Characterization of T cell responses against mycobacteria in Cystic Fibrosis patients and in an *in vitro* infection model

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I. LIST OF ABBREVIATIONS

$^{-}$	Negative for the described parameter (read “negative”)
$^{+}$	Positive for the described parameter (read “positive”)
ATS	American Thorax Society
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
BLI	Bioluminescence imaging
BSL	Biosafety level
CR	Complement receptor
CD	Cluster of differentiation
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
CFU	Colony forming unit
IFNγ	Interferon gamma
IGRA	IFNγ release assay
IL	Interleukin
LD	Live/dead
LTBI	Latently-infected individuals
MABSC	*Mycobacterium abscessus* complex
MAC	*Mycobacterium avium* complex
MAS	*Mycobacterium avium* Sensitin
MDM	Monocyte-derived macrophages
MTC	*Mycobacterium tuberculosis* complex
MOTT	Mycobacteria other than tuberculosis
NK	Natural killer

NTM	Non-tuberculous mycobacteria
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PPD	Purified protein derivatives
PRR	Pattern recognition receptor
RGM	Rapidly growing mycobacteria
SGM	Slow growing mycobacteria
TB	Tuberculosis
Th	T-helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TST	Tuberculin skin test

II. SUMMARY

This study focuses on the basics of anti-mycobacterial immune response in humans. In the first part of this PhD thesis, we aimed to clarify why infections with non-tuberculous mycobacteria (NTM) are successfully controlled by the immune system of immune competent people and do not lead to clinical symptoms, while people with certain genetic predispositions – studied in Cystic Fibrosis (CF) patients – have an increased susceptibility to NTM like *Mycobacterium abscessus*. For this, we developed a novel immunological test to detect *M. abscessus*-specific immune responses in CF patients. Our test circumvents both the collection of sputum samples – usually not available in paediatric patients – and the cultivation of the mycobacteria – which are frequently overgrown by other microorganisms. Blood samples of CF patients were collected at the University Children´s Hospital Düsseldorf, restimulated with purified protein derivatives (PPD) of different mycobacteria and mycobacteria-specific T cell responses were analysed. T cell immunity induced by *M. abscessus*-specific PPD detected acute *M. abscessus* infection in a subset of CF patients with positive *M. abscessus* culture (as accessed by routine diagnostics). Comparisons of different mycobacterial antigens suggested the presence of *M. abscessus*-specific epitopes. Phenotypic analysis of T cells reacting to *M. abscessus*-specific PPD revealed an increased production of CD40 positive T cells lacking interleukin-(IL)-2 in CF patients with acute *M. abscessus* colonization, although a functional relevance of these cells is still unclear.

Functional assays might help to evaluate candidate biomarkers in the context of host-pathogen interactions, when applied during clinical studies. In the second part of this PhD thesis, we established a novel polychromatic flow-cytometry-based functional *in vitro* assay to characterize anti-mycobacterial immune responses and interactions between infected macrophages and host T cells. In contrast to available assays, our novel assay enabled concomitant evaluation of mycobacterial growth inhibition by effector T cells and cytotoxic side effects on host macrophages. Our functional *in vitro* assay was based on infection of monocyte-derived macrophages (MDM) with a *M. bovis* BCG reporter strain (LD-BCG). Co-culture of infected MDM with polyclonal-activated or mycobacteria-specific effector T cells markedly reduced the viability of both LD-BCG and MDM in a concentration- and stimulus-dependent manner. Stimulation of $CD4^+$ or $CD8^+$ T cells with polyclonal activators or

mycobacteria-specific antigens increased the cytotoxic effect of these cells, which only differ in their capacity to induce MDM apoptosis. In particular, PPD-specific $CD4^+$ T cells induced significantly higher levels of infected MDM in early or late apoptotic stage than did PPD-specific $CD8^+$ T cells.

Taken together, our novel immunological test might improve the diagnosis of mycobacterial infections in CF patients, in particular in young children unable to expectorate sputum. Using this test, we pointed out the potential of $CD40L^+$ and IL-2^- T cells as candidate markers to identify CF patients with an acute *M. abscessus* infection. The functional relevance of these cells during – as well as of other biomarker candidates for – mycobacterial infections might be evaluated using the newly established functional *in vitro* assay.

III. ZUSAMMENFASSUNG

Diese Studie beschäftigt sich mit den Grundlagen der anti-Mykobakterien-Immunantwort beim Menschen. Im ersten Teil dieser Doktorarbeit soll die Frage geklärt werden, warum Infektionen mit nicht-tuberkulösen Mykobakterien (NTM) durch das Immunsystem von immunkompetenten Menschen erfolgreich kontrolliert werden und nicht zu klinischen Symptomen führen, während Menschen mit bestimmten genetischen Prädispositionen – untersucht an Mukoviszidose (CF) Patienten – eine erhöhte Anfälligkeit für NTM wie *Mycobacterium abscessus* haben. Hierfür entwickelten wir einen neuartigen immunologischen Test, um *M. abscessus*-spezifische Immunantworten in CF-Patienten nachzuweisen. Unser Test umgeht sowohl das Sammeln von Sputum-Proben – in der Regel nicht verfügbar bei pädiatrischen Patienten – und die Kultur der Mykobakterien – welche in der Regel von anderen Mikroorganismen überwuchert sind. Blutproben von CF-Patienten wurden an der Universitäts-Kinderklinik Düsseldorf gesammelt, mit Proteinderivate (PPD) verschiedener Mykobakterien restimuliert und Mykobakterien-spezifische T-Zell-Antworten wurden analysiert. T-Zell-Immunität induziert durch *M. abscessus*-spezifische PPD detektierte akute *M. abscessus*-Infektionen in einer Untergruppe von Patienten mit positiver *M. abscessus*-Kultur (durch Routinediagnostik nachgewiesen). Vergleiche verschiedener mykobakterieller Antigenen deutete auf die Anwesenheit von *M. abscessus*-spezifische Epitope. Phänotypische Analyse von T-Zellen, welche auf *M. abscessus*-spezifische PPD reagieren, zeigte eine erhöhte Produktion von CD40L-positive T-Zellen, welche das Interleukin (IL)-2 fehlen, in CF-Patienten mit akuter *M. abscessus*-Kolonisierung. Eine funktionelle Bedeutung dieser Zellen ist jedoch noch unklar.

Angewendet in klinische Studien, würden funktionelle Assays dazu beitragen, die Bedeutung von Biomarker-Kandidaten während Wirt-Pathogen-Interaktionen zu bewerten. Im zweiten Teil dieser Doktorarbeit haben wir einen neuartigen Durchflusszytometrie-basierten funktionellen *in vitro* Assay etabliert, um anti-mykobakterielle Immunantworten, sowie Interaktionen zwischen infizierte Makrophagen und Wirts-T-Zellen zu charakterisieren. Im Gegensatz zu verfügbaren Assays ermöglicht unseren neuen Assay die gleichzeitige Analyse von mykobakteriellen Wachstumshemmung durch Effektor-T-Zellen und zytotoxische Effekte auf Makrophagen. Der Assay beruhte auf Infektion von *in vitro* generierten Makrophagen (MDM) mit einem *M. bovis* BCG

Reporterstamm (LD-BCG). Co-Kultur von infizierten MDM mit polyklonal-aktivierten oder Mykobakterien-spezifische Effektor-T-Zellen zeigte eine konzentrations- und Stimulus-abhängige signifikante Reduzierung der Lebensfähigkeit von LD-BCG und MDM. Stimulation von $CD4^+$ oder $CD8^+$ T-Zellen mit polyklonalen Aktivatoren oder Mykobakterien-spezifische Antigene erhöhte die zytotoxische Wirkung dieser Zellen, die sich nur in ihrer Fähigkeit unterscheiden, MDM-Apoptose zu induzieren. Insbesondere induzierten PPD-spezifische $CD4^+$ T-zellen höhere Proportionen an infizierte MDM in der frühen oder späten Apoptose.

Zusammengefasst, unser immunologischer Detektionstest Test würde die Diagnose von mykobakteriellen Infektionen bei Mukoviszidose-Patienten verbessern, speziell bei Kleinkindern, die kein Sputum auswerfen können. Mit diesem Test wiesen wir auf das Potenzial von $CD40L^+$ und $IL\text{-}2^-$ T-Zellen als Kandidatenmarker für die Identifizierung von CF-Patienten mit einer akuten *M. abscessus* Infektion hin. Die funktionelle Bedeutung dieser Zellen während – sowie von anderen Biomarker-Kandidaten für – mykobakterielle(n) Infektionen könnte mit Hilfe unser neuen funktioneller *in vitro* Assay untersucht werden.

1. INTRODUCTION

1.1. Mycobacteria and mycobacterial diseases

Mycobacteria are gram positive bacteria, which can be divided into three groups. The *Mycobacterium tuberculous* complex (MTC) includes microorganisms like *M. tuberculosis*, *M. bovis* and *M. africanum*, which, with the exception of *M. bovis* BCG, can cause Tuberculosis (TB) in humans or animals. Primary pathogenic mycobacteria like *M. leprae* and *M. ulcerans* affect the skin and subcutaneous tissues, and respectively cause Leprosy and Buruli ulcer disease in humans. The group of non-tuberculous mycobacteria (NTM), earlier called "atypical mycobacteria" (ATM) or "mycobacteria other than tuberculosis" (MOTT) contains about 150 species, including organisms of the *M. avium* (MAC) and the *M. abscessus* complex (MABSC). They are ubiquitously found in the nature and mostly affect immune compromised persons or persons who suffer from previous pulmonary diseases. Depending on their growth rate, NTM can be divided into rapidly (RGM) and slow growing mycobacteria (SGM). In subculture, RGM are able to build colonies within seven days, whereas SGM need more than seven days to grow. The three most common RGM that can cause disease in humans are *M. abscessus, M. chelonae* and *M. fortuitum*. These organisms cause skin/soft tissue infection as well as chronic lung disease, manifested by bronchiectasis, nodules and cavitations.

Organisms of the MABSC can be divided into three genome species: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (Sassi and Drancourt, 2014). *M. abscessus* subsp. *Abscessus* (*M. abscessus*) was first isolated from subcutaneous abscess-like lesions in 1953 (Moore and Frerichs, 1953). It was first considered as a subspecies of *M. chelonae* (Kubica et al., 1972), until it was recognized as an independent species in 1992 (Kusunoki and Ezaki, 1992). *M. abscessus* have long been recognized as having rough (R) and smooth (S) colony phenotypes when grown on solid agar (Fregnan and Smith, 1962; Howard et al., 2006), and these morphotypes were associated with more or less severe human infections respectively (Rottman et al., 2007). Indeed, in contrast to the S variant, the R variant is able to persist and multiply in human monocytes, thereby residing in a phagosome with a virulent *M. tuberculosis*-typical tightly adherent phagosomal membrane (Byrd and Lyons, 1999). The mechanism responsible for

this switch is currently unknown, although it might involve genetic instability due to mobile elements, as described in *M. avium* (Belisle et al., 1993) and *M. smegmatis* (Kocincova et al., 2008).

1.2. The immune response against mycobacteria

Mycobacteria pathogens are the cause of a wide range of health problems, including pulmonary disease. Alveolar macrophages and dendritic cells are the first cells of the innate immune system to encounter the mycobacteria. In macrophages, complement receptor (CR) 3, as well as CR1, CR4, Mannose receptors, CD14 and Scavenger receptors have been implicated to play a role during phagocytosis, CR3 being described as the main receptor (Bhatt and Salgame, 2007). Once internalized, the pathogen resides in a phagosome. Virulent mycobacteria pathogens, including *Mycobacterium tuberculosis*, have evolved different strategies to arrest the phagosome maturation and prevent their further biogenesis and acquisition of lysosomal compartments, which enables survival within the host´s macrophages (Meena and Rajni, 2010).

Besides expressing phagocytic receptors, macrophages also express pattern recognition receptors (PRR) that recognize conserved molecular patterns expressed on pathogens. Among PPRs, toll-like receptors (TLRs) seem to play the major role and are believed to serve as a link between innate and adaptive immune defense against infection with mycobacteria, as they initiate an intracellular signaling cascade in host cells, that induces production of cytokines and chemokines, crucial factors to elicit the adaptive immune response against mycobacteria (Bhatt and Salgame, 2007; Hossain and Norazmi, 2013). Cytokine secretion then promotes activation of macrophages by natural killer (NK) cells and T cells. This initiates phagosome maturation and promotes anti-mycobacterial effector mechanisms such as autophagy, interferon inducible GTPase such as LRG47 and induces production of nitric oxide and other RNIs, leading to intracellular mycobacterial growth inhibition. Both $CD4^+$ and $CD8^+$ were associated with protection against mycobacterial infections. CD8 T cells produce cytolytic molecules like granzymes, Fas-L, granulysin and perforin, that kill both host cells and intracellular mycobacteria (Stenger et al., 1998; Kaufmann, 2013). Mechanisms by which cytolytic T cells can confer protective immunity

include the release of cytokines (e.g. IFNγ) that can activate macrophages to kill intracellular pathogens, as well as the induction of target cell apoptosis and target cell lysis (Stenger, 2001). However, there is a controversy as to whether the second mechanism is beneficial or detrimental, since not all of these forms of macrophage death resulted in killing of intracellular mycobacteria (Santucci et al., 2000). Not only CD8, but also CD4 T cells possess a cytolytic potential, which enables them to lyse infected macrophages (Canaday et al., 2001; Boom et al., 2003). In addition, CD4-depleted T cells had a reduced ability to restrict mycobacterial growth in both mice and humans (Appelberg et al., 1994; Tena et al., 2003; Chan et al., 2010). The role of $CD4^+$ T cells in immune control against mycobacteria is also highlighted by study on AIDS patients where children infected with AIDS showed a significant impairment in the control of mycobacterial growth as compared to uninfected ones, and this was assigned to their $CD4^+$-T cells deficiency (Tena et al., 2003).

Another mechanism how $CD4^+$ T cells contribute to protection against mycobacteria is the activation of effectors function in macrophages, which lead to production of Interferon-gamma (IFNγ) and Tumor necrosis factor (TNF) α (Kaufmann, 2013). TNFα is important in maintaining the structural integrity of granulomas (Flynn et al., 1995; Kaufmann, 2001). Indeed, granulomas that do form in the absence of TNFα or TNFα receptor are disorganized, with fewer activated or epitheloid macrophages (Flynn et al., 1995) and lymphocyte co-localization with macrophages is impaired (Bean et al., 1999). Moreover, an increased risk of Tuberculosis reactivation was described in patients with rheumatoid arthritis who were undergoing anti-TNFα-therapy (Kaufmann, 2001; Harris and Keane, 2010). IFNγ is thought to be the most important cytokine in control of mycobacterial infection. This is highlighted by the fact that individuals which are deficient in IFNγ and IFNγ receptor genes have an increased susceptibility to mycobacterial infections (Ottenhoff et al., 1998). The role of IL-12 for protective immune response against mycobacteria is probably due to the fact that this cytokine drives development of Th1 responses with production of IFNγ (Cooper et al., 1997), so that mutations in the IL-12 as well as the IFNγ pathways are believed to increased susceptibility to mycobacterial infections (Chan et al., 2010; Lake et al., 2016). However, the effect of IL-12 seems also to be dependent on TNFα and granulocyte macrophage colony stimulating factor (GM-CSF), as treatment of supernatant from IL-12-stimulated NK cells with anti-TNFα or anti-GM-CSF antibodies reduced the ability of these supernatants to limit *M. avium* growth within macrophages

(Bermudez et al., 1995). Multifunctional CD4$^+$ T cells producing IFNγ, TNFα and IL-2 were found to be elevated in patients with active *M. tuberculosis* infection compared to latently infected individuals (LTBI) (Mueller et al., 2008; Caccamo et al., 2010), highlighting a role of these cells for protective immune response against tuberculosis. Also, reduced proportions of these T helper 1 (Th1) cytokines were found in peripheral blood mononuclear cells (PBMC) from patients with NTM lung disease caused by *M. abscessus* or *M. avium* complex when compared to healthy controls. Altogether, these studies and others highlighted the association of polyfunctional T cells with bacterial loads, implying their role as indicative marker for active mycobacterial disease (Prezzemolo et al., 2014). Although polyfunctional T cells seem to be necessary for protection against mycobacteria, they are not sufficient for prevention of the infection, as children who received a prime-boost of the MVA85A vaccine – with expansion of polyfunctional T cells – were not better protected from mycobacterial infections than control children without prime-boosting (Tameris et al., 2013).

Altogether, the host immune response to mycobacteria pathogens mostly rely on the Th1 type independent on the mycobacterial class. However, there are some subtle differences, not yet fully understood differences were described (Orme and Ordway, 2014). These differences are partly responsible for our difficulties to understand the pathogenesis of these organisms.

1.3. Cystic Fibrosis and NTM infections

Cystic Fibrosis (CF) is an autosomal recessive hereditary disease caused by mutations of both copies of the gene that encodes the Cystic Fibrosis Transmembrane Regulator (CFTR) protein (O'Sullivan and Freedman, 2009). CFTR not only function as a chloride channel, but is also involved in bicarbonate-chloride exchange. The lack of secreted bicarbonate observed in CF patients might be responsible for impaired calcium removal, abnormal mucin expansion and stasis of mucus in the ducts and on the luminal surfaces of affected organs in these patients (Quinton, 2008). CFTR mutations can be categorized into five classes, whereas the absence of the amino acid phenylalanine at the position 508 (ΔF508), a class II mutation, accounts for about two-thirds of mutated alleles in northern European and North American populations (O'Sullivan and Freedman, 2009).

Increasing numbers of infections with NTM were described in CF patients. Reported prevalence rates are ranging from 3.7 to 24 %, depending on the geographical region and the patient population (Hill et al., 2012), with organisms of the MABSC belonging to the most frequently isolated species (Roux et al., 2015). Factors leading to increased susceptibility of CF patients to NTM infections are not known, but impaired mucus clearance and CFTR-dysfunction, as well as co-infection with other organisms and CF treatment – with azithromycin or steroids for example – are likely to play a central role (Ramsey et al., 1999; Equi et al., 2002; Mussaffi et al., 2005; Renna et al., 2011; Verregghen et al., 2012; Catherinot et al., 2013a; Catherinot et al., 2013b). Catherinot and co-workers analysed the differences between infections with MAC and with MABSC in a CF patient collective (Catherinot et al., 2013b), and found that MABSC patients were younger than MAC patients and had the ΔF508 / ΔF508 CFTR mutation. MABSC infection has an impact on CF disease course (decline in lung function, lethal disease course) and is a contraindication for lung transplantation (Sanguinetti et al., 2001; Olivier et al., 2003; Esther et al., 2010; Harris and Kenna, 2014; Martiniano et al., 2014). An English study used whole-genome sequencing to analyse transmission of MABSC between patients with CF and found evidence for between-patient transmission of *M. abscessus* subsp. *massiliense* in a CF centre in UK, indicating that person-to-person transmission of MABSC is possible, probably via fomite contamination or aerosol generation during chemotherapy and lung function testing (Bryant et al., 2013).

NTM diagnosis and treatment in CF patients is based on criteria defined by the American Thorax Society (ATS) (Griffith et al., 2007). However, these criteria do not specifically address the diagnosis and treatment of this particular patient population. Indeed, pulmonary symptoms are unspecific and cannot be differentiated from symptoms associated with CF pulmonary conditions (Griffith et al., 2007; Weiss and Glassroth, 2012). Also radiological criteria overlap with CF features and are not specific to the causative mycobacterium (McEvoy et al., 2012). Therefore, NTM detection often relies on the microbiological detection method to identify the specific NTM species. Microbiological methods are based on the examination of expectorated sputum or bronchoalveolar lavage (BAL) fluids. Two separately expectorated sputa or one BAL-sample positive for NTM are needed, in order to fulfil microbiological diagnostic criteria (Griffith et al., 2007). However, some patients, mostly children, are not able to expectorate sputum, which hinders the

microbiological detection of NTM in these patients (De Bel et al., 2013). A multitude of microbiological methods for detection of NTM were described, which all need decontamination of sputa to avoid overgrowth of mycobacteria by other microorganisms (Whittier et al., 1993), as well as concomitant cultivation of mycobacteria in solid or liquid media (Griffith et al., 2007). However, despite decontamination, some culture still fails to detect the mycobacteria (Bange et al., 1999), as decontamination procedures were shown to inhibit the growth of mycobacteria (Radhakrishnan et al., 2009). This reflects the need for novel assays to detect MABSC infection in CF patients.

Tests to detect the immune response to mycobacterial antigens, like for example the Tuberculin skin test (TST) and Interferon-gamma Release Assays (IGRAs), are well established in the diagnosis of Tuberculosis, but are not available for NTM species and *M. abscessus* in particular. However, false-positive reactions of the TST may occur, as healthy persons infected with NTM might respond to Tuberculin, due to the common antigens shared by mycobacterial species. Dual skin testing with *M. avium* Sensitin (MAS) and purified protein derivatives of *M. tuberculosis* (PPD) was conducted in patients with pulmonary disease due to *M. avium* or *M. tuberculosis* and was able to discriminate between both patient groups with 97 % specificity (von Reyn et al., 1998). Also discrimination between different NTM species appears possible using this method (Bierrenbach et al., 2001). Therefore, T cell immunity-based assays might represent potential tools for diagnosis of NTM. In contrast to microbiological detection methods, specific immunological tests might also help to evaluate disease severity and to predict treatment outcome.

1.4. Biomarkers in mycobacterial research

New goals of the World Health Organization include a 95% reduction in TB deaths, as well as a 90% reduction in TB cases by 2035, with the vision to get a TB-safe world by 2050 (WHO, 2015). To achieve these goals, new tools, including improved diagnostics and treatment as well as new and more effective vaccine are urgently needed. About 16 different TB vaccine candidates are currently in preclinical development or have already passed the first rounds of clinical trials (WHO, 2015). For the majority of these vaccine

candidates, development relies on recombinant expression or subunits boosting of the BCG vaccine (Cayabyab et al., 2012; Davenne and McShane, 2016; Fletcher and Schrager, 2016; Scriba et al., 2016). Testing of the candidates often relies on measurement of the immune response to the vaccination, for example through IFNγ response in $CD4^+$ T cells. In addition, also available diagnostic tests for TB including IGRAs like Quantiferon TB Gold relie on IFNγ response following stimulation of T cells with two *M. tuberculosis* immunogenic proteins early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) (Mazurek et al., 2005). However, there is a controversy, as to whether IFNγ is the "holy grail" for immune protection against TB, as many studies described the involvement of other cell types in TB immunology (Abebe, 2012).

Biomarkers can be indicators of disease or of immune protection, and might facilitate the evaluation of candidate vaccines, when applied as endpoints during clinical trials (Fletcher and Dockrell, 2016). Reliable biomarkers to predict immune protection against mycobacteria pathogens or progression to for example active TB disease are missing. One explanation for this is the restriction to animal models for biomarker identification, since most models only partially reflect human disease. The MVA85A (modified Vaccinia Ankara virus expressing the immunodominant *M. tuberculosis* protein antigen 85A) vaccine was developed as a heterologous boost for BCG vaccination (McShane et al., 2004) and improved BCG-induced protection against mycobacterial challenge in different animal models, including mouse, guinea pigs, rhesus macaque and cattle (Goonetilleke et al., 2003; Williams et al., 2005; Verreck et al., 2009; Vordermeier et al., 2009). However, vaccination of infants with MVA85A to boost previous BCG vaccination did not prevent incidence of TB disease nor impede further infection with *M. tuberculosis* (Tameris et al., 2013). Another explanation is that studies on human TB are vastly restricted to surrogate tissue analyses, which may not adequately reflect the situation at affected tissue sites. In addition, the majority of human TB studies identified "statistically significant" candidates (e.g. differentially expressed T-cell factors during disease course) but functional analyses to determine the role in the context of host-pathogen interaction are rare. Consequently, the "biological significance" of candidate factors often remains elusive. Appropriate functional *in vitro* assays, that would allow the analysis of host-pathogen interactions and predict the efficacy of biomarkers candidates, are missing.

The first developed functional *in vitro* assays allowed quantification of mycobacterial eradication by colony forming units (CFU) count, following infection of monocytes or macrophages cultures (Bonecini-Almeida et al., 1998; Silver et al., 1998; Worku and Hoft, 2000; Brookes et al., 2003; Green et al., 2013). Killing of the mycobacteria was usually referred to as a reduction in the CFU number at the end of the culture period as compared to on culture onset or to a control. Obviously, such changes in net numbers of CFU might be due to any other event, like for example the failure to replicate (Elkins et al., 2011). In addition, mycobacteria culture is long-lasting and other methods which circumvent culture of the mycobacteria were needed.

Measurement of bioluminescence emitted by viable bioluminescent mycobacteria enables a more rapid detection of viable mycobacteria and is as sensitive as CFU determination (Bonay et al., 1999). Kampmann and colleagues developed an *in vitro* functional assay, the BCG-lux assay, to monitor the growth of a *M. bovis* BCG strain carrying the luxAB genes in human peripheral blood cultures (Kampmann et al., 2000; Newton et al., 2011). Burl *et al*. established a modification of the BCG-lux assay to make it suitable for paediatric studies and found that a 2-fold reduction in the volume of blood sample used in the BCG-lux assay makes no significant difference in the bacterial growth ratios (Burl et al., 2013). In vivo bioluminescence imaging (BLI) was first applied in the context of mycobacteria research by Heuts and colleagues, who proved that BLI can be used to study bacterial dissemination in tissues, the efficacy of antimycobacterial drugs and the role of adaptive immune responses in the control of mycobacterial infections in vivo (Heuts et al., 2009). A year later, the use of the three main luciferin/luciferase systems (firefly, Gaussia and bacterial luciferases) in mycobacteria was optimized and Andreu et al. reported for the first time the functional expression of the whole bacterial lux operon in *M. tuberculosis* and *M. smegmatis*, thereby allowing the development of auto-luminescent mycobacteria and the detection of the produced signal in the lungs of infected mice by bioluminescence imaging (BLI) (Andreu et al., 2010). Macdonald *et al.* designed an infection assay, using fluorescence microscopy as the readout (Macdonald et al., 2012).

Altogether, these assays only give information about the viability of the mycobacteria, but no information about the killing mechanism or the mode of killing induced. In addition, concomitant determination of side effects of the candidate biomarkers on the host

immune cells, as well as analysis of the immune response they triggered is not possible. An essay which provides these informations might significantly improve the evaluation of candidate biomarkers for mycobacterial research.

1.5. Objectives and Approaches

Detection and characterization of non-tuberculosis mycobacteria (NTM) species in Cystic Fibrosis (CF) patients is crucial for disease management. Available detection methods require the culture of the mycobacteria from sputum or BAL samples, which is time consuming, and sometimes not available in paediatric patients. The first part of this project, aimed

- ➔ to develop a novel immunological test to specifically detect NTM in CF patients.

For this, CF patients´ peripheral blood T cells were re-stimulated *in vitro* with purified protein derivatives (PPD) of different mycobacterial species.

- ➔ Analyses of mycobacterial-specific immune responses

were performed, in order to get a better understanding of the mechanisms responsible for the increased susceptibility of these patients to NTM infections.

Biomarkers of a disease might be used for identification, prevention, treatment of or vaccine development against the relevant disease. Identification of biomarkers in mycobacterial research is partly hampered by the absence of functional assays, which would allow the analysis of host-pathogen interactions during the infection. In this second part of my PhD project, the aim was

- ➔ to establish a novel flow-cytometry-based *in vitro* infection assay

based on infection of monocyte-derived macrophages (MDM) with a *M. bovis* BCG reporter strain, in order to optimize the evaluation of biomarker candidates in mycobacterial research. Effector $CD4^+$ and $CD8^+$ T cells were co-cultured with infected MDM, in order

- ➔ to allow a quick and detailed characterization of the host macrophage / effector T cell response during mycobacterial infections.

2. PUBLICATIONS AND RESULTS

2.1. Chapter 1 – Rapid Detection and Immune Characterization of Mycobacterium *abscessus* Infection in Cystic Fibrosis Patients

Contribution to this publication: 40 %

- *In vitro* restimulation studies
- flow cytometry measurements and data analysis
- writing of the manuscript

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RESEARCH ARTICLE

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Abstract

Cystic fibrosis patients are highly susceptible to infections with non-tuberculous mycobacteria. Especially *Mycobacterium abscessus* infections are common but reliable diagnosis is hampered by non-specific clinical symptoms and insensitive mycobacterial culture. In the present study we established novel methods for rapid detection and immune characterization of *Mycobacterium abscessus* infection in cystic fibrosis patients. We performed *Mycobacterium abscessus* specific DNA-strip- and quantitative PCR-based analyses of non-cultured sputum samples to detect and characterize *Mycobacterium abscessus* infections. Concomitantly *in vitro* T-cell reactivation with purified protein derivatives (PPDs) from different mycobacterial species was used to determine *Mycobacterium abscessus* specific T-cell cytokine expression of infected cystic fibrosis patients. Four of 35 cystic fibrosis patients (11.4%) were *Mycobacterium abscessus* culture positive and showed concordant DNA- strip-test results. Quantitative PCR revealed marked differences of mycobacterial burden between cystic fibrosis patients and during disease course. Tandem-repeat analysis classified distinct *Mycobacterium abscessus* strains of infected cystic fibrosis patients and excluded patient-to-patient transmission. *Mycobacterium abscessus* specific T-cells were detected in the blood of cystic fibrosis patients with confirmed chronic infection and a sub- group of patients without evidence of *Mycobacterium abscessus* infection. Comparison of cytokine expression and phenotypic markers revealed increased proportions of CD40L positive T-cells that lack Interleukin-2 expression as a marker for chronic *Mycobacterium abscessus* infections in cystic fibrosis patients. Direct sputum examination enabled rapid di- agnosis and quantification of *Mycobacterium abscessus* in cystic fibrosis patients. T-cell *in vitro* reactivation and cytokine expression analyses may contribute to diagnosis of chronic *Mycobacterium abscessus* infection.

Introduction

Mutations on both alleles of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) are the genetic cause of Cystic Fibrosis (CF), the most common single-gene caused disease in Caucasians [1]. CF pathology affects multiple organs, but pulmonary disease predominantly influences morbidity and mortality of CF patients. Chronic pulmonary infections are a typical feature of CF [2]. Non-tuberculous mycobacteria are rarely pathogenic for immunocompetent individuals but frequently colonize vulnerable pulmonary epithelia of CF patients [3]. Closely coherent extrinsic factors (e.g. frequent infection with other opportunistic bacteria, viruses, and fungi) and intrinsic factors (e.g. continuous inflammation, dysregulation of innate and adaptive immune response) contribute to increased susceptibility against non-tuberculous mycobacteria but the exact mechanism remains elusive [4, 5].

Mycobacterium (M.) avium complex (MAC) and *M. abscessus complex* (MABSC) are the non-tuberculous mycobacterial species most commonly detected in the sputum of CF patients [6]. MABSC has recently been classified as an independent species [7]. Since then, MABSC has been found to be the most frequent 'rapid growing' human pathogenic mycobacterial species [8]. MABSC shares some similarities with the highly pathogenic species of the *M. tuberculosis complex* (MTC). Most intriguingly MABSC is able to cause persistent lung disease characterized by development of caseous lesions, a hallmark of human tuberculosis [9]. Recently evidence for direct transmission of MABSC between CF patients has been found [10]. Although the mode and likelihood of patient-to-patient transmission of MABSC is unclear, this finding will have major implications for clinical routine. In addition, the possibility of direct transmission renders early detection and characterization of MABSC in CF patients crucial.

The reported prevalence of MABSC infections in CF patients differs markedly between studies of different regions ranging from 3.4 to 24% [3]. These differences are at least in part due to difficulties in the diagnosis and detection [11]. A few major factors contribute to this divergence. First, clinical symptoms of MABSC infections are shared with several other infections and imaging methods are often inconclusive [11]. Second, mycobacterial culture from pulmonary samples (predominantly sputum) is time consuming and fails in many cases primarily due to fast growing colonizing bacteria such as pseudomonas and staphylococci [12]. Third, decontamination of sputum samples, a prerequisite for detection of mycobacteria, reduces the sensitivity for detection of mycobacteria [13]. Immunological tests for MABSC infections are not available and cross-reactivity of immune responses against different mycobacteria hampers the development of specific assays [14]. Previous studies have used purified protein derivatives of MTC or MAC for skin tests or *in vitro* assays to discriminate between different mycobacterial infections with encouraging results [15].

In the present study we established PCR-based methods (i.e. DNA-strip test, quantitative PCR) for rapid *ex vivo* detection and quantification of MABSC from sputum of CF patients. A T-cell assay based on *in vitro* reactivation with different mycobacterial antigens was performed to distinguish and characterize immunity to mycobacterial infections. This approach may help to define immune characteristics that lead to increased susceptibility of CF patients against non-tuberculous mycobacteria and may improve early diagnosis of infection.

Methods

Study subjects and design

35 patients diagnosed with CF were recruited in the Department for General Pediatrics, University Children´s Clinic, Duesseldorf and enrolled in the study in 2013 (starting in March). Clinical characteristics of the CF patients are reported in Table 1. Heparinized blood (3 ml)

Detection of *Mycobacterium abscessus* Infection in CF Patients

Table 1. Clinical characteristics of CF patients

	CF patients
number	35
Gender distribution (f/m)	18/17
Age mean (range)	13,1 (3–25)
FEV1% mean (range)	78,2 (37,2–124,1)
BMI mean (range)	17,9 (12,3–27)

n: number of patients; f/m: number of females/males; FEV: forced expiratory volume; BMI: body mass index

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and sputum samples were collected as part of routine evaluation. Routine culture for detection of non-tuberculous mycobacteria has been performed for all CF patients able to expectorate sputum samples. From one CF patient, sputum and blood samples were taken consecutively over a period of 200 days. Children unable to expectorate sputum donated peripheral blood samples. Written informed consent was obtained from all subjects or their guardians.

Ethics Statement

This study was approved by the ethics committee of the University Hospital Duesseldorf (Internal Study No. 4505).

MABSC-DNA-strip test

Sputum samples were immediately processed after expectoration or stored at -20°C for up to 60 days. A minimal volume of 50 µl was used for DNA isolation. DNA was extracted using the enzymatic lysis-based InnuPrep Mycobacteria DNA Kit (Analytik Jena). The DNA-strip test was performed using GenoType Mycobacterium CM kit (Hain Lifescience) following the manufacturer's protocol. To adjust for estimated lower mycobacterial numbers (as compared to culture enriched mycobacteria), we modified PCR-settings by adding ten cycles.

Quantitative PCR (qPCR) for MABSC *rpoB*

TaqMan (Life Technologies) real-time PCR for the MABSC *rpoB* gene was performed. A region in the *rpoB* gene specific for MABSC (not present in *M. chelonae*) was targeted [16]. Primers and probe (Table 2) were designed using Primer Express (Applied Biosystems). To evaluate assay specificity we tested following mycobacteria strains (i.e. *M. chelonae*, *M. kansasii*, *M. gordonii*, *M. fortuitum*, *M. szulgai*, *M. marinum*, *M. avium*, *M. intracellulare*, *M. celatum*, *M. simiae*, *M. malmoense*) from patient isolates. None of these related mycobacteria strains were detected. As internal standards, plasmids containing the *rpoB*-PCR target se- quence were generated and added to each experiment. In brief, *rpoB* amplicons were ligated in plasmids and transformed to DH5α-*E.coli* using pGEM-T-Vector Kit (Promega) and isolated

Table 2. Primers and probe used for qPCR

Target	Accession No.	Primers and probe
rpoB	AY147164.1	3635(+)CGATAGAGGACTTCGCCTAACC
		3711(-)TCGAGCACGTAAACTCCCTTTC
		3660(+)HEX-CCACTGACCGAACATCTATCCCGC

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using the High Pure Plasmid Isolation Kit (Roche). Plasmid concentration was measured in a Nanodrop 1000 spectrophotometer (Thermo Scientific) and calculated plasmid concentrations of 10^5 and 10^2 copies were used

as standards. Sputum DNA samples (5 µl) were used for each qPCR performed according to manufacturer's instructions (QuaniTect Multiplex PCR NoROX, Qiagen) on a C1000 Thermocycler (Biorad). For MABSC spike-in experiments, spu-tum of a MABSC-negative CF patient was spiked with serial dilutions (10-fold) of a MABSC patient isolate. CFU were then determined in triplicates after 72h incubation on sheep-blood-agar. Concomitantly DNA-strip tests and qPCR were performed for spiked sputum samples (as described above).

Variable number tandem repeat (VNTR) analysis

Classification of culture-enriched MABSC from sputum was performed by variable number tandem repeat (VNTR) analysis according to Wong *et al*. [17] with minor modifications. In brief, six of the described tandem repeats (TR45, TR109, TR116, TR150, TR155, TR172) were used for PCR analysis and tandem-repeat lengths were analyzed using agarose gel (1%) electrophoresis.

Flow cytometry-based detection of MABSC-specific T-cells

Heparinized blood was processed freshly (within five hours of blood collection) to assure maximal sensitivity of this assay (own unpublished data). In brief, blood (100 µl) was diluted (1:1) in RPMI containing 1% L-Glutamine (Sigma Aldrich), 1% Penicillin/Streptomycin (Life Technologies) and supplemented with recombinant human (rh) IL-7 (20 ng/ml; Biolegend) for overnight culture in 96-well round-bottomed microtiter plates as described [15]. IL-7 has been shown previously to increase the sensitivity of IFNγ release assays (IGRAs) for detection of mycobacteria specific T cells [18]. IL-7 has a minimal cytokine-inducing effect in the absence of antigens. The following antigens were used for stimulation: purified protein derivative (PPD) of MABSC (termed abscessin), PPD of *M. avium/intracellulare* (sensitin), and PPD of *M. tuberculosis* (tuberculin). All PPDs were purchased from Statens Serum Institute. *Staphylococcus* enterotoxin b (SEB) (15 µg/ml, Sigma Aldrich) was used as a positive control. The following monoclonal fluorescence-labeled antibodies were used: CD4 (clone Okt4, BV510), CD45RA (clone HI100, FITC), CCR7 (clone 3D12, PE-Cy7), IFNɣ (clone B27, BV450), TNFα (clone Mab11, APC), IL-2 (clone MQ1–17H12, PerCP-Cy5.5), and CD40L (clone 24–31, PE). All antibodies (except CCR7: BD Biosciences) were purchased from BioLegend. A FACS Canto-II flow cytometer (BD Biosciences) was used for the measurements and FCS Express 4 Software (De Novo) was used for analyses. The background values [cytokine positive T-cell proportions of non-stimulated (rhIL-7 only) samples] were subtracted from each stimulus. Ratios of PPD specific T-cells were calculated by dividing the proportions of CD40L/IL-2 double positive T- cell proportions for abscessin/tuberculin and abscessin/sensitin. For calculation of normalized values, we set the sum of abscessin-, tuberculin-, and sensitin-specific CD40L/IL-2 double positive T-cells proportions to 1. For presentation of cytokine expression pattern (i.e. three cytokines and CD40L) graphical depiction is not possible because 4-dimensional presentation (followed by selection of subsets) is technically not feasible. As a consequence the events for each cytokine pattern expressing T-cell subpopulation (numbers of non-stimulated numbers were subtracted) were counted and the number of T cells expressing at least one cytokine per activation marker in response to *M. abscessus* was calculated. Calculated proportions of each subpopulation of all activated T cells are indicated for each individual donor and compared between study groups.

Statistical analyses

Statistical calculations were performed using SigmaStat (Systat Software). Parametric or non-parametric tests were chosen according to Kolmogorov-Smirnow normality test. Accordingly, the student t-test or the Mann-

Whitney Rank Sum Test was used and indicated in the text and figure legend. P-values <0.05 were considered to be significantly different.

Results

Direct sputum DNA-strip/*rpoB*-qPCR MABSC tests

Mycobacterial sputum culture detected MABSC infection in four of 35 enrolled CF patients (11.4%) at study onset. We performed a DNA-strip test on sputum without prior *in vitro* culture from all sputum expectorating CF patients (n = 23). DNA-strip test detected MABSC in the sputum of all culture positive CF patients (CF034, CF023, CF029, CF022) (Fig. 1A, Table 3). None of the culture-negative CF patients had a positive PCR and CF002, a patient with a history of MABSC infection, was also DNA-strip test negative (Fig. 1A and Table 3). Next we analyzed the sensitivity of DNA-strip test in sputum samples by establishing a MABSC-specific *rpoB*-qPCR. Serial dilutions of MABSC in sputum and concomitant analyses of CFU, DNA-strip test, and *rpoB*-qPCR revealed ten CFU at the 10^{-6} dilution step (Fig. 1B). The *rpoB*-qPCR detected about two DNA copies at the 10^{-5} dilution step (about 100 CFU) (Fig. 1B). Accordingly, a rough estimate of 50-fold lower sensitivity of *rpoB*-qPCR as compared to CFU was deduced and applied for calculation of MABSC concentration in CF sputum samples. Notably, the DNA-strip test was positive at 10^{-3} but not at lower concentration. This indicated an about 100-fold lower sensitivity of the DNA-strip test as compared to *rpoB*-qPCR and a detection limit of estimated $5x10^{3}$ CFU/ml sputum.

Sputum MABSC characterization of CF patients

Next, *rpoB*-qPCR was applied to determine MABSC concentrations of CF patient´s sputum samples. MABSC-confirmed CF patients had marked differences of MABSC sputum burden with concentrations ranging from $5.6x10^{5}$ to $3.9x10^{7}$ bacteria per ml sputum (Fig. 1C). All MABSC-negative CF patients (including CF002; Fig. 1C) were *rpoB*-qPCR negative (Table 3). For one CF patient (CF034), *rpoB*-qPCR and MABSC sputum culture were performed repeatedly during the study period. Initially CF034 had the highest concentrations of MABSC in sputum (Fig. 1C, D) and was culture positive. At the next routine visits (97 and 200 days after study onset), CF034 had MABSC-negative in sputum cultures (Fig. 1D) and was also *rpoB*- qPCR negative (Fig. 1D).
The high prevalence of MABSC-positive sputum samples from CF patients in the present study suggested a common source of infection or direct transmission between patients [19]. Consequently, we characterized MABSC strains from CF patients by VNTR-analysis [17]. Altogether six regions were analyzed but none of the MABSC isolates from CF patients had identical patterns (Fig. 2). These results rendered transmission of MABSC strains between CF patients unlikely. Therefore our novel approaches enabled us to detect marked interindividual differences and changes of MABSC sputum concentrations during disease course caused by different MABSC strains.

MABSC-specific T-cells in CF patients

Immune-based tests for MABSC infection are not available. Hence, we established an *in vitro* whole blood assay to detect MABSC-specific T-cells using flow cytometry analysis of intracellular cytokines (for details see Methods section and Fig. 3). Ten CF patients showed positive

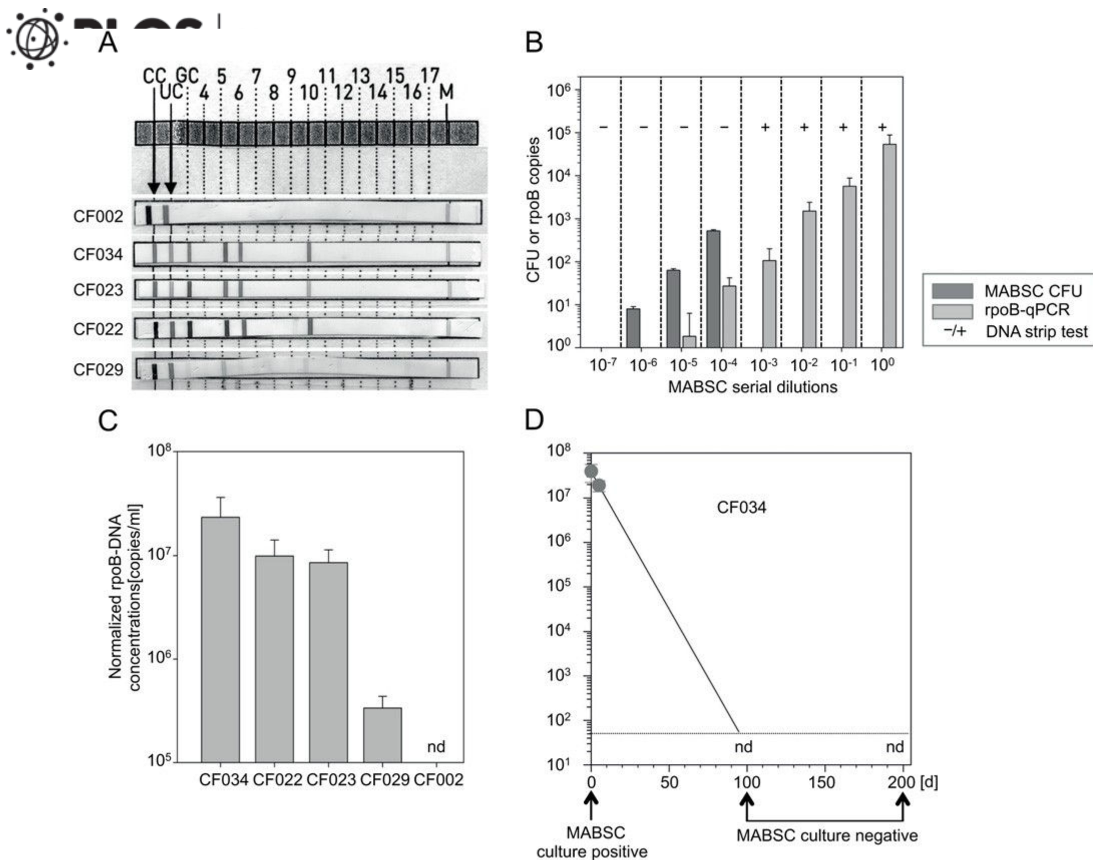

Fig 1. Direct detection and quantification of MABSC by CFU, DNA-strip, and rpoB-qPCR of non-cultured CF sputum samples. (A) Direct *ex vivo* PCR strip results of sputum from MABSC-positive CF patients (i.e. 034, 023, 022, 029) and CF patient 002 (previous MABSC infection but unclear infection status). A band pattern at positions 3, 5, 6, and 10 indicates MABSC (the same pattern but absence of a band at position 3 and 6 indicates either *M. abscessus* or *M. chelonae*). The CC band is a conjugate control, the UC band indicates bacterial DNA with a high GC-content, the GC band indicates DNA of the genus *mycobacterium*. (B) CFU (colony forming units), *rpoB*-qPCR (both at the y-axis), and PCR strip test results (positive tests indicated as +, negative as-) for serial dilution (10-fold) of MABSC in sputum. Dark grey bars indicate CFU numbers. Bright grey bars indicate normalized *rpoB* copies. Mean values of triplicate samples and standard deviations are given. One representative experiment of two is shown. (C) *RpoB*-qPCR results of sputum from MABSC- positive CF patients (i.e. 034, 023, 022, 029) and CF patient 002 are depicted. Calculated MABSC sputum concentrations (adjusted for 50-fold lower sensitivity of rpo-qPCR as compared to MABSC culture) are given. (D) Time course analyses of *rpoB*-qPCR and mycobacterial sputum culture from CF patient CF034. Circles depict *rpoB*-qPCR values and arrows indicate time points of MABSC sputum culture. nd (not detectable) indicates *rpoB*-qPCR runs where MABSC *rpoB* was not detected. The dotted line indicates the detection limit of qPCR.

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cytokine responses to MABSC-specific PPD (abscessin) (Fig. 4A). These comprised all confirmed MABSC-infected CF patients (CF022, CF023, CF029, CF034, CF002) and five CF patients (CF026, CF028, CF030, CF036, CF039) without evidence for MABSC infection ($CF_{non\text{-}confirmed}$) (Fig. 4A, Table 3, S2 Fig.). Three patients of the $CF_{non\text{-}confirmed}$ patient group (CF026, CF028, CF036) were unable to expectorate sputum and therefore mycobacterial culture or PCR-based analyses were not possible. Cross-reactive mycobacterial antigens may confound these results but concomitant testing of PPDs from different mycobacteria (i.e.

Table 3. Study results.

	CF patients (n = 35)		
sputum samples	available (n = 23)		not available (n = 12)
MABSC sputum culture	positive (n = 4(5*))	negative (n = 18)	na
DNA-strip test (pos/neg)	4/1	0/18	na
MABSC rpo-qPCR (pos/neg)	4/1	0/18	na
MABSC immune test (pos/neg)	5/0	2/16	3/9

* One CF patient (CF002) had a history of previous MABSC infection but an unclear infection status at study onset due to persistent culture contamination. For sputum PCR analyses this patient was classified as MABSC negative (confirmed by our assays). n: number of patients; na: not applicable; pos: positive; neg: negative.

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M. tuberculosis, tuberculin; *M. avium*, sensitin) was shown to reveal the causative mycobacteria [15]. Hence we compared T-cell responses between abscessin and tuberculin (Fig. 4B, left graph), as well as abscessin and sensitin (Fig. 4B; right graph), and detected higher T-cell proportions specific for abscessin (ratio > 1) for all *M. abscessus* confirmed cases (CF_{MABSC}, green triangles) (Fig. 4b). In contrast, in CF patients without indication of MABSC infection (blue circles) only one patient (CF030) had a stronger abscessin specific response (Fig. 4B). In acco dance, T-cell responses against all three PPDs detected more than 50% of abscessin-specific T-cells for confirmed cases (Fig. 4C). Again, only CF030 had an abscessin dominant T-cell response similar to confirmed cases. We concluded that T-cell response ratios confirmed MABSC infection and suggested non-MABSC related causes in the majority of $CF_{non\text{-}confirmed}$ patients.

Cytokine-expression pattern discriminate confirmed MABSC cases (CF_{MABSC})

Cytokine-expression pattern characterize functional T-cell differences and may reflect different infection stages (e.g. acute and previous MABSC infection). Three cytokines (i.e. IFNγ, TNFα, IL-2) and the T-cell activation marker CD40L were analyzed concomitantly in abscessin- specific T-cells. Expression patterns revealed significant differences between CF_{MABSC} and $CF_{non\text{-}confirmed}$ patients (Fig. 5A,B). CF_{MABSC} had higher levels of CD40L/IFNγ/TNFα triple-positive ($p = 0.004$), CD40L/IFNγ ($p = 0.01$), and CD40L/TNFα ($p = 0.02$) double-positive abscessin-specific T-cells. Notably, differences applied to T-cell populations that expressed CD40L but not IL-2 (Fig. 5B,C). Comparison of all CD40L-positive/IL-2-negative T-cell pro- portions revealed non-overlapping differences between CF_{MABSC} (median 32.8%, range 6%) and $CF_{non\text{-}confirmed}$ patients (median 21.7%, range 3.3%) ($p<0.001$) (Fig. 5C). We concluded that increased MABSC-specific T-cell expression of CD40L in the absence of IL-2 production is an immune marker of MABSC infection in CF patients.

Discussion

Here we characterized MABSC-specific T-cell responses in CF patients and revealed cytokine expression pattern indicative of chronic MABSC infection. In addition, novel PCR-based methods for rapid and reliable *ex vivo* diagnosis of MABSC infection in CF patients were established. DNA-strip test and *rpoB*-qPCR based detection of MABSC revealed concordant results with mycobacterial culture. This finding has potential important implications for diagnosis of MABSC infection because direct PCR-based analysis of CF sputum samples bypasses NTM

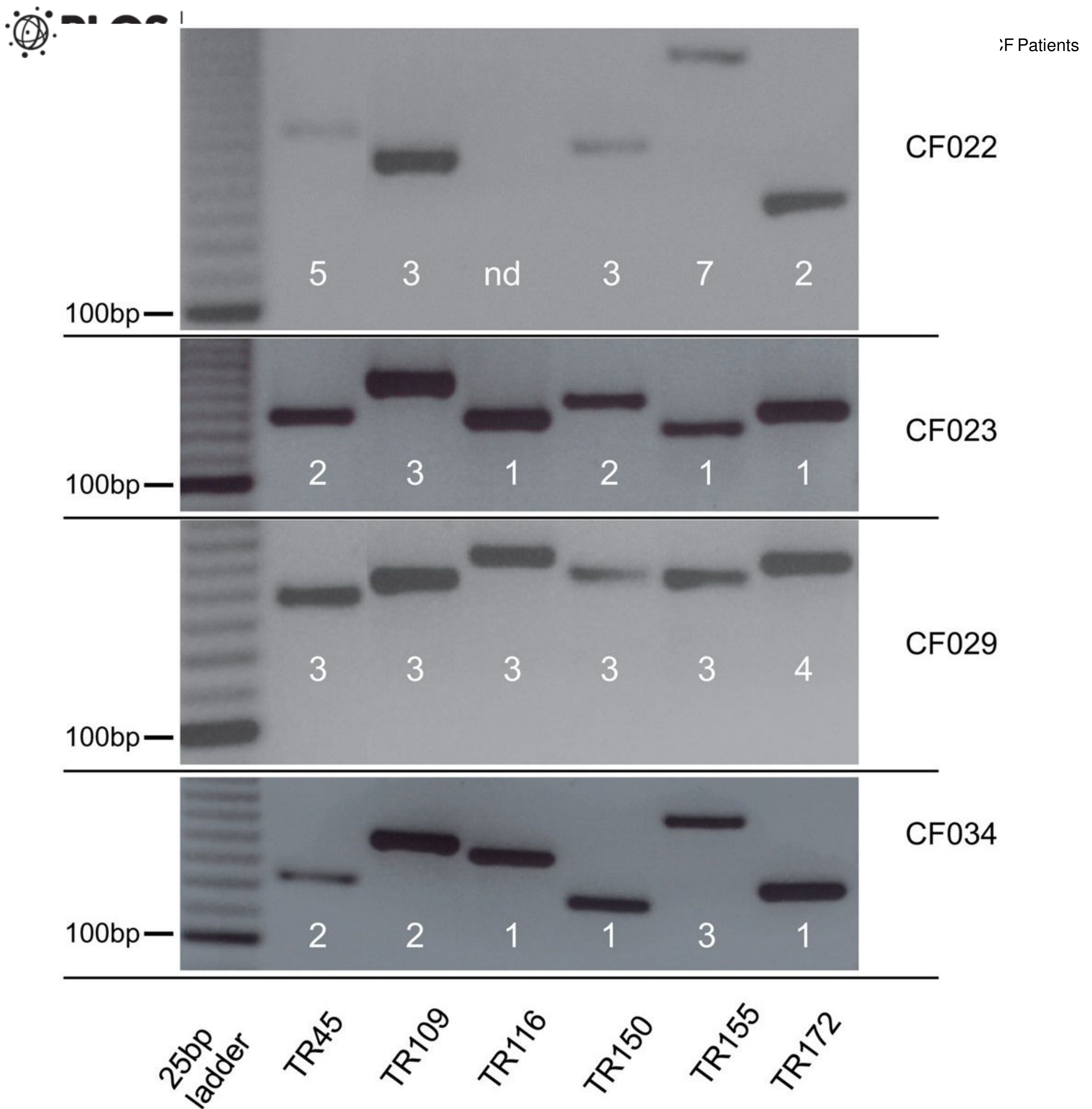

Fig 2. Characterization of MABSC strains from CF patients by VNTR (Variable Number Tandem Repeat) analysis. VNTR-PCR results of MABSC- confirmed CF patients are depicted. Agarose gel electrophoresis for six tandem repeat regions are shown. Differences in the band pattern indicate MABSC strain specific differences.

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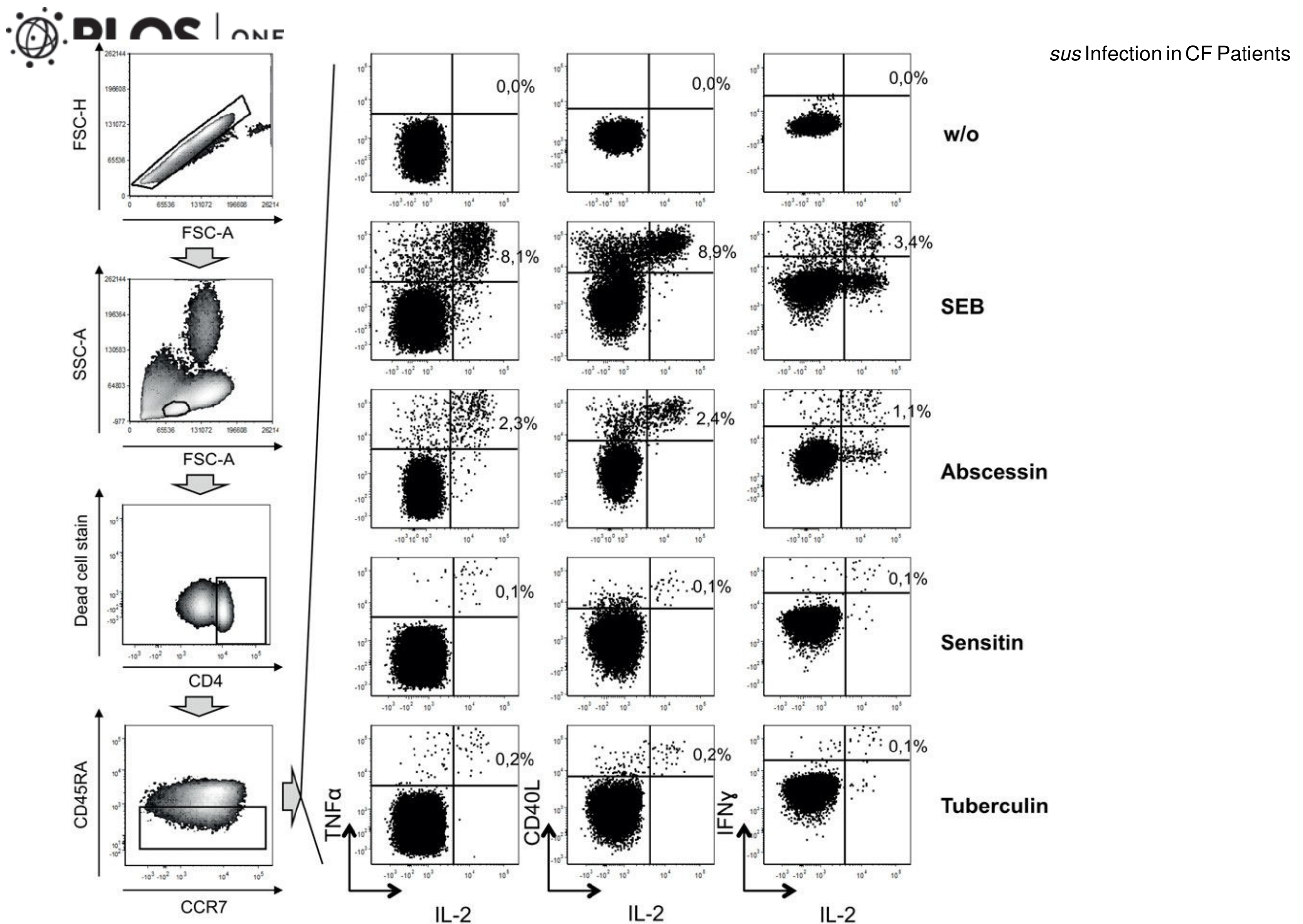

Fig 3. Gating strategy of cytokine-expressing T-cell proportions. Density plots (left graphs) and dot plots (right graphs) depict flow cytometry analyses of data of whole blood *in vitro* restimulation. Results of stimulation with different mycobacterial antigens, i.e. abscessin of *M. abscessus* (MABSC), sensitin of *M. avium* (MAC), and tuberculin of *M. tuberculosis* (MTB), the non-clonal T-cell activator SEB (Staphylococcus enterotoxin B), and without stimulation (w/o) are shown. Grey block-arrows indicate the sequence of analyses steps. After excluding cell doublets by comparing FSC-A (forward scatter area) and FSC-H (forward scatter height) parameters (upper left density plot), lymphocytes were selected on the basis of cellular size (FSC-A) and granularity (SSC-A; side scatter area). Viable CD4$^+$ memory T-cells (CD45RA negative) were selected for cytokine analysis. Proportions of TNFα/IL-2, CD40L/IL-2, and IFNγ/IL-2 double positive T-cells were determined for each stimuli (right graphs). A representative analysis of a MABSC infected CF patient is shown.
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culture that is time consuming and frequently fails [20]. In addition, reduced sample volumes (about 100 μl) were sufficient for DNA-strip tests whereas mycobacterial culture usually requires at least 10-fold larger volumes [21]. Thus, the DNA-strip test would be particularly im- portant for CF patients with low sputum sample volumes (e.g. young children).

MABSC-specific *rpoB*-qPCR had a 100-fold higher sensitivity as compared to the DNA-strip test and allowed quantification of mycobacteria in sputum. MABSC sputum burden varied markedly between CF patients and initial analyses during disease course of one CF patient

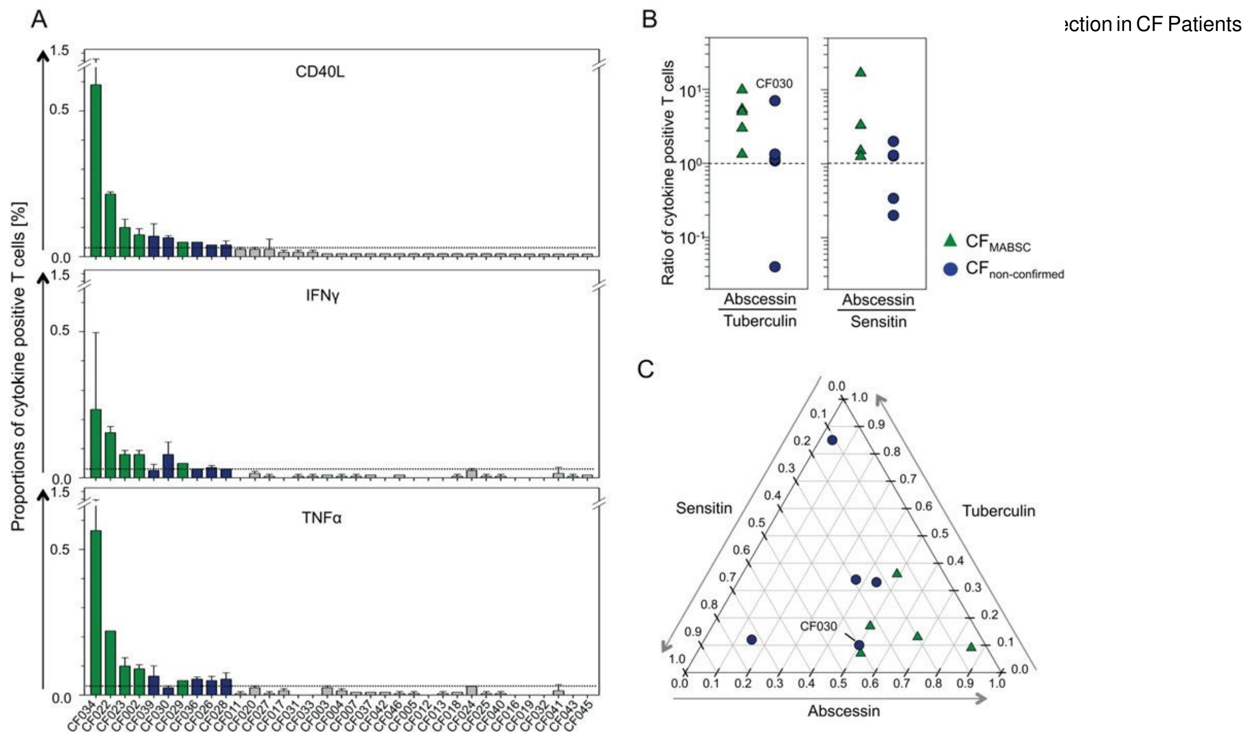

Fig 4. Cytokine expression of abscessin specific T-cells. MABSC induced cytokine-expressing T-cell proportions of CF patients (n = 35). (A) Abscessin specific T-cells that produce IL-2 together with either CD40L (upper graph), or IFNɣ (middle graph), or TNFα (lower graph) are shown. Bars indicate mean and standard deviation of measured duplicates for each individual patient (ID label on the x-axis). Green color indicates MABSC confirmed cases. Blue color indicates CF patients without MABSC infection (current and past) but a positive T-cell response against MABSC, defined by at least two of three cytokine combinations values [mean proportions] above 0.03% [3-times the assumed flow cytometry detection limit] (B) Comparisons of T-cell cytokine responses induced by abscessin, tuberculin, and sensitin. Ratios (abscessin / tuberculin or abscessin / sensitin) for CD40L/IL-2 positive T-cells are shown for confirmed MABSC infected CF patients (green triangles) and non-confirmed MABSC responders (blue circles). Each symbol represents ratios calculated of mean proportions for an individual donor. The dotted lines indicate equal T-cell responses (ratio = 1) (C) Relative T-cell responses against abscessin, tuberculin, and sensitin are depicted in a ternary plot. The sum of T-cell proportions for each individual donor was set to 1. Relative values of specific T-cells for each PPD are indicated in a ternary plot. MABSC infected CF patients are depicted as green triangles and non-confirmed MABSC responders as blue circles. The MABSC non-confirmed CF patient CF030 is highlighted who has 50% abscessin, 40% sensitin, and 10% tuberculin specific T-cells.
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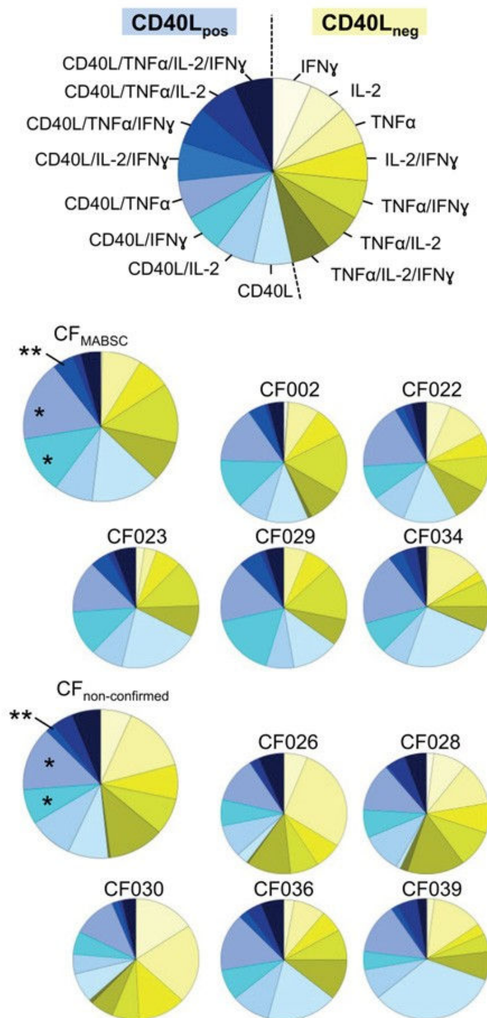
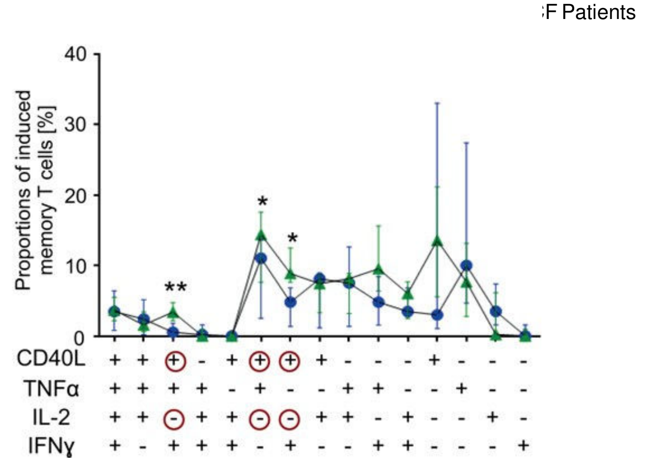
indicated fluctuating MABSC concentrations between 10^7 and 'not detectable' low numbers (below the detection limit of estimated 50 bacteria) of mycobacteria per ml sputum. Potentially, quantification of mycobacteria in CF sputum samples may become an important diagnostic tool to monitor efficacy of challenging MABSC therapy. Since standard therapy regimes for MABSC infection are not available, such marker would be of paramount clinical relevance. Ongoing studies apply DNA-strip tests for sputum samples in enlarged CF patient cohorts to confirm reliability of the novel assays.

In our study, all patients with current or past MABSC infection had MABSC-specific and- dominant T-cell responses. This finding indicated a high sensitivity of this assay, rendering it a putative strong tool for exclusion of MABSC infection in CF patients. However, MABSC-specific T-cell responses could be observed for patients without confirmed MABSC infection as well. Indeed, immunological detection of MABSC infection may always be confounded by





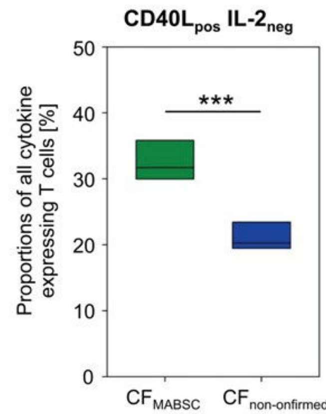


Fig 5. Cytokine expression patterns of MABSC-specific T-cells. Cytokine expression pattern of MABSC-specific T-cells for TNFα, IFNɣ, IL-2, and CD40L are shown. Data were normalized for each individual donor by setting the number of all cells expressing at least one cytokine to 100%. (A) CD40L positive (blue colors) and CD40L negative (yellow colors) subpopulations with different cytokine expressing subpopulations are indicated. An explanatory pie chart is depicted at the top. Pie charts represent data of individual CF patients (with the patient ID indicated above) and mean values for each study group (upper left pies). (B) CF patient groups with confirmed MABSC infection (green triangles) and non-confirmed MABSC responders (blue circles) are compared for cytokine expression pattern of MABSC-specific T-cells (i.e. quadruple, triple, double, single positive). Line and scatter plots indicate median expression levels and standard deviation (y axis) of distinct cytokine pattern expressing subsets (x-axis). Red circles highlight CD40L and IL-2 expression status for significantly different subsets. The student *t* test was used to evaluate data between study groups and significant differences are indicated as asterisks;
*: $P<0.05$ **, $P<0.01$. (C) A box plot shows the sum of all $CD40L_{positive}$ and $IL\text{-}2_{negative}$ T-cell subsets for MABSC infected CF patients (green) and non- confirmed MABSC responders (blue). The student *t* test was used to evaluate data between study groups. Significant differences are indicated as asterisks;***, $P<0.001$.
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several environmental and population specific factors that induce cross-reactive T-cells including i) Bacille-Calmette Guérin (BCG) vaccination; ii) latent *M. tuberculosis* infection; iii) previous contact to atypical mycobacteria. Although we were able to exclude the first two factors [BCG vaccination is not performed in Germany and IGRAs were negative for CF patients with a positive T-cell response against mycobacteria (data

not shown), a previous contact with atypical mycobacteria cannot be excluded. Therefore we assume that MABSC non-confirmed CF patients underwent asymptomatic infection (e.g. colonization) with different atypical mycobacteria in the past. This assumption is supported by the heterogeneity of PPD specificity in this group as compared to MABSC confirmed CF patients that suggested different causative mycobacteria. To avoid or at least minimize mycobacterial cross-reactivity for the detection of MABSC infection, identification of MABSC-specific immunogenic proteins would be prerequisite. Since such proteins are not identified so far, our comparative approach to concomitantly analyze abscessin-, tuberculin-, and sensitin-specific T-cells is a promising alternative. In addition, marked differences in the level of T-cell response against abscessin (as compared to tuberculin and sensitin) indicated that MABSC-specific immunogenic proteins with limited cross-reactivity exist. Identification of such proteins would be an important step towards the development of a specific *in vitro* T-cell test for MABSC infection (comparable to IGRAs used for detection of *M. tuberculosis*).

Our data indicated that besides antigen-specificity, cytokine expression differed between T- cells from MABSC confirmed and non-confirmed CF patients. Cytokine expression pattern analyses revealed increased CD40L-positive / IL-2-negative T-cell proportions in confirmed MABSC infected CF patients. Evidence for CD40L as a marker of acute mycobacterial disease is provided by studies on human tuberculosis [22]. Streitz *et al*. described increased proportions of *M. tuberculosis* specific T-cells expressing CD40L in tuberculosis patients although *M. tuberculosis* specific T-cells were generally diminished in patients. Notably, CD40L expressing T-cells were optimal for discrimination of tuberculosis patients from healthy latently *M. tuberculosis* infected contacts in this study [22]. IL-2 was described previously as a marker of 'polyfunctional' T-cells which are crucial for protection against tuberculosis [23, 24]. We detected increased CD40L-positive / IL-2-negative MABSC-specific T-cells in CF patients with chronic MABSC infection. Therefore the presence of this MABSC specific T-cell subset may reflect the inability of these CF patients to eradicate MABSC. Possible functional deficiencies of CD40L-positive / IL-2-negative T-cells as well as causative factors for the expansion of this T-cell subset (e.g. immune evasion as described for $CD8^+$ effector T-cells in tuberculosis [25]) will have to be elucidated. Furthermore, the question remains whether this T-cell subset is a general marker of increased CF patients susceptibility to recurrent pulmonary infections [26], e.g. caused by immune modulating effects of the underlying CFTR mutations [5, 27].

12 of 35 CF patients recruited in this study did not expectorate sputum samples. According to ATS criteria [11], none of these CF patients had evidence for MABSC infection. However, this assessment relies completely on the absence of clinical symptoms, which is limited by low specificity. Notably, our immune test indicated previous infection with different mycobacterial species for three sputum-negative CF patients. None of these immune responses was MABSC-dominant and cytokine expression patterns suggested no persistent infection (as compared to MABSC-confirmed CF patients). Obviously this thesis cannot be confirmed without invasive diagnostics (e.g. bronchoalveolar lavage) followed by mycobacterial culture and probably direct PCR analysis of sputum specimen, which is not indicated in asymptomatic patients without radiological evidence for a mycobacterial infection. Hence we were not able to prove our hypothesis in this regard. Future studies should perform long-term consecutive studies of larger CF patient's cohorts to determine if immune tests allow identification of CF patients with early MABSC infection and to evaluate the individual risk to suffer from MABSC exacerbation.

In conclusion immunological and PCR-based assays may help to diagnose the NTM infection especially early during MABSC infection and exacerbation. This is of special importance for the large group of patients not able to expectorate sputum. Our novel tests may help to decide whether additional diagnostics (e.g. bronchoalveolar lavage) should be performed. As a consequence the complicated treatment of MABSC infection can be started earlier with likely better efficacy and prognosis.

Supporting Information

S1 Fig. Flow cytometry results from a MABSC-negative CF patient. A representative analysis of flow cytometry results from a MABSC-negative CF patient is depicted. Results of stimulation with different mycobacterial antigens, i.e. abscessin of *M. abscessus* (MABSC), sensitin of *M. avium* (MAC), and tuberculin of *M. tuberculosis* (MTB), the non-clonal T-cell activator SEB (Staphylococcus enterotoxin B), and without stimulation (w/o) are shown.
(EPS)

S2 Fig. CD40L and IL-2 expression of M. abscessus stimulated T cells. Flow cytometry dot plots indicating for confirmed MABSC infected CF patients (CF_{MABSC}) and non-confirmed MABSC T-cell responders ($CF_{non\text{-}confirmed}$) are shown. Memory T cells are gated as indicated in Fig. 3. Proportions of memory T cells expressing CD40L, IL-2, and both are given.
(EPS)

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Author Contributions

Conceived and designed the experiments: MS VN MJ. Performed the experiments: MS VN. Analyzed the data: MS VN DS MJ. Contributed reagents/materials/analysis tools: CM EM. Wrote the paper: EM CM DS MJ.

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2.2. Chapter 2 – A Novel Mycobacterial in vitro Infection Assay Identifies Functional Differences of Induced Apoptosis between $CD4^+$ and $CD8^+$ T Cells

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- Isolation and culture of immune cells
- Infection assay
- FACS analysis
- Culture of the mycobacteria
- Microscopy
- Data analysis and Statistics
- Writing of the manuscript

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A Novel Mycobacterial *in vitro* Infection Assay Identifies Functional Differences of Induced Apoptosis between CD4^{+} and CD8^{+} T Cells

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Abstract

Alveolar macrophages are natural host cells for pathogenic mycobacteria, like *Mycobacterium tuberculosis*. Immune surveillance by T cells and interaction with *M. tuberculosis* infected macrophages is crucial for protection against *M. tuberculosis* reactivation and development of active tuberculosis. Several factors play a role in the control of *M. tuberculosis* infection but reliable biomarkers remain elusive. One major obstacle is the absence of appropriate functional *in vitro* assays, which allow concomitant determination of i) mycobacterial eradication; ii) cytotoxic effects on host macrophages; and iii) effector T-cell functions.

We have established a novel functional *in vitro* assay based on monocyte-derived macrophages (MDM) infected with a *Mycobacterium bovis* BCG reporter strain that contains a live/dead plasmid (LD-BCG). Short-term co-incubation with effector T cells or antibiotic treatment allowed determination of mycobacterial eradication, MDM viability and phenotype characterization by flow cytometry. Count beads normalized analysis of proportional and absolute MDM infection reflected mycobacterial colony forming units and fluorescence microscopy results. Co-culture with pre-activated effector T cells reduced viability of mycobacteria and MDM in a concentration-dependent manner. *M. tuberculosis* protein specific $CD4^+$ and $CD8^+$ T-cell subsets contributed quantitatively similar to anti-mycobacterial cytotoxicity but differed in the capacity to induce MDM apoptosis.

This novel assay enabled rapid quantification of anti-mycobacterial effector functions and effects on MDM viability. In addition, qualitative differences in cytotoxic mechanisms relevant for effector T cell/MDM interaction were identified. Our functional *in vitro* assay has the potential to contribute to the identification of biomarkers for protective T-cell responses against tuberculosis.

Author Summary

Alveolar macrophages represent the primary host cells of *Mycobacterium* t*uberculosis,* which colonize the lung of Tuberculosis (TB) patients. Reliable biomarkers to predict immune protection against this pathogen or progression to active TB disease are missing, partly due to the fact that functional *in vitro* assays, that would allow the analysis of host-pathogen interactions and predict the efficacy of biomarkers candidates, are not available.

We have established a functional *in vitro* infection assay for characterization of anti-mycobacterial immune responses and interactions between infected macrophages and host T cells. The assay makes use of *in vitro* generated macrophages, as well as of a *M. bovis* BCG reporter strain, which allows differentiation between viable and dead mycobacteria in FACS. Co-culture of infected macrophages with antibiotics, as well as with activated T cells reduced the viability of the mycobacteria, and results strongly correlated with colony forming units. Both $CD4^+$ and $CD8^+$ T cells were cytotoxic for mycobacteria, but these cells differently induce apoptosis of macrophages. Overall, our novel enables the evaluation of the functional relevance of T-cell effector molecules and possible side effects can be estimated.

Introduction

Tuberculosis (TB) is a major public health issue with worldwide incidence of about nine million cases and estimated two billion *Mycobacterium tuberculosis* infected individuals [1]. Immune surveillance is central for protection of about 90% of *M. tuberculosis* infected individuals but prediction of protection/disease progression remains elusive. The interaction of alveolar macrophages – the predominant host cell of *M. tuberculosis* – and effector T cells is decisive for the outcome of infection. Multifaceted mechanisms are involved in this process including i) activation of macrophage effector functions e.g. by T cells producing IFNγ and TNFα; ii) reversal of *M. tuberculosis* induced endosome maturation blockade; iii) inhibition of mycobacterial growth; iv) T cell-mediated killing of intracellular *M. tuberculosis* and, eventually, infected host macrophages (reviewed in [2]). It is a matter of debate whether killing of macrophages is beneficial or detrimental, since macrophage death is not necessarily accompanied by killing of intracellular mycobacteria [3]. The pathway of cytotoxicity is decisive and there is compelling evidence that exclusively apoptotic cell death together with certain effector molecules (i.e. granulysin) is able to kill mycobacteria [4, 5]. Against this background of complex and fine-tuned immune effector mechanisms, it is tempting to assume that methods applied to test novel candidates of host/pathogen interaction need to incorporate this complexity. In addition 'functional assays' determining the effect (e.g. of vaccine candidates or immune modulation) should be concomitantly tested for effects on macrophages/T cell interaction. Besides mycobacterial growth inhibition, analyses of harmful effects on immune cell viability may improve prediction of efficacy.

Functional anti-mycobacterial assays were previously mainly based on mycobacterial growth inhibition determined by colony-forming units (CFU) [6, 7, 8,

9, 10]. CFU analysis is laborious (about two to three weeks) and solely focuses on mycobacterial growth whereas concomitant analyses of host immune cells and effector molecules are not possible. The group of Kampmann et al. greatly promoted the progress of functional assay development by introducing a *M. bovis* BCG reporter strain (i.e. BCG-*lux*) to monitor the mycobacterial growth in human whole blood cultures [11, 12]. This comprised three major advantages i) incorporation of the majority of immune populations involved in anti-mycobacterial host response; ii) rapid luminescence-based readout to avoid long-term mycobacterial culture; iii) avoidance of sophisticated immune cell purification techniques to assure broad applicability. Furthermore the usage of *M. bovis* BCG allowed the application also in biosafety level (BSL)-1 facilities (whereas *M. tuberculosis* requires BSL-3). Subsequent studies modified the BCG-lux assay to analyse effects of cytokines during *M. tuberculosis* infection [13], immune responses to BCG vaccination [14], and the effect of vitamin D supplementation on anti-mycobacterial immunity [15]. Mycobacterial fluorescent reporter strains have rarely been used to determine viability due to the long half-life of fluorescent proteins. To circumvent this obstacle Martin *et al.* generated a *M. tuberculosis* H37Rv strain harboring a life-dead reporter plasmid [16]. The life-dead H37Rv strain constitutively expresses mCherry and – on induction by tetracyclin derivates such as anhydrotetracycline (ATC) – concomitantly expresses GFP. This strain enabled quantification of viable and dead mycobacteria inside macrophages and results strongly correlated with CFU [16].

In the present study we applied multi-colour flow cytometry to establish an *in vitro* assay of mycobacterial host interactions based on a life-dead *M. bovis BCG* (LD-BCG) infection. This assay enabled us to exactly quantify mycobacteria growth inhibition by antibiotics as well as effector T cells in a short-term (about 24h) co-culture assay. Concomitantly viability and phenotype of infected monocyte-derived

macrophages (MDMs) and effector T cells were determined. In addition the functional relevance of cytotoxic mechanisms in T-cell subpopulations were evaluated. To our knowledge this is the first mycobacterial infection assay that allows quantification of anti-mycobacterial cytotoxicity and characterization of involved functional mechanisms concomitantly.

Results

A live-dead reporter BCG strain (LD-BCG) allowed quantification of infected MDMs by flow cytometry

Mycobacterial reporter strains expressing fluorescent proteins can be used for flow cytometry-based analysis of monocyte-derived macrophage (MDM) infection. The fluorescent life-dead reporter *M. tuberculosis* strain H37Rv, described by Martin *et al.* (Martin et al., 2012), allowed furthermore determination of mycobacterial viability. We used the same construct to generate a live-dead *M. bovis BCG* reporter strain (LD-BCG) (for details see Methods section) that permanently expresses mCherry and, if viable, can be induced to co-express GFP by the tetracycline derivative ATC. ATC induced GFP in the vast majority of mCherry positive LD-BCG indicating high proportions of viable mycobacteria in culture (Fig 1A). Treatment of MDMs infected with LD-BCG with ATC induced GFP only in a subset of infected MDMs (Fig 1B). This could be due to decreased viability or suboptimal condition for ATC mediated induction. To test this, we added ATC for different co-culture periods (10 h; 24 h) and detected markedly higher proportions of GFP positive infected MDMs after 24 h (48%) as compared to 10 h (11 %) (Fig 1b). Therefore MDM infected LD-BCG mediated mycobacterial cytotoxicity in the absence of antibiotics or effector T cells. Next we applied different LD-BCG MOI and detected increased MDM infection rates at higher MOI levels (Fig 1C, left graph). In addition the relative proportion of viable LD-BCG increased at higher MOIs and reached a plateau at an MOI of 20:1 (Fig 1C, right graph). Accordingly we applied an MOI of 20:1 for further experiments.

Quantification of LD-BCG infected MDMs and rifampicin-mediated mycobacterial growth inhibition revealed results comparable to CFU

Rifampicin mediated LD-BCG eradication was used to evaluate anti-mycobacterial efficacy and possible side effects on MDM. Increasing concentrations of rifampicin steadily reduced the proportion of MDM containing viable LD-BCG whereas MDMs with dead LD-BCG increased (Fig 2A). At higher rifampicin concentrations (0.5 – 1 µM) the proportion of MDM infected with dead LD-BCG remained relatively stable. This could be due to rifampicin-mediated cytotoxic side effects on MDM. To analyse this absolute quantification of MDM numbers by flow cytometry is prerequisite. This approach required standards to normalize sample acquisition. In this regard we applied fluorescent bead counts to analyse comparable sample volumes by flow cytometry (Fig 2B, upper plots) (for details see Methods section). MDM numbers were similar at low concentrations (0.02 - 0.2 µM) and without rifampicin (Fig 2B, lower left graph). However at higher rifampicin concentrations (0.5 – 1 µM) diminished MDM numbers were detected per sample indicating cytotoxic rifampicin effects on MDM (Fig 2B, lower left graph). The combination of proportional infection results with absolute MDM cell numbers revealed decreased numbers of MDM infected LD-BCG (mainly due to decreased numbers of MDM containing viable LD-BCG) whereas MDM numbers containing dead LD-BCG are slightly increasing with low rifampicin concentrations (Fig 2B, lower right graph). At high rifampicin concentrations, hardly any viable LD-BCG containing MDM were detectable and also the number of dead LD-BCG containing MDM was decreased (Fig 2B, lower right graph). Therefore our assay allows for discrimination between proportional and absolute differences of infected MDM infected with live or dead BCG. In addition, anti-mycobacterial killing with or without concomitant cytotoxicity against MDM can be measured and discriminated.

To determine the reliability of flow cytometry-based quantification of LD-BCG viability, we compared viable LD-BCG infected MDM numbers with colony forming

units (CFU) from rifampicin-treated cultures. CFU and MDM infected with viable LD-BCG correlated strongly ($r = 0.84$, $p < 0.01$) (data not shown) but CFU counts were at average higher as compared to flow cytometry-based prediction at different rifampicin concentrations (Fig 2C). Multiple LD-BCG infections of single MDM may account for these differences and, therefore, we determined the number of viable LD-BCG per MDM by fluorescence microscopy (Fig 2D). LD-BCG infected MDM had a mean infection rate of 3.4 (SD = 0.7) and adjustment for this factor largely reflected differences between CFU and flow cytometry data (Fig 2C, grey circles). We concluded that flow cytometry analysis reliably predicted viability of LD-BCG and that adjustment for multiple MDM infection revealed CFU comparable results.

M. tuberculosis-specific and polyclonal activated effector T cells reduce LD-BCG and MDM viability

Effector T cells promote MDM activation during mycobacterial infection and can directly kill mycobacteria within MDM. We generated effector T cells of PBMC *in vitro* using *M. tuberculosis* antigen (i.e. PPD) and the polyclonal T-cell activator staphylococcus enterotoxin B (SEB). A defined number of effector T cells was then co-cultured for 24 hours with LD-BCG infected MDMs at different Effector T-cell to MDM (E:M) ratios (i.e. 1:9; 1:3; 1:1). Fig 3A depicts the experimental procedure. The highest proportions of infected MDMs were generally detected with non-stimulated T cells (Fig 3B, upper left graph). Increasing numbers of T cells (higher E:M ratios) resulted in decreased proportions of viable LD-BCG infected MDMs for non-stimulated and *in vitro* activated 'effector' T cells (Fig 3B, upper left graph). Effector T cells also reduced MDM numbers in a concentration and stimuli dependent (Fig 3B, lower left graph). This resulted in markedly reduced MDM numbers by polyclonal activated or mycobacteria specific T cells as compared to non-stimulated T cells

(Fig 3B, lower left graphs). Calculated numbers of MDM infected with live (Fig 3B, upper right graph) and dead LD-BCG (Fig 3B, lower right graph) revealed marked differences between *in vitro* activated and non-stimulated T cells. Since both measures, differential LD-BCG infected MDM proportions and numbers, characterize anti-mycobacterial T-cell functions, we applied calculated LD-BCG infected MDM numbers for further analyses.

Comparisons of anti-mycobacterial cytotoxicity between different experiments (or individuals) require normalization for differential MDM infection efficacy. Therefore the number of LD-BCG infected MDMs – without effector T cells – was set to 100% and the relative differences (≅ cytotoxic efficacy) for each effector T-cell population were calculated. T cells from healthy donors showed E:M ratio dependent anti-mycobacterial cytotoxic efficacy (ANOVA, $p< 0.001$ for all stimuli) (Fig 4A, upper graph). In addition significant higher cytotoxic efficacy after SEB induced polyclonal T-cell activation ($p=0.04$, $p=0.02$, $p<0.001$ for 1:9, 1:3, 1:1, respectively) as well as PPD-specific stimulation (for 1:3, $p=0.04$; for 1:1 $p=0.02$) as compared to non-stimulated samples was detected (Fig 4A, lower graph). Therefore, E:M ratio as well as stimulation dependent differences in cytotoxic efficacy of T cells significantly affected viability of LD-BCG infected MDMs. Since PBMC induced effector cell contain different subpopulations of T cells and other immune populations (e.g. NK cells, B cells). We further characterized relevant subpopulations by purifying $CD4^+$ and $CD8^+$ T cells before *in vitro* culture.

$CD4^+$ and $CD8^+$ T cells exert comparable cytotoxic effects but differ in PPD induced infected MDM kill mechanisms

$CD4^+$ and $CD8^+$ T cells were separately stimulated and co-cultured with infected MDM. Both, $CD4^+$ and $CD8^+$ T cells, exerted cytotoxic effects on LD-BCG infected

MDMs in a titration dependent manner (ANOVA, $p< 0.001$ for $CD4^+$ as well as $CD8^+$ T cells and for all stimuli) (Fig 4B, upper graph). As shown for PBMC, PPD and SEB stimulation induced enhanced cytotoxic efficacy of $CD4^+$ T cells (for p-values see Table 1) (Fig 4B, lower graph). The stimuli induced differences for $CD8^+$ T cells were not significant likely due to increased cytotoxicity of non-stimulated cells at higher E:M ratios (Fig 4B, lower graph and Table 1). Notably, no significant differences were detected between $CD4^+$ and $CD8^+$ T cells for different stimuli and at any E:M ratio (Table 1).

Table 1: Stimulus dependent reduction of MDM with viable LD-BCG by co-culture with $CD4^+$ and $CD8^+$ T cells

E:M	Comparison	$CD4^+$ T cells		$CD8^+$ T cells	
		p value	Significance	p value	Significance
1:9	w/o vs PPD	0.093	ns	0.132	ns
	w/o vs SEB	0.002	**	0.132	ns
	PPD vs SEB	0.258	ns	0.999	ns
1:3	w/o vs PPD	0.093	ns	0,387	ns
	w/o vs SEB	0.004	**	0.132	ns
	PPD vs SEB	0.028	*	0.571	ns
1:1	w/o vs PPD	0.015	*	0.571	ns
	w/o vs SEB	0.002	**	0.132	ns
	PPD vs SEB	0.017	*	0.474	ns

Mann-Whitney U-test results are indicated; E:M: Effector T-cell/MDM ratio; ns: not significant; *: $p<0.05$, **: $p<0.01$

A central T-cell dependent anti-mycobacterial effector function is the induction of apoptosis in infected macrophages. Therefore we determined the expression of

early and late apoptotic markers in infected and non-infected MDMs after co-culture. Co-culture with non-stimulated $CD4^+$ and $CD8^+$ T cells induced only marginal expression of early and late apoptotic markers in MDMs infected with viable or dead LD-BCG (Fig 4C, upper panel). But whereas SEB stimulated $CD4^+$ and $CD8^+$ T cells induced increased expression of early and late apoptotic markers in MDM (Fig 4C, lower panel; Table 2), only PPD specific $CD4^+$ effector T cells (Fig 4C, middle panel) induced increased expression of early apoptosis markers in MDM infected with viable (p=0.003) and dead LD-BCG (p=0.01) and increased late apoptotic markers in MDM infected with dead LD-BCG (p<0.001) as compared to $CD8^+$ effector T cells. We concluded that PPD induced comparable cytotoxic efficacy of $CD4^+$ and $CD8^+$ T cells against LD-BCG infected MDM, but cytotoxic mechanisms differed between T-cell subsets with regard to apoptosis induction. We concluded that flow cytometry-based characterization of effector T-cell/MDM interaction in mycobacterial infection can elucidate relevant functional differences, not detectable in conventional viability assays.

Table 2: Comparison of expression of apoptotic markers induced by differentially activated $CD4^+$ and $CD8^+$ T cells in MDM with viable BCG (E:M 1:1)

	Comparison	Apoptosis	$CD4^+$ T cells		$CD8^+$ T cells	
			p-value	Significance	p-value	Significance
viable	w/o vs PPD	early	<0.001	***	0.99	ns

LD-BCG		late	0.08	ns	0.86	ns
	w/o vs SEB	early	<0.001	***	0.004	**
		late	0.25	ns	0.25	ns
	PPD vs. SEB	early	0.21	ns	0.007	**
		late	0.90	ns	0.16	ns
dead LD-BCG	w/o vs PPD	early	0.006	**	0.92	ns
		late	<0.001	***	0.29	ns
	w/o vs SEB	early	0.001	**	0.08	ns
		late	<0.001	***	<0.001	***
	PPD vs. SEB	early	0.39	ns	0.07	ns
		late	0.43	ns	0.001	**

Mann-Whitney U-test results are indicated; ns: not significant; *: p<0.05, **: p<0.01, ***: p<0.001

Discussion

Functional *in vitro* assays are important tools to evaluate the relevance of immune biomarkers in mycobacterial infections. The present assay extends the potential of previous functional assays by characterizing the immune cell interplay and possible harmful side effects on mycobacteria hosting macrophages. The application of flow cytometry to detect a fluorescent BCG reporter strain allows rapid detection of viable and dead mycobacteria within MDM and circumvents the need for long-term mycobacteria culture. Normalization of flow cytometry measures allows absolute quantification of surviving and killed MDM with or without mycobacterial infections. Concomitantly characterization of immune cell phenotype is possible to compare e.g. cytotoxic mechanisms as performed for apoptosis in the present study.

We generated a BCG fluorescent reporter strain based on a previous publication [16]. The usage of the BCG vaccine strain permits implementation of this assay in the majority of facilities including hospitals and research institutions in low-income countries whereas the use of virulent *M. tuberculosis* strains requires biosafety level 3 facilities, which are rarely available. BCG mycobacteria do not contain the virulence associated RD1 region of *M. tuberculosis*. As a consequence, maturation blockade of early phagosomes – a typical feature of MDM infected with virulent *M. tuberculosis* [2] – is less prevalent in BCG. In accordance MDM infected with LD-BCG alone (without rifampicin or activated effector T cells) reduced the viability of mycobacteria in an MOI associated manner (Fig 1B). However, further reduction of viability was dependent on MDM co-culture with activated T cells or antibiotic treatment. Future studies will determine if the results of the LD-BCG based MDM kill assay reflect immune response in the context of *M. tuberculosis* MDM infection.

We demonstrated that LD-BCG viability in MDM (indicated by GFP expression) strongly correlates with CFU, the gold standard to determine mycobacterial viability. The comparison of bead-normalized numbers of MDMs infected with viable LD-BCG revealed lower numbers as compared to CFU (Fig 2C). Mainly three reasons may account for this. First, ATC-induced GFP expression of LD-BCG within MDM may be suboptimal since longer incubation increased GFP expression (Fig 1B) whereas extracellular LD-BCG showed discriminant GFP expression in the presence of ATC (Fig 1A). Therefore not all LD-BCG may respond to ATC when engulfed by MDM and the calculated proportions/absolute numbers of viable LD-BCG containing MDM may be underestimated. Obviously general reduced viability of LD-BCG in MDM (accompanied by decreased GFP expression) confounds estimation of this effect. Second, MDM inherent background fluorescence in the GFP channel (especially detected in $mCherry_{high}$ MDM not treated with ATC) limited the detection sensitivity for viable LD-BCG in MDM. Initial FACSort experiments to purify MDM with different level of GFP expression (data not shown) support the notion that one of these explanations affects analyses. Third, multiple LD-BCG infection of single MDM may account for differences between flow cytometry and CFU based calculation. Measurement of single MDM mycobacterial burden by fluorescence microscopy revealed a mean 3.4-fold LD-BCG infection rate. Therefore the effect of multiple LD-BCG infection of single MDMs largely accounts for differences between flow cytometry and CFU.

Previous functional mycobacterial *in vitro* assays focussed on quantification of pathogen viability [6, 7, 8, 9, 10], but characterization of host monocytes/macrophages and effector T cells concomitantly has not been addressed. The novel assay allows i) characterization of MDM infected with viable or dead LD-BCG as well as without infection; ii) absolute and/or proportional

quantification of MDM after co-culture to determine the influence of sample conditions on host MDM viability and infection status; iii) analysis of effector T-cell populations e.g. in the context of anti-mycobacterial killing efficiency; iv) analyses anti-mycobacterial effects of novel immune cell molecules or drug candidates. Discrimination between MDM infected with viable and/or dead bacteria, as well as non-infected cells renders identification of phenotypic changes of MDM relevant for effective mycobacterial eradication possible. This way we detected increased proportions of MDM showing apoptosis-associated markers induced by PPD-specific $CD4^+$ T cells not detected for $CD8^+$ T cells induced by PPD. This is in accordance with previous findings that apoptosis is crucial for mycobacterial killing [17] and suggested that $CD4^+$ and $CD8^+$ T cells differ in anti-mycobacterial killing mechanisms. In addition this rendered a role of direct cytotoxic effects of $CD8^+$ effector T cells (Stenger et al., 1998) and/or cell death-independent macrophage effector mechanisms (e.g autophagy, efferocytosis) [16, 18] likely.

Different effector cell populations and T-cell stimulations can be compared for their cytotoxic and anti-mycobacterial effects. Since *in vitro* activation precedes the co-culture, we can largely control for confounding effects like differential proliferation by adjusting cell numbers before co-culture. Multiple colour flow cytometry enabled us to characterize effector cell populations concomitantly. Identification of effector cell mechanisms associated e.g. with mycobacterial killing or MDM viability is therefore feasible. As an initial approach we purified $CD4^+$ and $CD8^+$ T cells before *in vitro* stimulation and showed that both populations exert anti-mycobacterial killing but that effector mechanism and side effects may differ.

This functional *in vitro* assay can be considered a bridge between descriptive patient’s studies and animal models to identify relevant biomarkers for host immunity against mycobacterial infections.

Methods

Ethics statements

This study was approved by the ethics committee of the University Hospital Duesseldorf (Internal Study No. 4505). Written informed consent was obtained from all participating donors.

Isolation of Peripheral blood mononuclear cells (PBMC) and purification of CD4$^+$ and CD8$^+$ T cells

Buffy coats (about 40 ml) from healthy donors were purchased from the Institute for Transfusion Medicine of the University Hospital Duesseldorf. PBMC were isolated by density gradient using Biocoll Separating Solution (Biochrom) following manufacturer´s instructions. The average cell number was between 0.7 to 1 x 10^9 PBMC. CD4$^+$ and CD8$^+$ T cells were enriched by magnetic cell sorting (IMag, BD Biosciences) of freshly isolated PBMC using magnetic beads labelled with CD4 or CD8 specific antibodies (BD Biosciences) following manufacturer´s instructions. The purity of enriched cells was > 95% as accessed by flow cytometry.

Generation of effector cells

Freshly isolated PBMC or enriched CD4$^+$ and CD8$^+$ cells were cultured in round-bottomed 96-well plates (Greiner) at 1.5 x 10^5 cells/well in RPMI 1640 (Gibco) supplemented with 1% L-Glutamin (Sigma-Aldrich), 10% heat inactivated human AB serum (Sigma-Aldrich) and 10 mM HEPES (Lonza) (for simplicity reasons called complete medium in the remaining manuscript) for 5 days at 37°C and 5% CO_2. Cells were stimulated with purified protein derivatives of *M. tuberculosis*. (PPD; 10 µg/ml; Statens Serum Institute), or *Staphylococcus* Enterotoxin B (SEB; 1.5

µg/ml; Sigma-Aldrich). Thereafter the cells were washed to remove antigens and counted.

Enrichment of monocytes and monocyte-derived macrophage (MDM) differentiation

Autologous monocytes were enriched from freshly isolated PBMC using CD14-labeled magnetic beads using the IMag system (IMag, BD Biosciences) following manufacturer´s instructions. The purity of enriched monocytes populations was > 90% as controlled by flow cytometry. Enriched monocytes (1 x 10^5 cells/well) were cultured in 96 well flat-bottomed plates (Greiner) in 200 µl complete medium for 5 days at 37°C and 5% CO_2 to generate MDMs. Thereafter the number of viable MDMs per well was about 2-3 x 10^4 as assessed by cell counting.

Generation and culture of LD-BCG

The Live-Dead (LD) reporter plasmid harbours a gene encoding mCherry fluorescent protein constitutively expressed from the hsp60 promotor, a gene encoding green fluorescent protein (GFP) under the control of a tetracycline-inducible promotor, as well as a hygromycin resistance cassette. It allows differentiation between viable and dead mycobacteria (Martin et al., 2012). Cells of *M. bovis* strain BCG Pasteur were grown to log-phase in Middlebrook 7H9 medium (BD Biosciences) supplemented with 10% ADC (BD Biosciences) and 50 µg/ml hygromycin (Gentaur), electroporated with the LD reporter plasmid at 2.5 kV, 1000 Ω and 25 µF using a Biorad Gene Pulser, and plated on 7H11 agar supplemented with 10 % OADC and 50 µg/ml hygromycin for selection. Individual clones were picked after 20 days of incubation, cultured in liquid media, and glycerol stocks were frozen at -80°C. For infection experiments, cryopreserved LD-BCG

bacteria were inoculated in 7H9 culture medium and cultured at 37°C and 90 rpm until an $OD_{600\ nm}$ of 0.7 to 0.8 was reached.

In vitro infection of macrophages with LD-BCG and co-culture with effector T cells

Following 5 days of MDM culture, medium was removed and 100 µl complete medium (pre-warmed to 37°C) was added to the wells. 5 ml of the LD-BCG culture was centrifuged, washed in complete medium, and titer was estimated based on OD-measurement, with an $OD_{600nm} = 1$ corresponding to 3×10^8 CFU/ml. MDM were infected at different MOIs (molecules of infection) by adding the respective number of LD-BCG containing complete medium (100µl per well) or left non-infected by adding complete medium only. After centrifugation at 209 x g for 3 min at room temperature, the cells were incubated for 3 h at 37°C and 5% CO_2.

LD-BCG-infected MDM cultures were washed with complete medium (pre-warmed to 37°C) to remove extracellular bacteria. Effector cell cultures were washed in 1 ml pre-warmed complete medium and counted in Neubauer cell chambers. Thereafter infected MDMs were incubated for 24 h effector cells at an effector to MDM (E/M) ratio of 1:9, 1:3 and 1:1 in a final volume of 200 µl/well. Anhydrotetracycline hydrochloride (ATC, 0.2 µg/ml final concentration; Sigma-Aldrich) was added for induction of GFP expression in viable LD-BCG.

Cell surface staining and FACS analysis

After infection of MDM and co-culture with effector cells, cells washed in PBS containing 10 mM EDTA and 0.5% BSA (washing medium; on ice), the supernatant was discarded, and the cells were stained with antibodies against CD11b (PE-Cy7, clone ICRF44, BioLegend) and viability dye eFluor 780 (eBioscience) in the residual volume for 30 min on ice. After washing in washing medium, cells were resuspended

and directly measured by flow cytometry or, for cell death analyses, cells were stained for Annexin V (BV421; BioLegend) and 7-AAD (BioLegend) according to manufacturer's instructions. Flow cytometry measurements were performed using a LSR Fortessa flow cytometer (BD Biosciences). Before measurement, 123-count eBeads (10 µl; stock: 1000 beads/µl; eBioscience) of were added to each sample. Flow cytometry measure stops automatically when 9000 beads were acquired. FlowJo software (Version 10; Tree Star) was used for data analysis. Analyses details and gating procedure is indicated as part of figure 1, 2, and 3. ATC-independent GFP expression was subtracted from each individual sample.

To determine the absolute numbers of MDM infected with viable or dead LD-BCG, the absolute number of MDM of each sample was multiplied with the respective proportion of infected MDM with viable or dead LD-BCG.

For interindividual comparisons of anti-mycobacterial T cell responses, the number of MDM infected with viable or dead BCG was set to 100% for the sample with MDM infected with LD-BCG alone (without activated effector T cells) in each experiment, and the relative change was calculated for each of the effector cell-treated samples within the experiment.

Determination of colony forming units (CFU)

Following infection of MDM and co-culture with effector cells, cells were centrifuged (800 x g for 5 min) and supernatant was removed. 120 µl sterile PBS containing 0.5% Tween80 (Sigma-Aldrich) was added to lyse MDMs. The plates were then incubated for 30 min on ice and serial dilutions were performed in PBS / 0,5% Tween. 50 µl of each dilution were plated on 7H11 agar supplemented with 10 % OADC and 50 µg/ml hygromycin. CFU were counted after 14 to 18 days of incubation at 37℃.

Microscopic analysis

Cell culture (generation of MDMs and effector cell co-culture) was performed in 8-well Permanox®-mounted chamber slides (Lab-Tek). Thereafter medium was removed, cells were fixed with fixation buffer (50 µl/well; BioLegend) at room temperature for 15 min, and washed with PBS (Gibco). After incubation with serum-free Protein Block buffer (Dako) for 15 min at RT, cell nuclei were stained with DAPI Dihydrochloride (Calbiochem) following manufacturer's instructions. Then mounting medium was added and cells were covered with cover glass. An Axio Observer.Z1 fluorescent microscope (Carl Zeiss) with ZEN Pro Software (Carl Zeiss) was used for imaging and analysis.

Calculations and statistical analyses

Proportions and absolute numbers of MDMs (non-infected or infected with viable/dead LD-BCG) were directly deduced from flow cytometry analyses. Absolute numbers could be compared since same sample volumes were acquired (as defined by count bead normalization, see above). Combinations of different effects (i.e. proportional and absolute reduction of MDM subsets) were then calculated and depicted (examples are shown as Fig 2B, lower right graph and as figure 3b right graphs). Inter-individual comparisons of cytotoxic efficacy required normalization between different experiments (confounded otherwise by different infection efficacy and MDM/effector cell viability). We therefore set the numbers of MDM infected with LD-BCG without effector cells to 1 (≈100%) and calculated relative differences of samples co-cultured with different effector cells and E/M ratios. This value is termed 'cytotoxic efficacy' throughout the manuscript.

Statistical calculations were performed using GraphPad Prism 6 (GraphPad Software). Non-parametric Mann-Whitney U-test was chosen for cytotoxic efficacy comparisons of different effector cell stimuli and proportions of early and late apoptotic markers expressing MDMs. The two-way ANOVA test was applied for comparisons of effector cell titration-dependent cytotoxic efficacy. *P*-values < 0.05 were considered to be significantly different.

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Legends to figures

Fig 1: Optimization of Live/Dead (LD)-BCG infection of human Monocytes Derived Macrophages (MDM) and tetracyclin (ATC) induced GFP expression.

(A) Zebra plots from flow cytometry analysis indicate LD-BCG (left graphs) or gated LD-BCG infected MDM (middle and right graphs) characterized by mCherry expression. **(B)** ATC induced GFP expression at different time periods for infected MDM (lower graphs) was compared to culture without ATC (upper graphs). A representative example of flow cytometry based gating procedure for MDM infected with viable or dead LD-BCG is depicted. Size (FSC) and granularity (SSC) gating of MDM is followed by CD11b and mCherry based detection of LD-BCG infected MDM. **(C)** MDM infected with LD-BCG at different molecules of infection (MOI) are shown. Proportions of MDM infected with viable and non-viable LD-BCG are indicated by stacked box plots (upper graph) and as ratios of (viable vs. non-viable BCG in MDM). Median with range of triplicates are depicted. A representative example of five independent experiments is shown.

Fig 2: Rifampicin induced proportional and absolute differences in viability of LD-BCG and infected MDM

(A) Flow cytometry analyses of MDM infected with LD-BCG treated with rifampicin at different indicated concentrations. Proportions of infected MDM with viable (grey) or non-viable LD-BCG (open) are shown as stacked boxes. **(B)** Count bead-based normalization for absolute quantification of MDM numbers by flow cytometry is depicted as gating procedure (upper zebra plots), deduced MDM numbers (lower left graph), and combined absolute MDM numbers with proportional differences (from (A)) (lower right graph) at different rifampicin concentrations are shown.

Median with range of triplicates are depicted. **(C)** Comparison of flow cytometry determined infected MDM numbers and mycobacterial culture determined by CFU from a representative experiment are shown. Median with range of triplicates are depicted. (**D**) Fluorescence microscopy pictures of MDMs infected with LD-BCG (middle panel (without ATC), lower panel (with ATC)) or non-infected (upper panel) are shown. Blue color indicates MDM nuclei; red color indicates mCherry expressing LD-BCG; green color indicates GFP-expressing viable LD-BCG.

Fig 3: Effector T-cell (E) generation and co-culture with LD-BCG infected MDM (M) at different E/M ratios

(A) Workflow depiction for the generation of effector T cells ①, LD-BCG infected MDM ②, Effector T cell/MDM-LD-BCG co-culture ③, analysis of different subpopulations and beads by flow cytometry ④**. (B)** Co-culture of effector T cells stimulated with different polyclonal activators (i.e. *Staphylococcus* Enterotoxin B (SEB), *M. tuberculosis* derived Purified Protein Derivative (PPD), and without stimulation (w/o). Different Effector T-cell (E) to MDM (M) ratios are shown on the x-axes. Proportions of infected MDM with viable (grey) or non-viable LD-BCG (open) are shown as stacked boxes (upper left graph). Absolute numbers of MDM are shown as symbols (lower left graph). Combined values (i.e. absolute numbers adjusted for proportional differences) for infected MDM with viable (grey) or non-viable LD-BCG (open) are shown as stacked boxes (right graphs). Median with range of a representative experiment are depicted.

Fig 4: Effector T cells influence on LD-BCG infected MDM viability and markers of cell death. (**A and B**) MDMs were infected with LD-BCG and treated with 200 ng/ml ATC. Effector T cells (i.e. PBMCs (for (A)) or enriched $CD4^+$ (grey

bars in (B)) or CD8$^+$ (open bars in (B)) were added at different E:M ratios to infected MDMs and the rate of MDMs infected with viable LD-BCG was accessed by flow cytometry. Combined values (i.e. absolute numbers adjusted for proportional differences) for infected MDM with viable (lower graphs) LD-BCG are shown. Mean and standard deviation are shown. n = 8 for (a); n = 6 for (b) **(C)** MDM were infected with LD-BCG and treated with 200 ng/ml ATC. Enriched CD4$^+$ or CD8$^+$ T cells were stimulated with SEB or PPD or left unstimulated and cocultured with infected MDM at an E:M ratio of 1:3. The rate of non apoptotic (open), early apoptotic (bright grey) or late apoptotic (dark grey) MDM with dead (lright), viable LD-BCG (middle) or non-infected (left) were acessed by flow cytometry. Mean of five independent experiments are depicted as pie chart. The Mann-Whitney-U Test was applied for group comparison. Asterisks indicate significant differences between CD4$^+$ and CD8$^+$ T cells (***: $p<0.001$; **: $p<0.01$; *: $p<0.05$).

Fig 1

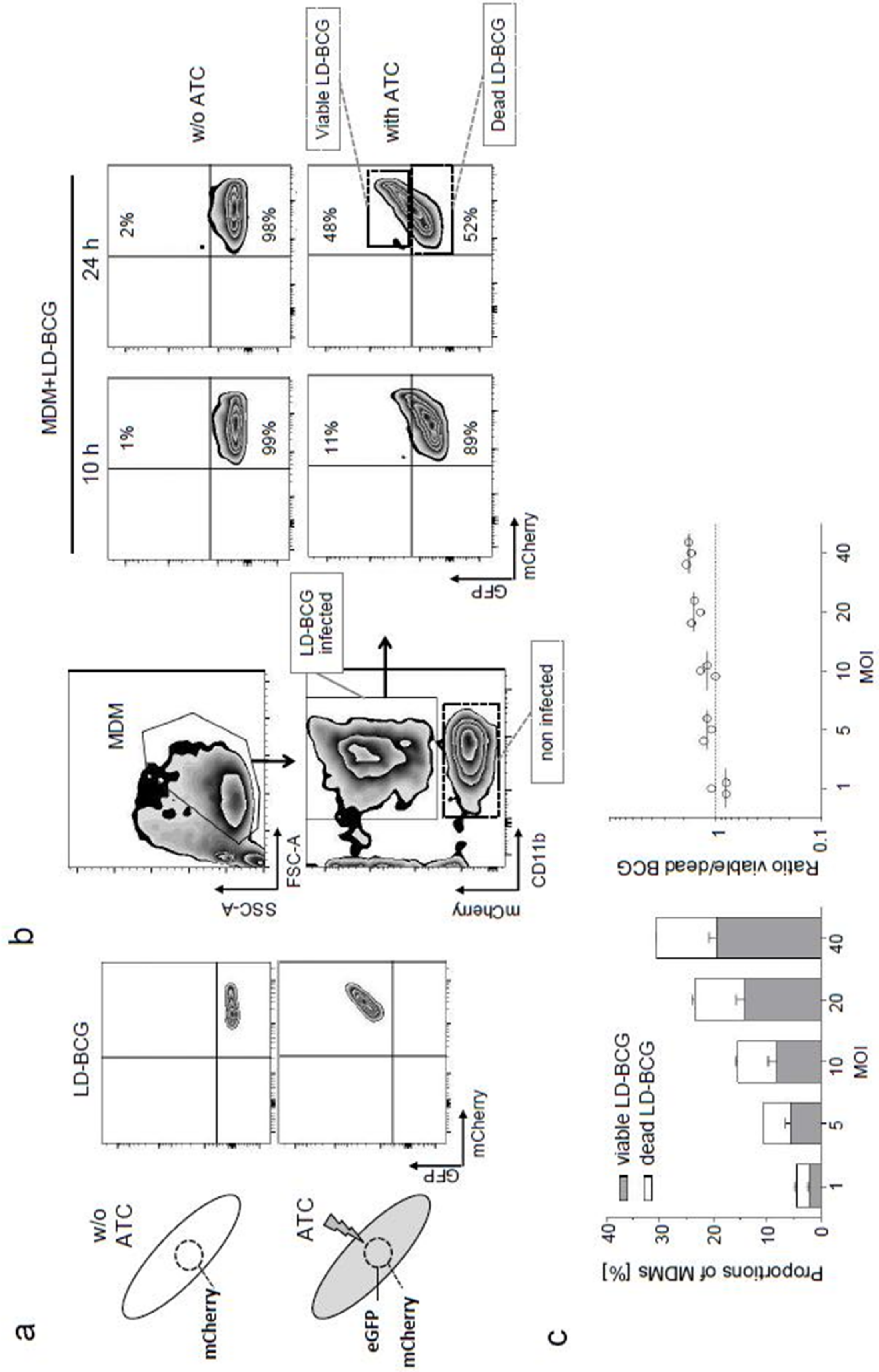

a
w/o ATC
ATC
mCherry
eGFP
mCherry
LD-BCG
GFP
mCherry
b
MDM
SSC-A
FSC-A
LD-BCG infected
non infected
mCherry
CD11b
MDM+LD-BCG
10 h
24 h
1%
99%
2%
98%
11%
89%
48%
52%
w/o ATC
with ATC
Viable LD-BCG
Dead LD-BCG
GFP
mCherry
c
Proportions of MDMs [%]
viable LD-BCG
dead LD-BCG
MOI
Ratio viable/dead BCG
MOI

Fig 2

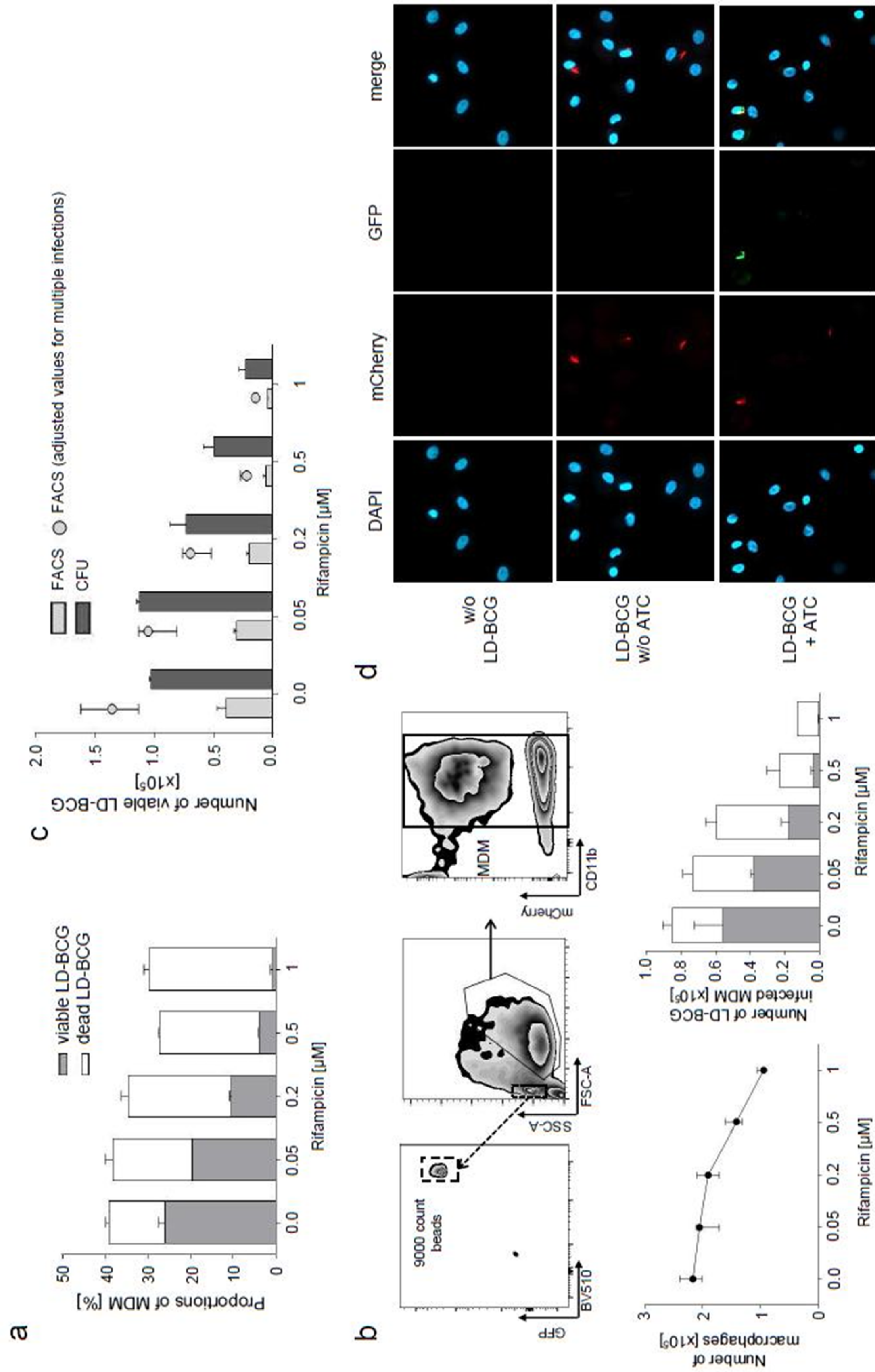


Fig 3

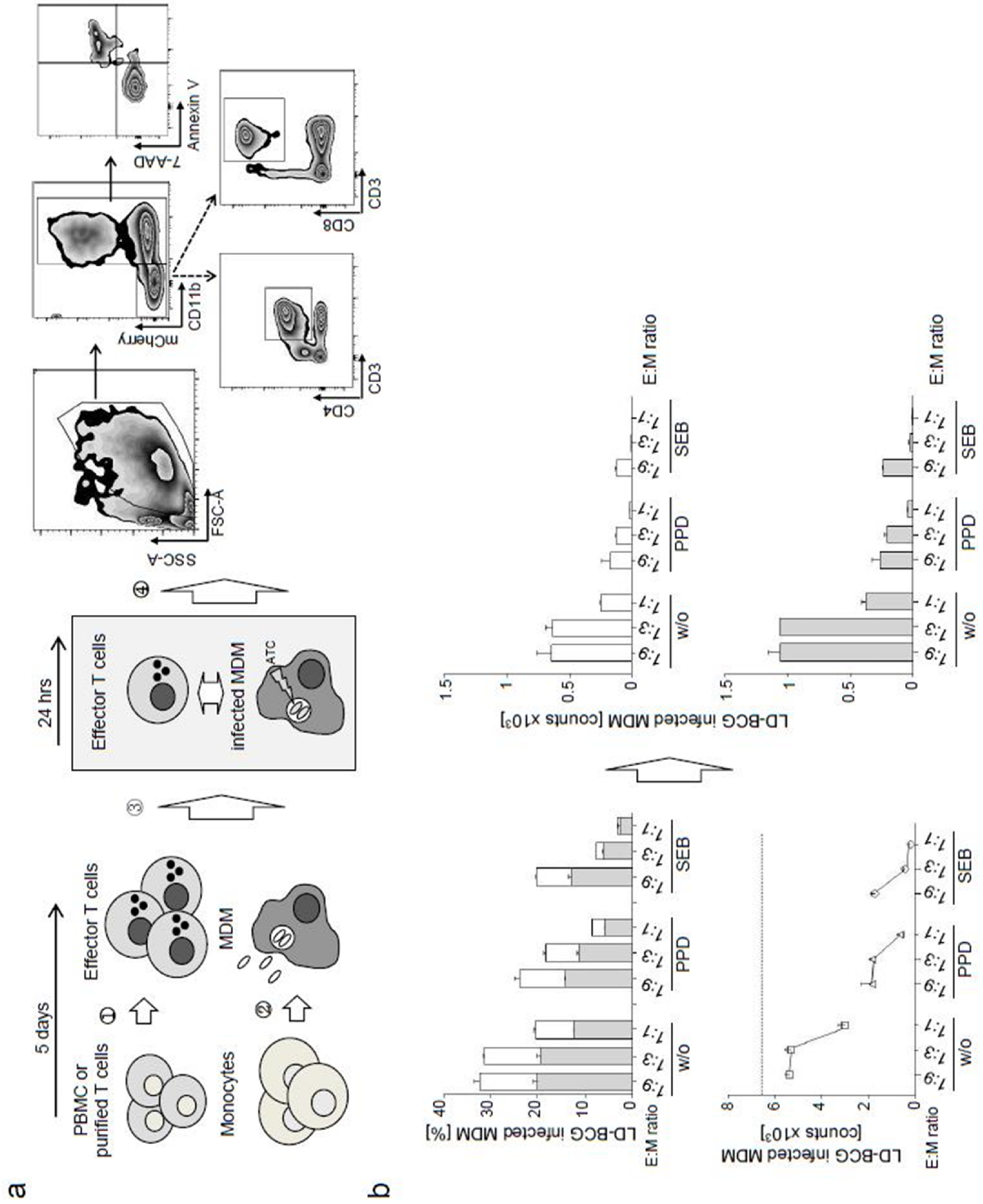
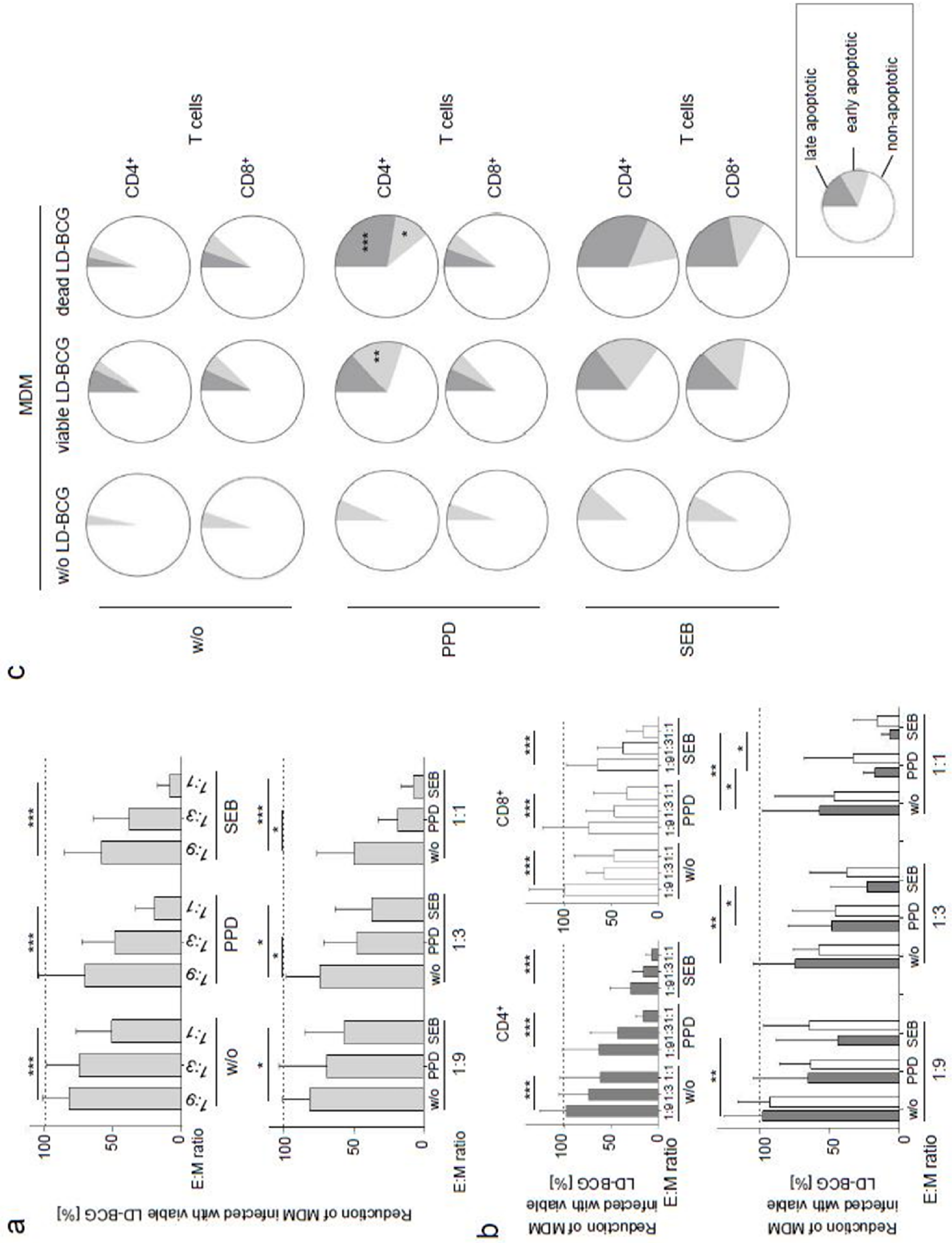


Fig 4


a
Reduction of MDM infected with viable LD-BCG [%]
E:M ratio
1:9
1:3
1:1
w/o
PPD
SEB
b
CD4+
CD8+
c
MDM
w/o LD-BCG
viable LD-BCG
dead LD-BCG
T cells
late apoptotic
early apoptotic
non-apoptotic

3. DISCUSSION AND OUTLOOK

Increasing numbers of NTM infections are reported in CF patients, whereby the prevalence varies, depending on the geographical region. Detection of infections relies on criteria defined by the ATS and mostly depends on the culture of mycobacteria from sputum or BAL samples, which are not always available, especially in paediatric patients. We developed a novel immunological test to detect *M. abscessus* in CF patients. To our knowledge, this is the first *M. abscessus*-specific immunological test. Our test circumvents the culture of the mycobacteria and makes use of small volumes of blood (about 1 ml), which renders it also practical and reliable for use in small children. In addition, our novel immunological test might predict disease severity in the patients and monitor treatment response, in contrast to available IFNγ release assays (IGRAs) developed for *M. tuberculosis* (Adekambi et al., 2015). However, the test applies *in vitro* restimulation of T cells with *M. abscessus*-specific PPD, so that blood samples have to be processed within a short term following blood withdrawal, as maintaining the blood samples in the blood collection tubes for periods longer than one hour was shown to affect the number of cytokine (mainly IFNγ) producing T cells (Hanekom et al., 2004). This might be problematic for the application of the test in clinical studies where processing of samples are often delayed, due to the non-availability of care personal. To reduce the time between blood withdrawal and beginning of the assay, we used to prepare the antigens in the stimulation-plates and store them at -20°C for up to 4 weeks. The so-prepared plates were then thawed shortly before collection of blood samples and used for the test. This had no effect on assay results (data not shown) and supports the application of our test in field studies (Hanekom et al., 2004).

Using our test, we were able to detect infections with *M. abscessus* in a subset of CF patients with positive *M. abscessus* culture, as accessed by routine diagnostics, indicating a high prevalence rate of *M. abscessus* (11.4 %) in our patient cohort. Comparisons of different mycobacterial antigens exhibited the evidence of *M. abscessus*-specific epitopes. Such epitopes might be useful for the development of novel *M. abscessus*-specific immunologic tests comparable to the IGRAs described for *M. tuberculosis* (Mazurek et al., 2005). Identification of these epitopes and evaluation of their potential as immunogenic proteins for *M. abscessus* will be the focus of a project funded by the Research Committee of the Heinrich-Heine-University Duesseldorf to Dr. med. Mathis Steindor. Furthermore,

we found patients with acute *M. abscessus* infection to have increased proportions of T cells positive for the activation marker CD40L, but negative for the cytokine IL-2, compared to non-infected CF patients. However, the biological significance of these cells in this context remains unclear and a bigger patient cohort might be necessary to evaluate if the conclusions from this study can be generalized.

Functional assays are designed to be reproducible and easily manipulative alternative to animal models, in order to determine the involvement of each protein or factor in a particular cellular pathway or biological process. They have been adapted for different human pathogens, including bacteria, viruses and pathogenic fungi, to evaluate either host cell cytotoxicity or pathogen replication (Kampmann et al., 2004; Wu et al., 2014; Chiang et al., 2015). Available assays either apply CFU quantification, fluorescence microscopy, real time PCR or measurement of bioluminescence as read out, and do not allow simultaneous evaluation of the host immune responses and pathogen or host cell viability.

We have established a novel polychromatic flow cytometry-based functional *in vitro* assay, which, in addition to exact quantification of mycobacterial growth-inhibition by effector T cells, allowed evaluation of their possible side effects on host macrophages in a short-term co-culture assay. Furthermore, the functional relevance of biomarker candidates for mycobacterial infection and prevention can be estimated. The advantage of using flow cytometry relies in the ability to rapidly analyse and quantify single viable cells, and also to get informations about cell populations. The assay relied on infection of monocyte-derived macrophages (MDM) with a – non-virulent – live/dead *M. bovis* BCG reporter strain (LD-BCG). The LD-BCG strain expressed a mCherry fluorescent protein and, on induction with tetracycline or its derivatives, a green fluorescent protein (GFP). This allowed detection of both viable and death mycobacteria within MDM by flow cytometry. However, 4-laser multiparameter flow cytometry devices are necessary for optimal application of the assay. Such devices are very expensive, which might hinder the application of our functional assay in rural field studies. Further optimization of the assay might apply other fluorochromes for labelling of the mycobacteria, in order to make the assay also suitable for “basic” flow cytometry devices. An alternative, if many parameters have to be analyzed, would be to prepare more samples as needed, so that staining might be performed in parallel, instead of staining all the parameters within one sample. But this would also means that the

amount of blood needed for purification of the MDM will have to be increased. We performed the assay in 96-well flat-bottomed plates and applied 1 x 10^5 MDM per well, as we found lower numbers of MDM per well to affect both MDM viability and phagocytic capacity (data not shown). One could try to use 384-well flat-bottomed, where less numbers of MDM per well will be needed, in order to circumvent the need for higher volumes of blood.

In addition to bypass cultivation in biosafety level 3 (BSL-3) laboratories, *M. bovis* BCG shares genetic sequence homologies with *M. tuberculosis*. However, it remains unclear, if our findings may apply to virulent mycobacteria including *M. tuberculosis.* To test this hypothesis, a live/dead *M. tuberculosis* H37Rv reporter strain (LD-H37Rv) was generated by the group of Prof. Dr. Rainer Kalscheuer and will be applied in future experiments by our group. A current study performed in our group makes use of the assay to analyse the functional role of candidate biomarkers and factors (including IL-7, IL-15, IL-2 and the cytokine inducible SH2 containing protein [CISH]) during infections with mycobacteria. Recently, a panel of 13 genes was identified, which were upregulated after co-culture of macrophages infected with either *M. bovis* BCG or *M. tuberculosis* with peripheral blood monocytes (PBL) or splenocytes from BCG-vaccinated mice (Kurtz and Elkins, 2015). Further characterization of these effector molecules with our novel assay might improve our understanding in anti-mycobacterial immune responses. Furthermore, the live/dead reporter plasmid might be cloned into *M. abscessus* and the so generated LD-MABSC might be applied to analyse the functional role of CD40L$^+$ and IL-2$^-$ T cells, which we found to be upregulated during the colonization of CF patients´ lungs by *M. abscessus.* We also found that co-culture of infected MDM with mycobacteria-specific CD4$^+$ or CD8$^+$ T cells significantly reduced the viability of both MDM and LD-BCG. In the same way, our novel functional *in vitro* assay might be applied to analyse the contribution of other cell populations to mycobacterial growth inhibition. For example, in an attempt to characterize mechanisms by which Glutathione (GSH) contribute to enhance the functions of NK cells in inhibiting the growth of *M. tuberculosis* inside human monocytes, Guerra *et al* found that treatment of NK cells with the GSH enhancing agent N-acetyl cysteine (NAC) in combination with IL-2 and IL-12 resulted in control of *M. tuberculosis* infection. The growth inhibitory effect correlated with increased expressions of the NK cytotoxic ligands FasL and CD40L, the NK activating receptor NKG2D and the cytotoxic receptors NKP30 and NKP44 on the

surface on the NK cells (Guerra et al., 2012). In the same way as for $CD4^+$ and $CD8^+$ T cells and as for $CD40L^+$/$IL\text{-}2^-$ T cells, the functional relevance of NK cells and of these NK cells factors during mycobacterial infection might be analysed using our novel functional *in vitro* assay.

Altogether, by applying the assays developed during this thesis, we might get a deeper insight, not only into factors leading to increased susceptibility to mycobacterial infections in persons with certain genetic predispositions, but also into anti-mycobacterial immune responses in general. This might help for the development of novel vaccines and improved treatments against mycobacteria.

4. LITERATURE

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EIDESSTATTLICHE ERKLÄRUNG

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der “Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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