1-independent manner. If TCR engagement and the formation of immunological synapses between MAIT cells and target cells are at all dispensable, undirected release of cytotoxic molecules could cause tissue damage and excessive inflammation ultimately leading to decreased epithelial barrier function. However, MR1-dependent MAIT responses characterized in this study might be even stronger within the intestinal mucosa where numerous cell types do express MR1 and potentially contribute to the MAIT cell stimulation (Tsukamoto et al.,

2013; Le Bourhis et al., 2013). Furthermore, activated tissue-resident MAIT cells can produce IL-17 (Gibbs et al., 2017; Dusseaux et al., 2011) that would additionally promote neutrophil-mediated inflammatory responses (Liu et al., 2016). Together, MAIT cells, although generally able to directly kill infected target cells, might be instrumental for *C. difficile* to overcome mucosal barriers and therefore might play a so far unrecognized detrimental role in inflammation-driven immunopathology observed in CDAC.

The hypothesis that MAIT cells might play a role in inflammation-driven immunopathology in CDAC is further supported by the finding that hypervirulent *C. difficile* ribotypes, although producing only moderate levels of riboflavin during anaerobic *in vitro* culture, induced superior MAIT cell cytotoxicity. However, based on this result, riboflavin concentration of *C. difficile* is not considered to sufficiently predict the concentration of MR1-binding intermediates. The generation of MAIT cell-activating ligands depends on several parameters including the activity of *ribD* as well as non-enzymatic reactions, whereas further processing depends on *ribE* (Corbett et al., 2014). When activity of *ribE* is higher compared to *ribD*, MR1-binding ligand (5-AR-U) is converted into riboflavin. The MR1-binding ligand (5-AR-U) is converted into 5-OP-RU and 5-OE-RU by non-enzymatic reactions with glyoxal and methylglyoxal (Schmaler et al., 2018). 5-OP-RU and 5-OE-RU have been shown to possess the highest MAIT-activating potency (Schmaler et al., 2018). So far there is no experimental data quantifying MR1-binding metabolites (5-A-RU, 5-OP-RU, and 5-OE-RU) in *C. difficile*, because selective enrichment and analysis of intermediates is technically difficult due to intermediates' short half-life and instability when not bound to MR1 (Corbett et al., 2014). Schmaler et al. (2018) were able to measure intermediate concentrations in *E. coli*, however, in contrast to *E. coli*, *C. difficile* does not constitutively express genes of the riboflavin biosynthesis pathway. Instead, riboflavin genes are organized in an operon and their expression is tightly regulated (Vitreschak, 2002). Due to the very specific (and not very efficient) metabolism of *C. difficile*, far lower concentrations of intermediates of any vitamin biosynthesis can be detected compared to model organisms like *E. coli*, *S. cerevisiae* or *C. glutamicum*. In particular, when *ribD* activity is relatively high, MAIT cell-activating metabolites would accumulate in *C. difficile* which could result in superior activation of MAIT cells as observed for hypervirulent *C. difficile* isolates despite only moderate riboflavin concentrations. However, a better understanding of the correlation between the generation of MR1-antigen and clostridial riboflavin metabolism is needed. To address this issue, a *rib* gene deletion mutant (based on *C. difficile* strain 630Δerm) has been generated that can not produce MR1-binding intermediates, and preliminary data indicate that riboflavin synthesis is not essential for *C. difficile* growth (data not shown). The use of such mutant strain will facilitate future functional and *in vivo* studies.

Beside antigen-specific differences between hypervirulent and less virulent *C. difficile* ribotypes one might speculate that the hypervirulent *C. difficile* ribotypes induce, by means of their toxins, a different cytokine pattern in epithelial and myeloid cells than less vir-

ulent (toxigenic) strains, resulting in altered TCR-independent MAIT cell activation by cytokines (Slichter et al., 2016; Ussher et al., 2014). In the present study, MAIT cell cytotoxic response was demonstrated to depend on the proinflammatory cytokines IL-12 and IL-18. Moreover, hypervirulent *C. difficile* ribotypes induce a higher IL-12 secretion by immune cells in the PBMC culture than less virulent *C. difficile* ribotypes. Kurioka et al. (2015) reported that granzyme B and perforin expression in *E. coli*-stimulated MAIT cells depend on IL-12 but not IL-18. This might also be a possible explanation for the superior cytotoxic response of MAIT cells by hypervirulent *C. difficile* isolates.
In conclusion, data obtained by in-depth characterization of the *C. difficile*-induced MAIT cell effector phenotype implies a multifaceted role of MAIT cells during CDI. On the one hand, MAIT cells might have a protective role by strengthening immunological barrier, and on the other hand, these cells could have a detrimental role since they might help *C. difficile* to overcome epithelial barriers at the intestinal mucosa. On top, superior MAIT cell activation by hypervirulent *C. difficile* isolates suggests MAIT cells as novel decision makers for disease severity in CDAC.

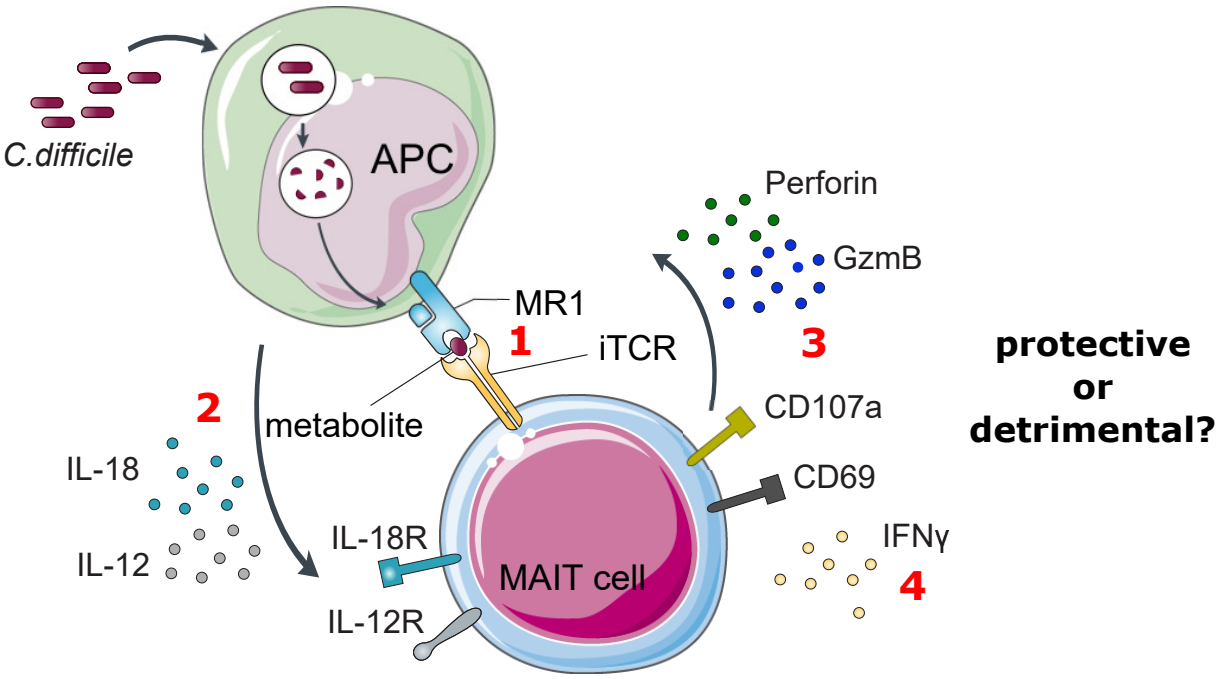

Figure 25: *C. difficile*-specific mechanisms in MAIT cell activation. Stimulation of PBMCs with *C. difficile* induces (1) MR1-dependent and (2) cytokine-dependent MAIT cell activation (CD69) including (3) cytotoxic response (GzmB and perforin) and degranulation (CD107a), and (4) proinflammatory response (IFNγ).

4.4 *C. difficile* toxins manipulate MAIT cell response

In the present study, we identified enzymatically active TcdA and CDT, but not TcdB, to be competent to induce activation of blood-derived human MAIT cells. Although TcdB is described to be more competent than TcdA in inducing cell rounding and apoptotic

cell death in various cell types (Chaves-Olarte et al., 1997; Donta et al., 1982), only TcdA induced a MAIT cell effector phenotype. The fact that TcdB differs from TcdA only in its receptor-binding domain suggests that target cells expressing TcdB-binding receptors are probably absent in human blood. In general, only a few *C. difficile* toxin-binding host receptors have been identified so far for TcdA, TcdB, and CDT, which have all been described to be expressed on human colonic epithelial cells (Tao et al., 2016; Hemmasi et al., 2015; LaFrance et al., 2015; Teneberg et al., 1996). However, in our study enzymatically active TcdA and CDT were capable to intoxicate blood circulating immune cells and thereby induce MAIT cell activation. There is only limited knowledge regarding toxin receptor expression on PBMCs and whether there exist further yet unknown receptors. Moreover, it was demonstrated here that already low toxin doses are sufficient to induce maximal MAIT cell response. One could speculate that high sensitivity of MAIT cells for *C. difficile* toxins in the blood is necessary to prevent development of systemic CDI or sepsis. In severe CDI in mice and piglets, toxemia was shown to correlate with an elevated proinflammatory cytokine pattern in sera and the development of systemic CDI (Steele et al., 2012). Very recently, toxemia was also identified in CDI patients (Yu et al., 2015), highlighting the importance to decipher in more detail whether MAIT cells that are highly abundant in human blood play a beneficial or rather detrimental role in the pathogenesis of CDI.

For *in vitro* MAIT cell stimulation, TcdA and CDT concentrations (100 ng/ml) being four-times higher than the one identified to be sufficient for MAIT cell activation (25 ng/ml) were used. The reason behind this was that this concentration has been shown (i) to induce effective innate immune response, (ii) to have no significant cytotoxic effect on PBMC culture , and (iii) to range within concentrations present in fecal specimen of CDI patients (Cowardin et al., 2016b; Ryder et al., 2010; Lee et al., 2009; Rocha et al., 1997). Toxin-induced MAIT cell activation appeared to require accessory immune cells. However, since *C. difficile* toxins induced less pronounced activation of conventional T cells, the effect was MAIT cell-specific, indicating that MAIT cells play a unique and so far unknown role in toxin-mediated host response in CDI. MAIT cells exhibit innate-like properties, including the immediate degranulation of cytotoxic granules following activation, and we have previously shown that MAIT cells directly respond to *C. difficile* in an MR1-dependent mechanism (Bernal et al., 2018). Unexpectedly, toxin-induced MAIT cell activation also depended on MR1 antigen presentation despite no metabolic antigen of the bacteria being available. Based on this observation one could speculate that TcdA and CDT exhibit super-antigenic activity, linking the MAIT cell TCR with MR1 protein on antigen presenting cells and resulting in the antigen-independent activation of MAIT cells. However, previous studies did not uncover a super-antigenic activity of *C. difficile* toxins on conventional T cells (Wanahita et al., 2006). In order to elicit cytotoxic effects, *C. difficile* toxins have to be internalized into the host cell via endocytosis and require acidification of endosomes to enter the cytosol (Henriques et al., 1987; Mitchell et al.,

1987; Florin and Thelestam, 1983). Within the endosome, toxins get in close proximity to MR1 proteins, which are as well expressed in endosomes, and can bind to them ((Harriff et al., 2016; Huang et al., 2008). Following (non-)specific binding, MR1 undergoes conformational change and translocates to the cell surface of intoxicated host cells. Thus, it is conceivable that the MAIT cell TCR might be (cross-)linked to the toxin-MR1-complex resulting in MAIT cell activation. This would represent a so far unknown mechanism of toxin-induced innate-like immune response in CDI, and indeed this hypothesis is well in line with the finding that an antibody against MR1 almost completely blocked toxin-induced MAIT cell activation. Alternatively, one might speculate that MR1 presents so far undefined toxin-derived or endogenous antigens released during toxin-induced host cell apoptosis. This would expand the steadily increasing number of MR1-binding non-bacterial antigens (Lepore et al., 2017; Keller et al., 2017).

It was demonstrated that TcdA and CDT induced IL-12 and IL-18 secretion in PBMCs, respectively. IL-12 together with IL-18 is required to induce IFNγ expression in MAIT cells independent of TCR stimulation (Ussher et al., 2014) thus explaining the TcdA-specific IFNγ response. IFNγ is an important effector molecule in cellular immunity, promoting antigen recognition and presentation by macrophages and dendritic cells, and driving macrophage and Th1 cell differentiation. High IFNγ levels in sera of CDI patients are associated with less severe disease courses (Yu et al., 2017). Additionally, recent studies have suggested a beneficial role for IFNγ in mediating host protection during *C. difficile* infection in mice (Abt et al., 2015). In the present study, it was shown that combined stimulation with TcdA and CDT resulted in enhanced IFNγ expression. Based on this one might speculate that MAIT cells would enhance their protective potential when it comes to an infection with a hypervirulent *C. difficile* strain. The fact that CDT alone did not induce IFNγ provides evidence for different MAIT cell activation mechanisms, probably based on different host cell intoxication strategies and related innate immune cell responses being induced. However, for both toxins, MR1-dependent cell contact was fundamentally required for MAIT cell activation, suggesting that cytotoxic response is targeted to intoxicated cells in order to rapidly clear them from the blood. According to these results, MAIT cell effector function in the blood might be considered as beneficial for prevention of toxemia in CDI patients.

Stimulation with recombinant *C. difficile* toxins revealed that TcdA- and CDT-induced MAIT cell activation is mainly cell contact- (MR1-) dependent but as well depends on IL-12 and IL-18. In CDI patients, intestinal epithelial cells (IECs) are the first target cell population for the toxins. IECs express MR1 and constitutively produce IL-18 (Le Bourhis et al., 2013; Takeuchi et al., 1997). Thus, it is conceivable that during *in vivo* CDI intoxicated IECs induce in a cell contact- and cytokine-dependent mechanism MAIT cell cytotoxicity resulting in targeted IEC killing. IFNγ expression by MAIT cells can only be induced when gut-resident macrophages, dendritic cells, neutrophils, or B cells in response to microbial or toxin stimuli secrete IL-12 (Kim et al., 2014; Sartori et al., 1997; Heufler

et al., 1996). However, despite the proposed protective role of IFNγ in CDI (Abt et al., 2015), MAIT cell-secreted IFNγ could also contribute to targeted IEC killing because IFNγ was shown to induce IEC apoptosis (Schuhmann et al., 2011). This would result in rapid clearance of *C. difficile* infected/intoxicated cells thereby preventing systemic pathogen spreading. However, IEC apoptosis might result in a loss of barrier function thus facilitating the adhesion of *C. difficile* and subsequent penetration of the bacteria through the intestinal barrier and thereby the manifestation of clinical CDI (Kasendra et al., 2014).

In conclusion, *C. difficile* toxin-induced MAIT cell effector phenotype in the blood implies a beneficial role for MAIT cells during CDI preventing systemic pathogen spread. However, at the site of infection, *C. difficile* toxin-induced MAIT cell effector response might facilitate *C. difficile* to overcome epithelial barrier at the mucosa. Based on this and the results discussed in chapter 4.3, MAIT cells are considered to have a multifaceted role in CDI.

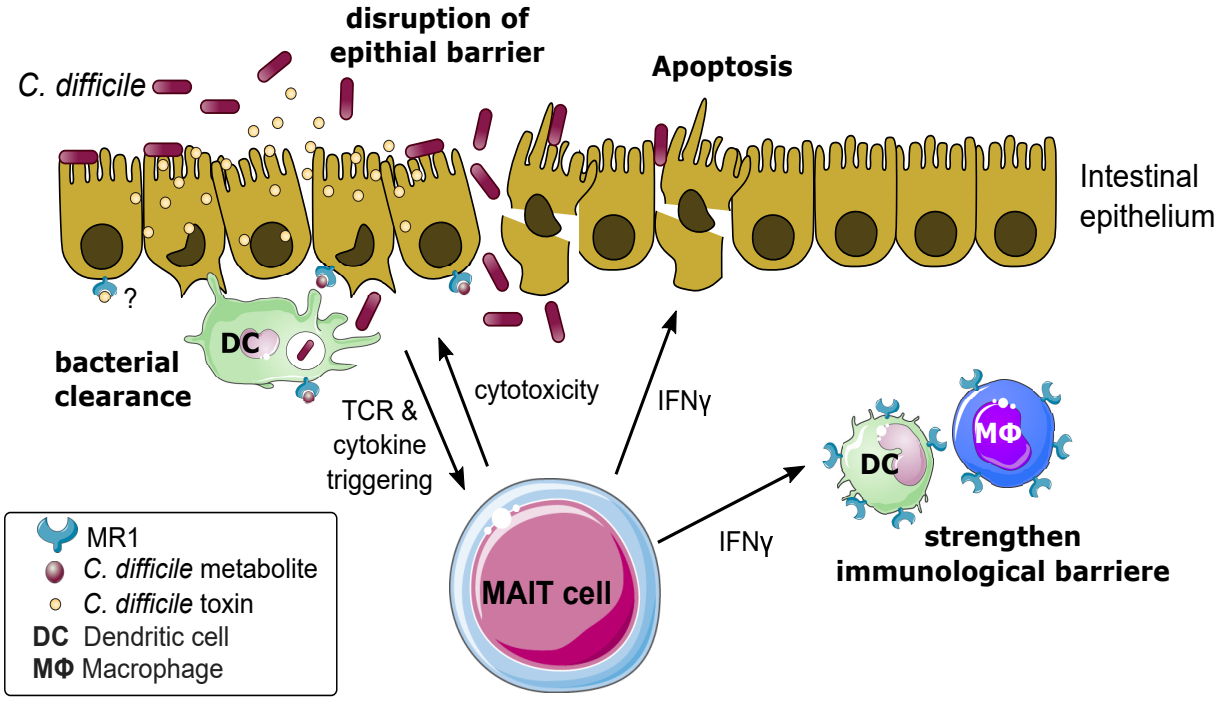

Figure 26: Proposed model for the multifaceted MAIT cell functions in CDI. *C. difficile* toxins disrupt the intestinal epithelial barrier and thereby facilitate *C. difficile* penetration into the mucosa. Intoxicated epithelial cells secrete cytokines and present *C. difficile*- or toxin-derived antigens. Innate immune cells such as DCs and macrophages are activated and provide MR1-dependent TCR and cytokine stimulation to activate gut-resident MAIT cells. Upon activation, MAIT cells directly kill *C. difficile*-infected cells thereby contributing to bacterial clearance but also to further disruption of the epithelial barrier. Moreover, MAIT cells produce IFNγ that promotes antigen recognition and presentation by DCs and macrophages to strengthen the immunological barrier. At the same time IFNγ induces epithelial cell apoptosis contributing to the breakdown of the intestinal epithelial barrier in CDI.

4.5 Outlook

4.5.1 Functional characterization of protein candidates

MAIT cells from healthy aged individuals showed increased expression of STING and BCL2 that have antiproliverative functions in conventional T cells, implying that MAIT cells of aged individuals might exhibit reduced proliferative capacity. To investigate this, proliferation assays with MAIT cells from aged and young individuals following stimulation with *C. difficile* should be performed. As an experimental approach, a monocyte cell line serving as antigen presenting cell should be stimulated with fixed *C. difficile* bacteria and co-cultured with purified primary MAIT cells from young or aged individuals. MAIT cell proliferation can be tracked with cell tracing dyes as already described in proliferation assays for *E. coli*-stimulated MAIT cells (Dias et al., 2016).

4.5.2 *In vitro* MAIT cell stimulation

C. difficile has been shown to possess a functional riboflavin biosynthesis pathway and to exhibit the capacity to MR1-dependently activate MAIT cells. As mentioned above, *C. difficile* is considered to express also a riboflavin transporter by which it is able to take up riboflavin from the environment. If *C. difficile* takes up riboflavin from the environment and shuts down biosynthesis of endogenous riboflavin, one might speculate that *C. difficile* can escape MR1-dependent MAIT cell activation. To further analyze the correlation between *C. difficile* riboflavin metabolism and riboflavin uptake from the environment, different *in vitro* culture conditions in the presence or absence of riboflavin should be tested with *C. difficile* wildtype strain (630Δerm) and ribZ mutant (based on 630Δerm) followed by examination of their MAIT cell activating capacity. The *C. difficile* ribZ mutant has already been generated in collaboration with Prof. Dieter Jahn (TU Braunschweig) and is currently characterized in more detail *in vitro*.
It was demonstrated that MAIT cell effector phenotype was induced MR1- and partly cytokine-dependently following stimulation of PBMCs with *C. difficile* bacteria or *C. difficile* toxins TcdA and CDT. Moreover, it was demonstrated that *C. difficile* bacteria and toxins induce a specific cytokine secretion patterns in PBMCs. To better understand the underlying mechanisms of MAIT cell activation, antigen presenting and cytokine-producing cells contained in PBMC cultures that contribute to MAIT cell activation need to be identified. To this end, the MR1 expression profiles of professional antigen presenting cells (APCs) such as dendritic cells, macrophages, and B cells, should be investigated following stimulation with *C. difficile* bacteria or TcdA and CDT using a multicolor FACS staining panel (Yu et al., 2016b). APCs that show increased MR1 expression upon *C. difficile* stimulation should be sorted from PBMCs and stimulated with fixed *C. difficile* bacteria or toxins followed by quantification of MAIT cell activating cytokines to identify APC-specific IL-12 and IL-18 secretion patterns. In a bi-cellular MAIT cell stimulation system, purified MAIT cells should be co-cultured with *C. difficile*-stimulated APCs to

dissect APC-specific MR1-dependent effects on MAIT cell effector phenotype. Moreover, APC-specific effects on MAIT cells mediated via the cytokines IL-12 and IL-18 can also be dissected in more detail by blocking IL-12 and IL-18 signaling via neutralizing antibodies.

4.5.3 *In vivo* CDI mouse model

To answer the question whether MAIT cells are detrimental or beneficial in CDI, the MAIT cell phenotype following *C. difficile* stimulation was investigated demonstrating a predominently cytotoxic response that can be either beneficial by preventing bacterial spread or detrimental by decreasing epithelial barrier function. To decipher *in vivo* MAIT cell function in more detail, a CDI mouse model will be used to investigate several aspects of the disease course (in cooperation with PD Dr. Matthias Lochner, Twincore Hanover). In this *in vivo* CDI model, antibiotic treatment is required for successful *C. diffcile* colonization. Since antibiotic treatment leads to lysis of the majority of bacteria in the gut, a high number of metabolites is released. Thus one should investigate antigen-dependent (pre-)activation of MAIT cells that might have effects on MAIT cell effector phenotype during CDI. After antibiotic treatment, mice are in a susceptible state (Theriot et al., 2014) for CDI and will be inoculated with *C. difficile* spores of *C. difficile* 630Δerm and a ribBA mutant strain (based on 630Δerm) that have been generated together with Prof. Dr. Dieter Jahn (TU Braunschweig). Using the ribBA mutant strain, which is no longer able to produce riboflavin, one could investigate MR1-independent effects on MAIT cell effector phenotype during CDI and evaluate potential changes in disease pathogenesis related to this. In this context, one should also investigate whether mice show altered immunopathology when infected with the ribZ mutant strain which is not able to take up external riboflavin.

As another interesting aspect for future studies one could investigate whether MAIT cell IFNγ response can be boosted by treating the mice with rIL-12 and r-IL-18 and wether an increased IFNγ response would contribute to enhanced bacterial clearance. By infecting WT C57BL/6 mice and MR1$^{-/-}$ mice, which lack MAIT cells one could directly investigate the relevance of MAIT cells in CDI (van Wilgenburg et al., 2018). To this end, mice would be infected with *C. difficile* and weight loss and survival would be determined as read-out. In immunodeficient Rag2$^{-/-}\gamma$C$^{-/-}$mice in which Rag2 and the common γ chain are deleted resulting in the absence of T, B and natural killer (NK) cells as well as several cytokine signaling pathways (Wang et al., 2018), one could disentangle the either protective or detrimental role of MAIT cells. In this CDI model, *ex vivo*-isolated and purified colon MAIT cells would be adoptively transferred into recipient Rag2$^{-/-}\gamma$C$^{-/-}$mice followed by infection with a lethal *C. difficile* dose. Survival and bacterial burden (CFU) in the colon should be determined in order to determine the function of MAIT cells in CDI in the absence of T, B and natural killer (NK) cells.

4.5.4 CDI clinical research study

In addition to the *in vivo* CDI mouse model, MAIT cell phenotyping in CDI patients could confirm results obtained from *in vitro* studies described in this study. Therefore, a clinical research study has been initiated together with PD Dr. med. Alexander Link (Otto-von-Guericke University Hospital). In this study blood and colon biopsies from CDI patients, patients with inflammatory bowel dieseases (IBD: Crohn's disease and ulcerative colitis), and healthy control patients are currently collected. This study design aims to differentiate *C. difficile*-specific effects clearly from the effects observed in pathogen-free diseases such as IBD. Flow cytometric and *in situ* fluorescent staining analyses of these samples will uncover correlations between *C. difficile*-specific MAIT cell phenotype and observed clinical outcomes that might give evidence whether MAIT cells have a beneficial or detrimental role during CDI. Moreover, ribotype and toxinotype of *C. difficile* that is responsible for infection will be identified in fecal specimen of CDI patients to reveal potential correlations between toxin production and observed MAIT cell effector phenotype *in situ*.

4.5.5 Characterization of other potential MAIT cell functions in CDI

In the present study, analyses were focused on cytotoxic and proinflammatory MAIT cell responses. Recently, Leng et al. (2019) have shown that MAIT cells that are triggered TCR-dependently exert a tissue-repair phenotype including expression of furin, TNF-α, and CCL3. They suggested that MAIT cells in the intestinal epithelium play a protective role by maintaining barrier function, which goes hand-in-hand with the control of microbial invasion (Leng et al., 2019). Based on these findings, one should investigate whether *C. difficile*-stimulated MAIT cells express cytokines that are associated with tissue-repair and whether these secreted cytokines could contribute to wound-healing processes. This could be investigated in *in vitro* wound-healing assays, in which confluently grown intestinal epithelial cells are scratched and subsequently treated with supernatant derived from co-cultures of purified primary MAIT cells and *C. difficile* bacteria- or toxin-stimulated monocytes, followed by assessment of wound repair via imaging. Testing different conditions, e.g. pretreatment of the intestinal epithelial cells with *C. difficile* toxins, one could examine in a more physiological setting whether MAIT cells and cytokines secreted by them are beneficial or detrimental for epithelial barrier function during CDI.

5 Supplementary

5.1 MAIT cell protein expression shows higher variation in aged individuals

Table 17: Top ten list of MAIT cell proteins with high donor variation

Uniprot	Protein	SD(young)	SD(old)	ΔSD(old/young)
P78316	NOP14	0.01	0.44	39.60
P30483	HLA-B(45)	0.10	2.84	27.54
Q5EP54	DPB1	0.17	2.17	12.91
Q2NL82	TSR1	0.04	0.43	10.82
P18465	HLA-B(57)	0.24	2.62	10.70
Q8NAT1	PMGT2	0.07	0.74	9.89
Q9UPN7	PP6R1	0.05	0.46	9.87
Q8WYB5	KAT6B	0.05	0.45	8.79
P02786	TFR1	0.05	0.41	8.67
Q9Y6Y0	NS1BP	0.06	0.41	8.33

5.2 *C. difficile* induces activation and effector functions in primary human MAIT cells

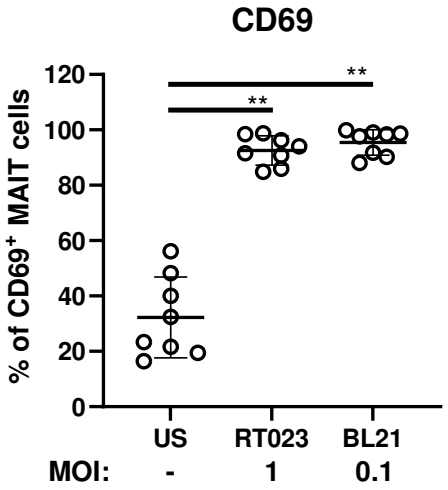

Figure 27: MAIT cell activation following stimulation with *C. difficile* or *E.coli*. PBMCs were isolated from healthy donors and stimulated with paraformaldehyde-fixed *C. difficile* isolate of ribotype RT023 and *E. coli* BL21 for 20 h followed by flow cytometric analyses of CD69 surface expression. Mean percentages ± SD are shown. Cells were gated on $CD161^{++}Va7.2^{+}CD3^{+}$ T cells (MAIT cells). Wilcoxon signed rank test for paired samples was used to detect significant differences and determine p-values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

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