Out of the

Division of Infectious Diseases and Tropical Medicine

Exploring sputum independent host biomarkers for detection of Tuberculosis disease and monitoring treatment

Doctoral Thesis
for the awarding of a Doctor of Philosophy (Ph.D.)
at the Medical Faculty of
Ludwig-Maximilians-Universität, Munich

submitted by

Mohamed Ibraheem Mahmoud Ahmed

born in

Khobar, Saudi Arabia

submitted on

30 April 2019

Supervisors LMU:	Dr. med. Norbert Heinrich
Habilitated Supervisor	PD, Dr. rer. nat. Christof Geldmacher
Direct Supervisor	Dr. med. Ulrich von Both

Reviewing Experts:

1st Reviewer	PD, Dr. rer. nat. Christof Geldmacher
2nd Reviewer	Dr. med. Ulrich von Both

Dean:	Prof. Dr. med. dent. Reinhard Hickel

Date of Oral Defense:	15.10.2019

Table of Contents

Acknowledgment

This work would have not been possible without God first and foremost. Nobody has been more important to me throughout my long journey than my family. I would like to thank them for the love and support they have provided me with. They are the best role models one can try to aspire towards.

I would like to thank our funding agencies, the German Center for Infection Research (DZIF), the European & Developing Countries Clinical Trials Partnership (EDCTP) and the German Ministry for Education and Research (BMBF), whom without this work would not have been possible. In addition, I would like to thank the EU Horizon 2020 program – for funding the HIV peptide array studies within the European HIV Vaccine Alliance (EHVA) project. I would like to thank the Center for International Health (CIH) for accepting me in their Phd program and the knowledge and support they have provided me. Also their continuous patience even when I was late for certain tasks.

I would like to thank the director of the Institute for Infectious Diseases and Tropical Medicine Prof. Dr. med. Michael Hölscher. I am very grateful to my supervisors Dr. Christof Geldmacher, Dr. med. Ulrich von Both and Dr. med. Norbert Heinrich for accepting me as their Phd student and providing me with continuous help and support and showing patience during this long and strenuous task. I would like to thank them for their personal and professional guidance and scientific knowledge that they have shared with me. They have truly shown me how a good scientist should be. I am also grateful for having the best colleagues one can wish for in a lab; Dr. Kathrin Held, Claudia Bräu-Heberger and Sabine Rappe. I would like to thank them for their support, for stepping in to help me at the times when the path was not simple and for sharing with me their extensive experience in the scientific field. I am thankful to all those with whom I had the pleasure to work with during this and other projects.

I would like to thank the Pan African Consortium for the Evaluation of Antituberculosis Antibiotics (PanACEA) comprising of the following individuals and institutions: Medical Centre of the University of Munich, Munich, Germany (Anna Maria Mekota, NH, Andrea Rachow, ES, MH); University of St. Andrews, St Andrews, United Kingdom (Stephen Gillespie); Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands (Angela

Colbers, Rob Aarnoutse, Martin Boeree); University College of London, London, UK (Anna Bateson, Timothy McHugh, Kasha Singh, Robert Hunt, Alimuddin Zumla); MRC Clinical Trials Unit at UCL, London, UK (Andrew J Nunn, Patrick PJ Phillips, Sunita Rehal); University of Cape Town, Cape Town, South Africa (Rodney Dawson, Kim Narunsky); University of Stellenbosch, Cape Town, South Africa (Andreas Diacon, Amour Venter, Sven Friedrich); University of the Witswatersrand, Johannesburg, South Africa (Ian Sanne, Karla Mellet, Eefje de Jong); The Aurum Institute, Johannesburg, South Africa (Gavin Churchyard, Salome Charalambous, Vinodh Edward, Madulagotla Sebe, Lungile Mbata, Robert Wallis); University of Zambia, Lusaka, Zambia (Peter Mwaba); NIMR-Mbeya Medical Research Centre, Mbeya, Tanzania (Nyanda Elias Ntinginya, Chacha Mangu, Christina Manyama, Bariki Mtafya); Ifakara Health Institute, Bagamoyo, Tanzania (Lilian T. Minja,Mohamed Sasamalo); Swiss Tropical and Public Health Institute, Basel, Switzerland, University of Basel, Basel, Switzerland (Klaus Reither, Levan Jugheli); Kilimanjaro Clinical Research Institute, Moshi, Tanzania (Blandina T. Mbaga, GK, HS, SM); Medical Research Unit - Albert Schweitzer Hospital, Lambarene, Gabon (Abraham Alabi, Ayola Akim Adegnika); Kenya Medical Research Institute, Nairobi, Kenya (Evans Amukoye); Makerere University, Kampala, Uganda (Alphonse Okwera) for their work in obtaining and providing me with the samples required to conduct the study.

I would like to thank all study participants who partook in our studies.

Furthermore, I would like to express my great appreciation for the support received by the UK HIV Vaccine Research Consortium (UKHVC) during the peptide array mapping studies of the *Spoke 03* HIV vaccine trial samples.

Abbreviations

AB	Antibody
AFB	Acid Fast Bacilli
ART	Anti-retroviral Therapy
aTB	Active Tuberculosis
BCG	Bacillus Calmette-Guérin
Bcl-2	B cell lymphoma 2
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CFP-10	culture filtrate protein-10
CRP	C-Reactive Protein
CXCR	CXC Chemokine Receptor
DNA	Deoxyribonucleic Acid
DosR	Dormancy of Survival Regulon
DST	Drug Susceptibility Testing
ELISA	enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked Immunospot Assay
EMB/E	Ethambutol
Env	HIV Envelope protein
EPTB	Extra-pulmonary Tuberculosis
ESAT-6	early secretory antigenic target-6
G0/1/2	Gap 0/1/2
GAG	HIV Group-Specific Antigen
GLA-AF	Glucopyranosyl Lipid Adjuvant-Aqueous Formulation
gp	HIV glycoprotein
HBHA	Heparin-Binding Hemagglutinin
HC	Healthy Control
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen-DR subtype
IFNγ	interferon gamma

IgG	Immunoglobulin Isotype G
IGRA	Interferon Gamma Release Assay
IL	Interleukin
INH/H	Isoniazid
IP-10	interferon γ inducible protein-10
IRIS	Immune Reconstitution Inflammatory Syndrome
LJ	Lowenstein-Jensen
LTBI	Latent Tuberculosis Infection
M	Mitosis
M7H10	Middlebrook 7H10
MDR-TB	Multi-drug Resistant Tuberculosis
MFI	Mean Fluorescent Intensity
MGIT	Mycobacterial Growth Indiacator Tube
MHC	Major Histocompatibility Complex
Mip-1β	macrophage inflammatory protein-1β
MTB	Mycobacterium Tuberculosis
MTBC	Mycobacterium Tuberculosis Complex
MVA	Modified vaccinia Virus Ankara
NTM	Nontuberculous Mycobacteria
PAS	4-aminosalicylic acid
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1
PETCT	Positron emission tomography computed tomorgraphy
POL	Polymerase
PPD	Purified Protein Derivative
PTB	Pulmonary Tuberculosis
PZA/Z	Pyrazinamide
QFT-Gold	QuantiFERON-TB Gold In-Tube
QFT-Plus	QuantiFERON-TB Gold Plus
qPCR	Quantitative Polymerase Chain Reaction

RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
RIF/R	Rifampin
RNA	Ribonucleic Acid
S	Synthesis
SDG	Sustainable Developmental Goals
TAM	T cell activation marker assay (TAM)
TB	Tuberculosis
TNF	Tumor Necrosis Factor
TST	Tuberculin Skin Test
UN	United Nations
WHO	World Health Organization
XDR-TB	Extensively Drug Resistant Tuberculosis

Publication List:

[1] Ahmed MIM, Ntinginya NE, Kibiki G, Mtafya BA, Semvua H, Mpagama S, Mtabho C, Saathoff E, Held K, Loose R, Kroidl I, Chachage M, von Both U, Haule A, Mekota A-M, Boeree MJ, Gillespie SH, Hoelscher M, Heinrich N and Geldmacher C (2018) Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy. *Front. Immunol*. 9:2247. doi: 10.3389/fimmu.2018.02247

[2] Joseph S, Quinn K, Greenwood A, Cope AV, McKay PF, Hayes PJ, Kopycinski JT, Gilmour J, Miller AN, Geldmacher C, Nadai Y, Ahmed MIM, Montefiori DC, Dally L, Bouliotis G, Lewis DJM, Tatoud R, Wagner R, Esteban M, Shattock RJ, McCormack S and Weber J (2017) A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes. *Front. Immunol.* 8:149. doi: 10.3389/fimmu.2017.00149

[3] Ahmed MIM, Ziegler C, Held K, Dubinski I, Ley-Zaporozhan J, Geldmacher C and von Both U (2019) The TAM-TB Assay—A Promising TB Immune-Diagnostic Test With a Potential for Treatment Monitoring. *Front. Pediatr.* 7:27. doi: 10.3389/fped.2019.00027

[4] Nadai Y, Held K, Joseph S, Ahmed MIM, Hoffmann VS, Peterhoff D, Missanga M, Bauer A, Joachim A, Reimer U, Zerweck J, McCormack S, Cope AV, Tatoud R, Shattock RJ, Robb ML, Sandstroem EG, Hoelscher M, Maboko L, Bakari M, Kroidl A, Wagner R, Weber J, Pollakis G and Geldmacher C (2019) Envelope-Specific Recognition Patterns of HIV Vaccine-Induced IgG Antibodies Are Linked to Immunogen Structure and Sequence. *Front. Immunol.* 10:717. doi: 10.3389/fimmu.2019.00717

1- Introduction

1.1- Tuberculosis

Tuberculosis (TB) is an old disease that was shown to be present in humans thousands of years ago. The bacterium causing this disease, *Mycobacterium tuberculosis* (MTB), was discovered in 1882 by Robert Koch. MTB is a highly aerobic, non-motile, pathogenic bacterium from the family Mycobacteriaceae. It contains an unusual, waxy coating on its surface made from mycolic acid; a virulence factor, responsible for its resistance to desiccation. It is a slow dividing bacterium that divides every ~20 hours. MTB is part of the Mycobacterium tuberculosis complex (MTBC), a family consisting of at least 9 genetically related members that all cause tuberculosis either in humans or other animals. Unlike MTBC, nontuberculous Mycobacteria (NTM), also known as environmental mycobacteria, do not lead to TB, however can cause pulmonary diseases that resemble TB.

TB is one of the top ten causes of deaths worldwide. In 2017, an estimated 1.7 billion people were infected with MTB. Of those, 10 million people had fallen ill, with 1.3 million deaths within the human immunodeficiency virus (HIV) negative population and 0.3 million deaths within the HIV positive group. HIV infection is an important driver of the TB epidemic, particularly in Sub-Saharan Africa. For example, exponentially increasing HIV infection rates in South Africa during the 1990s were linked to a quadrupled TB incidence rate during the same time period [5]. In fact, untreated HIV infection increases the risk to develop active TB 20 to 37-fold [6]. An efficacious HIV vaccine would therefore be able to reduce aTB incidence rates in global regions with a high burden of HIV-TB co-infection.

More adults (90%) are infected than children and more men than women, with a ratio of male to female of approximately 2:1 [7]. The majority of the disease burden seem to concentrate within three regions; South-East Asia (44%), Sub-Saharan Africa (25%) and the Western Pacific Region (18%) (Figure 1.1). The majority of the cases are pulmonary tuberculosis (PTB). Extra-pulmonary TB (EPTB) accounts for approximately 14% of active TB cases.

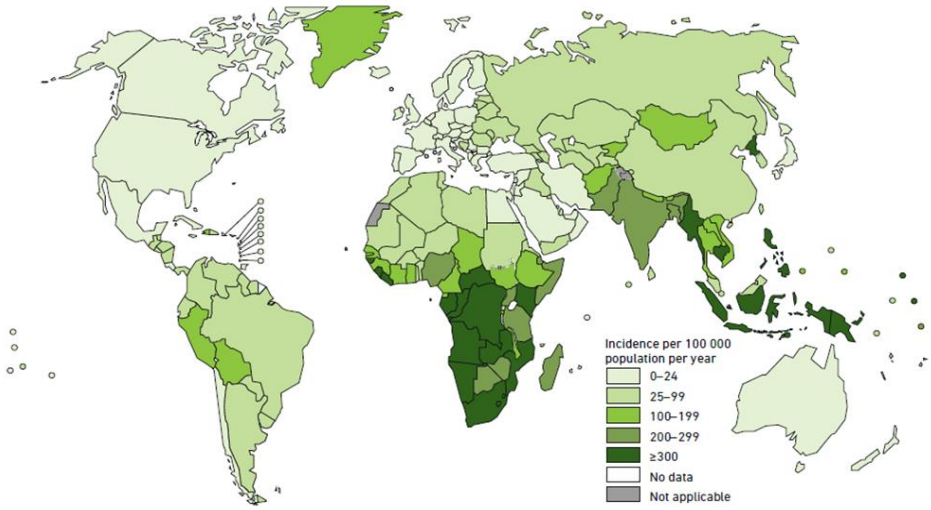

Figure 1.1: Estimated TB incidence rate for 2017. This image was reproduced from the WHO Global Tuberculosis Report 2018 [7].

1.2- Global Commitments

In 2016, the sustainable developmental goals (SDG) were enacted by the United Nations (UN). Under goal 3, which ensures healthy lives and promotes well-being for all at all ages, 13 other targets were defined one of which, point 3, mentions "By 2030, end the epidemics of AIDS, tuberculosis, malaria and neglected tropical diseases and combat hepatitis, water-borne diseases and other communicable diseases"[8]. A similar bill was passed by the World Health Organization (WHO) under the name "End TB Strategy" which sets the goal of ending the global TB epidemic by 2035. There are three main indicators for this, the number of deaths per year, the TB incidence rate and the percentage of TB-affected households that experience catastrophic costs as a result of TB disease. The main goal of this strategy is to reduce TB death by 95%, while reducing incidence rate by 90%, as compared to levels of 2015, by 2035 [7].

1.3- Pathophysiology

Mycobacterium tuberculosis (MTB) is transferred via air droplets from an individual that suffers from the active form of the disease (active tuberculosis, aTB) to another. The bacteria enter the lungs and cause the most common form of TB in adults and children,

called pulmonary tuberculosis (PTB). It can also cause extra-pulmonary tuberculosis (EPTB) (ex. lymph node TB, pleural TB etc.) where the disease develops outside of the lungs. The disease is usually less infectious when it occurs in children, mostly due to them having a paucibacillary disease, less forceful cough and less cavitary lesions [9]. On the other hand, children are more vulnerable to the infection and have a greater risk to progress to active disease once infection occurred, especially in children less than 5 years of age.

The classic thought of the tuberculosis disease spectrum is that it exists in two states, the metabolically inactive form called latent tuberculosis infection (LTBI) and the metabolically active form called active tuberculosis. It is assumed that 5-10% of all LTBI patients will develop aTB, especially within the first two years of getting infected. However, other situations may increase the chances of one progressing to aTB, such as HIV co-infection or other immunodeficient conditions; the reason being that CD4 T cells producing IFNγ play a crucial role in controlling MTB and thus their depletion increases the likelihood of progression to active TB [10, 11]. As time progressed, scientists gained a greater understanding about the disease pathophysiology and it has become clear that TB is, rather, a continuous disease with more than two states [12-16]. In order to understand the pathophysiology better, scientists have added three additional groups; eliminated TB infection, incipient TB infection and subclinical TB infection [17]. Elimination of TB infection occurs when an individual gets exposed to viable MTB and his innate/adaptive immunity clears the infection. LTBI occurs when a healthy person gets exposed to MTB, but rather than eliminating it, the immune system controls it reducing the likelihood of aTB occurring. This is performed by immune cells such as CD4 T cells which release cytokines that attract neutrophils and macrophages, surrounding the bacteria and isolating it in what is called a granuloma. The CD4 T cells are crucial for granuloma formation as they maintain its organization [18]. Incipient TB infection is an infection that is likely to cause aTB, however no signs appear radiologically or microbiologically to suggest such an event from occurring. During subclinical TB, the bacterium causes asymptomatic disease, which can be detected radiologically or microbiologically. Finally, during active TB the disease causes clinical symptoms that can also be radiologically and microbiologically detected [17].

Following the establishment of LTBI an individual has several pathways by which the disease may progress. The disease may remain in an equilibrium form within a granuloma, where

there is constant elimination occurring to excess bacteria. The disease may progress slowly or rapidly through incipient and subclinical disease to develop aTB. One theory, behind the cause of this, is that the granulomas start bursting, overloading the immune system of the person. Lastly, the disease may cycle between incipient and subclinical disease, before either getting eliminated, or developing active TB (Figure 1.3). It is clear that, although LTBI was considered a metabolically inactive state, several studies have shown that there is continuous activity occurring within an individual even after being cured [15, 19].

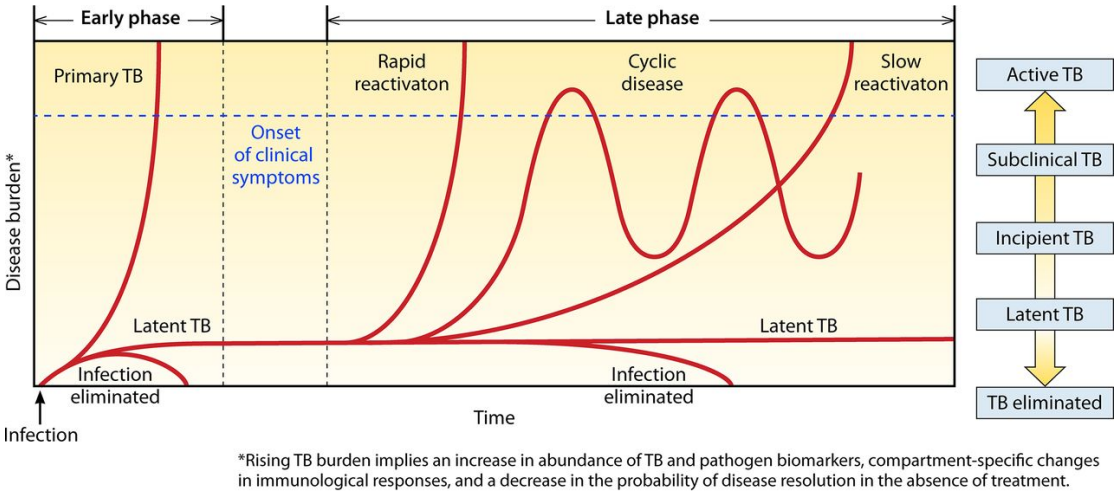

Figure 1.3: Life cycle of TB disease. The pathway taken by the disease to progress between the different pathophysiological states is shown. This image was reproduced from Drain et al. [17]

1.4- Anti-TB Therapy

One of the first drugs to be developed for anti-tuberculosis therapy (TB) was PAS (4-aminosalicylic acid), back in 1943 [20]. This was followed by the discovery of the effect of streptomycin against *Mycobacterium tuberculosis* by Selman Waksman in 1944 [21]. However, this drug had to be injected and had serious side effects such as hearing loss. Finally in 1951, isoniazid was discovered by three pharmaceutical companies; this compound was found to be 10 times more potent than its predecessors. By then people had already realized the importance of combination therapy, and thus streptomycin, isoniazid and PAS were used in combination to treat TB. By 1957, rifmaycins were discovered, with rifampin being used clinically in 1966. Unfortunately, for the next 5 decades no other drugs

were discovered until in 2012 two new drugs were introduced, delamanid and bedaquiline, for the use against drug resistant tuberculosis [22-24].

The current standard anti-TB therapy for new patients with drug-susceptible tuberculosis involves the use of 3-4 different drugs daily during the first 2 months (intensive phase), isoniazid (H/INH), rifampin (R/RIF), ethambutol (E/EMB) and pyrazinamide (Z/PZA), followed by 2 drugs (H and R) daily for the next 4 months (continuous phase), to complete a total of 6 months of treatment therapy [25]. The concentrations of these drugs are weight dependent. With the 6 month standard therapy, 3-5% of the subjects relapse [26]. As for those patients that require retreatment (relapse), they must initially undergo a drug susceptible testing to determine whether the bacterium is resistant to isoniazid and preferably rifampin or not. If no resistance is documented, the standard therapy can be applied again, however if resistance occurs, then treatment must be changed and lengthened depending on the resistance observed, whether it is multi-drug resistance (MDR-TB) or extensively drug resistant (XDR-TB) (Figure 1.4) .

The problems that arise with a “one-size-fits-all” approach to standard therapy are that the patient may suffer from physiological conditions, such as hepatotoxicity, liver toxicity and neurological disorders [27, 28]. This leads to non-compliance from the patient and the development of resistant mycobacterial strains, which eventually results in even longer treatment durations. Several studies have tried to reduce the treatment period for TB therapy. Previous studies, consisting of 2 randomized control studies and 2 observational studies, have looked at 4 month treatment durations [29-33]. Of interest, for those individuals presenting with culture negative pulmonary tuberculosis, the relapse rate within all four studies was low (1-3%). Thus, those presenting with a milder form of tuberculosis at pre-treatment would not have to undergo the full 6 month of therapy. Recently, 4 studies consisting of 3 phase 3 trials using fluoroquinolones, gatifloxacin and moxifloxacin, have tried to reduce therapy from 6 to 4 months for drug-susceptible tuberculosis [34-37]. Although, the majority of the patients (80%) had been cured after 4 months without any relapse after a follow up of 18 months, the relapse rate was higher than in the standard therapy and thus declared unsuccessful. Currently, a study called SHINE (shorter treatment for minimal tuberculosis in children) is recruiting children in India and Africa in order to observe the effectiveness of a 4 month versus a 6 month anti-TB treatment therapy [38].

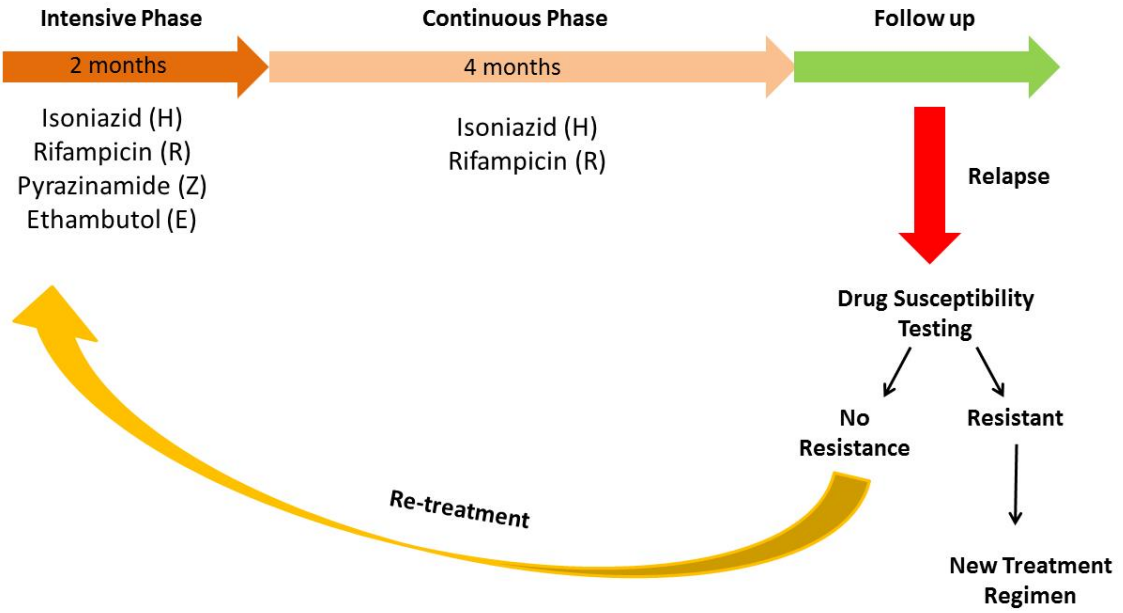

Figure 1.4: Treatment strategy towards Pulmonary Tuberculosis (PTB). Anti-TB therapy requires 6 month of treatment where 4 drugs are used during the first 2 months (intensive phase) followed by 2 drugs for 4 months (continuation phase). A follow up period ensues after end of treatment. If relapse were to occur, drug susceptibility is performed on the bacterium. A resistant strain would undergo a new treatment regimen, while a drug susceptible/non-resistant bacterium would repeat the same treatment.

1.5- Current Diagnostic Tools

A summary of the current diagnostic tools and which TB states they are useful in detecting is presented in figure 1.5.

1.5.1- Chest X-ray

After taking the medical history and doing a physical exam, a chest X-ray is performed if TB is suspected. The health worker can examine the lungs in this manner and thus see if any cavitation may have occurred in the lung tissue. Usually, the cavities are present in the upper part of the lung. The issue with chest X-rays is that they have poor specificity and they can, on their own, neither confirm nor exclude TB diagnosis. Rather they are used to confirm a suspicion.

1.5.2- Tuberculin Skin Test (TST)

The Mantoux tuberculin skin test is the process by which one tests whether an individual has been infected with *Mycobacterium tuberculosis* or not. It is one of the earliest techniques developed in the 19th century that is still in use. The procedure involves the intradermal injection of tuberculin purified protein derivative (PPD) into the inner forearm of a person. After 48 hours the person comes back in order to determine the results, by measuring the diameter of the swelling. The reasoning that underlines this process is that T cells are sensitized towards tuberculin by prior infection. Thus, a delayed hypersensitivity reaction occurs after the injection, where T cells are recruited to the site and release their cytokines causing an inflammatory reaction. The Mendel-Mantoux test only measures the hypersensitivity towards tuberculin and not TB immunity. One of the disadvantages of this test is that BCG-vaccinated individuals could also provide a positive result (false positive). Furthermore, one can not determine whether a subject has active TB or not. False negative results could be provided by infants and those only recently infected by tuberculosis and lastly, the technique itself requires trained personal [39].

1.5.3- Acid-Fast Bacilli (AFB) smear microscopy

Acid-Fast Bacilli smear is a microscopic process where a sample is stained with a flurochrome in order to detect Mycobacteria. If mycobacteria (acid-fast bacilli) are present in the sample they will be seen under the microscope following an acid wash due to their ability to resist decolourization via the mycolic acid in the cell wall thus retaining the primary stain. The advantages of this technique are that it is a rapid, inexpensive and easy procedure, where different types of specimen can be tested (tissue, sputum, body fluids, etc.) [40]. Furthermore, it could be used as a first indication of a mycobacterial infection; perhaps even tuberculosis disease if a positive result is obtained. However, confirmatory diagnoses are required, since it lacks in sensitivity, due to its pre-requisite of a high bacterial load for detection [40-42]. It also has limitations in its specificity, since all mycobacteria are acid-fast, thus the identification of the species is not possible, unless taking into account the local prevalence of MTB and nontuberculous mycobacteria (NTM). The determination of the resistance of the bacteria is also not possible. In addition its use in treatment monitoring is limited, as it can not differentiate between viable and dead organisms.

1.5.4- Sputum Culture Method

Sputum culture involves the direct detection of *Mycobacterium tuberculosis* on either solid or liquid medium. It is currently the gold standard for sensitivity and specificity and unlike the smear method can be used for species identification and drug susceptibility testing (DST). Furthermore, although not optimal, it is used for monitoring TB treatment response. Both the liquid and solid medium are recommended for use. The solid medium consists of two methods, one is the Lowenstein-Jensen (LJ, egg based) and the other is the Middlebrook 7H10 (M7H10, agar based). Each one has its own advantages and disadvantages, with the M7H10 medium allowing faster culture growth and allowing easier colony quantification, while the LJ suffers from less contamination. Liquid medium although even easier to contaminate than the solid medium, can provide fast detection time of mycobacteria. Although, used in tuberculosis diagnosis, the method is far from perfect; their reliance on sputum, which is obtained from non-sterile regions, leads to contamination issues [43]. Furthermore, it is difficult to obtain sputum from infants [44]. False negative arises in paucibacillary aTB patients and those where aTB lesions do not have access to the airways, for example in extra-pulmonary TB patients [45, 46]. MTB cultures have low sensitivity, even sequential bacterial load measurement by culture suffer from poor sensitivity [47]. The major disadvantage of the culture method is that it requires a long time (>2 weeks) for result to be obtained.

1.5.5- Interferon Gamma Release Assay (IGRA)

IGRA is used to detect the immune response towards *Mycobacterium tuberculosis* (MTB). The method relies on the fact that most people, who have been previously exposed to MTB, produce specific immune cells that upon re-exposure to that antigen produce interferon gamma (IFNγ). There are two ways for measuring this marker and that is either by enzyme-linked immunosorbent assay (ELISA, QuantiFERON assays) or via enzyme-linked immunospot assay (ELISPOT, T-SPOT.TB assay)[48]. Unlike the QFT, which measure IFNγ in the supernatant of antigen stimulated cells, the T-SPOT.TB assay detects individual effector T-cells that produce IFNγ in response to the MTB antigens. Currently, there are two products in the market, QuantiFERON-TB Gold Plus (QFT-Plus) and T-SPOT.TB. The QuantiFERON-TB Gold In-Tube (QFT-Gold) has been recently discontinued (June 2018) and replaced by the QFT-Plus. The additional benefit of the QFT-Plus, over the QFT-Gold, is that it constitutes of

4 tubes instead of 3, with the additional tube containing antigens that can stimulate both CD4+ and CD8+ T cells instead of CD4+ only. One of the benefits of the IGRA, over TST, is its use of the early secretory antigenic target (ESAT)-6/ culture filtrate protein (CFP)-10 antigens that are specific to the MTB. Thus, individuals who had taken the BCG vaccine (false positive in the TST) would not obtain any result in the IGRA assays. However, similar to the TST, both techniques suffer from poor sensitivity (~80%) and can not differentiate between active TB and LTBI, even when using a different marker such as interferon γ inducible protein (IP-10) [49-52]. Unlike the TST though, the techniques have a higher specificity (~90% vs. ~60%) [53]. Furthermore it is believed that QuantiFERON assays may not be beneficial for HIV infected individuals. Lastly, its requirement of a lab makes it impossible for its use as a point of care diagnostic technique.

1.5.6- GeneXpert MTB/RIF

The GeneXpert is a new, nucleic acid amplification TB diagnostic technique that works by measuring the deoxyribonucleic acid (DNA) of the Mycobacterium tuberculosis complex (MTBC) within a sputum sample via PCR [54]. The process takes less than 2 hours and has an additional benefit of measuring the genetic mutations associated with resistance to the drug rifampicin. The process is fully automated and thus minimal technical training is required. However, since it measures the DNA of MTBC, it suffers from a lag during treatment monitoring and detection of end of treatment, as it can not differentiate live from dead bacilli [55]. As other sputum dependent diagnostic techniques it has a poor sensitivity in detecting certain extra-pulmonary tuberculosis [56].

	TB eliminated		Latent TB	Incipient TB	Subclinical TB	Active TB
	innate immune response	adaptive immune response				
TST	Negative	Positive	Positive	Positive	Positive	Positive
IGRA	Negative	Positive	Positive	Positive	Positive	Positive
Culture	Negative	Negative	Negative	Negative	Intermittently positive	Positive
Smear	Negative	Negative	Negative	Negative	Negative	Positive
GeneXpert	Negative	Negative	Negative	Negative	Negative	Positive

Figure 1.5: Tuberculosis Pathophysiology in comparison to current diagnostic tools in use. The different pathophysiological stages of tuberculosis are shown compared to the diagnostic tools used and the stages at which each respective tool can be used. The colour signifies the severity of the disease as you go from left to right.

1.6- Prospective Diagnostic Tools for aTB

1.6.1- Novel antigens for T cell based Assays

As mentioned above, several of the current diagnostic tests suffer from lack of sensitivity and thus fail to differentiate active tuberculosis (aTB) from latent TB infection (LTBI). Antigens used in these experiments include the purified peptide derivative (PPD), which lacks also in specificity as it cross reacts with BCG-vaccinated individuals and nontuberculous mycobacteria (NTM) and ESAT-6/CFP-10. Due to the previous shortcomings, several studies have tried to identify different antigens in order to differentiate aTB from LTBI; the majority coming from the dormancy of survival regulon (DosR), which consists of genes expressed during latency. One of the antigens that show promise is the Heparin-Binding Hemagglutinin (HBHA), which induces the production of IFNγ by cells when the bacterium is contained (LTBI), while during aTB no IFNγ is produced [57-59]. Although promising, the antigen does seem to have shortcomings when it comes to HIV co-infection, where HIV-LTBI patients seem to produce low IFNγ response [60, 61]. Furthermore, children that suffered from asymptomatic tuberculosis also produced IFNγ [59]. Although several antigens have been studied, some showing sensitivities and specificities similar to IGRAs, future issues may still arise since several of them are added in vaccines, and thus cross reactivity may always occur [62].

1.6.2- Plasma Markers

Measuring soluble cytokine levels within the blood of aTB, LTBI and healthy control (HC) patients as a method to discriminate between these different states has also been explored. Some studies use unstimulated whole blood, which renders the plasma markers concentration observed not specific; since the change observed might not necessarily be due to tuberculosis, but an underlying factor or disease. Some studies do look at stimulated whole blood, however several markers, such as macrophage inflammatory protein (Mip)-1β, C-Reactive Protein (CRP), IFNγ, interleukin (IL) -2, IL-10, IL-12, IL-17 interferon gamma inducible protein -10 (IP-10) and RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted) were not able to discriminate between aTB and LTBI [63-67]. Similar results (no difference) were observed for some of the markers when used in TB treatment monitoring [68]. However, markers such as IP-10, may be useful at discriminating between those that were exposed to MTB (aTB and LTBI) and healthy controls [67].

Although, on their own, each marker may not be able to differentiate aTB from LTBI, different approaches where one may utilize combinations to obtain better results can be explored. One such approach is the use of an IL-2/IFNγ ratio, which has been shown to differentiate aTB from LTBI after a 72 hour stimulation [69]. Another such example is a study that looked at a 6 cytokine biosignature [70].

1.6.3- Transcriptional Markers

Genome wide RNA expression profile is a new tool that provides information on the response from the host towards *Mycobacteria* tuberculosis [71]. The process involves the extraction of RNA from unstimulated whole blood or PBMCs and performing next generation sequencing and big data analysis to identify which genes are up- or downregulated within an individual. Although, still in its infancy, the technique has provided interesting findings, such as a transcriptional signature dominated by interferon inducible genes being over-represented within active TB patients [72]. This was confirmed by other studies [73]. Bloom et al. were able to show that active tuberculosis differed from LTBI by 320 genes. Furthermore, it was observed that this 320 gene signature changed significantly within the first two weeks of anti-TB chemotherapy, and continued to decrease as treatment progressed to 6 months [74]. One of the issues with the transcriptomics studies is that they rely on unstimulated whole blood, questioning whether the genes detected are specific to tuberculosis or not. Another study by Bloom et al. and Kaforou et al. were able to show that certain gene signatures were able to discriminate between an individual infected with tuberculosis or other diseases, such as pneumonias, lung cancers pulmonary sarcoidosis and HIV [73, 75]. In general, RNA expression profiling is regarded as a powerful way to understand and diagnose diseases and to elucidate immunological processes [71]. However, in both experiments only samples from culture confirmed tuberculosis was included. Anderson et al. were also able to show that a 51 transcript signature was able to differentiate tuberculosis from other diseases in African children with 82.9% sensitivity and 83.6% specificity [76]. The problem with these initial studies, is that they used several tens to hundred genes for diagnosing TB, however in order to develop a point of care diagnostic involving a PCR or qPCR, the number of genes required to differentiate active TB from LTBI and other diseases must be reduced. Thus, a recent study by Sweeney et al. combined the datasets from several previous studies containing a heterogeneous population with

different diseases and obtained a three transcript signature that was able to differentiate active TB from healthy controls, LTBI and other diseases [77, 78]. Another study by Zak et al. was trying to determine gene signatures that would predict which individuals infected with Mycobacterium would develop active TB disease [79]. A 16 gene signature was discovered with a sensitivity of 66.1% and a specificity of 80.6% 12 months preceding tuberculosis diagnosis. Due to this poor sensitivity, the genes would most probably be used in high risk settings and to predict those individuals who have a high chance of not developing active TB. Recent studies try to look at genes within certain cell populations in whole blood such as CD4+ T cells to try and obtain further insight into the function and phenotype within these populations [80]. One of the issues with the transcriptional studies is the computational methods used for each study differs than the rest, in other words the computational landscape is still developing and thus there is little overlap in the genes discovered between each study [81]. Furthermore, the signatures need to be compared to other diseases to make sure they do not overlap with them. However, as these machine learning algorithms mature and the number of gene signatures required decrease, these transcriptomic markers could proof to be very promising and useful when transferred into a simpler test [82].

1.6.4- Flow-Cytometric Markers

A summary of the process by which T cells are first stimulated and then acquired by the flow cytometric technique works is shown in figure 1.6.4.

1.6.4.1- TAM-TB assay

The T cell activation marker assay (TAM)-TB assay is a sputum independent, whole blood assay that relies on the phenotypic (markers) changes that occur intracellularly and on the surface of MTB-specific CD4 T cells, as an individual progresses from an active TB disease to a latent TB infection (LTBI). The underlying reason behind this process relies on the fact that phenotypic changes are a reflection to changes in T cell function as a consequence to different disease status. The rarity of the CD8 T cell response to PPD, especially within the LTBI population, made it difficult to be incorporated into this assay [83-87].

The first marker to be used in the TAM-TB assay is CD27. This “maturation” marker, a member of the TNF-receptor superfamily and involved in co-stimulation, is downregulated as a T cell changes from a naïve (antigen inexperienced) to a memory T cell (antigen

experienced) [88]. Several studies in TB infected mice models have shown the downregulation of this marker on IFNγ producing CD4 T cells within the lungs [89, 90]. This marker was first described by Streitz et al. for its use as a diagnostic marker for TB; where it was shown that the % of PPD stimulated CD27- CD4 T cells, in those subjects with smear or culture positivity, was higher in aTB than those both BCG exposed and LTBI subjects [91]. Another observation within this study was that the frequency of CD27 expression recovery (CD27+) for a great portion of subjects required more than a year after treatment initiation [91]. This could mean that although such a marker may be beneficial in differentiating active and latent TB, it can't be used to monitor or to determine end of TB therapy. Further evidence for the use of CD27 as a marker for TB diagnostic was buttressed by Schuetz et al., where the observed phenotypic changes in the MTB-specific CD27 cells was observed regardless of HIV infection status [92], this was also confirmed by Riou et al. [85]. However, TB+ and TB- subjects showed greater overlap in the frequency of CD27 marker within the HIV+ group. Reasons behind this, although largely unknown, could be due to low level of TB re-activation occurring in diseases of chronic infection such as HIV, leading to a more diverse CD27 expression in TB asymptomatic patients. One interesting case was observed, where an individual had been downregulating CD27 expression prior to developing symptomatic TB. Although too early to conclude, this could add another function to the CD27 marker, where it not just differentiates active TB from LTBI, but also could determine the likelihood of LTBI re-activation within an individual (predictive marker). A recent paper by Nikitina et al. has shown that the accumulation of these MTB-specific IFNγ+ producing CD27- cells in peripheral blood, correlated with factors such as lung destruction and clinical TB severity [93]. In addition, although CD27- reverts slowly, for those subjects that did show a faster decline in CD27- cells with treatment progression, the aforementioned symptoms decreased [93]. A recent prospective cohort study was performed on paediatric children, using CD27 as a diagnostic marker for active TB. Although, rather than relying on the % of MTB-specific CD27 cells, they looked at the mean fluorescent intensity (MFI). This method was compared with the culture method and the Xpert MTB/RIF assay [94]. Portevin et al. was able to show that in a sample size of 113, a sensitivity of 83.3% and specificity of 96.8% was achieved using the MFI [94]. Of interest though, is the fact that the TAM-TB assay was able to detect 8 subjects that were both culture and Xpert MTB/RIF negative. This could imply that using CD27 alone in the TAM-TB assay is a great addition to the current diagnostic tools for TB. Of

the three not detected via the TAM-TB assay, two were malnourished, while one died. Other papers have studied the diagnostic strength of the frequencies of MTB-specific CD27 cells, and shown that those subjects with a more persistent culture positivity and X-ray lesions seem to have a stronger CD27 expression. Although, the definition of persistent TB is different from each paper, with one describing culture positivity after at least 6 months of standard TB therapy [95] and the other, after 2 months of therapy [96].

The next promising markers to be used in the assay are 2 activation markers and a proliferation marker; CD38, a transmembrane glycoprotein involved in the catalysis and synthesis of NAD or cyclic ADP-ribose, HLA-DR, an MHC class II antigen presenting cell surface receptor, and Ki67, a nuclear protein expressed in G1, S, G2 and M phase of the cell cycle, however not in G0, thus used to provide evidence for proliferating cells. Adekambi et al was the first to provide evidence of the use of these three markers to distinguish active TB from LTBI [84]. All three markers showed an activated phenotype during active TB (% of MTB-specific CD38+, HLA-DR+, Ki67+ CD4+ T cells), while LTBI subjects had a downregulation of these markers [84, 97]. A validation cohort showed that the predictive value for diagnosing active TB was 100% for two of the markers, CD38 and HLA-DR, while Ki67 had an 80% predictive value. Furthermore, a change in the dynamics of the markers was observed as treatment progressed, with the largest downregulation in both CD38 and Ki67 occurring within the first two months after treatment initiation, while HLA-DR showed a gradual decrease. Unlike CD27, which required a long duration of time after TB treatment in order to revert to its pre-TB infected phenotype, these three markers were able to achieve the LTBI phenotype within the treatment window. Thus, these markers would be better suited for treatment monitoring. A correspondence by Wilkinson et al. has examined these markers and their ability to differentiate active TB from LTBI within a population co-infected with HIV [98]. Although similar phenotypes were observed for active and LTBI for the frequencies of all three markers on ESAT-6/CFP-10 stimulated IFNγ+ MTB-specific CD4 T cells, Ki67 and CD38 performed poorer in the TB+/HIV+ group. A reason behind this could be due to the influence of bacterial load on these markers, where the majority of smear negative samples had a low CD38 and Ki67 frequencies (similar to LTBI), while HLA-DR was not influenced with the smear status. Similar results were observed by riou et al for HLA-DR [85].

1.6.4.2- Other Flow-Cytometric Markers

Some markers that were not included in the TAM-TB assay did not show any phenotypic difference between active TB and LTBI. The homing receptors, CCR4, CCR6 and CXCR3 that were presented in the study by Riou et al, did not show any difference in their frequencies on CD4+ IFNγ+ cells between active TB and LTBI, with or without HIV [85]. Although, a more recent study agrees with the results observed for the CCR4 marker on ESAT-6/CFP-10, it does show a significant difference for PPD stimulated cells (not tested by Riou et al.) [99]. Latorre et al. tests both CD27 and CCR4 on CD4+ IFNγ+ cells and goes a further step by using both concomitantly to try and decrease marker overlap between active TB patients and LTBI. Nevertheless, using CD27 alone was able to show a similar sensitivity and specificity as in combination [99]. Although the other two markers (CXCR3 and CCR6) failed to differentiate between active TB and LTBI, CXCR3+CCR6- CD4+ T cells did present themselves as useful markers for differentiating HIV co-infected TB patients that develop immune reconstitution inflammatory syndrome (IRIS) 2-6 weeks after starting anti-retroviral therapy (ART) and those that do not. This condition, IRIS, occurs when a patient worsens clinically or radiologically despite suppression of the virus by ART [100].

Another marker studied was CD161, a C-type transmembrane lectin. Yang et al. was not able to show that the marker alone was able to differentiate active TB from LTBI from household contacts. However, by calculating a score, where the %CD4+/CD8+/CD3+ CD161+ is multiplied against the lymphocyte to monocyte ratio, a better sensitivity and specificity value was obtained; with CD8+ CD161+ showing the best values. One of the issues with this study is the use of unstimulated whole blood and thus the specificity of the change observed in the marker might occur with other diseases and not just during *Mycobacterium tuberculosis* (MTB) infection [101]. However, Riou et al. has shown that at least with ESAT-6/CFP-10 stimulated cells, no difference was observed for the %frequency of CD161 on IFNγ+ cells [85].

The frequencies of two other markers, on MTB-specific CD4+IFNγ+, were shown to be significantly different during active TB and LTBI; the anti-apoptotic molecule Bcl-2 and pro-apoptotic molecule CD95 (Fas) [97]. Both were shown to work vice versa, where the CD95 frequency was high during active TB and decreased with time, while Bcl-2 frequency was low during active TB and increased with time. Although, markers such as CD57 and CD127

did show a significant difference between active TB and LTBI, this difference was only observed on CD8+ IFNγ+ cells. This poses a difficulty due to the low IFNγ secretion in CD8 T cells.

CD244 or 2B4, a member of the signaling lymphocyte activation molecule family, when expressed at high levels activates an inhibitory signal and vice versa; thus in a paper by Yang et al., a higher MFI and % of CD244 was observed on CD4+ T cells for those that had failed anti-TB treatment compared to those that had been cured [102]. No significant difference was observed between LTBI and active TB for ESAT-6/CFP-10 stimulated cells. The difference between active TB and those undergoing retreatment was especially obvious for those with an initial positive culture. Henceforth, although this marker may not be beneficial in differentiating active TB from LTBI, it could be used to determine those that might need to undergo longer duration of treatment due to standard anti-TB chemotherapy failure. Another paper by Saharia et al. showed that for PPD stimulated cells, the % frequency of CD244 cells did not show a significant difference between treated and untreated TB, although a downregulation was observed as treatment progressed [103]. However, for those individuals co-infected with HIV, there was a significant upregulation in CD244 specific CD4 T cells as anti-TB therapy progressed. Hence, HIV could play a disruptive role in this marker being used for treatment failure or success. The only issue with this study is the low number of individuals tested for each group ($n < 12$).

PD-1, an inhibitory receptor, was shown by Adekambi et al. to be upregulated on LTBI patients as compared to BCG vaccinated individuals [104]. This was further expanded by Saharia et al., where a significant downregulation of PD-1 was observed as anti-TB therapy progressed within HIV/TB co-infected individuals [103]. The same result was not observed for TB infected patients. However, the large overlap observed makes the use of this marker difficult. A recent study by Day et al. showed that for ESAT-6/CFP-10 and TB 10.4 stimulated cells, a significant difference between LTBI and smear+ TB in MFI of PD-1+IFNγ+CD4+ T cells was observed. While, no difference was observed between LTBI and smear- samples. This could indicate that the overlap observed in the previous study could have been due to smear- patients.

Intracellular cytokines have also shown their importance for use as diagnostic tools, even though IFNγ+ MTB-specific CD4+ T cells alone were not sufficient to diagnose aTB (as shown in IGRA) [85]. However, a combination of three cytokines (IL-2, IFNγ and TNF-α) may play a more beneficial role. The issue that arises with such studies is the conflicting results obtained on the combination that can differentiate aTB from LTBI, especially since the antigen used for stimulation can play a vital role. An earlier study by Harari et al. was able to determine that TNFα+ and TNFα+ IL2+ CD4+T cells stimulated by a variety of MTB-specific antigens was able to differentiate aTB from LTBI [105]. However, Kim et al. was not able to observe this difference for TNFα+ CD4+ T cells when stimulated with a cocktail of MTB-specific antigens (ESAT-6/CFP-10 and TB7.7) [106]. This was followed up by Rozot et al., who was not able to observe any difference between aTB and LTBI for ESAT-6/CFP-10 stimulated MTB-specific CD4+ cytokine markers for either of the cytokine combinations (TNFα, IL-2 and IFNγ) [86]. However, a study by Saharia et al. was able to show that PPD stimulated TNFα+ and TNFα+ IL2+ CD4+T cells were able to differentiate treated and untreated TB patients, with the TB treated group having a higher TNFα+ IL2+ CD4+T cell frequency [103]. The same study was able to show that TNFα+IL2+IFNγ+ CD4+ T cells could also differentiate TB and TB/HIV co-infected treated and untreated patients. Another study by Riou et al. performed the experiment on ESAT-6/CFP-10 stimulated cells, however no difference in %frequency was observed for TNFα+IL2+IFNγ+ and TNFα+ CD4+ T cells. However, the TNFα+ IL2+ CD4+T cell frequencies were able to differentiate the different TB and TB/HIV co-infected aTB and LTBI patients [85]. Latore et al. was able to confirm the previous results, showing that the frequency of TNFα+ CD4+ T cells did not show a difference between aTB and LTBI when it came to ESAT-6/CFP-10 stimulation, however a difference was observed for PPD stimulated cells [99].

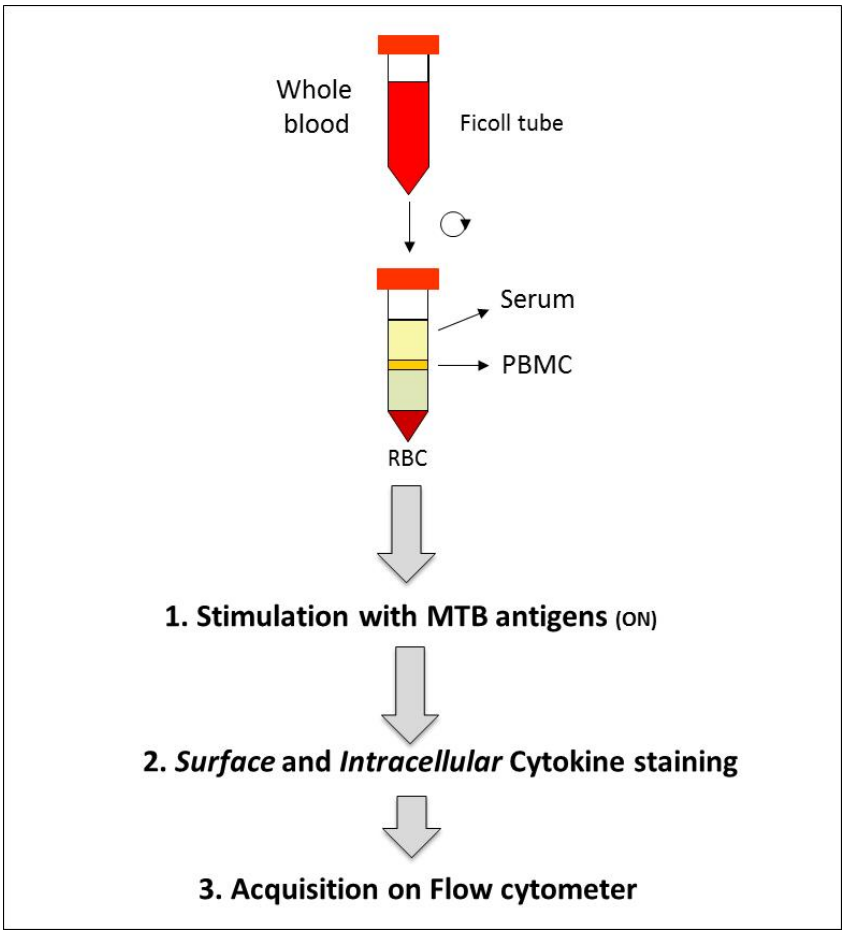

Figure 1.6.4: Summary of the flow cytometric method for diagnostic marker identification: A general summary of the assay used to identify new flow cytometry diagnostic markers for tuberculosis, involve the primary process of PBMC isolation, followed by overnight (ON) stimulation with MTB-specific antigens. The cells are then surface and intracellularly stained with the required antibodies and acquired on the flow cytometer.

1.7- HIV vaccines and immune correlates of protection from HIV acquisition

As mentioned before, an efficacious HIV vaccine would dramatically reduce TB incidence and the associated need for TB treatment in regions of high HIV prevalence. After 3 decades of HIV vaccine research, only one HIV vaccine trial – the Rv144 trial – showed moderate but significant efficacy to reduce HIV acquisition in a low risk population in Thailand [107]. The Rv144 vaccine consisted of 4 injections with a recombinant Canarypox viral vector that encoded for the HIV proteins Envelope, Gag and Pol, with additional injections of the recombinant AIDSVAX B/E (gp120) during the last two injections [107]. The Rv144 study also

resulted in the identification of one immune correlate of protection from HIV acquisition; an embedded case-control study showed that strong IgG recognition of the HIV Envelope protein variable region 2 correlated with a reduced risk of HIV acquisition [108]. Furthermore, fine mapping of vaccine-induced IgG Envelope recognition by a peptide array approach identified a 15 amino acid long region located in close proximity to the HIV co-receptor α4β7 Integrin (representative peptide sequence; TELRDKKQRVYALFY) [109]. Peptide array studies by Gottardo et al. confirmed these results and also identified, in a subset analyses of subjects with lower levels of HIV Envelope-specific plasma IgA responses, that the Rv144 vaccine-induced recognition of the highly immunodominant variable region 3 correlated with immune protection [110].

2- Contribution

This dissertation contains work that was conducted during the period of October 1st 2016 to April 30th 2019 at the Department of Tropical Medicine and Infectious Diseases, University Hospital, Ludwig-Maximilians-University, Munich, Germany

Publication 1 (Ahmed et al. 2018)

In this publication we present how the TAM-TB markers, on MTB-specific CD4 T cells, change as TB treatment progresses. Furthermore, not all markers are required for future studies as some show redundancy in results. Lastly, the change in %frequency on MTB-specific CD4 T cells correlate with the time to stable culture conversion. In this study I performed the assay itself, starting from thawing the PBMCs, stimulating the cells overnight, surface and intracellularly staining the cells and lastly acquiring it on the flow cytometry. I analyzed the data and generated the majority of the figures (except Figure 5). I contributed in writing the manuscript.

Publication 2 (Joseph et al. 2017)

In this publication we compare the immunologic response in participants taking two different vaccine strategies. I contributed to the peptide array experimental work, data analysis, generating the graphs and article figures.

Publication 3 (Ahmed et al. 2019)

In this publication we present how the TAM-TB assay may be used for TB treatment monitoring on an individual suffering from an extra-pulmonary TB (hip TB). In this study I performed the TAM-TB assay, from isolating the PBMCs, stimulating the cells overnight, surface and intracellularly staining the cells and lastly acquiring it on the flow cytometry. I analyzed the data and generated figure 2. I also contributed in writing the manuscript.

3- Objectives

3.1- Article 1

Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy

We have shown that current diagnostic tools, although effective in combination at recognizing symptomatic tuberculosis, still lack in several aspects when it comes to TB treatment, such as determining the end of treatment, monitoring therapy, diagnosing asymptomatic and extra-pulmonary TB (especially in children) and determining prospectively who from the LTBI patients would most likely develop aTB. These issues arise mainly since tools such as the GeneXpert and the sputum culture method concentrate on recognizing the pathogen itself within sputum samples. Thus, false negatives are observed in paucibacillery aTB (ex. children) and those cases where the lesions do not have access to the airways (ex. exta-pulmonary TB). Furthermore, the GeneXpert technique suffers from a lag in positivity, mainly due to its recognition of dead bacilli, while the sputum culture method has a low sensitivity and positive predictive value estimate [55]. Techniques such as the sputum culture also suffer from the length required to achieve results. Furthermore, sequential sputum bacterial load measurement by culture showed insufficient sensitivity for detecting unfavorable outcomes [47]. The IGRA test was shown to lack the ability to distinguish aTB from LTBI rendering it useless for TB diagnosis and monitoring. All these issues impede the accurate diagnosis of TB leading to a presumptive diagnosis.

Several studies have shown that a high percentage of patients are cured before the 6 month standard therapy [34-36]. A blood based immunodiagnostic technique, called the T cell activation marker assay (TAM), was developed to try and overcome the current problems and thus improve TB diagnosis and treatment monitoring. We have mentioned that the TAM-TB assay consisted of the maturation marker CD27, the proliferation marker Ki67 and the activation markers CD38 and HLA-DR. We have also provided previous evidence of proof of concept for the CD27 marker [91, 92, 94, 111]. Furthermore, we have shown that during active TB, the activation and proliferation markers are upregulated on MTB-specific CD4+IFNγ+ T cells, while the maturation marker is downregulated. In addition, evidence has been provided of the change in frequency in activation and proliferation markers as TB

treatment is initiated. Thus, we could exploit a dual-marker expression profile between a marker that requires extended period of time for reversion and one that changes quickly, in order to further characterize the different TB infection stages: active TB, "cured" TB (6 months after treatment initiation) and LTBI (table 1). Furthermore, information still lacks on how the change in markers as treatment progresses correlates with the in vivo mycobacterial load.

The purpose of this study, as stated in our article "is to obtain a more detailed understanding of the relationship between TAM-TB assay results, the MTB infection status, and mycobacterial treatment response. Here, we have therefore studied activation (CD38 and HLA-DR), proliferation (Ki67) and maturation (CD27) marker profiles on IFNg+ MTB-specific CD4 T cells in subjects with LTBI, and in aTB patients before and after TB treatment initiation in comparison to the mycobacteriological treatment response. The patients were tightly monitored using MGIT culture on a weekly basis until week 12 and on 4 additional time points until the end of treatment at week 26 and showed no relapse during a 6 months follow-up after the end of treatment"[1].

Cell marker	for	Active TB disease	End of Treatment	LTBI
CD27	maturation	-	-	+
CD38	activation	+	-	-
HLA DR	activation	+	-	-
Ki67	cell cycle	+	-	-

Table 3.1: The markers used in the TAM-TB assay and their phenotype on MTB-specific CD4 T cells during the different diseases states.

3.2- Article 2

A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes

The UKHVC SPOKE03 trial compared two different prime-boost vaccination strategies as follows; the standard group first received 3 vaccinations of HIV-DNA followed by two boosts of Modified vaccinia virus Ankara (MVA)-C and an additional two boosts with the recombinant protein CN54gp140 in 5ug of the novel adjuvant GLA-AF [2]. The accelerated group also received the identical DNA vaccine prime, but the recombinant protein boosts were administered simultaneously to the MVA-C. The overall purpose of the study was to compare immunogenicity of both vaccination strategies, which included both humoral and cellular immunology endpoints, including peptide array mapping analyses of Env-specific IgG recognition, which was performed in Munich.

3.3- Article 3

The TAM-TB Assay—A Promising TB Immune-Diagnostic Test With a Potential for Treatment Monitoring

Childhood tuberculosis is very difficult to diagnose due to the child's inability to produce sputum easily (especially infants). Furthermore, children usually suffer from the paucibacillary form of the disease, rendering several diagnostic tools ineffective. A further complication more frequently observed in younger children as well as in immunocompromised patients, is the extra-pulmonary (EPTB) form of the disease. Due to the assays nature of indirectly diagnosing active TB, it may be likely that the TAM-TB could be a very useful tool for EPTB situations. However, it is unknown whether this form of tuberculosis would also render the TAM-TB assay ineffective or not. It has been shown that markers such as CD27 present in peripheral blood have been correlated with lung tissue damage, this may not necessarily be indicative that other forms of TB produce similar results. This is especially true for EPTB, since some forms have a farther proximity to the circulatory system than the lungs.

We have previously shown that the TAM-TB assay has the potential to differentiate aTB from LTBI, however little information is available on its ability to monitor TB treatment. This is the first study to show the treatment monitoring potential of the TAM-TB assay in a TB patient. Thus, the purpose of this study was to test the TAM-TB assay on a hip EPTB case, in order to determine whether diagnosis would be possible (using cut-offs from the previously published work on PTB) and to monitor the patient as he undergoes TB therapy to identify whether improvement in patient condition can be monitored using the assay.

4- Discussion

Diagnostic tools such as the tuberculin skin test (TST), acid fast bacilli (AFB) smear microscopy method, the sputum culture method, the interferon gamma release assay (IGRA) and the GeneXpert are currently used in combination to diagnose TB and determine treatment outcome. Techniques such as the TST and IGRA, which detect the immune response towards the bacterium, are quick methods that determine whether an individual was infected with MTB, with the IGRA being more specific. However these techniques are unable to differentiate aTB from LTBI, thus rather used as an aid for diagnosing MTB. For unvaccinated children below the age of 5, the TST has a superior sensitivity than the IGRA for detecting LTBI [112]. The remaining techniques rely on the direct detection of MTB, mainly in sputum. The AFB smear technique, which involves the direct detection of MTB, is a quick method that determines whether an individual suffers from MTB, however due to the requirement of an initial high bacterial load, it suffers from poor sensitivity [41]. The sputum culture method is the only method available to monitor treatment response during TB therapy. However, it suffers from the extended time period required to obtain results, high contamination rate due to its reliance on sputum and false negatives observed in paucibacillery aTB and cases where the lesions do not have access to the airways [43, 44, 46, 113]. Furthermore, sequential sputum bacterial load measurement by culture showed insufficient sensitivity for detecting unfavorable outcomes [47]. The GeneXpert technique is a new diagnostic PCR technique that provides results within 2 hours and shows high specificity in detecting aTB, especially in smear positive samples. It has the added benefit of measuring MTB resistance and does not require trained personal (automatic). Furthermore, the recent GeneXpert Ultra showed a higher sensitivity in children (64% vs 53%) and smear negative patients (64% vs 46%) as compared to its predecessor GeneXpert, although showing a slight decrease in specificity [114, 115]. This technique however, suffers when it comes to monitoring treatment efficacy due to its lag in positivity, the reason being its detection of dead bacilli [55]. Thus, in conclusion the diagnosis of a patient is usually based on a presumptive basis [116].

The T cell activation marker assay is a sputum independent whole blood technique that depends on the phenotypic changes that occur on MTB-specific CD4 T cells. It is a rapid assay that requires a single day to process the results. It contains a maturation marker

(CD27) that has been shown, in several studies, to be able to differentiate aTB and LTBI within adults and children [91, 92, 94, 111]. Furthermore, this differentiation was observed in HIV co-infected patients [92]. The CD27 marker has also provided additional information to both the sputum culture and the GeneXpert method by identifying children with aTB that were not detected via these current methods [94]. The assay contains two activation markers (CD38 and HLA-DR) and a proliferation marker (Ki67), which have also been shown to differentiate between aTB and LTBI [84].

We were able to show similar phenotypes during aTB and LTBI as stated by previous studies for both pulmonary tuberculosis (PTB) and extra-pulmonary tuberculosis (EPTB) [84, 91]. During aTB, the % frequencies of the activation and proliferation markers on MTB-specific CD4 T cells were high, while that of the maturation marker was low. During LTBI, the inverse was observed; the % frequencies of the activation and proliferation markers were low, while the maturation marker was high. Furthermore, we were able to show a substantial reduction in the %frequency of activation (CD38 and HLA-DR) and proliferation markers (Ki67) on MTB-specific CD4 T cells within the first few weeks of treatment initiation. This, however, was not true for the maturation marker (CD27) throughout the treatment period (26 weeks) in PTB. While in EPTB, a gradual increase was observed albeit slower than the other markers. These changes observed, due to tuberculosis therapy, were specific to the MTB-specific compartment, since the same markers on total CD4 T cells did not show any difference as compared to the pretreatment status. A plausible reason behind this slow CD27 reversion could be the residual TB disease activity at the end of TB treatment and during LTBI as observed in a recent positron emission tomography computed tomography (PETCT) study [15]. Similar observations were observed in non-human primates with LTBI, where metabolic activity in MTB lesions was observed [117, 118].

Next, we assessed the combination of markers that would provide the most information on treatment and disease/infection status. The CD38 marker was the best at differentiating aTB from LTBI in PTB and correlated with the activation (HLA-DR) and proliferation (Ki67) marker; thus, the determination of CD38 would suffice. CD27, however, was the best at differentiating treated TB from LTBI in PTB, while showing no correlation with CD38. Hence, the simultaneous assessment of both %frequency of CD38 and CD27 on MTB-specific CD4 T cells could help differentiate phenotypically the three MTB statuses as described in table 1.

Where, aTB presented with a high %frequency of CD38+ and low CD27+ on MTB-specific CD4 T cells, while end of treatment presented with a low CD38+ and CD27+ on MTB-specific CD4 T cells and finally LTBI presented with a low CD38+ and high CD27+ on MTB-specific CD4 T cells. However, for EPTB, the fact that only one patient was involved makes this conclusion less concrete, since a gradual increase in the %frequency of the CD27 marker into the LTBI range was observed. The reason behind this could be that the treatment period for EPTB is 12 month as compared to the standard therapy for PTB of 6 months, allowing more time for CD27 to revert [111]. Another reason might be that the patient responded well to treatment, so one could argue that he could have potentially discontinued treatment earlier than at 12 months.[34-36]. Further and more elaborate studies in large clinical cohorts are underway to evaluate this important finding that with the TAM TB assay we may have the first TB specific test that allows treatment monitoring.

Another major hurdle in the tuberculosis pandemic is the duration of treatment. The current standard therapy for pulmonary tuberculosis is 6 months, while that for extra-pulmonary tuberculosis may range from 9-12 months. This hampers compliance and may also lead to psychological and physiological side effects as well as financial burden on the patient. Furthermore, the premature discontinuation of the treatment may lead to the rise of resistant forms of the bacterium. Several studies have tried to address this issue, albeit unsuccessful due to the high relapse in the shorter treatment therapy [34-36]. Current diagnostic tools such as the IGRA, TST, GeneXpert and even the gold standard, the sputum culture method, fail to monitor TB treatment and determine end of treatment. Thus, we tried to obtain a greater understanding at how our markers may be influenced by mycobacterial load by comparing our method with the sputum culture method. Weekly culture results were available for the first 12 weeks, followed by cultures at week 14, 17, 22 and 26. The problem however, is the high contamination rate observed in the sputum culture method, making it very difficult to determine the time to stable culture conversion. Thus, in order to define more accurate treatment end-points, we placed a cut-off, where the time to culture positivity and the time to stable culture negativity (two consecutive culture negative samples), were less than or equal to 4 weeks. Initially, single time-points, post treatment initiation, were used to determine the end of treatment. Individuals that responded well to treatment and could supposedly could end treatment early would be

below a pre-defined cut-off for a specific marker, while those that had to continue treatment, would have a % marker frequency above that cut-off; however no correlation was observed with the time to stable culture conversion. Thus, a different strategy was used, where early changes, from baseline to week 9 or 12, in the TAM expression profiles on MTB-specific CD4 T cells, were calculated and correlated to the sputum culture data. The results correlated and thus, the change in TAM expression profiles might act as a surrogate marker for treatment efficacy, since it reflects the clearance of viable mycobacteria in vivo. This method could circumvent the high contamination rate observed in the sputum culture method.

Diagnostic techniques still under development, such as the measurement of cytokine levels in the plasma of aTB patients, the determination of RNA expression profile of different disease states (transcriptomics) and the identification of novel antigens that can differentiate aTB from LTBI, although interesting with promising results, have unavailable data when it comes to TB treatment monitoring. In addition, genes identified via the transcriptomic method, need to be processed into simpler tests, such as PCR, in order to avoid the sophisticated resources required for these tests.

Current limitations in our study (Ahmed et al. 2018) involved the lack of X-ray scores and disease severity assessment, such as the number of lesions in the lungs, available for the pulmonary TB patients. Although, a high proportion of TB patients are co-infected with HIV, our cohorts did not include any of these patients and thus the effect of HIV on the TAM-TB markers was not assessed. HIV is associated with increased levels of systemic T cell activation; however a previous study has shown little influence on our TAM-TB markers [85, 119, 120]. Larger, well characterized cohorts are needed to clarify the influence of different parameters on the TAM-TB assay results. While the results of the extra-pulmonary patient look promising, the study still represents a single patient for a heterogeneous group of diseases. Furthermore, the cut-off values of the markers on MTB-specific CD4 T cells from the EPTB patient were referenced to PTB patients and thus, modifications need to be made to the cut-off values in order to obtain a better interpretation; since EPTB is a different clinical entity.

Although the TAM-TB assay has several benefits, it remains an expensive and complex technique requiring specialized instrumentation and trained personal to operate. Thus, its use as a point of care diagnostic technique and in resource poor settings may be difficult. Rather, it could be incorporated in reference laboratories. Current studies are looking at simplifying the assay, by using whole blood instead of peripheral blood mononuclear cells [121]. This would be especially important for vulnerable patients such as infants and children, as it reduces the volume of blood required to be drawn. Recently, in order to reduce and simplify the steps required to process the samples thus reducing the hands on time required by the personal, we further developed our whole blood TAM-TB assay. By using whole blood instead of PBMCs we were able to avoid the long process of isolating these cells. Secondly, we developed two pre-prepared lyophilized tubes, one containing our stimulating peptides and the other containing the antibodies for the surface and intracellularly antigens we would like to stain. From our results (Ahmed et al. 2018), we were able to reduce the number of markers required in the assay, thus limiting the number of lasers required to acquire the data by the flow cytometry. Furthermore, by limiting the number of lasers we were able to use instrumentation with a smaller size. We have recently tested this new whole blood assay in the ReFuScreen study, which is based on determining TB incidence within the population of people entering Germany, with positive results [122].

In conclusion, our data on the TAM-TB assay, based on determining treatment efficacy and diagnosing TB using phenotypic profiles on MTB-specific CD4 T cells as a surrogate marker, warrants further research in order to translate the results we obtained into broader clinical application leading to its use in standard clinical settings and during TB drug trials for both PTB and those difficult to diagnose cases, such as EPTB.

As mentioned before, HIV infection is a risk factor for the development of aTB and hence an efficacious HIV vaccine would reduce the TB burden in global regions of HIV endemicity. HIV vaccines that induce strong HIV Env-specific IgG recognition of the variable regions 2 and also 3 are good candidates to be tested in expensive phase 3 HIV vaccine trials. During the Spoke03 trial, the recognition of linear HIV envelope regions by IgG for the standard and accelerated vaccination groups was tested. Our results show that both regimens induce frequent recognition of the same regions within gp120, but virtually no frequent recognition

of any regions within gp41. The accelerated regimen appears to be comparable to the standard regimen with a reduced number of vaccination time points and would therefore be preferable in a real life setting. Furthermore, our results do not provide any evidence that the important V2 region is being recognized. However, several relatively conserved regions within gp120 were strongly recognized, which may contribute to protective efficiency. Several of the same more conserved regions were also targeted during the Rv144 study and recognition of these were not linked at all to the protective efficiency observed in this trial [110]. One possible explanation for this is that these conserved regions are only accessible in the gp120 monomer, but may be occluded in the trimeric Envelope protein, which is present on the infectious HIV virion [123]. Further studies also showed that IgG recognition of the V3 region was broadly cross-reactive. These are promising results, as IgG recognition of Env V3 was associated with protection from HIV acquisition, in an Rv144 subgroup analysis [110].

5- References

1. Ahmed, M.I.M., et al., *Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy.* Front Immunol, 2018. **9**: p. 2247.
2. Joseph, S., et al., *A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes.* Front Immunol, 2017. **8**: p. 149.
3. Ahmed, M.I.M., et al., *The TAM-TB Assay-A Promising TB Immune-Diagnostic Test With a Potential for Treatment Monitoring.* Front Pediatr, 2019. **7**: p. 27.
4. Nadai, Y., et al., *Envelope-Specific Recognition Patterns of HIV Vaccine-Induced IgG Antibodies Are Linked to Immunogen Structure and Sequence.* Front Immunol, 2019. **10**: p. 717.
5. Geldmacher, C., A. Zumla, and M. Hoelscher, *Interaction between HIV and Mycobacterium tuberculosis: HIV-1-induced CD4 T-cell depletion and the development of active tuberculosis.* Curr Opin HIV AIDS, 2012. **7**(3): p. 268-75.
6. *Global Tuberculosis Control: A short update to the 2009 report;Geneva: World Health Organization.* 2009.
7. *Global Tuberculosis Report 2018. Geneva: World Health Organization.* 2018.
8. Economic, U.N.D.o. and S. Affairs, *The Sustainable Development Goals Report 2018*. 2018.
9. Piccini, P., et al., *Clinical peculiarities of tuberculosis.* BMC Infect Dis, 2014. **14 Suppl 1**: p. S4.
10. Geldmacher, C., et al., *Preferential infection and depletion of Mycobacterium tuberculosis-specific CD4 T cells after HIV-1 infection.* J Exp Med, 2010. **207**(13): p. 2869-81.
11. Gallegos, A.M., E.G. Pamer, and M.S. Glickman, *Delayed protection by ESAT-6-specific effector CD4+ T cells after airborne M. tuberculosis infection.* J Exp Med, 2008. **205**(10): p. 2359-68.
12. Barry, C.E., 3rd, et al., *The spectrum of latent tuberculosis: rethinking the biology and intervention strategies.* Nat Rev Microbiol, 2009. **7**(12): p. 845-55.
13. Chee, C.B.E., et al., *Latent tuberculosis infection: Opportunities and challenges.* Respirology, 2018. **23**(10): p. 893-900.
14. Esmail, H., et al., *Characterization of progressive HIV-associated tuberculosis using 2-deoxy-2-[(18)F]fluoro-D-glucose positron emission and computed tomography.* Nat Med, 2016. **22**(10): p. 1090-1093.
15. Malherbe, S.T., et al., *Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure.* Nat Med, 2016. **22**(10): p. 1094-1100.
16. Schnappinger, D. and S. Ehrt, *A broader spectrum of tuberculosis.* Nat Med, 2016. **22**(10): p. 1076-1077.
17. Drain, P.K., et al., *Incipient and Subclinical Tuberculosis: a Clinical Review of Early Stages and Progression of Infection.* Clin Microbiol Rev, 2018. **31**(4).
18. Silva Miranda, M., et al., *The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria?* Clin Dev Immunol, 2012. **2012**: p. 139127.
19. Gideon, H.P., et al., *Variability in tuberculosis granuloma T cell responses exists, but a balance of pro- and anti-inflammatory cytokines is associated with sterilization.* PLoS Pathog, 2015. **11**(1): p. e1004603.
20. Todd, R.M., *Treatment of primary pulmonary tuberculosis with P.A.S.* Br Med J, 1953. **1**(4822): p. 1247-9.
21. Schatz, A., E. Bugie, and S.A. Waksman, *Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944.* Clin Orthop Relat Res, 2005(437): p. 3-6.

22. Gler, M.T., et al., *Delamanid for multidrug-resistant pulmonary tuberculosis.* N Engl J Med, 2012. **366**(23): p. 2151-60.
23. Murray, J.F., D.E. Schraufnagel, and P.C. Hopewell, *Treatment of Tuberculosis. A Historical Perspective.* Ann Am Thorac Soc, 2015. **12**(12): p. 1749-59.
24. Chakraborty, S. and K.Y. Rhee, *Tuberculosis Drug Development: History and Evolution of the Mechanism-Based Paradigm.* Cold Spring Harb Perspect Med, 2015. **5**(8): p. a021147.
25. *Guidelines for treatment of drug-susceptible tuberculosis and patient care, 2017 update. Geneva: World Health Organization;.* 2017.
26. Romanowski, K., et al., *Predicting tuberculosis relapse in patients treated with the standard 6-month regimen: an individual patient data meta-analysis.* Thorax, 2019. **74**(3): p. 291-297.
27. Long, Q., et al., *Patient medical costs for tuberculosis treatment and impact on adherence in China: a systematic review.* BMC Public Health, 2011. **11**: p. 393.
28. Saukkonen, J.J., et al., *An official ATS statement: hepatotoxicity of antituberculosis therapy.* Am J Respir Crit Care Med, 2006. **174**(8): p. 935-52.
29. Teo, S.K., K.K. Tan, and T.K. Khoo, *Four-month chemotherapy in the treatment of smear-negative pulmonary tuberculosis: results at 30 to 60 months.* Ann Acad Med Singapore, 2002. **31**(2): p. 175-81.
30. *A controlled trial of 3-month, 4-month, and 6-month regimens of chemotherapy for sputum-smear-negative pulmonary tuberculosis. Results at 5 years. Hong Kong Chest Service/Tuberculosis Research Centre, Madras/British Medical Research Council.* Am Rev Respir Dis, 1989. **139**(4): p. 871-6.
31. Chan, S.L., P.C. Wong, and C.M. Tam, *4-, 5- and 6-month regimens containing isoniazid, rifampicin, pyrazinamide and streptomycin for treatment of pulmonary tuberculosis under program conditions in Hong Kong.* Tuber Lung Dis, 1994. **75**(4): p. 245-50.
32. Dutt, A.K., D. Moers, and W.W. Stead, *Smear- and culture-negative pulmonary tuberculosis: four-month short-course chemotherapy.* Am Rev Respir Dis, 1989. **139**(4): p. 867-70.
33. Hamada, Y., et al., *Proposed Approach for 4-Month Treatment of Culture-Negative Pulmonary Tuberculosis in Adults.* Ann Am Thorac Soc, 2016. **13**(9): p. 1657-8.
34. Jindani, A., et al., *High-dose rifapentine with moxifloxacin for pulmonary tuberculosis.* N Engl J Med, 2014. **371**(17): p. 1599-608.
35. Gillespie, S.H., et al., *Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis.* N Engl J Med, 2014. **371**(17): p. 1577-87.
36. Merle, C.S., et al., *A four-month gatifloxacin-containing regimen for treating tuberculosis.* N Engl J Med, 2014. **371**(17): p. 1588-98.
37. Fox, W., G.A. Ellard, and D.A. Mitchison, *Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications.* Int J Tuberc Lung Dis, 1999. **3**(10 Suppl 2): p. S231-79.
38. Chabala, C., et al., *Shorter treatment for minimal tuberculosis (TB) in children (SHINE): a study protocol for a randomised controlled trial.* Trials, 2018. **19**(1): p. 237.
39. Nayak, S. and B. Acharjya, *Mantoux test and its interpretation.* Indian Dermatol Online J, 2012. **3**(1): p. 2-6.
40. Davis, J.L., et al., *Diagnostic accuracy of same-day microscopy versus standard microscopy for pulmonary tuberculosis: a systematic review and meta-analysis.* Lancet Infect Dis, 2013. **13**(2): p. 147-54.
41. Singhal, R. and V.P. Myneedu, *Microscopy as a diagnostic tool in pulmonary tuberculosis.* Int J Mycobacteriol, 2015. **4**(1): p. 1-6.
42. Cattamanchi, A., et al., *Sensitivity of direct versus concentrated sputum smear microscopy in HIV-infected patients suspected of having pulmonary tuberculosis.* BMC Infect Dis, 2009. **9**: p. 53.
43. Sloan, D.J., et al., *Optimizing outpatient serial sputum colony counting for studies of tuberculosis treatment in resource-poor settings.* J Clin Microbiol, 2012. **50**(7): p. 2315-20.

44. Zar, H.J., et al., *Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study.* Lancet, 2005. **365**(9454): p. 130-4.
45. Perez-Velez, C.M. and B.J. Marais, *Tuberculosis in children.* N Engl J Med, 2012. **367**(4): p. 348-61.
46. Marais, B.J., et al., *The bacteriologic yield in children with intrathoracic tuberculosis.* Clin Infect Dis, 2006. **42**(8): p. e69-71.
47. Horne, D.J., et al., *Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis.* Lancet Infect Dis, 2010. **10**(6): p. 387-94.
48. Lalvani, A. and M. Pareek, *Interferon gamma release assays: principles and practice.* Enferm Infecc Microbiol Clin, 2010. **28**(4): p. 245-52.
49. Hofland, R.W., et al., *QuantiFERON-plus does not discriminate between active and latent tuberculosis.* Infect Dis (Lond), 2018. **50**(6): p. 479-482.
50. Bartalesi, F., et al., *The role of Quantiferon-TB Gold in-Tube in the diagnosis and treatment monitoring of active tuberculosis.* Infect Dis (Lond), 2017. **49**(6): p. 474-477.
51. Du, F., et al., *Prospective Comparison of QFT-GIT and T-SPOT.TB Assays for Diagnosis of Active Tuberculosis.* Sci Rep, 2018. **8**(1): p. 5882.
52. Petrone, L., et al., *Evaluation of IP-10 in Quantiferon-Plus as biomarker for the diagnosis of latent tuberculosis infection.* Tuberculosis (Edinb), 2018. **111**: p. 147-153.
53. Borkowska, D.I., et al., *From Latent Tuberculosis Infection to Tuberculosis. News in Diagnostics (QuantiFERON-Plus).* Pol J Microbiol, 2017. **66**(1): p. 5-8.
54. Lawn, S.D. and M.P. Nicol, *Xpert(R) MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance.* Future Microbiol, 2011. **6**(9): p. 1067-82.
55. Friedrich, S.O., et al., *Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment.* Lancet Respir Med, 2013. **1**(6): p. 462-70.
56. Kohli, M., et al., *Xpert((R)) MTB/RIF assay for extrapulmonary tuberculosis and rifampicin resistance.* Cochrane Database Syst Rev, 2018. **8**: p. CD012768.
57. Hougardy, J.M., et al., *Heparin-binding-hemagglutinin-induced IFN-gamma release as a diagnostic tool for latent tuberculosis.* PLoS One, 2007. **2**(10): p. e926.
58. Wyndham-Thomas, C., et al., *Key role of effector memory CD4+ T lymphocytes in a short-incubation heparin-binding hemagglutinin gamma interferon release assay for the detection of latent tuberculosis.* Clin Vaccine Immunol, 2014. **21**(3): p. 321-8.
59. Sali, M., et al., *Combined use of Quantiferon and HBHA-based IGRA supports tuberculosis diagnosis and therapy management in children.* J Infect, 2018. **77**(6): p. 526-533.
60. Chiacchio, T., et al., *Immune characterization of the HBHA-specific response in Mycobacterium tuberculosis-infected patients with or without HIV infection.* PLoS One, 2017. **12**(8): p. e0183846.
61. Delogu, G., et al., *Lack of Response to HBHA in HIV-Infected Patients with Latent Tuberculosis Infection.* Scand J Immunol, 2016. **84**(6): p. 344-352.
62. Meier, N.R., et al., *A Systematic Review on Novel Mycobacterium tuberculosis Antigens and Their Discriminatory Potential for the Diagnosis of Latent and Active Tuberculosis.* Front Immunol, 2018. **9**: p. 2476.
63. Santos, V.S., et al., *Acute phase proteins and IP-10 as triage tests for the diagnosis of tuberculosis: systematic review and meta-analysis.* Clin Microbiol Infect, 2019. **25**(2): p. 169-177.
64. Sigal, G.B., et al., *Biomarkers of Tuberculosis Severity and Treatment Effect: A Directed Screen of 70 Host Markers in a Randomized Clinical Trial.* EBioMedicine, 2017. **25**: p. 112-121.
65. Zhao, Y., et al., *IP-10 and RANTES as biomarkers for pulmonary tuberculosis diagnosis and monitoring.* Tuberculosis (Edinb), 2018. **111**: p. 45-53.

66. Clifford, V., et al., *Cytokine biomarkers for the diagnosis of tuberculosis infection and disease in adults in a low prevalence setting.* Tuberculosis (Edinb), 2019. **114**: p. 91-102.
67. Tebruegge, M., et al., *Mycobacteria-Specific Cytokine Responses Detect Tuberculosis Infection and Distinguish Latent from Active Tuberculosis.* Am J Respir Crit Care Med, 2015. **192**(4): p. 485-99.
68. Leem, A.Y., et al., *Changes in cytokine responses to TB antigens ESAT-6, CFP-10 and TB 7.7 and inflammatory markers in peripheral blood during therapy.* Sci Rep, 2018. **8**(1): p. 1159.
69. Sun, Q., W. Wei, and W. Sha, *Potential Role for Mycobacterium tuberculosis Specific IL-2 and IFN-gamma Responses in Discriminating between Latent Infection and Active Disease after Long-Term Stimulation.* PLoS One, 2016. **11**(12): p. e0166501.
70. Wang, S., et al., *Screening and identification of a six-cytokine biosignature for detecting TB infection and discriminating active from latent TB.* J Transl Med, 2018. **16**(1): p. 206.
71. von Both, U., et al., *Understanding immune protection against tuberculosis using RNA expression profiling.* Vaccine, 2015. **33**(40): p. 5289-93.
72. Berry, M.P., et al., *An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis.* Nature, 2010. **466**(7309): p. 973-7.
73. Bloom, C.I., et al., *Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers.* PLoS One, 2013. **8**(8): p. e70630.
74. Bloom, C.I., et al., *Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy.* PLoS One, 2012. **7**(10): p. e46191.
75. Kaforou, M., et al., *Detection of tuberculosis in HIV-infected and -uninfected African adults using whole blood RNA expression signatures: a case-control study.* PLoS Med, 2013. **10**(10): p. e1001538.
76. Anderson, S.T., et al., *Diagnosis of childhood tuberculosis and host RNA expression in Africa.* N Engl J Med, 2014. **370**(18): p. 1712-1723.
77. Sweeney, T.E., et al., *Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis.* Lancet Respir Med, 2016. **4**(3): p. 213-24.
78. Warsinske, H.C., et al., *Assessment of Validity of a Blood-Based 3-Gene Signature Score for Progression and Diagnosis of Tuberculosis, Disease Severity, and Treatment Response.* JAMA Netw Open, 2018. **1**(6): p. e183779.
79. Zak, D.E., et al., *A blood RNA signature for tuberculosis disease risk: a prospective cohort study.* Lancet, 2016. **387**(10035): p. 2312-2322.
80. Burel, J.G., et al., *Transcriptomic Analysis of CD4(+) T Cells Reveals Novel Immune Signatures of Latent Tuberculosis.* J Immunol, 2018. **200**(9): p. 3283-3290.
81. Singhania, A., et al., *The value of transcriptomics in advancing knowledge of the immune response and diagnosis in tuberculosis.* Nat Immunol, 2018. **19**(11): p. 1159-1168.
82. Burel, J.G., et al., *Host Transcriptomics as a Tool to Identify Diagnostic and Mechanistic Immune Signatures of Tuberculosis.* Frontiers in Immunology, 2019. **10**(221).
83. Streitz, M., et al., *Tuberculin-specific T cells are reduced in active pulmonary tuberculosis compared to LTBI or status post BCG vaccination.* J Infect Dis, 2011. **203**(3): p. 378-82.
84. Adekambi, T., et al., *Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response.* J Clin Invest, 2015. **125**(5): p. 1827-38.
85. Riou, C., et al., *Analysis of the Phenotype of Mycobacterium tuberculosis-Specific CD4+ T Cells to Discriminate Latent from Active Tuberculosis in HIV-Uninfected and HIV-Infected Individuals.* Front Immunol, 2017. **8**: p. 968.
86. Rozot, V., et al., *Combined use of Mycobacterium tuberculosis-specific CD4 and CD8 T-cell responses is a powerful diagnostic tool of active tuberculosis.* Clin Infect Dis, 2015. **60**(3): p. 432-7.
87. Day, C.L., et al., *PD-1 Expression on Mycobacterium tuberculosis-Specific CD4 T Cells Is Associated With Bacterial Load in Human Tuberculosis.* Front Immunol, 2018. **9**: p. 1995.

88. Appay, V., et al., *Phenotype and function of human T lymphocyte subsets: consensus and issues.* Cytometry A, 2008. **73**(11): p. 975-83.
89. Kapina, M.A., et al., *CD27low CD4 T lymphocytes that accumulate in the mouse lungs during mycobacterial infection differentiate from CD27high precursors in situ, produce IFN-gamma, and protect the host against tuberculosis infection.* J Immunol, 2007. **178**(2): p. 976-85.
90. Lyadova, I.V., et al., *CD4 T cells producing IFN-gamma in the lungs of mice challenged with mycobacteria express a CD27-negative phenotype.* Clin Exp Immunol, 2004. **138**(1): p. 21-9.
91. Streitz, M., et al., *Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis.* PLoS One, 2007. **2**(8): p. e735.
92. Schuetz, A., et al., *Monitoring CD27 expression to evaluate Mycobacterium tuberculosis activity in HIV-1 infected individuals in vivo.* PLoS One, 2011. **6**(11): p. e27284.
93. Nikitina, I.Y., et al., *Mtb-specific CD27low CD4 T cells as markers of lung tissue destruction during pulmonary tuberculosis in humans.* PLoS One, 2012. **7**(8): p. e43733.
94. Portevin, D., et al., *Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study.* Lancet Infect Dis, 2014. **14**(10): p. 931-8.
95. Jiang, J., et al., *Reduced CD27 expression on antigen-specific CD4+ T cells correlates with persistent active tuberculosis.* J Clin Immunol, 2010. **30**(4): p. 566-73.
96. Gadallah, M., et al., *Evaluation of CD27 Expression on Mycobacterial AntigenSpecific CD4+ T Cells as an Immunological Marker for Diagnosis of Active Pulmonary Tuberculosis*. Vol. 27. 2018.
97. Day, C.L., et al., *Patients with tuberculosis disease have Mycobacterium tuberculosis-specific CD8 T cells with a pro-apoptotic phenotype and impaired proliferative capacity, which is not restored following treatment.* PLoS One, 2014. **9**(4): p. e94949.
98. Wilkinson, K.A., et al., *Activation Profile of Mycobacterium tuberculosis-Specific CD4(+) T Cells Reflects Disease Activity Irrespective of HIV Status.* Am J Respir Crit Care Med, 2016. **193**(11): p. 1307-10.
99. Latorre, I., et al., *Study of CD27 and CCR4 Markers on Specific CD4(+) T-Cells as Immune Tools for Active and Latent Tuberculosis Management.* Front Immunol, 2018. **9**: p. 3094.
100. Silveira-Mattos, P.S., et al., *Differential expression of CXCR3 and CCR6 on CD4(+) T-lymphocytes with distinct memory phenotypes characterizes tuberculosis-associated immune reconstitution inflammatory syndrome.* Sci Rep, 2019. **9**(1): p. 1502.
101. Yang, Q., et al., *Discriminating Active Tuberculosis from Latent Tuberculosis Infection by flow cytometric measurement of CD161-expressing T cells.* Sci Rep, 2015. **5**: p. 17918.
102. Yang, B., et al., *Involvement of CD244 in regulating CD4+ T cell immunity in patients with active tuberculosis.* PLoS One, 2013. **8**(4): p. e63261.
103. Saharia, K.K., et al., *Tuberculosis Therapy Modifies the Cytokine Profile, Maturation State, and Expression of Inhibitory Molecules on Mycobacterium tuberculosis-Specific CD4+ T-Cells.* PLoS One, 2016. **11**(7): p. e0158262.
104. Adekambi, T., et al., *Distinct effector memory CD4+ T cell signatures in latent Mycobacterium tuberculosis infection, BCG vaccination and clinically resolved tuberculosis.* PLoS One, 2012. **7**(4): p. e36046.
105. Harari, A., et al., *Dominant TNF-alpha+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease.* Nat Med, 2011. **17**(3): p. 372-6.
106. Kim, C.H., et al., *Comparative analysis of whole-blood interferon-gamma and flow cytometry assays for detecting post-treatment immune responses in patients with active tuberculosis.* Cytometry B Clin Cytom, 2014. **86**(4): p. 236-43.
107. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. **361**(23): p. 2209-20.

108. Haynes, B.F., et al., *Immune-correlates analysis of an HIV-1 vaccine efficacy trial.* N Engl J Med, 2012. **366**(14): p. 1275-86.
109. Zolla-Pazner, S., et al., *Analysis of V2 antibody responses induced in vaccinees in the ALVAC/AIDSVAX HIV-1 vaccine efficacy trial.* PLoS One, 2013. **8**(1): p. e53629.
110. Gottardo, R., et al., *Plasma IgG to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlate with a reduced risk of infection in the RV144 vaccine efficacy trial.* PLoS One, 2013. **8**(9): p. e75665.
111. Petruccioli, E., et al., *Assessment of CD27 expression as a tool for active and latent tuberculosis diagnosis.* J Infect, 2015. **71**(5): p. 526-33.
112. Doan, T.N., et al., *Interferon-gamma release assay for the diagnosis of latent tuberculosis infection: A latent-class analysis.* PLoS One, 2017. **12**(11): p. e0188631.
113. Wallis, R.S., et al., *Tuberculosis biomarkers discovery: developments, needs, and challenges.* Lancet Infect Dis, 2013. **13**(4): p. 362-72.
114. Sabi, I., et al., *Xpert MTB/RIF Ultra assay for the diagnosis of pulmonary tuberculosis in children: a multicentre comparative accuracy study.* J Infect, 2018. **77**(4): p. 321-327.
115. Dorman, S.E., et al., *Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study.* Lancet Infect Dis, 2018. **18**(1): p. 76-84.
116. Lawn, S.D., B. Afful, and J.W. Acheampong, *Pulmonary tuberculosis: diagnostic delay in Ghanaian adults.* Int J Tuberc Lung Dis, 1998. **2**(8): p. 635-40.
117. Capuano, S.V., 3rd, et al., *Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection.* Infect Immun, 2003. **71**(10): p. 5831-44.
118. Coleman, M.T., et al., *Early Changes by (18)Fluorodeoxyglucose positron emission tomography coregistered with computed tomography predict outcome after Mycobacterium tuberculosis infection in cynomolgus macaques.* Infect Immun, 2014. **82**(6): p. 2400-4.
119. Hazenberg, M.D., et al., *T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART).* Blood, 2000. **95**(1): p. 249-55.
120. Giorgi, J.V., et al., *Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage.* J Infect Dis, 1999. **179**(4): p. 859-70.
121. Musvosvi, M., et al., *T-cell biomarkers for diagnosis of tuberculosis: candidate evaluation by a simple whole blood assay for clinical translation.* Eur Respir J, 2018. **51**(3).
122. *The T-Cell Activation Marker for TB (TAM-TB): A New Diagnostic Tool for Paediatric TB*, in *11th ESPID Research Masterclass*. 2019: Ljubljana, Slovenia.
123. Nadai, Y., et al., *Envelope-Specific Recognition Patterns of HIV Vaccine-Induced IgG Antibodies Are Linked to Immunogen Structure and Sequence.* Frontiers in Immunology, 2019. **10**(717).

6- Appendix

[1] Ahmed MIM, Ntinginya NE, Kibiki G, Mtafya BA, Semvua H, Mpagama S, Mtabho C, Saathoff E, Held K, Loose R, Kroidl I, Chachage M, von Both U, Haule A, Mekota A-M, Boeree MJ, Gillespie SH, Hoelscher M, Heinrich N and Geldmacher C (2018) Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy. *Front. Immunol*. 9:2247. doi: 10.3389/fimmu.2018.02247

[2] Joseph S, Quinn K, Greenwood A, Cope AV, McKay PF, Hayes PJ, Kopycinski JT, Gilmour J, Miller AN, Geldmacher C, Nadai Y, Ahmed MIM, Montefiori DC, Dally L, Bouliotis G, Lewis DJM, Tatoud R, Wagner R, Esteban M, Shattock RJ, McCormack S and Weber J (2017) A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes. *Front. Immunol.* 8:149. doi: 10.3389/fimmu.2017.00149

[3] Ahmed MIM, Ziegler C, Held K, Dubinski I, Ley-Zaporozhan J, Geldmacher C and von Both U (2019) The TAM-TB Assay—A Promising TB Immune-Diagnostic Test With a Potential for Treatment Monitoring. *Front. Pediatr.* 7:27. doi: 10.3389/fped.2019.00027

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 September 2018
doi: 10.3389/fimmu.2018.02247

Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy

Mohamed I. M. Ahmed[1,2,3], Nyanda E. Ntinginya[4], Gibson Kibiki[5], Bariki A Mtafya[4], Hadija Semvua[5], Stellah Mpagama[5], Charles Mtabho[5], Elmar Saathoff[1,2], Kathrin Held[1,2], Rebecca Loose[1,2], Inge Kroidl[1,2], Mkunde Chachage[1,4], Ulrich von Both[1,2,6], Antelmo Haule[4], Anna-Maria Mekota[1,2], Martin J. Boeree[7], Stephen H. Gillespie[8], Michael Hoelscher[1,2†], Norbert Heinrich[1,2†] and Christof Geldmacher[1,2] on behalf of the Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics (PanACEA)*

[1] Division of Infectious Diseases and Tropical Medicine, Klinikum of the University of Munich (LMU), Munich, Germany, [2] German Center for Infection Research (DZIF), Partner Site Munich, Munich, Germany, [3] CIH LMU Center for International Health, Klinikum of the University of Munich, Munich, Germany, [4] National Institute for Medical Research—Mbeya Medical Research Center, Mbeya, Tanzania, [5] Kilimanjaro Christian Medical Centre, Kilimanjaro Clinical Research Institute, Moshi, Tanzania, [6] Dr. von Hauner Children's Hospital, Division of Paediatric Infectious Diseases, Klinikum of the University of Munich (LMU), Munich, Germany, [7] Department of Lung Diseases, Radboud University Medical Centre Nijmegen, Nijmegen, Netherlands, [8] Infection and Global Health Research Division, University of St Andrews, St Andrews, United Kingdom

OPEN ACCESS

Edited by:
Heinrich Korner,
University of Tasmania, Australia

Reviewed by:
Ronan O'Toole,
University of Tasmania, Australia
Florian Kern,
Brighton and Sussex Medical School, United Kingdom
Stephanie Wehrstedt,
Universitätsklinikum Ulm, Germany

***Correspondence:**
Christof Geldmacher
geldmacher@lrz.uni-muenchen.de

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: 17 April 2018
Accepted: 10 September 2018
Published: 28 September 2018

Citation:
Ahmed MIM, Ntinginya NE, Kibiki G, Mtafya BA, Semvua H, Mpagama S, Mtabho C, Saathoff E, Held K, Loose R, Kroidl I, Chachage M, von Both U, Haule A, Mekota A-M, Boeree MJ, Gillespie SH, Hoelscher M, Heinrich N and Geldmacher C (2018) Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy. Front. Immunol. 9:2247. doi: 10.3389/fimmu.2018.02247

Background: The analysis of phenotypic characteristics on *Mycobacterium tuberculosis* (MTB)-specific T cells is a promising approach for the diagnosis of active tuberculosis (aTB) and for monitoring treatment success. We therefore studied phenotypic changes on MTB-specific CD4 T cells upon anti-tuberculosis treatment initiation in relation to the treatment response as determined by sputum culture.

Methods: Peripheral blood mononuclear cells from subjects with latent MTB infection ($n = 16$) and aTB ($n = 39$) at baseline, weeks 9, 12, and 26 (end of treatment) were analyzed after intracellular interferon gamma staining and overnight stimulation with tuberculin. Liquid sputum cultures were performed weekly until week 12 and during 4 visits until week 26.

Results: T cell activation marker expression on MTB-specific CD4 T cells differed significantly between subjects with aTB and latent MTB infection with no overlap for the frequencies of $CD38^{pos}$ and $Ki67^{pos}$ cells (both $p < 0.0001$). At 9 weeks after anti-TB treatment initiation the frequencies of activation marker (CD38, HLA-DR, Ki67) positive MTB-specific, but not total CD4 T cells, were significantly reduced ($p < 0.0001$). Treatment induced phenotypic changes from baseline until week 9 and until week 12 differed substantially between individual aTB patients and correlated with an individual's time to stable sputum culture conversion for expression of CD38 and HLA-DR (both $p < 0.05$). In contrast, the frequencies of maturation marker CD27 positive MTB-specific CD4 T cells remained largely unchanged until week 26 and significantly differed between subjects with treated TB disease and latent MTB infection ($p = 0.0003$).

Discussion: Phenotypic changes of MTB-specific T cells are potential surrogate markers for tuberculosis treatment efficacy and can help to discriminate between aTB (profile: $CD38^{pos}$, $CD27^{low}$), treated TB ($CD38^{neg}$, $CD27^{low}$), and latent MTB infection ($CD38^{neg}$, $CD27^{high}$).

Keywords: TAM-TB assay, tuberculosis, treatment monitoring, Mycobacterium tuberculosis-specific T cells, serial sputum culture, biomarker

INTRODUCTION

Novel diagnostic tools for improved detection of active tuberculosis (aTB) and for monitoring TB treatment are urgently required to succeed in the WHO END TB strategy, which—in the abscence of an efficacious MTB vaccine - sets the ambitious target of a world free of tuberculosis by 2030 (1). Recent studies have highlighted the diagnostic potential of a flow cytometry based approach to detect and differentiate aTB disease from latent *Mycobacterium tuberculosis* (MTB) infection (LTBI) via phenotypic and/or functional characterization of MTB-specific T cells in adults and children (2–12). These "T cell activation and maturation marker assays" (TAM-TB assay) are sputum-independent, use easy-to-collect peripheral blood and—in contrast to the traditional immunodiagnostic Tuberculin skin test or Interferon gamma release assays (13)—allow highly specific detection of aTB (3, 5). TAM-TB assay results have been correlated with MTB loads in sputum (4, 9), with disease severity and with lung tissue destruction (4). Our previous study showed highly specific detection of childhood aTB in an endemic setting (3), potentially superior to sputum culture. Furthermore, TB treatment initiation decreases activation marker expression on MTB-specific CD4 T cells, probably reflecting the decrease of mycobacterial burden *in vivo* (5); which would make this a promising candidate marker for assessing TB treatment success.

While liquid culture and PCR are held to be the most sensitive tools to detect MTB, their widespread implementation for diagnosis and treatment monitoring is hampered by practical and methodological problems. Firstly, since these methods function by direct detection of the pathogen, they often remain false negative in paucibacillery aTB patients (14–16), and those where aTB lesions do not have access to the airways. As a consequence, TB treatment is often started on a presumptive diagnosis (17). Secondly, culture and PCR have shortcomings for monitoring of the TB treatment response. MTB culture methods have low sensitivity for unfavorable outcome and low positive predictive value estimates (18). The GeneXpert PCR shows a lag of positivity most likely due to detection of dead bacilli (19). The current treatment duration is that of a "one-size-fits-all" 6-months drug regimen without modifications based on treatment response monitoring. Past trials conducted by the MRC East Africa, and the more recent fluoroquinolone phase 3 studies, have demonstrated that more than 80% of TB patients will achieve cure after only 4 months of treatment (20–23). However to introduce a 4-months treatment as a blanket approach, it will be essential to discriminate between aTB patients who achieve cure already after 4 months and those in need of longer treatment. Sequential sputum bacterial load measurements by culture have been tested in this regard, but have insufficient sensitivity for detection of unfavorable treatment outcome on an individual basis (19, 24). Together, these shortcomings in mycobacteriological detection methods can impede accurate diagnosis of aTB, meaningful TB treatment monitoring and safe individualized treatment (21–23). The novel TAM-TB assay approach could potentially improve TB diagnosis and treatment monitoring; and hence help to overcome some of the challenges affecting diagnosis solely based on the direct detection of MTB bacilli in sputum. A prerequisite, herefore is a more detailed understanding of the relationship between TAM-TB assay results, the MTB infection status, and mycobacterial treatment response.

Here, we have therefore studied activation (CD38, HLA-DR, and Ki67) and maturation (CD27) marker profiles on IFNγ+ MTB-specific CD4 T cells in subjects with LTBI, and in aTB patients (25) before and after TB treatment initiation in comparison to the mycobacteriological treatment response. The patients were tightly monitored using MGIT culture on a weekly basis until week 12 and on 4 additional time points until the end of treatment at week 26 and showed no relapse during a 6 months follow-up after the end of treatment.

MATERIALS AND METHODS

Study Populations, Study Samples, and Ethics Statements

HIV^{neg} adult patients with culture confirmed aTB were enrolled at two clinical sites in Tanzania (NIMR-MMRC, Mbeya, and KCRI, Moshi) within the Panacea-TB MAMS treatment study (**Table 1**, **Figure 1**; clinicaltrials.gov identifier NCT01785186) (26). Four experimental treatments or the standard TB treatment were given for 12 weeks, followed by 8 weeks of standard treatment (rifampicin, isoniazid, pyrazinamide, and ethambutol) and by standard dose rifampicin and isoniazid to complete

TABLE 1 | Demography of study subjects.

	PanACEA	HISIS
Gender (Male/Female)	30/9	0/16
Median Age (Range)	34.4 (19.2–65.4)	32 (17.9–38)
Median BMI (Range)	19.2 (15.2–41.6)	not determined
HIV status (+/–)	0/39	0/16
AFB sputum smear positivity grade (1/2/3)	3/10/26	Not applicable
Median Days to Positivity Baseline (Range)	3.5 (1–7.5)	Not applicable

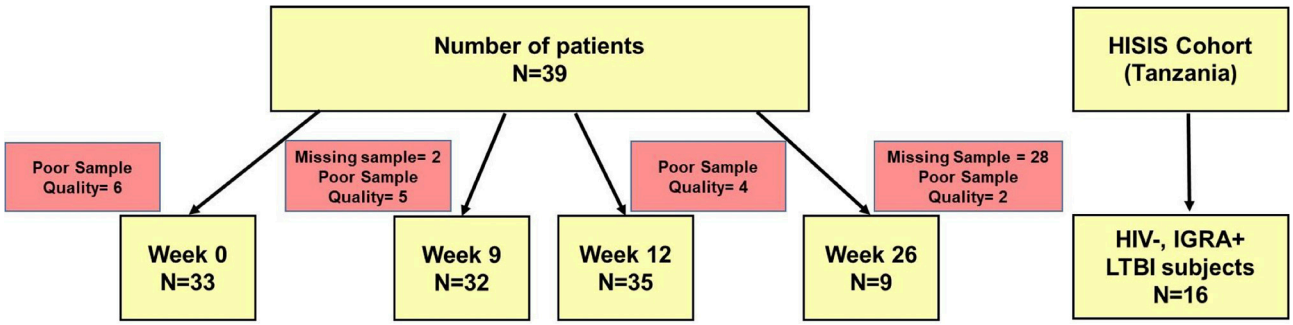

FIGURE 1 | Diagram of study subjects, time points and TAM-TB analyses. Peripheral blood mononuclear cell samples from aTB patients ($n = 39$) were collected at baseline, at week 9, 12, and 26 after TB treatment initiation and subjected to TAM-TB assay analyses. The numbers of missing samples and those excluded due to none responsiveness to the positive control antigen SEB ("poor quality") are indicated for each time point. PBMC samples ($n = 16$) from HIV- IGRA+ subjects were collected during the HISIS cohort study.

26 weeks of treatment. All study participants received further follow-up for 6 months after end of treatment by telephonic interviews and site visits. Of the patients included in this sub-study, 25 were in either one of the experimental treatment arms, whereas 14 subjects received the standard treatment. None of the patients relapsed during this 6-months follow-up. The protocol was approved by independent ethics committees of the sponsor, the trial sites, and the regulatory authorities of Tanzania and South Africa. Additional samples from HIV^{neg} IGRA+ healthy female bar workers from the HISIS study (27) were included as LTBI controls ($n = 16$). The HISIS study was conducted at the NIMR-MMRC in compliance with national guidelines and institutional policies, and informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the local ethic board at Mbeya (FWA no. 00002469) and the National Ethic Board at the National Institute for Medical Research (FWA no. 00002632).

TAM-TB Assay

Cryopreserved Peripheral Blood Mononuclear Cell (PBMC) samples from aTB patients ($n = 39$, **Figure 1**) from baseline, 9, 12, and 26 weeks were analyzed using the TAM-TB assay approach. PBMC samples from subjects with LTBI ($n = 16$) were included as additional controls. PBMCs were stimulated overnight at 37°C and 5% CO_2 with Purified Protein Derivative (PPD, 10 μg/ml, Serum Staten Institute), SEB (0.6 μg/ml, Sigma-Aldrich) as a positive control, or no added peptide as negative control in the presence of Brefeldin A (BFA, final concentration 5 μg/ml, Sigma) and the costimulatory antibodies anti-CD49d (L25, BD) and anti-CD28 (L293, BD). Cells were stained with anti-CD38 BV785 (clone HIT2, Biolegend), anti-CD4 APC (clone 13B8.2, Beckmann Coulter), anti-CD27 ECD (clone 1A4CD27, Beckmann Coulter), and anti-HLA-DR APC-H7 (clone G46-6, Beckmann Dickinson), followed by fixation and permeabilization using FoxP3 Perm/Fix buffer and diluent (eBioscience), and then stained intracellularly using anti-IFNγ FITC (clone B27, BD Pharmingen), anti-Ki67 BV421 (clone B56, BD Pharmingen), and anti-CD3 APC-A700 (clone UCHT1, Beckmann Coulter). Cells were acquired on a CytoFlex Flow cytometer (Beckman Coulter). Data analysis was performed using FlowJo_V10. MTB-specific CD4 T cell responses were defined by a frequency of ≥0.03% of IFNγ+ CD4 T cells after PPD stimulation and by ≥2-fold increase over the negative control. Furthermore, a cell count of greater than 25 IFNγ+ CD4 T cell events had to be recorded. Samples with no response to the positive control antigen Staphylococcal enterotoxin B (SEB) were excluded from the analyses ($n = 17$). Pestle and Spice software (28)were used to analyze combinatorial expresssion of the four phenotypic markers on IFNγ+ MTB-specific CD4 T cells.

Bacteriological Assessments

Patients submitted sputum during weekly visits until week 12, and at weeks 14, 17, 22, and 26. Sputum was decontaminated with NaCl-OH, and cultured in liquid media; the mycobacterial growth indicator tube (Bactec MGIT960), and on Löwenstein-Jensen (LJ) solid medium (22). The primary study endpoint was time from treatment initiation to the first of two consecutive negative weekly sputum cultures without an intervening positive or contaminated culture in liquid media.

Statistical Analysis

The Statistical analysis was performed using GraphPad prism software version 6. The tests used are indicated in the Figure Legends.

RESULTS

T Cell Activation Marker Expression Profiles on MTB-Specific CD4 T Cells Differentiate Between aTB and LTBI

At time of diagnosis, all aTB patients had detectable MTB-specific CD4 T cell responses upon PPD re-stimulation (median: 0.22%, range 0.03–4.3%). None-stimulated controls showed no or very little background in $IFN\gamma^+$ T cells (median: 0.008%, range 0.0–0.066%). SEB stimulated controls showed high frequencies of IFNg+ CD4 T cells (median: 1.92%, range: 0.6–9.3%). High frequencies of MTB-specific CD4 T cells expressed the activation

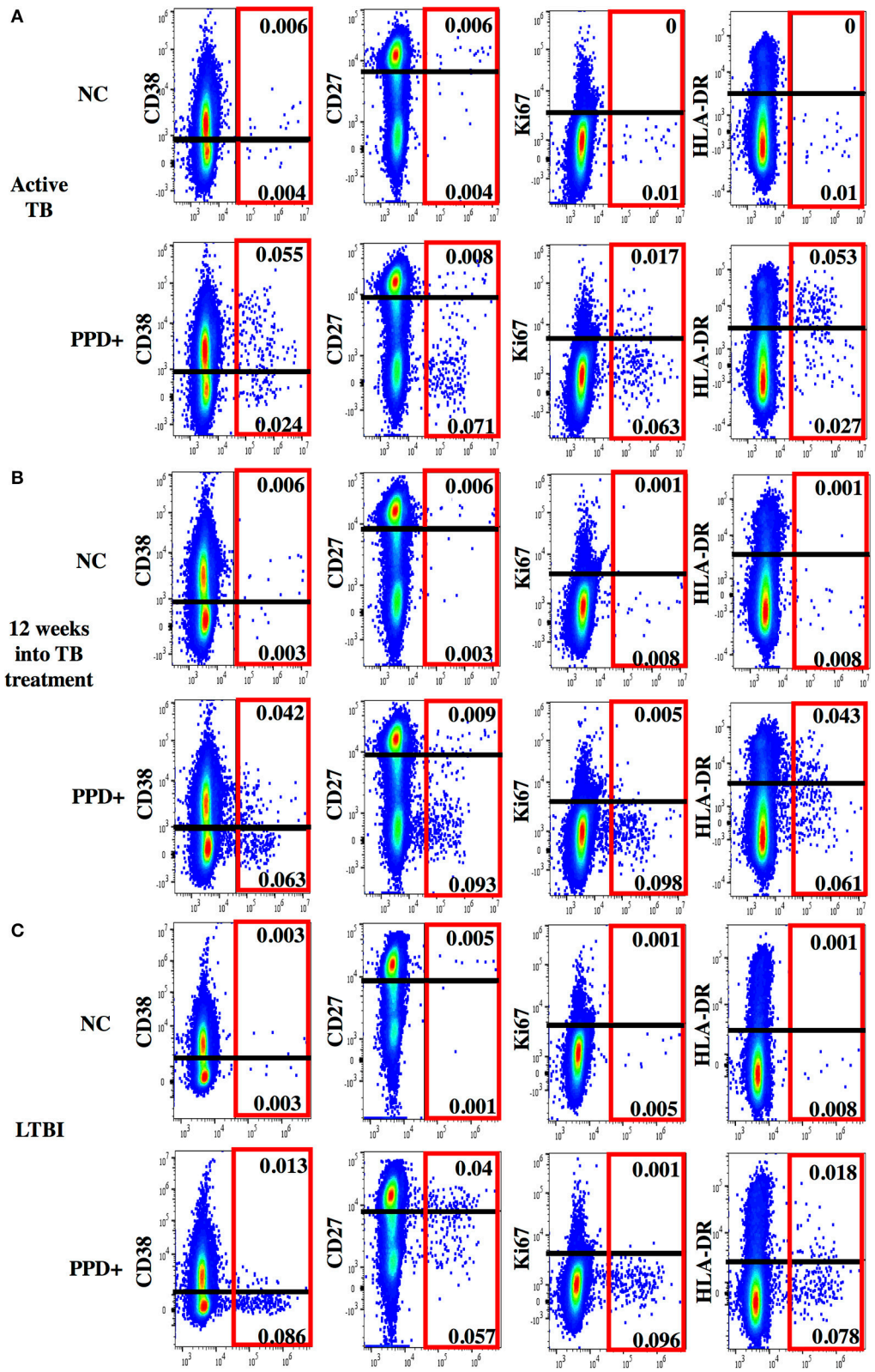

FIGURE 2 | Representative dot plots for phenotypic characterization of MTB-specific CD4 T cells. Shown are dot plots for active TB **(A)**, 12 weeks into TB treatment **(B)** and Latent TB Infection **(C)**. Dot plots are gated on CD4 T cells showing IFNγ (x-axis) and activation (CD38, HLA-DR, and Ki67) and maturation (CD27) marker staining (y-axis) without stimulation (upper panel) and after PPD stimulation (lower panel). IFNγ+ MTB-specific CD4 T cells are indicated (red box). The cut-off for the expression of each phenotypic marker is indicated as a black line.

markers CD38 (median: 71%, **Figure 3**, representative dot plots in **Figure 2**), HLA-DR (median: 49.3%) and Ki67 (median: 17.5%) with a predominance of $CD27^{low}$ cells (median: 91.3%), upon PPD stimulation. TAM expression on MTB-specific CD4 T cells differed significantly and showed no overlap for the frequencies of $CD38^{pos}$ and $Ki67^{pos}$ cells between aTB and LTBI (both $p < 0.0001$). Frequencies of $CD27^{high}$ and $HLA\text{-}DR^{pos}$ cells also differed (both $p < 0.0001$), but showed greater overlaps between aTB and LTBI. Receiver Operating Characteristic (ROC) curve analysis between aTB and LTBI (**Supplementary Figure 1**) confirmed that the frequency of $CD38^{pos}$ and $Ki67^{pos}$ MTB-specific CD4 T cells differentiated best between aTB and LTBI [Area under the Curve (AUC) = 1] with a defined cut-off of 31.55% for CD38 and 3.7% for Ki67 (**Supplementary Figure 1**).

T Cell Activation Marker Expression Profiles On MTB-Specific CD4 T Cells Change Rapidly Upon TB Treatment Initiation

Frequencies of activated MTB-specific CD4 T cells declined dramatically from pre-treatment to W9 post-treatment ($p < 0.0001$, **Figure 3**), while $CD27^{high}$ cell frequencies remained largely unchanged ($p = 0.8$). These treatment-induced changes varied substantially between individuals and were exclusively observed in the MTB-specific T cell compartment, but not in the non-specific CD4 T cells (**Figures 4A–D**, Ki67 not shown). The numeric data for the underlying Figure 4 is provided in **Supplementary Table 1**. Consequently the changes in CD38 and HLA-DR expression until week 9 differed significantly between MTB-specific and total CD4 T cells ($p < 0.0001$ for both markers, **Figures 4E,F**). Only comparatively moderate changes were observed between W9 and W26 for $CD38^{pos}$ and $HLA\text{-}DR^{pos}$ cell frequencies. The median frequency of $Ki67^{pos}$ cells declined further to 1.5% ($p = 0.01$) between W12 and 26, whereas the median frequency of $CD27^{low}$ cells remained unchanged between before and at the end of treatment (median: 91%, **Figures 3B,D**). Using SPICE analyses (28) combinatorial changes of the 4 phenotypic markers on MTB-specific CD4 T cells were analyzed (**Figure 5**). The pie chart arcs show that TB treatment induced substantial reductions in MTB-specific T cell frequencies co-expressing the activation markers CD38, HLA-DR, and Ki67 in different combinations as shown.

CD38 and CD27 Expression Phenotypes Differentiate 3 MTB Infection States: Active TB Disease, Treated TB, and Latent MTB Infection

We next addressed the question whether and to what degree the expression of activation and maturation markers on MTB-specific CD4 T cells correlate with one another and analyzed data

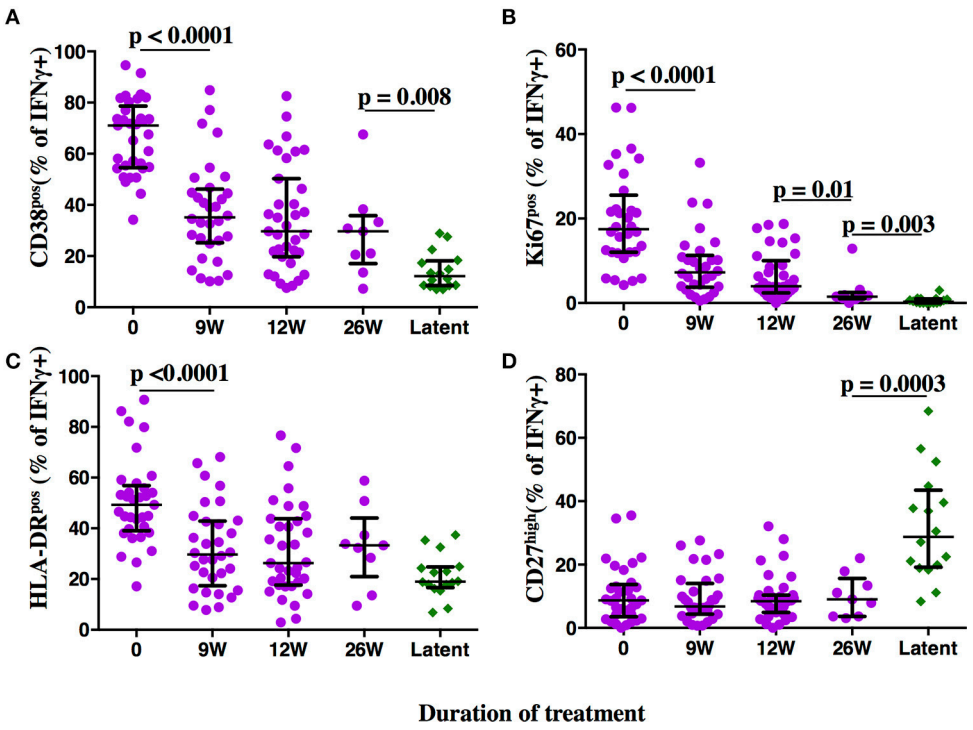

FIGURE 3 | Phenotypic profiles of MTB-specific CD4 T cells in subjects with aTB, after TB treatment initiation and during LTBI. The frequency of MTB-specific CD4 T cells expressing the activation markers CD38 **(A)**, Ki67 **(B)**, HLA-DR **(C),** and the maturation marker CD27 **(D)** is shown on the y-axis for pulmonary TB patients (purple circles) at baseline, 9, 12, and 26 weeks (x-axis) after TB treatment initiation. Subjects with latent MTB infection were included as controls (green diamonds). MTB-specific CD4 T cells were characterized after PPD stimulation. Statistical analyses were performed using the Mann-Whitney test. Median values, interquartile range and *p*-values below 0.05 are indicated.

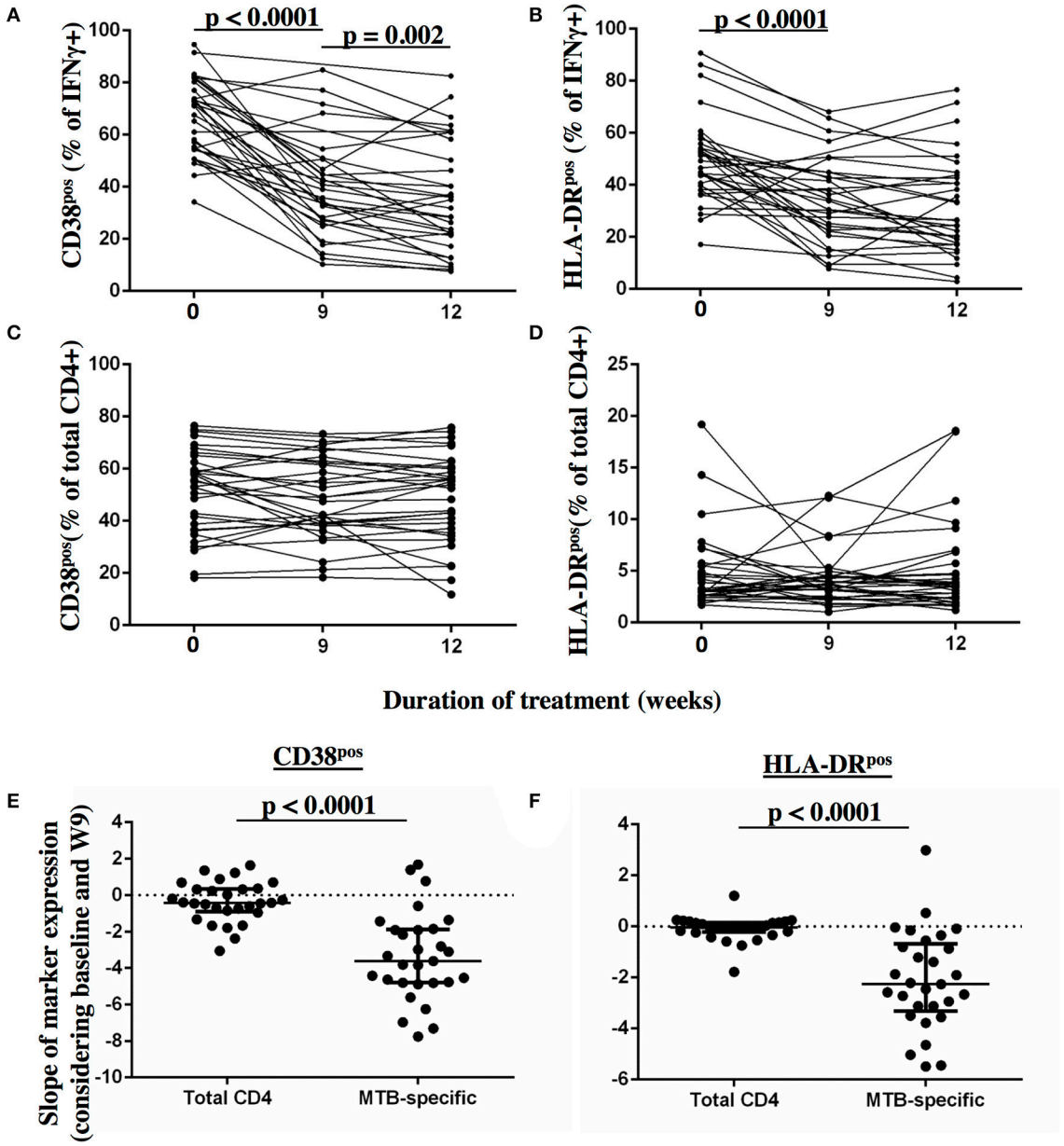

FIGURE 4 | Detection of dynamic changes in CD38 and HLA-DR expression upon TB treatment initiation on MTB-specific, but not total CD4 T cells. The frequency of T cells expressing the activation markers CD38 and HLA-DR (y-axis) are shown for MTB-specific CD4 T cells **(A,B)** and for total CD4 T cells **(C,D)** before and at 9 and 12 weeks after treatment for each subject. MTB-specific CD4 T cells were characterized after PPD stimulation. The slopes of the activation marker expression on MTB-specific and on total CD4 T cells were compared for CD38 **(E)** and HLA-DR **(F)** between baseline and week 9 ($n = 29$). Statistical analyses for paired data were performed using the Wilcoxon-signed rank paired test. None-paired data analyzed using the Mann-Whitney test. *P*-values below 0.05 are indicated.

from aTB patients before and after TB treatment ($n = 109$ subject visits, **Figure 6**). The frequency of all the activation marker positive cells correlated with each other (all $p < 0.0005$). The strength of correlation varied; CD38 and HLA-DR expression correlated most strongly (Spm Rho = 0.76). The correlation of Ki67 with either CD38 or HLA-DR was comparatively weak (Spm Rho = 0.35 and 0.33, respectively). A weak inverse correlation was detected for the frequencies of $CD27^{high}$ cells with HLA-DR^{pos} (data not shown, both $p < 0.05$). In contrast, frequencies of $CD27^{high}$ MTB-specific CD4 T cells did not correlate with the frequencies of $CD38^{pos}$ and $Ki67^{pos}$ cells (**Figure 6D** and data not shown, $p = 0.79$ and $p = 0.4$, respectively). Hence, simultaneous assessment of CD38 and CD27 holds most information on TB disease and treatment status. Indeed, by

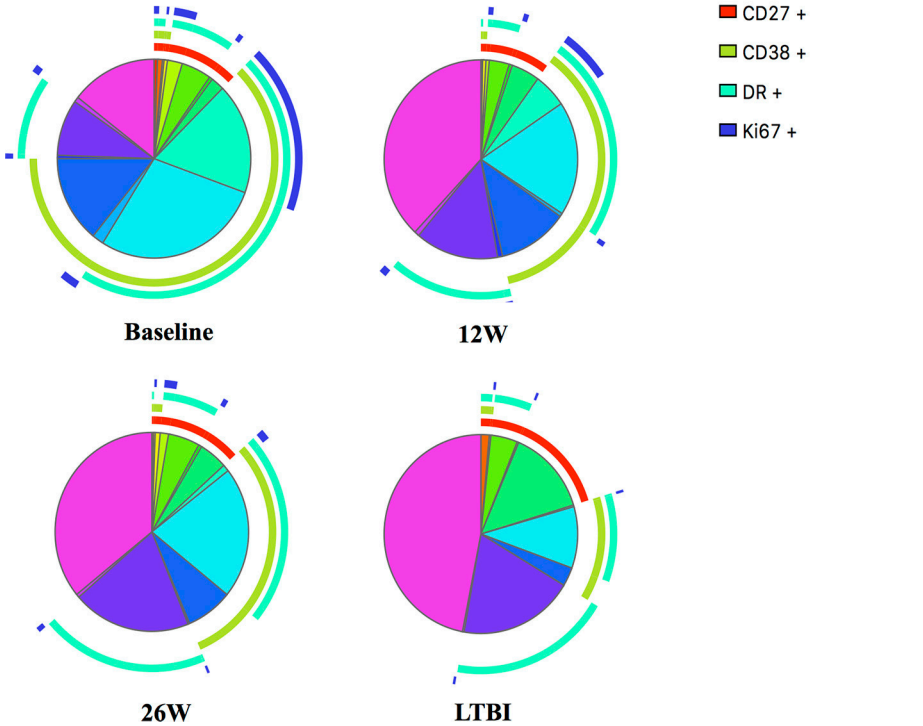

FIGURE 5 | SPICE analyses for in-depth phenotypic profiling of MTB-specific CD4 T cells. Shown are SPICE pie charts visualizing the mean frequency for each of the 16 possible phenotypic profiles of MTB-specific CD4 T cells. The arcs indicate the proportion of cells that express CD27 (red), CD38 (green), HLA-DR (light blue) and/or Ki67 (dark blue). The time point or LTBI infection status is indicated below each pie chart.

assessing only these two markers, the phenotypic characteristics of MTB-specific CD4 T cells at week 26 still differed from LTBI with median frequencies of 28.8 and 12.1% for $CD27^{high}$ ($p = 0.0003$) and $CD38^{pos}$ cells ($p = 0.008$), respectively. Hence, phenotypic profiles differed significantly between aTB (profile: $CD38^{pos}$, $CD27^{low}$), treated TB ($CD38^{neg/pos}$, $CD27^{low}$), and LTBI ($CD38^{neg}$, $CD27^{high}$).

Treatment Induced Reductions in TAM Expression Profiles on MTB-Specific CD4 T Cells Reflect Declining Bacterial Burden in Sputum

Using the cut-off of $<$ 31.55% $CD38^{pos}$ MTB-specific CD4 T cells to define LTBI, 37.5% (12 of 32) and 51.4% (18 of 35) of aTB patients had a TAM-TB profile consistent with "LTBI" at W9 and W12 post-treatment, respectively. To address whether such a phenotypic profile—determined at a single post-treatment time point—indicates *in vivo* mycobacterial clearance, we compared the time to last culture-positive result from these patients with this "cured TAM-TB assay profile" to those without such a TAM-TB profile. The two groups did not differ ($p = 0.58$, data not shown) and those with a "cured signature" still included 5 and 4 subjects, who were still culture positive at or after W9 and W12, respectively. Similar results were obtained using a more stringent cut off of $<$19.7% $CD38^{pos}$ MTB-specific CD4 T cells, which defined the lower quartile of $CD38^{pos}$ cells at W12 ($p = 0.26$). Hence, a "cured" TAM-TB signature measured at a single post-treatment time point was not suggestive for clearance of viable bacteria in sputum nor did it differentiate rapid from slow treatment responders.

We next addressed whether changes in the frequencies of T cell activation marker positive MTB-specific CD4 T cells between baseline and W9; and between baseline and W12 were linked to treatment-induced bacterial clearance using the primary PanACEA study endpoint—time to stable culture negativity. 32 of 39 subjects had TAM-TB results at baseline and at W12 and/or W9 and thus the slope for the change in expression of the individual TAMs after treatment initiation could be determined. 15 of these 32 subjects also had an accurate endpoint determination of $\leq$ 4 weeks between the last positive and stable culture conversion (**Figure 7**). In the other 17 subjects, determination of the accurate time to culture conversion was compromised due to increasing rates of culture contamination as treatment progressed—a common phenomenon (29). These either did not reach stable culture conversion or had large gaps between the last positive and stable culture conversion and hence were excluded from analyses. Taking into consideration baseline and W12 results, the slope of decline in expression of CD38 ($p = 0.0045$, Rho $= 0.7$) and HLA-DR ($p = 0.02$, Rho $= 0.61$) on MTB-specific CD4 T

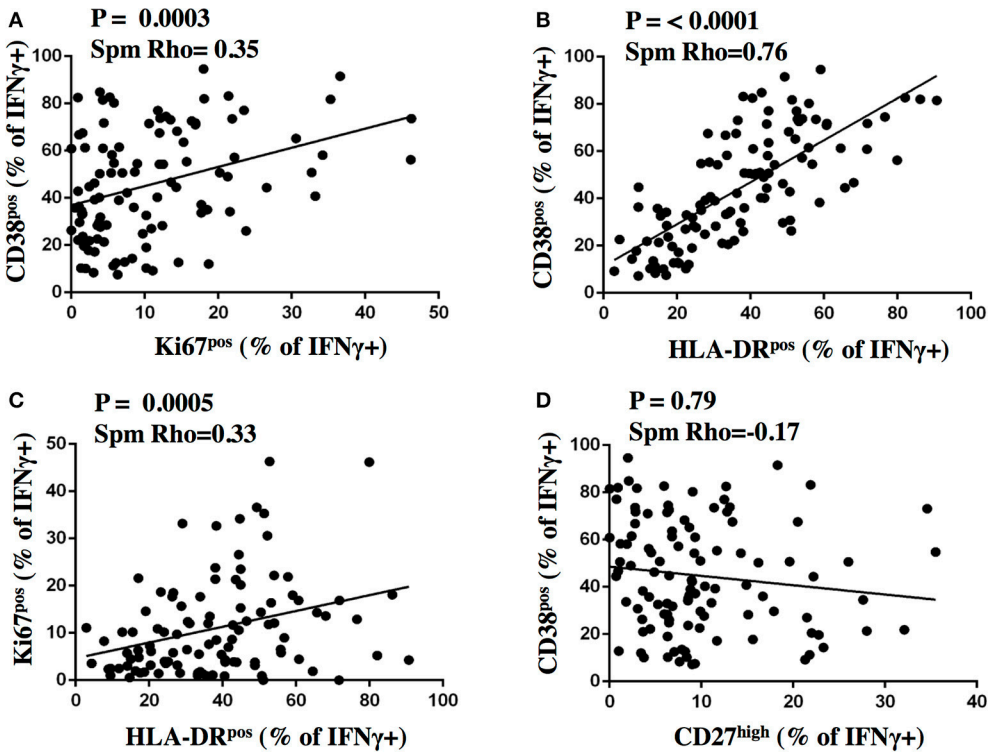

FIGURE 6 | Correlation analysis of activation and maturation marker expression on MTB-specific CD4 T cells. The proportion of IFNγ+ MTB-specific CD4 T cells expressing activation and maturation markers after stimulation were plotted for CD38 and Ki67 **(A)**, CD38 and HLA-DR **(B)**, Ki67 and HLA-DR **(C)**, CD38 and CD27 **(D)** on the y- and x-axis, respectively for samples from subjects with aTB from before and after treatment initiation ($n = 109$). The Spearman's rank test was used for statistical analysis.

cells correlated with the time to stable culture conversion (**Figures 8A,B**). Likewise, similar correlations were detected when considering measurements at baseline and week 9 ($n = 13$ subjects, CD38: $p = 0.015$, Rho $= 0.67$; HLA-DR: $p = 0.007$, Rho $= 0.72$, **Figures 8C,D**). No such correlations were detected for CD38 and HLA-DR expression on total CD4 T cells (data not shown).

DISCUSSION

Substantial reductions in the expression of activation markers on MTB-specific CD4 T cells were observed at W9 into TB treatment for most patients. In contrast, expression of these markers on total CD4 T cells remained comparable to pre-treatment values, showing that TB treatment-induced changes in T cell activation status are specific to the MTB-specific T cell compartment. Individual patients varied substantially in the early reduction in MTB-specific CD4 T cell activation until week 9 and week 12; and for both of these time intervals, the degree of change in activation correlated well with the mycobacterial response. Our data therefore provide evidence that early changes in TAM expression profiles on MTB-specific CD4 T cells reflect clearance of viable mycobacteria *in vivo*. These TAM-TB profiles may therefore serve as a surrogate marker for treatment efficacy, as originally proposed by Adekambi et al. (5) and now further substantiated in more aTB patients with more precisely determined time to culture conversion. This immunological approach therefore bypasses the issues associated with the collection of sputum specimen (e g., in young children, after TB treatment initiation) and increased culture contamination rates as treatment progresses (29); which renders treatment monitoring by serial cultures difficult and often imprecise on a per person level, as was observed in our patient subset. It is noteworthy, that all tested TB patients responded to the MTB antigen PPD, therefore enabling the determination of the activation status on MTB-specific CD4 T cells.

Shortening treatment is a major objective of TB drug development and has been tested in 4 recent studies (20–23); the majority (at least 80%) of patients treated in these trials for only 4 months had been cured without relapse within the defined follow up of 18 months; but nevertheless the trials were declared unsuccessful due to high relapse rates in the 4 months regimens. While speculative at this point, a TAM-TB assay approach based treatment-monitoring algorithm could therefore potentially help to personalize TB treatment duration by discrimination of patients who respond well to TB treatment marked by a substantial reduction in MTB-specific CD4 T cell activation—and those who do not.

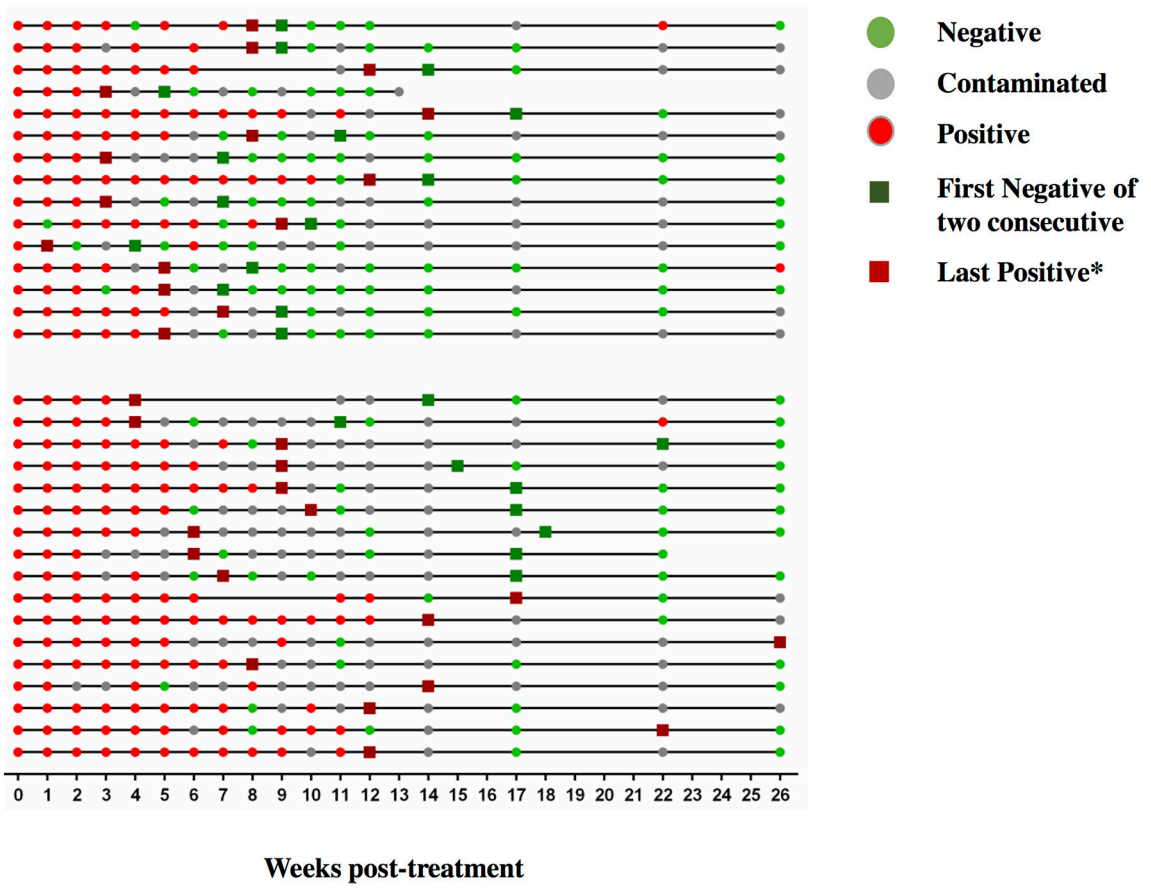

FIGURE 7 | Sequential MGIT culture results. Sequential MGIT culture results from 17 study visits from week 0 to week 26 (x-axis) are shown for each subject with a TAM-TB assay result from baseline and/or at week 9 and/or week12 ($n = 32$). The upper and lower line graphs indicate culture results from subjects with ≤ 4 ($n = 15$) and ≥ 4 weeks ($n = 17$) between the last positive and stable culture conversion, respectively. Red dots indicate a MTB positive culture result, green dots a negative culture result, and gray dots indicate a contaminated sample. Dark green squares indicate stable culture conversion defined as the first of two consecutive culture negative results. Dark red square*: last culture positive sample before 2 consecutive culture negative samples.

The dynamic changes in expression of T cell activation markers observed upon treatment initiation contrasted with the minor changes observed for the maturation marker CD27. We assessed, which combination of markers are most informative on treatment and disease/infection status. Consistent with previous results (5), expression of CD38 differentiated best between aTB and LTBI. The three activation markers correlated with one another and thus the determination of one activation marker—CD38—might suffice to differentiate active TB from LTBI and to monitor the treatment response. No correlation was detected between expression of CD38 and CD27 and the latter best differentiated treated TB at W26 from LTBI. Hence, simultaneous assessment of CD38 and CD27 expression on MTB-specific CD4 T cells, can help to differentiate between three MTB infection/disease states; (1) aTB—defined by high expression of activation markers, but low CD27 expression; (2) LTBI—defined by low expression of activation markers and high expression of CD27 and (3) treated TB—defined by low expression of activation markers and continuously low CD27 expression. Our data therefore support the concept that phenotypic characteristics of pathogen-specific T cells may not only differentiate between active TB and LTBI, but may also detect past episodes of pathogen activity, which were resolved through treatment or naturally.

Previous reports on CD27 expression on MTB-specific CD4 T cells also showed no substantial increase by 26 weeks of treatment (6). However, at 12 months after the end of TB treatment it appears to revert to levels characteristic for LTBI (8). It could be that residual TB disease activity at the end of TB treatment and during LTBI, might contribute to such a $CD27^{low}$ profile, as suggested by a recent positron emission tomography (PET-CT) study. This study showed persistent, active lesion activity in the majority of cured TB patients even until 6 months after TB treatment (30). A large range of bacterial loads and metabolic activity in MTB lesions (31–33) has also been observed in non-human primates with LTBI consistent with a broad spectrum between LTBI, treated and active disease (34, 35). We had previously reported on a HIV seroconverter where CD27 downregulation on MTB-specific T cells preceded active TB

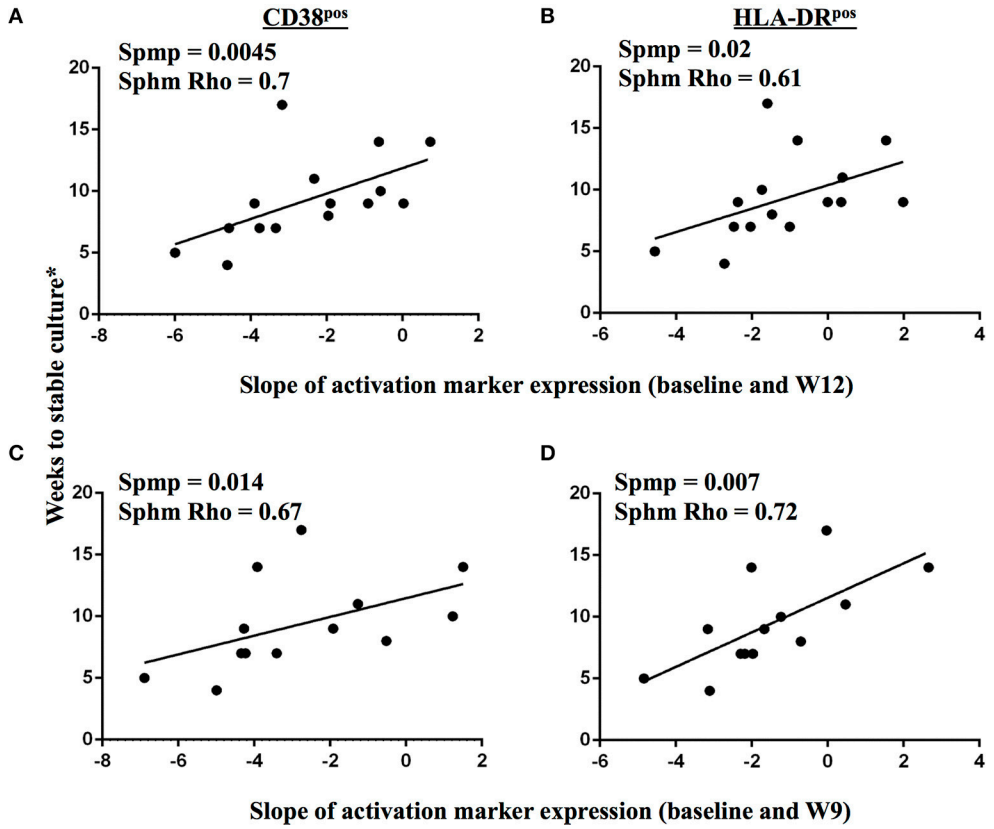

FIGURE 8 | Changes in TAM expression profiles on MTB-specific CD4 T cells upon treatment initiation reflect declining bacterial burden in sputum. A correlation analysis between time to stable culture conversion and the slope of CD38 and HLA-DR marker expression dynamics on IFNγ+ MTB-specific CD4 T cells is shown for the time interval from baseline to week 12 **(A,B**, $n = 15$), and from baseline to week 9 **(C,D**, $n = 13$), respectively, for subjects with accurately defined time point of less than 5 weeks between the last positive MGIT culture result and stable culture conversion. The Spearman's rank test was used for statistical analysis.

diagnosis by 6–9 months (2). It would therefore be of great interest to better define the range of expression of phenotypic markers on MTB-specific T cells and other host response markers (36–38) in relation to the presence and dynamics of TB lesions *in vivo* in subjects with LTBI and in patients after treatment initiation (30) with systematic follow up on active TB disease progression, and treatment failure and relapse, respectively.

It is known that males account to 65% of aTB patients globally (39). Our LTBI control group consisted only of females, which raised the concern whether gender might have confounded our results. To address this question, males (or females) were excluded in a sub-group analysis (**Supplementary Figure 2**); there was no overlap of CD38 and Ki67 in subjects with LTBI and aTB, when considering only females ($p < 0.0001$). Further, no gender-associated difference in the TAM-TB profile at baseline was detected, whereas significant reductions in T cell activation marker expression—but not for CD27 expression—were induced by TB treatment regardless of gender. Hence there was no evidence that gender alone influenced the TAM-TB profile of MTB-specific CD4 T cells in our study. It is however noteworthy that males had significantly higher frequencies of proliferating (Ki67+) MTB-specific CD4 T cells and a trend for increased expression of CD38 at 12 weeks post treatment initiation ($p = 0.09$) compared to females (data not shown). The latter finding is "consistent" with another finding of the Panacea-TB MAMS treatment study; females cleared mycobacteria significantly faster upon treatment initiation as compared to males (unpublished results). Hence, gender-associated differences in activation marker profiles of MTB-specific CD4 T cells at 12 weeks after treatment initiation probably reflect gender-related differences in the rate of mycobacterial clearance.

Limitations in this study were the lack of available X-ray scores as well as disease severity assessment. We were also not able to study the effect of HIV infection on TAM-TB assay profiles during treatment monitoring, because our cohort recruited exclusively HIV negative patients. HIV infection is associated with increased levels of systemic T cell activation (40, 41). Another study investigated the effect of HIV on MTB-specific CD4 T cell activation, and maturation, showing only little influence (7). Larger studies in well-characterized cohorts need to clarify the influence of these parameters on TAM-TB assay results and their changes upon treatment initiation. While the

need for flow cytometry and basic cell culture methods may limit the use of this approach in resource poor settings, our data show that more complex flow cytometry is in principle not needed. We and others have previously shown that by using whole blood this assay can be simplified substantially (2, 42). Other methods assessing the MTB-specific cytokine secretion upon whole blood stimulations may also allow specific diagnoses of active TB (43), whereas monitoring the TB treatment response using the latter approach so far appears less promising (44). The measurement of soluble biomarkers in the blood without prior stimulation also may hold valuable information on TB disease and treatment status (45). The diagnostic value of these needs further investigation.

In conclusion, our data on using phenotypic profiles of MTB-specific CD4 T cells as a surrogate marker for treatment efficiency warrant further research and methodological simplification to define its usefulness in standard clinical sttings and during TB drug trials.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript writing. NN, GK, BM, HS, SM, and CM were involved in design, conduct, and analysis of the underlying patient study at both participating clinical study centers. A-MM, MB, SG, MH, and NH conceived, planned, and managed the patient study from the study sponsor side. MA, MH, NH, ES, UB, IK, RL, and CG participated in data analysis. MA, KH, MC, AH, and CG contributed to experimental work. MH, NH, and CG conceived the immunological sub-study reported here.

FUNDING

This work was supported by the European & Developing Countries Clinical Trials Partnership (EDCTP) (PanACEA, grant numbers IP.2007.32011.011, IP.2007.32011.012, IP.2007.32011.013), by the German Ministry for Education and Research (BMBF; Grant No. 01KA0901) by the European Commission, DG XII, INCO-DC (grant ICA-CT-2002-10048) and by the German Center for Infection Research (DZIF).

ACKNOWLEDGMENTS

We would like to thank all study participants.

The Pan African Consortium for the Evaluation of Anti-tuberculosis Antibiotics (PanACEA) comprises of the following individuals and institutions: Medical Centre of the University of Munich, Munich, Germany (Anna Maria Mekota, NH, Andrea Rachow, ES, MH); University of St. Andrews, St Andrews, United Kingdom (Stephen Gillespie); Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands (Angela Colbers, Rob Aarnoutse, Martin Boeree); University College of London, London, UK (Anna Bateson, Timothy McHugh, Kasha Singh, Robert Hunt, Alimuddin Zumla); MRC Clinical Trials Unit at UCL, London, UK (Andrew J Nunn, Patrick PJ Phillips, Sunita Rehal); University of Cape Town, Cape Town, South Africa (Rodney Dawson, Kim Narunsky); University of Stellenbosch, Cape Town, South Africa (Andreas Diacon, Amour Venter, Sven Friedrich); University of the Witswatersrand, Johannesburg, South Africa (Ian Sanne, Karla Mellet, Eefje de Jong); The Aurum Institute, Johannesburg, South Africa (Gavin Churchyard, Salome Charalambous, Vinodh Edward, Madulagotla Sebe, Lungile Mbata, Robert Wallis); University of Zambia, Lusaka, Zambia (Peter Mwaba); NIMR-Mbeya Medical Research Centre, Mbeya, Tanzania (Nyanda Elias Ntinginya, Chacha Mangu, Christina Manyama, Bariki Mtafya); Ifakara Health Institute, Bagamoyo, Tanzania (Lilian T. Minja, Mohamed Sasamalo); Swiss Tropical and Public Health Institute, Basel, Switzerland, University of Basel, Basel, Switzerland (Klaus Reither, Levan Jugheli); Kilimanjaro Clinical Research Institute, Moshi, Tanzania (Blandina T. Mbaga, GK, HS, SM); Medical Research Unit - Albert Schweitzer Hospital, Lambarene, Gabon (Abraham Alabi, Ayola Akim Adegnika); Kenya Medical Research Institute, Nairobi, Kenya (Evans Amukoye); Makerere University, Kampala, Uganda (Alphonse Okwera).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02247/full#supplementary-material

REFERENCES

1. WHO. *End TB Strategy: Global Strategy and Targets for Tuberculosis Prevention, Care and Control After 2015*. Available online at: http://www.who.int/tb/post2015_strategy/en/ (Accessed on August 7, 2017).
2. Schuetz A, Haule A, Reither K, Ngwenyama N, Rachow A, Meyerhans A, et al. Monitoring CD27 expression to evaluate *Mycobacterium tuberculosis* activity in HIV-1 infected individuals *in vivo*. *PLoS ONE* (2011) 6:e27284. doi: 10.1371/journal.pone.0027284
3. Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya N, et al. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. *Lancet Infect Dis.* (2014) 14:931–8. doi: 10.1016/S1473-3099(14)70884-9
4. Nikitina IY, Kondratuk NA, Kosmiadi GA, Amansahedov RB, Vasilyeva IA, Ganusov VV, et al. Mtb-specific CD27low CD4 T cells as markers of lung tissue destruction during pulmonary tuberculosis in humans. *PLoS ONE* (2012) 7:e43733. doi: 10.1371/journal.pone.0043733
5. Adekambi T, Ibegbu CC, Cagle S, Kalokhe AS, Wang YF, Hu Y, et al. Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. *J Clin Invest.* (2015) 125:1827–38. doi: 10.1172/JCI77990
6. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. *PLoS ONE* (2007) 2:e735. doi: 10.1371/journal.pone.0000735
7. Riou C, Berkowitz N, Goliath R, Burgers WA, Wilkinson RJ. Analysis of the phenotype of *Mycobacterium tuberculosis*-specific CD4+ T cells to discriminate latent from active tuberculosis in HIV-uninfected and HIV-infected individuals. *Front Immunol.* (2017) 8:968. doi: 10.3389/fimmu.2017.00968
8. Petruccioli E, Petrone L, Vanini V, Cuzzi G, Navarra A, Gualano G, et al. Assessment of CD27 expression as a tool for active and latent

tuberculosis diagnosis. *J Infect.* (2015) 71:526–33. doi: 10.1016/j.jinf.2015.07.009
9. Riou C, Strickland N, Soares AP, Corleis B, Kwon DS, Wherry EJ, et al. HIV skews the lineage-defining transcriptional profile of *Mycobacterium tuberculosis*-specific CD4+ T cells. *J Immunol.* (2016) 196:3006–18. doi: 10.4049/jimmunol.1502094
10. Harari A, Rozot V, Bellutti Enders F, Perreau M, Stalder JM, Nicod LP, et al. Dominant TNF-alpha+ *Mycobacterium tuberculosis*-specific CD4+ T cell responses discriminate between latent infection and active disease. *Nat Med.* (2011) 17:372–6. doi: 10.1038/nm.2299
11. Perreau M, Rozot V, Welles HC, Belluti-Enders F, Vigano S, Maillard M, et al. Lack of *Mycobacterium tuberculosis*-specific interleukin-17A-producing CD4+ T cells in active disease. *Eur J Immunol.* (2013) 43:939–48. doi: 10.1002/eji.201243090
12. Rozot V, Vigano S, Mazza-Stalder J, Idrizi E, Day CL, Perreau M, et al. *Mycobacterium tuberculosis*-specific CD8+ T cells are functionally and phenotypically different between latent infection and active disease. *Eur J Immunol.* (2013) 43:1568–77. doi: 10.1002/eji.201243262
13. Fuhrmann S, Streitz M, Kern F. How flow cytometry is changing the study of TB immunology and clinical diagnosis. *Cytometry A* (2008) 73:1100–6. doi: 10.1002/cyto.a.20614
14. Perez-Velez CM, Marais BJ. Tuberculosis in children. *N Engl J Med.* (2012) 367:348–61. doi: 10.1056/NEJMra1008049
15. Zar HJ, Hanslo D, Apolles P, Swingler G, Hussey G. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. *Lancet* (2005) 365:130–4. doi: 10.1016/S0140-6736(05)17702-2
16. Marais BJ, Hesseling AC, Gie RP, Schaaf HS, Enarson DA, Beyers, N. The bacteriologic yield in children with intrathoracic tuberculosis. *Clin Infect Dis.* (2006) 42:e69–71. doi: 10.1086/502652
17. Lawn SD, Afful B, Acheampong JW. Pulmonary tuberculosis: diagnostic delay in Ghanaian adults. *Int J Tuberc Lung Dis.* (1998) 2:635–40.
18. Wallis RS, Kim P, Cole S, Hanna D, Andrade BB, Maeurer M, et al. Tuberculosis biomarkers discovery: developments, needs, and challenges. *Lancet Infect Dis.* (2013) 13:362–72. doi: 10.1016/S1473-3099(13)70034-3
19. Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, et al. Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. *Lancet Respir Med.* (2013) 1:462–70. doi: 10.1016/S2213-2600(13)70119-X
20. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946–1986, with relevant subsequent publications. *Int J Tuberc Lung Dis.* (1999) 3:S231–79.
21. Jindani A, Harrison TS, Nunn AJ, Phillips PP, Churchyard GJ, Charalambous S, et al. High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. *N Engl J Med.* (2014) 371:1599–608. doi: 10.1056/NEJMoa1314210
22. Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *N Engl J Med.* (2014) 371:1577–87. doi: 10.1056/NEJMoa1407426
23. Merle CS, Fielding K, Sow OB, Gninafon M, Lo MB, Mthiyane T, et al. A four-month gatifloxacin-containing regimen for treating tuberculosis. *N Engl J Med.* (2014) 371:1588–98. doi: 10.1056/NEJMoa1315817
24. Horne DJ, Royce SE, Gooze L, Narita M, Hopewell PC, Nahid P, et al. Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis. *Lancet Infect Dis.* (2010) 10:387–94. doi: 10.1016/S1473-3099(10)70071-2
25. Boeree MJ, Heinrich N, Aarnoutse R, Diacon AH, Dawson R, Rehal S, et al. High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial. *Lancet Infect Dis.* (2017) 17:39–49. doi: 10.1016/S1473-3099(16)30274-2
26. Svensson RJ, Aarnoutse RE, Diacon AH, Dawson R, Gillespie SH, Boeree MJ, et al. A population pharmacokinetic model incorporating saturable pharmacokinetics and auto-induction for high rifampicin doses. *Clin Pharmacol Ther.* (2017) 103:674–83. doi: 10.1002/cpt.778
27. Riedner G, Rusizoka M, Hoffmann O, Nichombe F, Lyamuya E, Mmbando D, et al. Baseline survey of sexually transmitted infections in a cohort of female bar workers in Mbeya Region, Tanzania. *Sex Transm Infect.* (2003) 79:382–7. doi: 10.1136/sti.79.5.382
28. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* (2011) 79:167–74. doi: 10.1002/cyto.a.21015
29. Sloan DJ, Corbett EL, Butterworth AE, Mwandumba HC, Khoo SH, Mdolo A, et al. Optimizing outpatient serial sputum colony counting for studies of tuberculosis treatment in resource-poor settings. *J Clin Microbiol.* (2012) 50:2315–20. doi: 10.1128/JCM.00043-12
30. Malherbe ST, Shenai S. Ronacher K, Loxton AG, Dolganov G, Kriel M, et al. Persisting positron emission tomography lesion activity and Mycobacterium tuberculosis mRNA after tuberculosis cure. *Nat Med.* (2016) 22:1094–100. doi: 10.1038/nm.4177
31. Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, et al. Variability in tuberculosis granuloma T cell responses exists, but a balance of pro- and anti-inflammatory cytokines is associated with sterilization. *PLoS Pathog.* (2015) 11:e1004603. doi: 10.1371/journal.ppat.1004603
32. Capuano SV III, Croix DA, Pawar S, Zinovik A, Myers A, Lin PL, et al. Experimental *Mycobacterium tuberculosis* infection of cynomolgus macaques closely resembles the various manifestations of human *M tuberculosis* infection. *Infect Immun.* (2003) 71:5831–44. doi: 10.1128/IAI.71.10.5831-5844.2003
33. Coleman MT, Maiello P, Tomko J, Frye LJ, Fillmore D, Janssen, C, et al. (2014). Early Changes by (18)Fluorodeoxyglucose positron emission tomography coregistered with computed tomography predict outcome after *Mycobacterium tuberculosis* infection in cynomolgus macaques. *Infect Immun.* 82:2400–4. doi: 10.1128/IAI.01599-13
34. Barry CE III, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol.* (2009) 7:845–55. doi: 10.1038/nrmicro2236
35. Ehlers S. Lazy, dynamic or minimally recrudescent? On the elusive nature and location of the mycobacterium responsible for latent tuberculosis. *Infection* (2009) 37:87–95. doi: 10.1007/s15010-009-8450-7
36. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. *Lancet* (2016) 387:2312–22. doi: 10.1016/S0140-6736(15)01316-1
37. Anderson ST, Kaforou M, Brent AJ, Wright VJ, Banwell CM, Chagaluka G, et al. Diagnosis of childhood tuberculosis and host RNA expression in Africa. *N Engl J Med.* (2014) 370:1712–23. doi: 10.1056/NEJMoa1303657
38. Bloom CI, Graham CM, Berry MP, Wilkinson KA, Oni T, Rozakeas F, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS ONE* (2012) 7:e46191. doi: 10.1371/journal.pone.0046191
39. WHO *Global Tuberculosis Report*. (2018). Available online at: http://www.who.int/tb/publications/global_report/en/
40. Hazenberg MD, Stuart JW, Otto SA, Borleffs JC, Boucher CA, de Boer RJ, et al. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* (2000) 95:249–55.
41. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine

coreceptor usage. *J Infect Dis.* (1999) 179:859–70. doi: 10.1086/314660

42. Musvosvi M, Duffy D, Filander E, Africa H, Mabwe S, Jaxa L, et al. T-cell biomarkers for diagnosis of tuberculosis: candidate evaluation by a simple whole blood assay for clinical translation. *Eur Respir J.* (2018) 51:1800153. doi: 10.1183/13993003.00153-2018
43. Sun Q, Wei W, Sha W. Potential role for *Mycobacterium tuberculosis* specific IL-2 and IFN-gamma responses in discriminating between latent infection and active disease after long-term stimulation. *PLoS ONE* (2016) 11:e0166501. doi: 10.1371/journal.pone.0166501
44. Leem AY, Song JH, Lee EH, Lee H, Sim B, Kim SY, et al. Changes in cytokine responses to TB antigens ESAT-6, CFP-10 and TB 7.7 and inflammatory markers in peripheral blood during therapy. *Sci Rep.* (2018) 8:159. doi: 10.1038/s41598-018-19523-7
45. Sigal GB, Segal MR, Mathew A, Jarlsberg L, Wang M, Barbero S, et al. Biomarkers of tuberculosis severity and treatment effect: a directed screen of 70 host markers in a randomized clinical trial. *EBioMedicine* (2017) 25:112–21. doi: 10.1016/j.ebiom.2017.10.018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer RT and handling Editor declared their shared affiliation.

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 February 2017
doi: 10.3389/fimmu.2017.00149

A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes

Sarah Joseph[1], Killian Quinn[2], Aldona Greenwood[3], Alethea V. Cope[2], Paul F. McKay[2], Peter J. Hayes[4], Jakub T. Kopycinski[4], Jill Gilmour[4], Aleisha N. Miller[5], Christof Geldmacher[6,7], Yuka Nadai[6,7], Mohamed I. M. Ahmed[6,7], David C. Montefiori[8], Len Dally[9], George Bouliotis[5], David J. M. Lewis[3,10], Roger Tatoud[2], Ralf Wagner[11], Mariano Esteban[12], Robin J. Shattock[2], Sheena McCormack[1] and Jonathan Weber[2]*

[1] MRC Clinical Trials Unit at UCL, Institute of Clinical Trials and Methodology, University College London, London, UK, [2] Department of Medicine, Imperial College London, London, UK, [3] Clinical Research Centre, University of Surrey, Guildford, UK, [4] IAVI Human Immunology Laboratory, Imperial College London, London, UK, [5] ICTU, Department of Public Health, Imperial College London, London, UK, [6] Department of Infectious Diseases and Tropical Medicine, Klinikum of the University of Munich, Munich, Germany, [7] German Centre for Infection Research (DZIF), Munich, Germany, [8] Duke University Medical Centre, Durham, NC, USA, [9] The EMMES Corporation, Rockville, MD, USA, [10] Clinical Research Facility, Imperial College Healthcare NHS Trust, London, UK, [11] University of Regensburg and University Hospital Regensburg, Regensburg, Germany, [12] National Center for Biotechnology, CSIC, Madrid, Spain

OPEN ACCESS

Edited by:
Marc H. V. Van Regenmortel, Centre national de la recherche scientifique (CNRS), France

Reviewed by:
Thorsten Demberg, Immatics Biotechnologies (Germany), USA
Daniela Santoro Rosa, Federal University of São Paulo, Brazil

***Correspondence:**
Sheena McCormack
s.mccormack@ucl.ac.uk

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *24 November 2016*
Accepted: *30 January 2017*
Published: *22 February 2017*

Citation:
Joseph S, Quinn K, Greenwood A, Cope AV, McKay PF, Hayes PJ, Kopycinski JT, Gilmour J, Miller AN, Geldmacher C, Nadai Y, Ahmed MIM, Montefiori DC, Dally L, Bouliotis G, Lewis DJM, Tatoud R, Wagner R, Esteban M, Shattock RJ, McCormack S and Weber J (2017) A Comparative Phase I Study of Combination, Homologous Subtype-C DNA, MVA, and Env gp140 Protein/Adjuvant HIV Vaccines in Two Immunization Regimes. Front. Immunol. 8:149. doi: 10.3389/fimmu.2017.00149

There remains an urgent need for a prophylactic HIV vaccine. We compared combined MVA and adjuvanted gp140 to sequential MVA/gp140 after DNA priming. We expected Env-specific CD4+ T-cells after DNA and MVA priming, and Env-binding antibodies in 100% individuals after boosting with gp140 and that combined vaccines would not compromise safety and might augment immunogenicity. Forty volunteers were primed three times with DNA plasmids encoding (CN54) env and (ZM96) gag-pol-nef at 0, 4 and 8 weeks then boosted with MVA-C (CN54 env and gag-pol-nef) and glucopyranosyl lipid adjuvant—aqueous formulation (GLA-AF) adjuvanted CN54gp140. They were randomised to receive them in combination at the same visit at 16 and 20 weeks (accelerated) or sequentially with MVA-C at 16, 20, and GLA-AF/gp140 at 24 and 28 weeks (standard). All vaccinations were intramuscular. Primary outcomes included ≥grade 3 safety events and the titer of CN54gp140-specific binding IgG. Other outcomes included neutralization, binding antibody specificity and T-cell responses. Two participants experienced asymptomatic ≥grade 3 transaminitis leading to discontinuation of vaccinations, and three had grade 3 solicited local or systemic reactions. A total of 100% made anti-CN54gp140 IgG and combining vaccines did not significantly alter the response; geometric mean titer 6424 (accelerated) and 6578 (standard); neutralization of MW965.2 Tier 1 pseudovirus was superior in the standard group (82 versus 45% responders, $p = 0.04$). T-cell ELISpot responses were CD4+ and Env-dominant; 85 and 82% responding in the accelerated and standard groups, respectively. Vaccine-induced IgG responses targeted multiple regions within gp120 with the V3 region most

immunodominant and no differences between groups detected. Combining MVA and gp140 vaccines did not result in increased adverse events and did not significantly impact upon the titer of Env-specific binding antibodies, which were seen in 100% individuals. The approach did however affect other immune responses; neutralizing antibody responses, seen only to Tier 1 pseudoviruses, were poorer when the vaccines were combined and while T-cell responses were seen in >80% individuals in both groups and similarly CD4 and Env dominant, their breadth/polyfunctionality tended to be lower when the vaccines were combined, suggesting attenuation of immunogenicity and cautioning against this accelerated regimen.

Keywords: HIV vaccine, phase I trial, DNA, MVA, envelope protein

INTRODUCTION

In an era of antiretroviral medication for the treatment and prevention of HIV, concerns around access, toxicity, and escalating cost suggest that a vaccine for HIV is still likely to be the most effective and sustainable way of reducing new infections (1, 2). Of the five HIV efficacy vaccine trials to date, there has only been only one to demonstrate significant, if modest efficacy; the RV144 "Thai" trial (3–8). This study with 16,402 subjects randomized to four immunizations with ALVAC given twice and then twice more with AIDSVAX B/E adjuvanted with ALUM, reported 31.2% protection (95% CI 1–51) against acquisition, without impacting HIV viral load or CD4+ T cell count (8). Subsequent immunological analyzes reported an inverse correlation between the levels of circulating polyclonal non-neutralizing antibodies and risk of infection, which has been associated with Fc receptor-mediated antibody effector functions (9–15). The results stimulated interest in prime-boost pox and protein combination vaccine approaches and the role of non-neutralizing antibodies.

Heterologous prime boost regimens employing DNA, viral vectors, and/or recombinant proteins have generated robust cellular and humoral responses maximizing breadth and potency while limiting the attenuating effects of vector specific immunity (16–20). DNA vaccines have been shown to prime cellular and humoral immune responses, upon boosting with recombinant vectors (21). The EuroVacc trials demonstrated that DNA prime, NYVAC boost increased the frequency, magnitude, and breadth of HIV-specific T-cell ELISpot responses (22, 23) and that three DNA priming immunizations were more immunogenic than two (24). A recent clinical trial comparing different prime boost regimens showed no benefit of DNA priming for Env-specific antibody responses but evidence of an improvement in T-cell responses, although overall immunogenicity was lower than seen previously in response to the same DNA and MVA vaccines (25).

In this study, the UK HIV Vaccine Consortium built upon these prior data showing enhanced immunogenicity of DNA prime, pox vector boost, and the protection seen in RV144 by protein boosting, to produce homologous DNA, MVA, and gp140 immunogens. We have made DNA plasmids and an MVA expressing matched HIV-1 subtype C (CN54)-derived inserts, and adjuvanted trimeric glycoprotein with a view for use in Sub-Saharan Africa. We believe this strategy is ideally suited to inducing Env-dominant CD4+ T-cell responses, favoring the development of high titer Env-specific antibody responses. The same trimeric recombinant CN54gp140 protein has already been administered to 469 individuals in a variety of trial settings (with and without DNA priming, or adjuvant and via different routes), showing excellent safety and induction of vaccine specific antibodies (26–28). When given systemically with glucopyranosyl lipid adjuvant—aqueous formulation (GLA-AF) after priming with heterologous DNA and MVA, high titer systemic binding antibodies were seen to the protein (28).

Prompted by the results of the RV144 trial, but with long-term feasibility in mind, we have explicitly assessed the impact of combining pox (MVA-C) and GLA-AF adjuvanted CN54gp140 protein after priming with DNA. We compared the safety and immunogenicity of two regimens using identical vaccines; given sequentially in one regimen (standard) and with the pox and protein combined in the other (accelerated). We shortened the regimens relative to our previous studies and RV144 by reducing the intervals between vaccinations, with 4 weeks between each of three DNA immunizations, 8 weeks between prime and first boost, and 4 weeks between subsequent boosts. We administered vaccinations intramuscularly (IM) for logistical ease and with a view to eventual roll out in resource limited settings.

The DNA and MVA-C were produced by UK HVC and based closely on those used previously (EV02 Eudract 2004-001802-28 and EV03 Eudract 2006-006141-13), with matched CN54/ZM96 subtype C-derived *gag pol nef* and *env* inserts. We anticipated Env-dominated CD4+ T-cell responses and modest Env-specific antibody responses after DNA and MVA, with the development of high titer binding and neutralizing antibody responses after boosting with adjuvanted CN54gp140 protein (29–31). Based on our previous studies, we expected that the immunogens would prove more potent B-cell immunogens than the ALVAC/AIDSVAX/ALUM used in RV144 and that the combined MVA/CN54p140/GLA might augment immunogenicity, offering the potential for a short regimen.

MATERIALS AND METHODS

Study Design and Conduct

This was a Phase I randomized open-label trial conducted at two UK centers: Imperial College London and Surrey Clinical Research Centre. Participants were recruited through advertising,

social media, and a dedicated website. The study documents were reviewed and approved by the NRES London—West London and GTAC Ethics Committee (13/LO/0115), and the UK Medicines and Healthcare products Regulatory Agency, and all participants gave fully informed written consent according to the Declaration of Helsinki before any study procedures were conducted. The trial was registered with the European Union Drug Regulating Authorities for Clinical Trials (EUDRACT TC 2012-003277-26) and Clinical Trials.gov (NCT01922284) and with the UK Clinical Trials Research Network (UKRN-14173). Laboratory personnel were blind to the allocation. Participants were block randomized centrally using a computer generated algorithm with a back-up manual procedure, and the randomization list was stratified by center and gender.

The primary objective was to compare the safety and immunogenicity of two vaccination regimens, one of which was shortened by 8 weeks (**Figure 1**) in healthy HIV-uninfected male and female volunteers aged 18–45 years at low risk of HIV infection. The primary outcomes were (i) a severe (grade 3) or worse local or systemic clinical or laboratory adverse event or an event that led to a clinical decision to discontinue vaccinations and (ii) the magnitude of the CN54gp140-specific IgG antibody response in serum 4 weeks after the final immunization. Secondary outcomes of interest included IFNγ T-cell ELISpot, intracellular cytokine, neutralizing antibody, CN54gp140-specific serum IgA, mucosal IgG, and IgA antibody responses.

Safety Evaluations

Local and systemic events recognized to be associated with licensed vaccines were solicited systematically at clinical centers prior to, 10 min and 1 h after each vaccination, and then 7 days later, and by diary card. Clinical and laboratory events were collected via an open question at each visit and through routine hematology and chemical pathology performed at screening, 1 week after each vaccination and at week 40 in both groups.

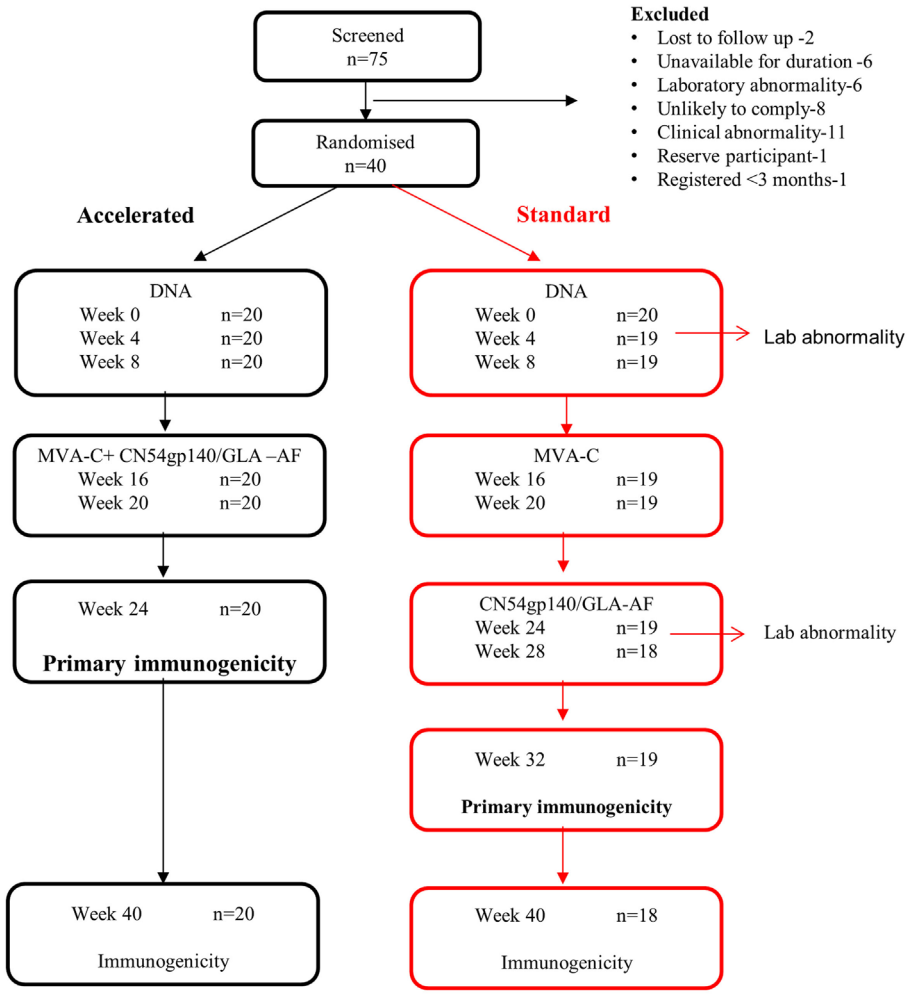

FIGURE 1 | Trial flow.

Immunological Specimens

Blood was taken for immunological assessments at weeks 0, 4, 8, 16, 20, and 24 and 40 for all participants and at weeks 28 and 32 for those in the standard group. Mucosal samples were collected at weeks 0 and 24 for the accelerated and at weeks 0 and 32 for the standard group. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient separation, frozen in a mixture of fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and DMSO (9:1 ratio) using a Kryo 560-16 rate controlled freezer (Planer, Sunbury-On-Thames, UK). PBMCs were shipped and stored in vapor phase liquid nitrogen as previously (32). Genital tract secretions from women were collected using the Instead Softcup™ (Evofem Inc.) and urethral swabs (Hunt Biologics, UK) from male volunteers and rectal Floq™ swabs were taken when possible from males and females, primarily to assess the feasibility of the sampling method. Vaginal samples were collected, processed, and analyzed as described previously (33). Urethral swabs were collected from male participants in clinic by inserting the swab and allowing it to absorb mucosal secretions for 2 min. Rectal Floq™ swabs were inserted into the anus and rotated to collect secretions. Rectal and urethral swabs were either snap frozen on receipt or processed directly. Processing involved addition of 300 µl of extraction buffer [250 mM NaCl, 1× protease inhibitor cocktail set 1 (Calbiochem) in phosphate buffered saline (1× DPBS)] to the swabs, vortexing for 1 min and placing on ice to 15 min. Rectal and urethral swabs were then placed in the top chamber of a spin X tube, centrifuged at max speed (10,000 *g*) for 2 min and the eluates either analyzed immediately or aliquoted and frozen at −80°C until analysis.

Vaccines and Schedule

The recombinant clade C HIV-1 envelope gp140 protein (CN54gp140) is a naturally cleavage resistant envelope clone of 97CN54 (34). The protein was manufactured to GMP specification (33) (Polymun Scientific Austria) generating a product which was >80% trimeric protein with a projected mass of 420 kD and a defined glycan (35). A total of 100 µg CN54gp140 was mixed with 5 µg GLA-AF (IDRI, Seattle, WA, USA) and administered in a volume of 0.4 ml as below. There were two DNA plasmids; one encoded (CN54) *env* and the other a (ZM96) *gag-pol-nef* fusion protein. Both open-reading frames were RNA and codon optimized (GeneArt AG, Regensburg, Germany). Both plasmids utilized a chimeric CMV enhancer/promoter with a human T-cell leukemia type 1 regulatory element to drive expression (36). The MVA-C (Mariano Esteban CSIC, Spain) expressed the CN54gp120 Env and Gag-Pol-Nef polyprotein from two back-to-back synthetic early/late transcriptional promoters (37, 38). All vaccinations were given IM into the deltoid muscles of the upper arms. 4 mg of each DNA plasmid was given to all participants at weeks 0, 4, and 8 in a volume of 1.0 ml (8.0 mg in total) with the same plasmid given into the same arm on each occasion (CN54 plasmid into right arm and ZM96 into left arm). In the "standard" group, 10^8 $TCID_{50}$ MVA-C was given at weeks 16 and 20 in a volume of 1.0 ml (into left arm) and then 100 µg CN54gp140 mixed with 5 µg GLA-AF at weeks 24 and 28 in a volume of 0.4 ml (into right arm). In the "accelerated" group, 10^8 $TCID_{50}$ MVA-C in 1.0 ml was given at the same time as 100 µg CN54gp140 mixed with 5 µg GLA-AF at weeks 16 and 20 in 0.4 ml IM as above (with MVA-C into left and CN54gp140/GLA-AF into the right arms as shown, see **Table 1**).

Humoral Assays

CN54gp140-Specific Antibody ELISA

Serum and mucosal binding antibodies against recombinant CN54gp140 were measured using a standardized ELISA with minor modifications. 96-well ELISA plates were coated with 50 µl per well of capture antigen CN54gp140 (1 µg/ml) (Polymun, Austria). Human standards (IgG or IgA) were captured by coating wells with a combination of α-Human κ and α-Human λ (1:1 ratio) capture antibodies. After incubation at 37°C for 1 h, plates were washed with PBST then blocked for 1 h at 37°C with 200 µl/well of assay buffer (PBS + 1% BSA) then washed, as above (26). Standards were prepared by adding the required concentration of either human IgG or IgA. Serum samples were screened at 1:100 dilution, Softcup cervical mucosal samples at 1:10 dilution. Samples, standards, and controls (normal human sera) were added to triplicate wells. Detection antibodies were added following incubation and washing, either goat α-Human IgG-HRP or goat α-Human IgA-HRP detection antibodies. After incubation and washing, plates were developed by the addition of TMB substrate (KPL) followed by addition of 50 µl of Stop Solution (KPL). Absorbencies were read immediately at 450 nm using a VersaMax plate-reader (Molecular Devices). A response detected for both IgG and IgA was defined as OD A450 nm value >0.2; samples below this value were deemed negative or response not detected. Samples were further diluted following screening assays if positive with a series of dilutions in order to extrapolate a concentration expressed as microgram per milliliter of specific IgG or IgA using the ELISA software SoftMax Pro v 5.4. Serum samples that were positive by the

TABLE 1 | Schedule of doses, formulation, and routes of immunization.

Group	Route of immunization; dose of vaccine		
	Weeks 0, 4, 8	**Weeks 16, 20**	**Weeks 24, 28**
1 (*n* = 20)	4 mg DNA (CN54) in 1 ml (right arm)	1×10^8 $TCID_{50}$ MVA-C in 0.5 ml (left arm) + [100 µg CN54gp140 + 5 µg glucopyranosyl lipid adjuvant—aqueous formulation (GLA-AF)] in 0.4 ml (right arm)	Nothing
	4 mg DNA (ZM96) in 1 ml (left arm)		
	Intramuscular (IM)	IM	
2 (*n* = 20)	4 mg DNA (CN54) in 1 ml (right arm)	1×10^8 $TCID_{50}$ MVA-C in 0.5 ml (left arm)	(100 µg CN54gp140 + 5 µg GLA-AF) in 0.4 ml (right arm)
	4 mg DNA (ZM96) in 1 ml (left arm)		
	IM	IM	IM

above method were also tested in a conventional endpoint titer assay as previously described (32).

Neutralizing Antibody Responses

Neutralizing antibody responses against a panel of Tier 1 (MW965.26, MN.3, 00836-2.5, ZM197M-PB7) and Tier 2 (Ce1176_A3, Ce703010217_B6, HIV-2510-2) pseudo viruses were measured using TZM-bl cells in the lab of David Montefiori as described previously (27, 39). Briefly, pseudoviruses (TZM-bl assay) were incubated with serial dilutions of sera and added to their respective target cells. Luciferase expression was measured after 2 days (TZM-bl), and IC_{50}s were determined as the serum concentration that reduced the background-subtracted relative light units by 50% compared to virus-only control wells.

Cellular Assays

IFNγ ELISpot

Cellular immunogenicity was assessed by standardized IFNγ ELISpot assay using frozen PBMCs as previously described (32, 39). One day prior to assay setup, PBMCs were thawed in and rested overnight in RPMI medium containing 20% heat-inactivated fetal calf serum (HIFCS), glutamine, penicillin, and streptomycin (R20) (all supplied by Sigma, Poole, UK) at 37°C, 5% CO_2. 96-well PVDF membrane (MSIPS4510 Millipore, UK) plates were coated with mouse anti-human IFNγ (10μg/ml; MabTech clone 1-D1K) in sterile PBS. On the day of assay setup, coated ELISpot plates were washed with sterile PBS and blocked with RPMI 10% HIFCS (R10) for at least 1 h. Synthetic peptides (15-mers overlapping by 11aa; HPLC purified >90%, JPT Germany) covering the HIV-1 gene inserts and CMV pp65 gene were dissolved and pooled in dimethyl sulfoxide (DMSO, Sigma), further diluted in PBS and R10 to achieve a final assay concentration of 1.5 μg/ml per peptide and 0.45% v/v DMSO. 100 μl volumes of HIV-1 peptide pools were added to ELISpot plate wells in quadruplicate. The CMV pp65 peptide pool and phytohemagglutinin (PHA, 10 μg/ml) were plated as positive controls in duplicate wells for each. For a negative control, quadruplicate wells containing a mock stimulus (0.45% v/v DMSO final concentration in R10) were used. Rested PBMCs were recovered and washed in R10 and viable cells counted using a Beckman Coulter Vi-Cell counter. A total of 200,000 viable PBMCs (in 50 μl) were added to all wells except for 1 well with R10 only (reagent control well). Plates were incubated at 37°C, 5% CO_2 overnight (16–24 h). All subsequent steps were performed at room temperature. Plates were washed six times with PBS/0.05% v/v Tween 20 (Sigma) and the production of IFNγ by T-cells was assessed by addition of 1 μg/ml biotinylated mouse-anti-human IFNγ antibody (clone 7-B6-1, Mabtech, Sweden) for 2–4 h. Plates were washed as before and ABC peroxidase–avidin–biotin complex (PK6100, Vector labs, UK) was added for 1 h, followed by three washes with PBS/Tween and three washes with PBS. Spots were developed with addition of filtered AEC/H_2O_2 substrate solution (Sigma) for 4 min. The reaction was stopped by washing plates under running tap water, plate underdrains removed, and plates allowed to dry overnight in the dark before spots in each well were counted using an automated AID ELISpot reader (AutoImmun Diagnostika, Germany).

A positive response was defined by the following criteria: (1) average number of background-subtracted spots in a single pool >specified cutoff of 38 SFC/10^6 PBMCs (40). The cutoffs were derived from assessing peptide pool responses in PBMCs from 178 HIV-1 seronegative individuals; (2) for each pool, if the number of replicates was 2 or ≥3, then the coefficient of variation (standard deviation/mean) between replicates had to be ≤50% or ≤70%, respectively; (3) mean count had to be >4 times mean background; (4) mean background had to be ≤55 SFC/10^6 PBMCs. Samples with mean background >55 SFC/10^6 PBMCs were considered failures, were repeated, and excluded from all analyses if failed a second time. The breadth of responses was described in terms of the number of individual peptide pools to which each individual responded.

Flow Cytometry

Antigen-specific cytokine secretion was assessed using a validated seven-color polychromatic flow cytometry panel assessed at the IAVI human immunology lab in London. Previously frozen PBMCs were coincubated with peptide pools matched to the inserts at 1.5 μg/ml (as previously described), 1 μg/ml SEB (Sigma-Aldrich, St. Louis, MO, USA), or mock stimuli and Brefeldin A (Sigma-Aldrich, Poole Dorset, UK) for 6 h at 37°C. Cells were stained for viability with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen, Eugene, OR, USA), fixed and then stained intracellularly using anti-CD4 PeCF594 (clone RPA-T4), anti-CD8 BV421 (RPA-T8), anti-CD3 APC-H7 (SK7), anti-IFNγ APC (B27), anti-IL2-PE (MQ1-17HI2), and anti-TNFα-FITC (Mab11) (all Becton Dickinson, San Jose, CA, USA). Cells were washed and acquired on the same day. At least 5,000 CD8 and CD4+ CD3+ viable, singlet lymphocyte events were acquired using BD Fortessa II instruments. Data were analyzed and presented using FlowJo (version 9.8 Treestar, Ashland, OR, USA). Samples were failed where fewer than 5,000 events in the predefined populations were acquired or where mock IFNγ responses were above 0.2% of either parental population. Flow cytometric analysis was performed at baseline, and 16 and 24 weeks for group 1, and additionally at 32 weeks for group 2.

Peptide Array Mapping

The microarrays were processed according to the manufacturers instructions with minor modifications (www.jpt.com). Briefly, the slides were pre-incubated with T20 blocking buffer (Thermo Fisher) for 10 min. Plasma samples were then added at a dilution of 1:100 in T20 blocking buffer and incubated for 2 h at room temperature with gentle shaking before washing five times with 2.5 ml TBS-Tween (0.5% Tween). The secondary mouse anti-human-IgG Dylight649 (JPT) was then incubated at room temperature for 1 h at a dilution of 1:5,000 in T20 blocking buffer. After five washings with 2.5 ml TBS-Tween, and five washes with double distilled de-ionized water, the slides were left to dry under a laminar flow hood. Samples from all timepoints from one individual were processed simultaneously. Slides were scanned on a GenePix 4000A scanner and processed using GenepixPro 6.0 software at 650 and 532 nm to generate a Tiff image file. The array lay out was then added using the .gal file JPT_2758_V04.gal provided by JPT. Accuracy of the array alignment was controlled

and individual features were adjusted or excluded manually when needed. After this QA/QC step, .gpr files were generated and processed further into .dat files using R-program and the R-script "MakeDat_V05r_stat.R" to generate one fluorescent intensity (FI) value for the peptide-specific IgG response from the triplicates. Individual IgG responses were mapped using the R-script "MapAlign_BG_V11.R" after subtraction of FI values from baseline plasma. The array included the immunogen sequence CN54gp140 and nine other sequences from acute phase primary HIV isolates of subtypes A, C, B, CRF01_AE, and CRF02_AG to maximize coverage of global HIV diversity (Ahmed *et al*., in preparation).

Statistical Analyses

All clinical and routine laboratory data were included in the safety analyses. Data sets included (i) modified intention to treat; all participants who were randomized and received at least one vaccination and (ii) per protocol (PP), all participants who completed vaccinations with no major protocol deviations. The primary safety outcome was expressed as a proportion of participants with 95% confidence interval, and groups were compared using Fisher's exact test. The primary immunological outcome was the magnitude of serum CN54gp140-specific IgG 4 weeks after the final vaccination and we assumed a 100% response rate in the standard (reference) arm. The sample size was calculated on the basis of the binding antibody responses distributions described in the RV144 trial. In this trial, the reciprocal GMT of binding antibodies to subtype E gp120 was ~1:15,000 (log10: 4.18) and for subtype B gp120 was ~1:30,000 (log10:4.5). On the basis of previous trials, we assumed that everyone would respond to the CN54gp140 and that the titer of subtype C-specific binding antibody responses would be in the same range and at least 1:15,000 in the standard group and that a four-fold increase in the magnitude of would be immunologically relevant. This translates to an absolute difference of 0.6 on the log10 scale. In the absence of raw data from the Rv144 trial, we have assumed a standard deviation of 0.58 on the log 10 scale in the distribution of the antibody responses (corresponding to a SD of ~33,400 in titer). Assuming this variation, 20 participants per group allowed for the detection of an absolute increase in titer of 0.60 with 90% power and 5% alpha. Comparison of the groups was made using the geometric mean (GM) ratios of the titer with their 95% CI and equality was assumed if these ratios included 1. Skewed data was log transformed for normality and then comparisons made using parametric tests. Secondary outcomes were compared by the response frequency per group, using chi-square tests if frequencies were adequate or the Fisher's exact 2-tailed test for small numbers. Comparison of the magnitude of T-cell ELISpot responses was made using the non-parametric Wilcoxon two-sample test. No corrections were made for multiple testing. For the flow cytometric analysis, responses are described relative to each mock-stimulated control. Two-by-two contingency tables were generated to compare the peptide stimulated versus the mock control for each cytokine and T-cell subset. One-sided Fisher's exact tests were then applied to each table to resolve whether the percentage of cytokines generated following peptide stimulation was greater than that compared following stimulation with mock antigen. Bonferroni corrections were applied to account for multiple testing. Heatmaps summarizing ICS analyses were generated using SPICE version 5.1 downloaded from http://exon.niaid.nih.gov (41).

RESULTS

Participant Accrual, Study Population, and Compliance with Schedule

Of 75 healthy, low-risk, HIV-negative volunteers screened between 19th June 2013 and 10th January 2014, 40 were enrolled; the reasons for the 35 who were screened out are summarized in **Figure 1**. Twenty participants were enrolled at each center, and baseline characteristics are summarized in **Table 2**. The majority were white, half were female and the median age was 32 years (IQR 23–39). All randomized participants received the first immunization but two in the standard group did not complete the immunization schedule due to adverse events. In addition, two participants from the standard group missed the final visit at weeks 40 and 1 also missed the primary immunogenicity endpoint visit at week 32 (**Figure 1**).

TABLE 2 | Baseline characteristics and median follow-up by treatment group.

	Accelerated *n* = 20	Standard *n* = 20	Total *n* = 40
Number	20	20	40
Age (SD)	31 (25–38)	32 (22–39)	32 (23–39)
Center			
Imperial College	10 (50%)	10 (50%)	20 (50%)
Surrey	10 (50%)	10 (50%)	20 (50%)
Gender			
Female	10 (50%)	9 (45%)	19 (47.5%)
Male	10 (50%)	11 (55%)	21 (52.5%)
Ethnicity			
Asian	2 (10%)	0 (0%)	2 (5%)
Mixed	0 (0%)	2 (10%)	2 (5%)
White	18 (90%)	18 (90%)	36 (90%)
Weight (kg)	73 (63–82)	72 (67–78)	72.8 (66–78)
Routine laboratory parameters			
Hemoglobin (g/dl)	14 (13–15)	14 (13–14)	14 (13–15)
White cell count (10^9/l)	7 (5.3–8.2)	6.4 (5.8–8.0)	6.5 (5.6–8.2)
Neutrophils (10^9/l)	4.1 (2.9–5.1)	3.6 (3.3–5.1)	3.8 (3.0–5.1)
Platelets (10^9/l)	250 (236–288)	259 (225–289)	250 (219–288)
Lymphocytes (10^9/l)	1.9 (1.6–2.3)	1.9 (1.6–2.3)	1.9 (1.5–2.3)
ALT (U/l)	20 (19–25)	19 (14–27)	21 (16–27)
AST (U/l)	21 (18–26)	23 (18–27)	23 (18–27)
Bilirubin (μmol/l)	10 (9–14)	11 (7–15)	10 (7–14)
Creatinine (μmol/l)	71 (58–78)	71 (59–84)	71 (60–80)
Glucose (mmol/l)	4.7 (4.4–5.0)	4.6 (4.5–4.9)	4.6 (4.4–4.9)
DNA/ANA antibodies			
Positive	4[a] (20)	0	4
Negative	16 (80)	20	36
Follow-up (weeks, range)	43 (40–46)	45 (30–50)	44 (30–50)

[a]*Positive only at a dilution of 1:160 which was deemed eligible.*

Primary Safety Endpoints

Four (20%; 95% CI 5.73–43.66) participants in the standard group and one (5%; 95% CI 0.13–24.87) participant in the accelerated group experienced a primary safety endpoint, $p = 0.342$ (two-tailed Fisher's exact test). Two of these were ≥grade 3 laboratory adverse events that resulted in discontinuation of vaccinations. Both occurred in males aged 20 years in the standard group. The first occurred after the first DNA; the 7-day blood test revealed asymptomatic transaminitis (AST 375: Grade 3, ALT 109: Grade 2), which was confirmed 6 days later. Other blood tests, a viral screen, and ultrasound scan were normal. Apart from an episode of tonsillitis between screening and enrollment, treated with penicillin V, there were no other risk factors. The levels spontaneously returned to normal during the following week, but due to the temporal nature, a relationship could not be excluded and so vaccinations were discontinued. The second case occurred after administration of the second MVA-C. In between the immunization visit and the safety review, the participant reported an episode of vomiting after drinking around 12–14 units of alcohol, and strenuous exercise. The 7-day blood test revealed a transaminitis (AST 530: Grade 4, ALT 184: Grade 2). He was not able to return for 28 days at which time both were within the normal range. investigations including an ultrasound scan and blood tests for causes of viral and non-viral hepatitides were normal. Although it was felt that the more likely cause of the transaminitis was a combination of unusually high alcohol intake and strenuous exercise, there was a temporal relationship with vaccination and so vaccinations were discontinued. There were five others ≥grade 3 adverse events reported by three participants on diary cards during the 7 days after vaccination: severe malaise in a female accompanied by a headache 2 days after the first MVA-C/CN54gp140 GLA-AF, severe malaise in a male 5 days after the first CN54gp140 GLA-AF, and a severe/extreme lumpy swelling in a female 6 days after receipt of the first MVA-C and then again 3 and 6 days after the second MVA-C reported by the same participant.

There was one serious adverse event during the study; a female randomized to the accelerated group was referred to hospital by her GP with pain and suspected appendicitis 2 days after receiving her first combined MVA-C and CN54gp140 vaccination. She was treated with paracetamol for the pain (moderate grade according to the toxicity table) before being discharged after an overnight stay when the pain had resolved. The participant continued in the trial and received her last immunization without a repeat of this problem.

Other Adverse Events

All of the remaining solicited local, systemic and other events were mild or moderate. There were 10 laboratory abnormalities other than those reported above, all of these were mild (5 raised ALT, 3 AST, 1 hyperglycaemica, and 1 bilirubin). There were differences in reporting between the centers (125 by Surrey; 50 by Imperial) and by gender (119 by females; 56 by males).

Immunogenicity

All analyses presented below are derived from the PP data set and include 20 from the accelerated group and 17 from the standard group unless otherwise stated (1 participant from the standard group who received all vaccinations did not attend the primary immunogenicity visit and 2 did not receive all vaccinations).

Primary Immunogenicity Endpoint

CN54gp140-Specific Binding Antibody

At the primary endpoint 4 weeks after the final vaccination, all individuals who completed the schedule made CN54gp140-specific IgG. The GM titer and concentration of specific binding antibody in the accelerated group at the primary endpoint were 6,424 (95% CI 4,391–9,396) and 10.46 μg/ml (95% CI 7.3–15.0) and in the standard group 6,578 (95% CI 3,927–11,020) and 12.76 μg/ml (95% CI 8.7.0–18.75), respectively. There was no significant difference between the groups; as assessed by the ratio of the GM titer (0.98, 95 CI 0.53–1.79, $p = 0.93$) or concentration (1.46, 95% CI 0.49–4.34, $p = 0.49$). The response was first detected in both groups 4 weeks after the first MVA-C (week 20) when 15/20 (75%) in the accelerated and 8/18 (44.4%) in the standard group were positive—with a greater response in the accelerated group, $p = 0.02$. In both groups, the response increased significantly after each subsequent vaccination (**Figure 2**). In the accelerated group, the GMT at week 20 was 517, and this increased to 6,424 at week 24. In the standard group, the GM titer was 17 at week 20, increasing to 246 at week 24, 3,596 at week 28, and 6,578 at week 32. In both groups, the response fell away again by week 40; to GMT 1,302 and 2,536 in the accelerated and standard groups, respectively, a difference which was significant ($p = 0.02$) (**Figure 2**). There was no CN54gp140-specific serum IgA detected at any time point.

Mucosal Antibody Responses

Of the mucosal sites sampled, CN54gp140-specific IgG was only detected in cervicovaginal secretions, with no specific responses detected in either urethral or rectal samples (**Figure 3**). The only samples included in the analyses of mucosal secretions were collected from women using Instead cups. There was no CN54gp140-specific IgG detected in samples at baseline, and at the primary endpoint there was no difference in the frequency of responders between the groups; 70% (7/10) women had detectable CN54gp140-specific IgG in the accelerated group as compared to 88% (7/8) in the standard group ($p = 0.59$, Fisher's exact test). The GM concentration of cervicovaginal CN54gp140-specific IgG was 0.11 μg/ml (95% CI 0.01–1.28) in the accelerated group compared with 0.43 μg/ml (95% CI 0.05–3.7) in the standard group 4 weeks after the final vaccination. There was no CN54gp140-specific IgA detected in cervicovaginal samples.

Neutralizing Antibody Responses

Neutralizing antibody responses were detected against two Tier 1A Env-pseudoviruses (**Figure 4**). At the primary endpoint 9/20 (45%) participants in the accelerated group showed neutralization of subtype C MW965.26 virus (closest match to CN54gp140) compared to 14/17 (82%) in the standard group, a difference which was statistically significant ($p = 0.04$, Fisher's exact test) with higher median titer neutralization in the responders from the standard relative to the accelerated group (median titer of 51 and 78, respectively). There was also a trend toward a higher

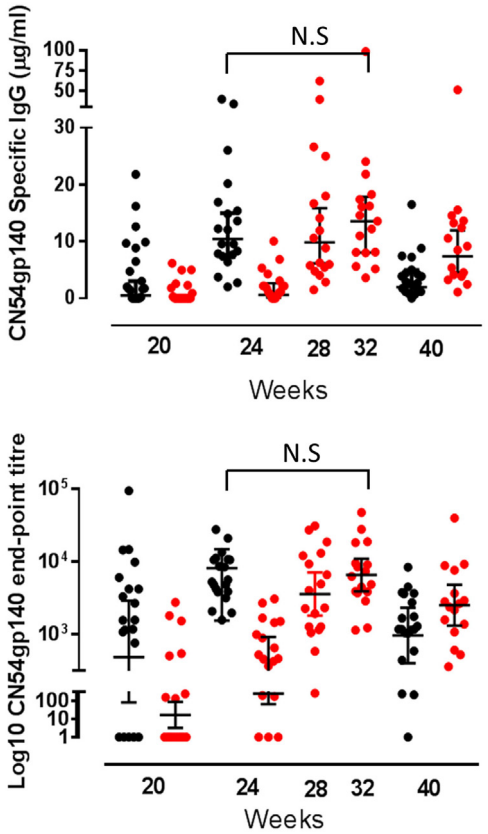

FIGURE 2 | Serum CN54gp140-specific binding antibody responses by group. CN54gp140-specific serum IgG responses in accelerated (black closed circles) and standard groups (red closed circles). Solid lines represent geometric mean (GM) values with 95% CI. Comparisons made using the GM ratio of titers and concentration by group at the primary endpoint, and there were no significant differences between the groups (comparison of week 24 for accelerated group and week 32 for standard group).

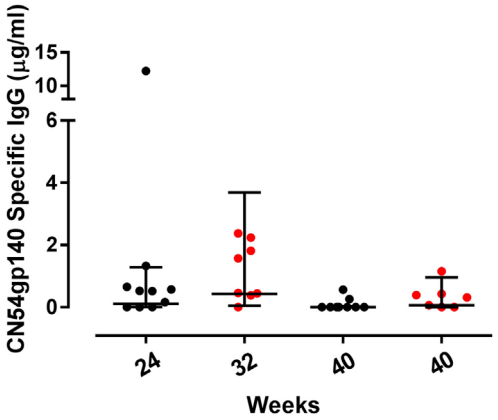

FIGURE 3 | Mucosal binding CN54gp140-specific binding antibody responses by group. Concentrations of CN54gp140-specific cervicovaginal IgG responses at the primary endpoint at week 24 for accelerated (black circles) and week 32 for standard groups (red circles) and at week 40 for both. Solid lines represent geometric mean (GM) values with 95% CI. Comparisons were made using non-paired *t*-tests using GM values, and there were no significant differences between the groups.

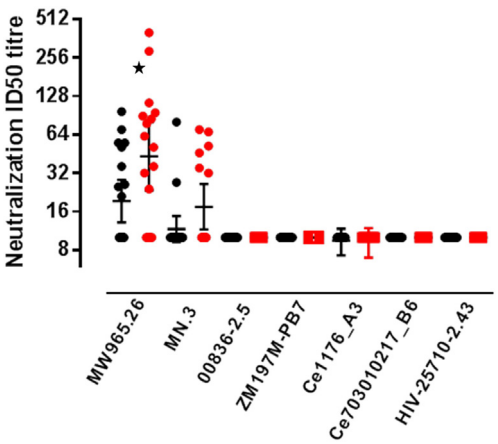

FIGURE 4 | Serum neutralizing antibody responses by group. Neutralizing antibodies measured in serum from accelerated and standard groups at the primary endpoint, 4 weeks after the final vaccination: accelerated group, closed black circles (measured at week 24) and standard group, closed red circles (measured at week 32). Virus strains; MW965.26 (Clade C, Tier 1A), MN.3 (Clade B, Tier 1A), 00836-2.5 (Clade C, Tier 1B), ZM197M-PB7 (Clade C, Tier 1B), Ce1176_A3 (Clade C, Tier 2), Ce703010217_B6 (Clade C, Tier 2), and HIV-25710-2.43 (Clade C, Tier 2). Solid lines represent geometric mean titer with 95% CI. The frequency of responders in each group was compared using the Fishers exact test, $^*p = 0.04$.

frequency response to MN.3 (Tier 1 subtype B) in the standard group (10 versus 35% positive, $p = 0.107$). There was no neutralization detected against two Tier 1B Clade C viruses (00836-2.5, ZM197M-PB7) or against 2 Tier 2 clade C viruses (Ce1176_A3, Ce703010217_B6, HIV-25710-2.43).

Peptide Array Mapping of the Env-specific Antibody Response

Vaccine-induced Env-specific IgG responses to linear 15-mer peptides (**Table 3**) were mapped using a custom designed peptide micro array approach in subjects of accelerated group ($n = 12$) and standard group ($n = 11$) 4 weeks after the final vaccination. The immunodominant regions (IDRs) targeted and magnitude of region-specific responses were largely similar between the two groups (**Figure 5**). IDRs were exlusively located within gp120 with little recognition of gp41. Basic characteristics of IDR-specific IgG responses including the representative peptide sequence targeted and mean fluorescence intensity within each group are summarized in **Figure 5**. Within gp120, four consecutive peptides covering the tip of the V3 region (indicated as peak 5, aa300 to 320 (HxB2 reference strain) were targeted by >90% of vaccinees with a high mean fluorescence intensity (MFI) (referring to the highest measured response, if multiple peptides covered for this region) of above 50,000 units in both groups. Other IDRs were located in the C1 region (peak 1; HxB2 aa position 104–124 and peak 2; HxB_aa117–136) with maximum MFIs of 43,000 and 25,000 in standard and accelerated groups, respectively; in the

TABLE 3 | Antigenic peaks of recognition in Env.

Peak number	HXB2	Env region	Representative sequence	MFI standard	MFI accelerated
1	104	C1a	MHEDIISLWDQSLKP	34650	19507
1	107	C1a	DVISLWDQSLKPCVK	43478	25183
1	109	C1a	ISLWDQSLKPCVKLT	41274	21634
2	119	C1b/V1	SVKLTPLSVTLNSTD	11993	x
2	121	C1b/V1	KLTPLCVTLNCTNAK	29195	x
3	200	C2	AITQACPKVTFDPIP	30593	25445
4	245	C2	VQCTHGIKPVVSTQL	33780	36173
4	249	C2	HGIKPVVSTQLLLNG	15699	20643
5	300	V3	GNNTRKSIRIGPGQT	44475	40678
5	301	V3	NNTRKSIHIGPGQAF	60570	59711
5	304	V3	RKSINIGPGRAFYAT	59915	59186
5	305	V3	TSIRIGPGQTFYATG	59589	56526
6	429	C4	EVGKAMYAPPIKGQI	x	15762
6	433	C4	AMYAPPIKGQIKCLS	x	19922
7	473	C5	GDMRNNWRSELYKYK	34577	22160
7	475	C5	MKDNWRSELYKYKVV	37522	22782
8	491	C5	IKPLGVAPTTTKRRV	35026	35758

Summary of HxB location, Envelope region, representative peptide sequences, and mean fluorescence intensity (MFI) of the most frequently recognized antigenic regions in Env displayed in ***Figure 5*** *and the MFI of each peak from standard (n = 11) and accelerated (n = 12) groups.*

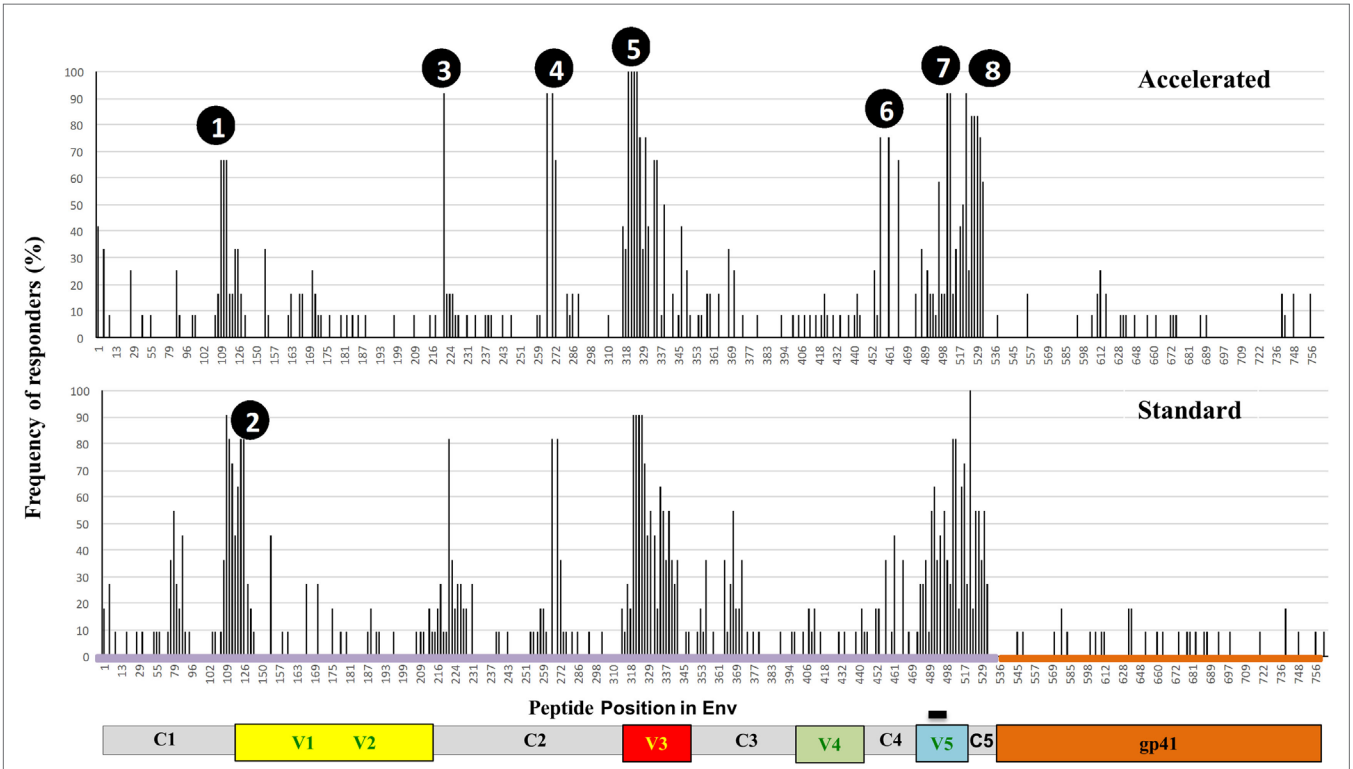

FIGURE 5 | Specificity of systemic binding antibody responses to Env by group. Frequency of recognition of linear overlapping peptides spanning the HIV envelope in plasma samples from the accelerated (*n* = 12, upper panel) and the standard groups (*n* = 11, lower panel). The *y* axis shows the proportion of individuals recognizing the specified peptides and the *x* axis denotes the distribution of linear peptides along the envelope with relevant regions of interest highlighted.

C2 region (peak 3; HxB_aa200–215 and peak 4; HxB_aa245–264) with MFIs between 25,000 and 35,000; and in the C4 (peak 6 HxB2_aa429–457) and C5 regions (peak 7, HxB2_aa473–490 and peak 8, HxB2_aa491–504) were targeted with MFIs between 20,000 and 38,000. All these IDRs were recognized by 70% of vaccines in at least one group. There was no significant differences between the groups in terms of the number of epitopes recognized, the magnitude of individual responses or the sum of fluorescence intensity values for all peptide variants recognized that were included in the array ($p = 0.21$).

T-Cell ELISpot Responses

There were no IFNγ ELISpot responses at baseline and 8 weeks after the third DNA vaccination (week 16) responses were seen in a minority of individuals to Env peptide pools (20.5% overall). At the primary endpoint, there was no difference in either the frequency or magnitude of ELISpot responses between groups; 85 and 82.4% responded to any pool in the accelerated and standard groups, respectively, at the primary endpoint ($p = 1.00$) with overall GM values of 111 and 147 SFC/10^6 PBMCs ($p = 0.44$) **Figure 6**. All responding individuals recognized one or both Env peptide pools (75.0 and 76.5% recognizing Env pool 1 and 65.0 and 64.7% recognizing Env pool 2 for the accelerated and standard groups, respectively, p values >0.99) and the magnitude of these responses was similar between groups [medians 70–189 SFC/10^6 PBMCs for the two Env pools ($p = 0.09$–0.32)]. Gag peptide-specific responses were relatively modest and detected in only 25% of the accelerated and 23.5% of the standard group ($p = 1.00$) with similar magnitude (median 58 and 86 SFC/10^6 PBMCs, $p = 0.28$). There were no responses to the 5′ Pol pool in either group and none of the accelerated group and 17.6% of the standard group responded to the 3′ Pol pool ($p = 0.09$, median 43 SFC/10^6 PBMCs). None of the accelerated group and 5.9% of standard group responded to Nef peptides ($p = 0.46$, 1 response of 179 of SFC/10^6 PBMCs in the standard group). At this time point, the mean number of peptide pools recognized (out of a total possible of 6) for each subject was similar between groups; 1.65 and 1.88 for the accelerated and standard groups respectively (median of 2 for both groups, $p = 0.63$). In terms of magnitude, responses to Env pools peaked 4 weeks after the second MVA-C/CN54gp140 (median 111 SFC/10^6 PBMCs) in the accelerated group and 4 weeks after the first MVA-C in the standard group (median 213 SFC/10^6 PBMCs). While the great majority of responses in both groups recognized Env or Env plus Gag peptides, recognition of ENV in combination with Nef or 3′Pol or of Gag peptides was only seen in the standard group—accounting for approximately 17% of all responses (data not shown). In the

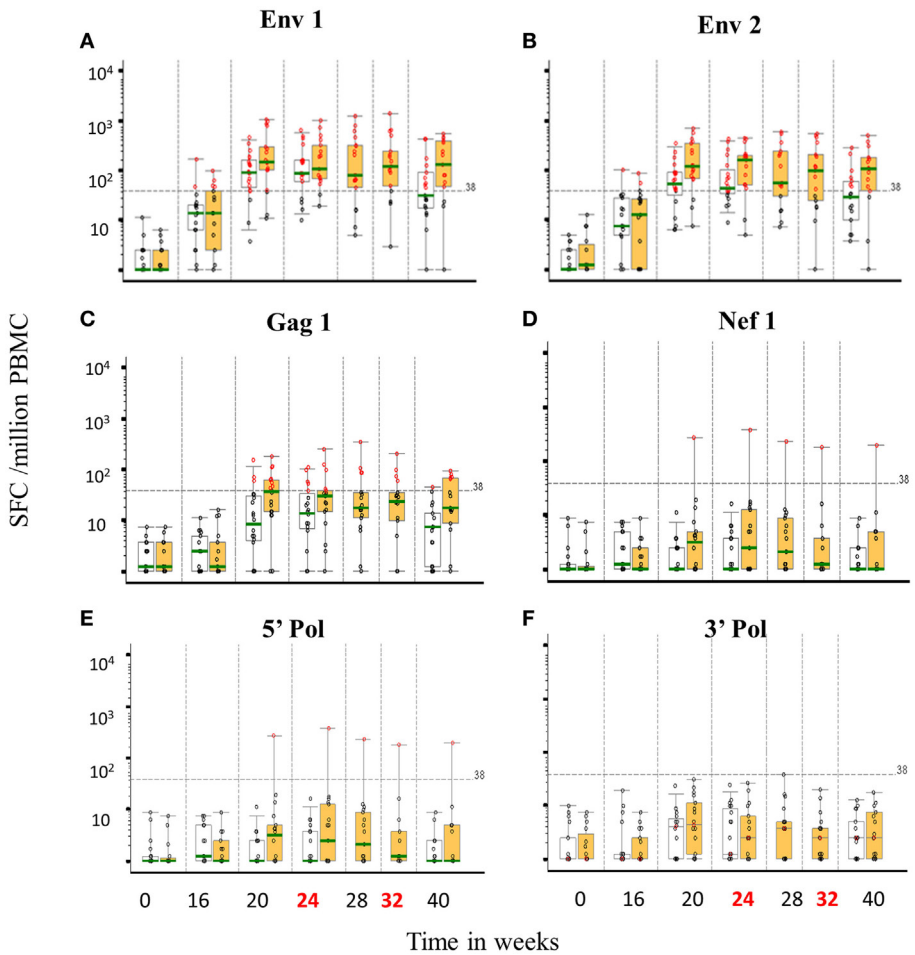

FIGURE 6 | T-cell ELISpot responses by group over time to vaccine encoded peptide pools. Distribution of IFNγ ELISpot responses (background subtracted; spot forming units per million PBMCs) prior to and following vaccine candidate administration for six HIV-1 peptide pools; CN54 1/2, Env 1 **(A)** and 2 **(B)**, ZM65 Gag **(C)**, Nef **(D)**, 5′ Pol **(E)**, and 3′ Pol **(F)**. Boxes represent the interquartile ranges, whiskers extend to the 5th and 95th percentiles and the green bar is the median. Red circles represent positive responses, black circles are negative responses. Accelerated group: open boxes, $n = 20$, Standard group: orange boxes, $n = 18$. Dashed line is the ELISpot assay positive response value (38 SFU/million PBMCs).

accelerated group, responses to Env and Gag peptides dropped between weeks 24 and 40 ($p = 0.04$, 0.02) whereas in the standard group there was no significant change ($p = 0.4$, 0.65). At week 40, the responses to Env and Gag peptide pools was of greater magnitude in the standard than the accelerated group ($p = 0.03$, 0.02, and 0.091, respectively) and more frequent to Env peptides, with 71% responding to both Env peptide pools in the former compared to 37 and 47% in the latter ($p = 0.09$ for responses to Env 1 pool) and this was also true of responses to Gag peptide pools with 30% responding in the standard and 5% in the accelerated group.

Intracellular Cytokine Responses

Overall, intracellular cytokine responses were modest and results are therefore descriptive. The majority of responses were polyfunctional and focused toward Env rather than Gag peptide pools. Response rates to *any* antigen at *any* post-baseline visit, for both CD4 and CD8 populations, were higher in the standard than the accelerated group, 50 versus 30% respectively for CD4+, and 33.3 versus 10% for CD8+ lymphocytes, but these differences were not statistically significant (data not shown). CD4 and CD8 responses to all antigens were predominantly polyfunctional, with IFNγ being the dominant cytokine (**Figure 7**). The standard group tended toward higher CD8 responses (IFNγ+TNFα+) while both groups had comparable CD4 responses (the majority being IL2+IFNγ+). In the standard group, responses seemed to peak at week 24 and wane at week 32 in CD4 lymphocytes but were more persistent in some CD8 lymphocytes (data not shown).

DISCUSSION

We have compared two vaccination regimens using identical DNA-C, MVA-C, and GLA adjuvanted CN54gp140 with the aim of assessing the safety and immunogenicity of a shortened regimen in which the MVA and adjuvanted protein were combined. We expected strong Env-specific CD4+ T-cells after DNA and MVA and Env-specific binding antibodies in everyone after adjuvanted gp140 and the study was powered to detect a four-fold difference in the magnitude of this response between groups. As expected, 100% individuals made strong CN54gp140-specific antibody irrespective of regimen, but combining the vaccines had no detectable impact on the magnitude or specificity of the antibody response as assessed by the recognition of linear peptides. This pattern was characterized by strong recognition of V3 with notably little recognition within gp41—in spite of its presence in the DNA and gp140 vaccines. The immunogens also induced recognition of linear epitopes within C2 and C4, but their significance, if any, is unknown. Despite these binding

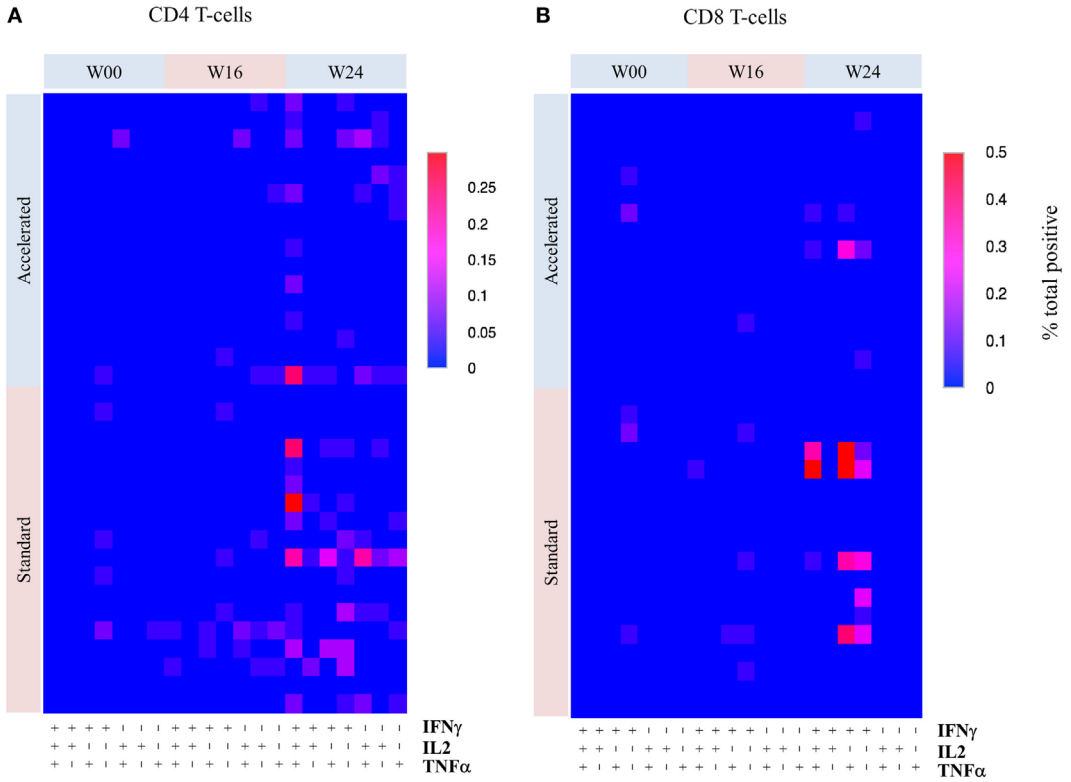

FIGURE 7 | Heatmaps showing the frequency of different cytokine responses by T-cell subset over time by group. Overall frequency of different combinations of three cytokines (IFNγ, IL2, and TNFα) produced by CD4 **(A)** and CD8 **(B)** T-cells responses to combinations of P5/6 Env peptide pools by week. The highest frequency (% of total) is shown in red and the lowest in blue (scale depends on maximal response). Due to the low frequency of responses, no formal statistical comparisons were made between the groups.

antibody responses, neutralizing antibody responses were disappointing and only seen to Tier 1A pseudoviruses, similar to our previous experiences using this adjuvanted protein (27, 28). Interestingly, however, the frequency and titer of the responses was inferior when vaccines were combined to two Tier 1A pseudoviruses. There was no difference in the overall frequency or specificity of T-cell ELISpot responses which were seen in >80% individuals irrespective of group and tended to be CD4+ and specific for Env peptide pools. While the great majority of IFNγ ELISpot responses were seen to Env peptide pools in both groups, recognition of Gag, Nef and Pol peptides was less frequent in the accelerated group suggesting that combining the vaccines might have led to further polarization of immune responses. Cytokine responses were relatively modest, but also less frequent or less polyfunctional in both CD4+ and CD8+ compartments when the MVA and CN54gp140 were combined. There was no significant impact of combining the vaccines upon tolerability. Although two participants experienced events that resulted in discontinuation of immunizations, these followed different immunogens (DNA-C, MVA-C) and there were alternative explanations for the asymptomatic elevation in transaminases in each case. The great majority of adverse events were mild in line with our previous experience using the same adjuvanted protein in different settings (27, 28).

Even though we controlled for variables such as site and gender, the study has limitations; overall statistical power was compromised by the fact that we did not have 40 in the final analyses as planned. Nevertheless, there was clear evidence that combining MVA and gp140 led to attenuation of certain T-cell and B-cell immune responses. Both regimens were shorter overall than used in previous studies using similar homologous (22–24) or heterologous DNA and MVA (26, 42) and it remains possible that the time between DNA prime and MVA boost (8 weeks) might have been too short to allow for the optimal maturation of immune responses. The use of a common and semi-quantitative assay for the measurement of CN54gp140-specific IgG antibody allows for direct comparison across our different trials. The median CN54gp140- specific binding antibody response seen here (12.8 μg/ml) exceeds that seen in the Mucovac 2 trial (UK HVC_001) after three doses of CN54gp140/GLA-AF IM in the absence of DNA priming (4.2 μg/ml) (27), but is lower than seen in the TaMoVac 01 trial (UK HVC_00 2) after boosting twice with GLA-AF adjuvanted CN54gp140 30–71 weeks after priming with DNA and MVA (17.8 μg/ml) (28). This supports the value of DNA/MVA priming and suggests that the long gap between prime and boost could be important. Sallusto and colleagues propose a minimum gap of 12 weeks and note that if boosting is too frequent, responding cells might be preferentially driven to terminal differentiation resulting in attenuation of immunity (43).

Our decision to use immunogens expressing matched subtype C inserts was driven by our commitment to a vaccine for use in Sub-Saharan Africa as well a belief that this approach would ellicit high titer binding antibodies and so favor functional/neutralizing antibodies. In addition to the logistical advantages offered by fewer vaccinations, we were interested in combining the pox and protein in light of the results of the RV144 trial which included combined canarypox and alum adjuvanted gp120 and was the first ever trial of an HIV vaccine to show (modest) efficacy. In UK HVC_003 overall, immunogenicity was somewhat disappointing and it remains possible that the combined MVA and adjuvanted gp140 protein may have overwhelmed the pool of antigen-specific/innate immune cells, as offered as one explanation of the attenuation in responses sometimes seen when certain pediatric vaccines are combined (44, 45) and which might be particularly associated with MVA at it has been shown to be highly immunogenic and to preferentially deplete antigen presenting cells (46–48). Immunogenicity may have been further compromised in the accelerated group as a result antigenic competition (even though the vaccines were administered into opposite arms) as has been suggested to occur in response to response to certain combinations of conjugated vaccines (49) and observed more recently in an HIV vaccine trial in South Africa (25). The choice of adjuvant was partly practical as we had access to GLA-AF through the UK HVC and had already used it with the same gp140 protein (27, 28). In our hands, the GLA-AF (MPLA) adjuvant has previously been shown to be potent for antibodies at equivalent (3 × 100 μg) and lower (3 × 20 μg) doses (27) (in the absence of DNA/MVA), and we had every reason to suspect that priming would further enhance these responses. We expected the immunogens to be at least as immunogenic as those used in RV144 in both groups and, based on our previous experiences with DNA and MVA, predicted that they would be more potent for T-cells.

In light of these findings, we remain cautious about the accelerated regimen, and feel that the combined MVA-C and adjuvanted GLA-AF warrants further evaluation in a regimen with a longer gap between priming and boost. We believe this trial provides further support for exploring the clinical efficacy of a priming regimen including DNA, with at gap of least 12 weeks prior to boosting. The precise contribution of adjuvanted protein (if any) is yet to be defined. While Churchyard and colleagues reported no clear benefit of DNA priming on Env-specific antibody responses, T-cell responses appeared augmented by the priming although overall immunogenicity in the trial was lower than seen previously using the same vaccines in a different population (25). In light of our previous experience with a variety of immunogens, we remain committed to the inclusion of DNA as we believe balanced immune responses highly desirable. We await data from a direct comparison of the specificity of antibody responses from trials using different heterologous prime boost regimens (including RV144 and Tamovac 01) with this one to inform our selection of immunogens to take forward. Non-neutralizing IgG antibody responses targeting V2 have been shown to correlate with a reduced risk of HIV using a variety of different approaches, although we did not see much response to linear peptides within this region (9, 50, 51) suggesting perhaps that they are associated with the subtype E gp120 protein. We did however see strong recognition of the V3 loop in both groups and this has been described as characteristic of the responses seen during natural infection (27) and also in other vaccine trials including RV144-when the response correlated with reduced risk of HIV acquisition in a subset of individuals

(51). In conclusion, the vaccines were potent irrespective of regimen, but immunogenicity was lower than anticipated, and we cannot exclude the possibility that this was due to the relatively short regimens. Our data suggest that combining MVA and CN54gp140/GLA-AF in this relatively short regimen had no significant impact on safety and also no impact on the magnitude of CN54gp140-specific systemic antibody responses and the strategy may have attenuated immunogenicity as reported previously (25).

AUTHOR CONTRIBUTIONS

SM, JW, RS, SJ, JG, DL, RW, and ME oversaw and/or designed the study/immunogens. KQ, AG, SM, AM, SJ, AC, RT, and DL were involved in the day to day running and conduct of the study. AC, PM, PH, JK, CG, YN, MA, and DM were involved in laboratory analyses, interpretation of results, and drafting figures. LD, SJ, and GB conducted the statistical analyses. SJ drafted the manuscript with editorial support and comment from PM, PH, SM, AC, RS, CG, JW, DM, RW, ME, LD, and GB.

ACKNOWLEDGMENTS

We would like to thank the volunteers who agreed to participate in the study and gave generously their time and the clinic teams at both centres. We would also like to thank Josephine Cox and Deborah King from IAVI and the Human Immunology Lab and Kate Skinner at Imperial College for their input. The neutralizing antibody responses were analysed as part of NIH HHSN27201100016C.

FUNDING

This study was funded through core funding from the Wellcome Trust via UKHVC (083844/Z/07/Z), MRC (G1001784), Imperial NIHR BRC, and NIH.

REFERENCES

1. Harmon TM, Fisher KA, McGlynn MG, Stover J, Warren MJ, Teng Y, et al. Exploring the potential health impact and cost-effectiveness of AIDS vaccine within a comprehensive HIV/AIDS response in low- and middle-income countries. *PLoS One* (2016) 11(1):e0146387. doi:10.1371/journal.pone.0146387
2. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, et al. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med* (2011) 365(6):493–505. doi:10.1056/NEJMoa1105243
3. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* (2005) 191(5):654–65. doi:10.1086/428404
4. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* (2006) 194(12):1661–71. doi:10.1086/508748
5. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* (2008) 372(9653):1881–93. doi:10.1016/S0140-6736(08)61591-3
6. Gray GE, Allen M, Moodie Z, Churchyard G, Bekker LG, Nchabeleng M, et al. Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. *Lancet Infect Dis* (2011) 11(7):507–15. doi:10.1016/S1473-3099(11)70098-6
7. Hammer SM, Sobieszczyk ME, Janes H, Karuna ST, Mulligan MJ, Grove D, et al. Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. *N Engl J Med* (2013) 369(22):2083–92. doi:10.1056/NEJMoa1310566
8. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* (2009) 361(23):2209–20. doi:10.1056/NEJMoa0908492
9. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* (2012) 366(14):1275–86. doi:10.1056/NEJMoa1113425
10. Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X, Hwang KK, et al. Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. *J Virol* (2012) 86(21):11521–32. doi:10.1128/JVI.01023-12
11. Tomaras GD, Ferrari G, Shen X, Alam SM, Liao HX, Pollara J, et al. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. *Proc Natl Acad Sci U S A* (2013) 110(22):9019–24. doi:10.1073/pnas.1301456110
12. Chung AW, Ghebremichael M, Robinson H, Brown E, Choi I, Lane S, et al. Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. *Sci Transl Med* (2014) 6(228):228ra38. doi:10.1126/scitranslmed.3007736
13. Li SS, Gilbert PB, Tomaras GD, Kijak G, Ferrari G, Thomas R, et al. FCGR2C polymorphisms associate with HIV-1 vaccine protection in RV144 trial. *J Clin Invest* (2014) 124(9):3879–90. doi:10.1172/JCI75539
14. Liao HX, Bonsignori M, Alam SM, McLellan JS, Tomaras GD, Moody MA, et al. Vaccine induction of antibodies against a structurally heterogeneous site of immune pressure within HIV-1 envelope protein variable regions 1 and 2. *Immunity* (2013) 38(1):176–86. doi:10.1016/j.immuni.2012.11.011
15. Pollara J, Bonsignori M, Moody MA, Liu P, Alam SM, Hwang KK, et al. HIV-1 vaccine-induced C1 and V2 Env-specific antibodies synergize for increased antiviral activities. *J Virol* (2014) 88(14):7715–26. doi:10.1128/JVI.00156-14
16. Hurwitz JL, Zhan X, Brown SA, Bonsignori M, Stambas J, Lockey TD, et al. A multi-vector, multi-envelope HIV-1 vaccine. *J Pediatr Pharmacol Ther* (2007) 12(2):68–76. doi:10.5863/1551-6776-12.2.68
17. Amara RR, Smith JM, Staprans SI, Montefiori DC, Villinger F, Altman JD, et al. Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified *Vaccinia virus* Ankara vaccine. *J Virol* (2002) 76(12):6138–46. doi:10.1128/JVI.76.15.7625-7631.2002
18. Lu S. Heterologous prime-boost vaccination. *Curr Opin Immunol* (2009) 21(3):346–51. doi:10.1016/j.coi.2009.05.016
19. Thorner AR, Lemckert AA, Goudsmit J, Lynch DM, Ewald BA, Denholtz M, et al. Immunogenicity of heterologous recombinant adenovirus prime-boost vaccine regimens is enhanced by circumventing vector cross-reactivity. *J Virol* (2006) 80(24):12009–16. doi:10.1128/JVI.01749-06
20. Zak DE, Andersen-Nissen E, Peterson ER, Sato A, Hamilton MK, Borgerding J, et al. Merck Ad5/HIV induces broad innate immune activation that predicts CD8(+) T-cell responses but is attenuated by preexisting Ad5 immunity. *Proc Natl Acad Sci U S A* (2012) 109(50):E3503–12. doi:10.1073/pnas.1208972109
21. Lu S, Wang S, Grimes-Serrano JM. Current progress of DNA vaccine studies in humans. *Expert Rev Vaccines* (2008) 7(2):175–91. doi:10.1586/14760584.7.2.175
22. McCormack S, Stohr W, Barber T, Bart PA, Harari A, Moog C, et al. EV02: a Phase I trial to compare the safety and immunogenicity of HIV DNA-C prime-NYVAC-C boost to NYVAC-C alone. *Vaccine* (2008) 26(25):3162–74. doi:10.1016/j.vaccine.2008.02.072
23. Harari A, Bart PA, Stohr W, Tapia G, Garcia M, Medjitna-Rais E, et al. An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J Exp Med* (2008) 205(1):63–77. doi:10.1084/jem.20071331
24. Levy Y, Ellefsen K, Stoehr W, Bart PA, Lilievre JD, Launay O, et al. *Optimal Priming of Poxvirus Vector-Based Regimens Requires 3 DNA Injections; Results of the Randomised Multicentre EV03/ANRS Vac20 Phase I/II Trial. 17th Conference on Retroviruses and Opportunistic Infections (78LB)*. San Francisco (2010).

25. Churchyard G, Mlisana K, Karuna S, Williamson AL, Williamson C, Morris L, et al. Sequential immunization with gp140 boosts immune responses primed by modified vaccinia Ankara or DNA in HIV-uninfected South African participants. *PLoS One* (2016) 11(9):e0161753. doi:10.1371/journal.pone.0161753
26. Nilsson C, Hejdeman B, Godoy-Ramirez K, Tecleab T, Scarlatti G, Brave A, et al. HIV-DNA given with or without intradermal electroporation is safe and highly immunogenic in healthy Swedish HIV-1 DNA/MVA vaccines: a phase I randomized trial. *PLoS One* (2015) 10(6):e0131748. doi:10.1371/journal.pone.0131748
27. Cosgrove CA, Lacey CJ, Cope AV, Bartolf A, Morris G, Yan C, et al. Comparative immunogenicity of HIV-1 gp140 vaccine delivered by parenteral, and mucosal routes in female volunteers; MUCOVAC2, a randomized two centre study. *PLoS One* (2016) 11(5):e0152038. doi:10.1371/journal.pone.0152038
28. Joachim A, Bauer A, Joseph S, Geldmacher C, Munseri PJ, Aboud S, et al. Boosting with subtype C CN54rgp140 protein adjuvanted with glucopyranosyl lipid adjuvant after priming with HIV-DNA and HIV-MVA is safe and enhances immune responses: a phase I trial. *PLoS One* (2016) 11(5):e0155702. doi:10.1371/journal.pone.0155702
29. Ledgerwood JE, Wei CJ, Hu Z, Gordon IJ, Enama ME, Hendel CS, et al. DNA priming and influenza vaccine immunogenicity: two phase 1 open label randomised clinical trials. *Lancet Infect Dis* (2011) 11(12):916–24. doi:10.1016/S1473-3099(11)70240-7
30. Wise MC, Hutnick NA, Pollara J, Myles DJ, Williams C, Yan J, et al. An enhanced synthetic multiclade DNA prime induces improved cross-clade-reactive functional antibodies when combined with an adjuvanted protein boost in nonhuman primates. *J Virol* (2015) 89(18):9154–66. doi:10.1128/JVI.00652-15
31. Hulot SL, Korber B, Giorgi EE, Vandergrift N, Saunders KO, Balachandran H, et al. Comparison of immunogenicity in rhesus macaques of transmitted-founder, HIV-1 group M consensus, and trivalent mosaic envelope vaccines formulated as a DNA prime, NYVAC, and envelope protein boost. *J Virol* (2015) 89(12):6462–80. doi:10.1128/JVI.00383-15
32. Keefer MC, Gilmour J, Hayes P, Gill D, Kopycinski J, Cheeseman H, et al. A phase I double blind, placebo-controlled, randomized study of a multigenic HIV-1 adenovirus subtype 35 vector vaccine in healthy uninfected adults. *PLoS One* (2012) 7(8):e41936. doi:10.1371/journal.pone.0041936
33. Lewis DJ, Fraser CA, Mahmoud AN, Wiggins RC, Woodrow M, Cope A, et al. Phase I randomised clinical trial of an HIV-1(CN54), clade C, trimeric envelope vaccine candidate delivered vaginally. *PLoS One* (2011) 6(9):e25165. doi:10.1371/journal.pone.0025165
34. Su L, Graf M, Zhang Y, von Briesen H, Xing H, Kostler J, et al. Characterization of a virtually full-length human immunodeficiency virus type 1 genome of a prevalent intersubtype (C/B') recombinant strain in China. *J Virol* (2000) 74(23):11367–76. doi:10.1128/JVI.74.23.11367-11376.2000
35. Pabst M, Chang M, Stadlmann J, Altmann F. Glycan profiles of the 27 N-glycosylation sites of the HIV envelope protein CN54gp140. *Biol Chem* (2012) 393(8):719–30. doi:10.1515/hsz-2012-0148
36. Barouch DH, Yang ZY, Kong WP, Korioth-Schmitz B, Sumida SM, Truitt DM, et al. A human T-cell leukemia virus type 1 regulatory element enhances the immunogenicity of human immunodeficiency virus type 1 DNA vaccines in mice and nonhuman primates. *J Virol* (2005) 79(14):8828–34. doi:10.1128/JVI.79.14.8828-8834.2005
37. Gomez CE, Najera JL, Jimenez V, Bieler K, Wild J, Kostic L, et al. Generation and immunogenicity of novel HIV/AIDS vaccine candidates targeting HIV-1 Env/Gag-Pol-Nef antigens of clade C. *Vaccine* (2007) 25(11):1969–92. doi:10.1016/j.vaccine.2006.11.051
38. Gomez CE, Perdiguero B, Jimenez V, Filali-Mouhim A, Ghneim K, Haddad EK, et al. Systems analysis of MVA-C induced immune response reveals its significance as a vaccine candidate against HIV/AIDS of clade C. *PLoS One* (2012) 7(4):e35485. doi:10.1371/journal.pone.0035485
39. Sarzotti-Kelsoe M, Bailer RT, Turk E, Lin CL, Bilska M, Greene KM, et al. Optimization and validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1. *J Immunol Methods* (2014) 409:131–46. doi:10.1016/j.jim.2013.11.022
40. Boaz MJ, Hayes P, Tarragona T, Seamons L, Cooper A, Birungi J, et al. Concordant proficiency in measurement of T-cell immunity in human immunodeficiency virus vaccine clinical trials by peripheral blood mononuclear cell and enzyme-linked immunospot assays in laboratories from three continents. *Clin Vaccine Immunol* (2009) 16(2):147–55. doi:10.1128/CVI.00326-08
41. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* (2011) 79(2):167–74. doi:10.1002/cyto.a.21015
42. Munseri PJ, Kroidl A, Nilsson C, Joachim A, Geldmacher C, Mann P, et al. Priming with a simplified intradermal HIV-1 DNA vaccine regimen followed by boosting with recombinant HIV-1 MVA vaccine is safe and immunogenic: a phase IIa randomized clinical trial. *PLoS One* (2015) 10(4):e0119629. doi:10.1371/journal.pone.0119629
43. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. *Immunity* (2010) 33(4):451–63. doi:10.1016/j.immuni.2010.10.008
44. Bar-On ES, Goldberg E, Fraser A, Vidal L, Hellmann S, Leibovici L. Combined DTP-HBV-HIB vaccine versus separately administered DTP-HBV and HIB vaccines for primary prevention of diphtheria, tetanus, pertussis, hepatitis B and *Haemophilus influenzae* B (HIB). *Cochrane Database Syst Rev* (2009) (3):CD005530. doi:10.1002/14651858.CD005530.pub2
45. Findlow H, Borrow R. Interactions of conjugate vaccines and co-administered vaccines. *Hum Vaccin Immunother* (2016) 12(1):226–30. doi:10.1080/21645515.2015.1091908
46. Liu L, Chavan R, Feinberg MB. Dendritic cells are preferentially targeted among hematolymphocytes by modified *Vaccinia virus* Ankara and play a key role in the induction of virus-specific T cell responses in vivo. *BMC Immunol* (2008) 9:15. doi:10.1186/1471-2172-9-15
47. Guzman E, Cubillos-Zapata C, Cottingham MG, Gilbert SC, Prentice H, Charleston B, et al. Modified *Vaccinia virus* Ankara-based vaccine vectors induce apoptosis in dendritic cells draining from the skin via both the extrinsic and intrinsic caspase pathways, preventing efficient antigen presentation. *J Virol* (2012) 86(10):5452–66. doi:10.1128/JVI.00264-12
48. Kastenmuller W, Drexler I, Ludwig H, Erfle V, Peschel C, Bernhard H, et al. Infection of human dendritic cells with recombinant *Vaccinia virus* MVA reveals general persistence of viral early transcription but distinct maturation-dependent cytopathogenicity. *Virology* (2006) 350(2):276–88. doi:10.1016/j.virol.2006.02.039
49. Buttery JP, Riddell A, McVernon J, Chantler T, Lane L, Bowen-Morris J, et al. Immunogenicity and safety of a combination pneumococcal-meningococcal vaccine in infants: a randomized controlled trial. *JAMA* (2005) 293(14):1751–8. doi:10.1001/jama.293.14.1751
50. Zolla-Pazner S, deCamp A, Gilbert PB, Williams C, Yates NL, Williams WT, et al. Vaccine-induced IgG antibodies to V1V2 regions of multiple HIV-1 subtypes correlate with decreased risk of HIV-1 infection. *PLoS One* (2014) 9(2):e87572. doi:10.1371/journal.pone.0087572
51. Gottardo R, Bailer RT, Korber BT, Gnanakaran S, Phillips J, Shen X, et al. Plasma IgG to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlate with a reduced risk of infection in the RV144 vaccine efficacy trial. *PLoS One* (2013) 8(9):e75665. doi:10.1371/journal.pone.0075665

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

PERSPECTIVE
published: 11 February 2019
doi: 10.3389/fped.2019.00027

The TAM-TB Assay—A Promising TB Immune-Diagnostic Test With a Potential for Treatment Monitoring

Mohamed I. M. Ahmed[1,2,3], *Christian Ziegler*[4], *Kathrin Held*[1,2], *Ilja Dubinski*[5], *Julia Ley-Zaporozhan*[6], *Christof Geldmacher*[1,2†] *and Ulrich von Both*[1,5,7*†]

[1] German Center for Infection Research (DZIF), Partner Site Munich, Munich, Germany, [2] Department of Tropical Medicine and Infectious Diseases, University Hospital, Ludwig-Maximilians-University, Munich, Germany, [3] CIH[LMU] Center for International Health, University of Munich, Munich, Germany, [4] Division of Orthopaedics, Ludwig-Maximilians-University, Munich, Germany, [5] Division of Paediatric Infectious Diseases, Dr. von Hauner Children's Hospital, University Hospital, Ludwig-Maximilians-University, Munich, Germany, [6] Department of Radiology, Paediatric Radiology, University Hospital, Ludwig-Maximilians-University, Munich, Germany, [7] Section of Paediatric Infectious Diseases and Allergy, Department of Medicine, Imperial College London, London, United Kingdom

OPEN ACCESS

Edited by:
Dimitri Van der Linden, Cliniques Universitaires Saint-Luc, Belgium

Reviewed by:
Danilo Buonsenso, Catholic University of Sacred Heart, Italy
Ben J. Marais, University of Sydney, Australia

***Correspondence:**
Ulrich von Both
Ulrich.von.Both@med.uni-muenchen.de

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Pediatric Infectious Diseases, a section of the journal Frontiers in Pediatrics

Received: 19 September 2018
Accepted: 22 January 2019
Published: 11 February 2019

Citation:
Ahmed MIM, Ziegler C, Held K, Dubinski I, Ley-Zaporozhan J, Geldmacher C and von Both U (2019) The TAM-TB Assay—A Promising TB Immune-Diagnostic Test With a Potential for Treatment Monitoring. Front. Pediatr. 7:27. doi: 10.3389/fped.2019.00027

Tuberculosis (TB) epidemiology is changing in Western and Central Europe due to the rise in immigration and refugees fleeing high-TB-burden areas of war and devastation. The change in local demography and the lack of sensitive and specific TB diagnostic and monitoring tools, especially for cases of childhood TB, leads to either missed cases or over-treatment of this group. Here we present a promising new diagnostic approach, the T cell activation marker (TAM)-TB assay, and its performance in a case of extra-pulmonary TB occurring in a 16 year old refugee from Afghanistan. This assay is based on the characterization of 3 activation markers (CD38, HLA-DR, and Ki67) and one maturation marker (CD27) on *M. tuberculosis*-specific CD4 T cells. It was performed at time-points T0 (10 days), T1 (1 month), T2 (6 months), and T3 (12 months) post-treatment initiation. All markers were able to detect active tuberculosis (aTB) within this patient at T0 and reverted to a healthy/LTBI phenotype at the end of treatment. Tantalizingly, there was a clear trend toward the healthy/LTBI phenotype for the markers at T1 and T2, indicating a potential role in monitoring anti-TB treatment in the future. This assay may therefore contribute to improved TB diagnostic algorithms and TB treatment monitoring, potentially allowing for individualization of TB treatment duration in the future.

Keywords: extra-pulmonary tuberculosis, treatment monitoring, TAM-TB assay, TB diagnostics, pediatric tuberculosis

INTRODUCTION

Childhood TB, particularly in its extra-pulmonary form, is very challenging to diagnose, let alone to monitor treatment response (1, 2). Predictive markers that would allow clinicians to differentiate between ongoing active disease and cure as well as specific correlates for protection are lacking (3). Thus, there is an urgent need for improved diagnostic tests. Traditional diagnostic tests such as the Tuberculin Skin Test (TST) or the Interferon Gamma Release Assays (IGRAs) are primarily detecting *Mycobacterium* tuberculosis (MTB) infection, however their inability to differentiate between aTB, LTBI and successfully treated TB makes them an unsatisfactory tool for diagnosis of active TB. Only two diagnostic methods in use form the current "gold standard," the liquid

culture method, and the PCR technique (GeneXpert MTB/RIF®, Cepheid) both of which involve the detection of MTB in sputum or other clinical samples. However, the reliance on sputum samples makes the liquid culture susceptible to contamination (4). Furthermore, obtaining sputum samples from pediatric patients, especially in infants, is difficult. Another, group of patients where microbiological detection of MTB tends to be difficult consists of extra-pulmonary cases who do not necessarily produce sputum containing MTB. In addition to the described sampling issues the liquid culture method is a long process, requiring weeks for results to be available, Furthermore, a major limitation of all currently available diagnostic tools is their inability to monitor treatment response. Liquid culture methods require weeks for results to be available, and as treatment progresses the production of sputum and detection of live MTB in sputum becomes more difficult (5). The GeneXpert MTB/RIF® method, though being a very sensitive method is hampered by its inability to differentiate between live and dead bacilli, thus not allowing for evaluating success of treatment (6). This has led to a presumed overtreatment of a majority of pulmonary TB patients, shown in previous studies reporting that at least 80% of pulmonary TB patients had been cured within 4 month of anti-TB treatment not showing any signs of relapse within the defined study period (7–9). An optimal monitoring tool would be rapid and dynamic to allow *in vivo* measurement of changes of biomarkers to mirror treatment progress in each individual patient, this opens the avenue toward a personalized medicine approach in TB care—particularly in children and adolescents.

AN EXEMPLARY CASE

A 16 year-old male refugee from Afghanistan with a self-reported unremarkable past medical history presented to our tertiary care hospital complaining of a 3 months history of worsening left-sided hip pain, while otherwise being clinically well without signs of fever. Four weeks prior to presenting to our institution he had been seen in a private surgical practice. An ultrasound of the hip revealed significant effusion and joint fluid aspirate obtained via subsequent puncture was sent for conventional bacterial cultures. The patient was discharged home on ibuprofen; conventional microbiological culture showed no growth of bacteria. On presentation to our A&E department he was unable to walk due to severe left-sided hip pain, while being otherwise clinically well. His routine bloods showed a mildly raised CRP (26 mg/l), normal FBC and chemistry. Conventional radiography and a subsequent MRI scan of the left hip showed blurring of cortical margins of the femoral head and acetabulum, hyper intensity of the left hip and acetabulum (oedema of the bone marrow) and marked joint effusion on the T2 weighted image with fat suppression as well as synovial thickening and enhancement on the post-contrast T1 weighted image (**Figures 1A–C**). A chest x-ray showed signs of adult-type TB, but the patient did not display any clinical evidence of active pulmonary tuberculosis (data not shown). Hence, this result was not followed up by a CT scan of the chest. In view of the prolonged, non-acute clinical course, and his background of being born and raised in a TB high-endemic country (national incidence, including HIV+TB: 189 per 100.000; source: http://www.who.int/tb/country/data/profiles/en/), work-up for suspected tuberculosis of the hip was initiated. IGRA testing (QuantiFeronGold®, Qiagen) was positive; AFBs were seen on microscopy from joint fluid aspirate, subsequently confirmed as *M. tuberculosis* (MTB) using PCR (GeneXpert MTB/RIF®, Cepheid) and culture. Since we were able to diagnose this patient on his joint fluid aspirate (microscopy results available on the same day), we deliberately refrained from a synovial biopsy, a more invasive diagnostic approach, generally recommended to obtain the gold standard specimen if joint involvement is suspected in tuberculosis. An HIV test was negative. The patient reported that he had never received any treatment for TB previously. He was started on isoniazid, rifampicin, pyrazinamide, and ethambutol for 2 months, followed by an additional 10 months period on rifampicin and isoniazid. Drug resistance testing showed a fully sensitive MTB isolate. Treatment was well tolerated and during the 12 months course, the patient showed continuous clinical improvement with regular ibuprofen required until month 3 of treatment. During this phase, treatment was supplemented by pantoprazole. Follow-up clinical visits revealed no signs of side effects and radiological assessment documented gradual improvement on MRI imaging but also unavoidable long-term damage to the left hip joint on conventional x-ray in terms of severe narrowing of the joint space, osteophytes and severe deformity of the femoral head was evident (**Figures 1D,E**). Though the patient was able to move slowly without support and almost free of pain from month 9 of anti-tuberculosis treatment, regaining his full range of movement was impossible due to extensive destruction of the left hip. Thus, almost 2 years following TB diagnosis he underwent hip-replacement surgery and is currently in perfect health having regained full mobility.

TAM-TB ASSAY RESULTS

After obtaining written informed consent from the patient, we tested a novel, rapid, sputum independent diagnostic approach, now referred to as the T cell activation marker (TAM)-TB assay. The TAM-TB assay has been shown to accurately differentiate between active pulmonary TB (aTB) and latent TB infection (LTBI) in different age groups by determination of the phenotypic and functional characteristics of MTB-specific CD4 T cells via flow cytometry (10–12); detection of high frequencies of activated ($CD38^+$, $HLA\text{-}DR^+$, and $Ki67^+$), $CD27^{low}$ effector memory MTB-specific CD4 T cells is indicative of active TB. Furthermore, results using this approach have been shown to correlate with disease severity and lung tissue destruction (13). Sequential blood samples were obtained from the time of diagnosis (T0, day 10 after treatment initiation), 1 month (T1), 6 months (T2) post-treatment initiation, and at end of treatment (12 months, T3) in order to determine expression levels of the T cell activation markers CD38, HLA-DR, and Ki67 and the maturation marker CD27 on $IFNy^+$ MTB-specific CD4 T cells. As previously described (14), the assay was performed

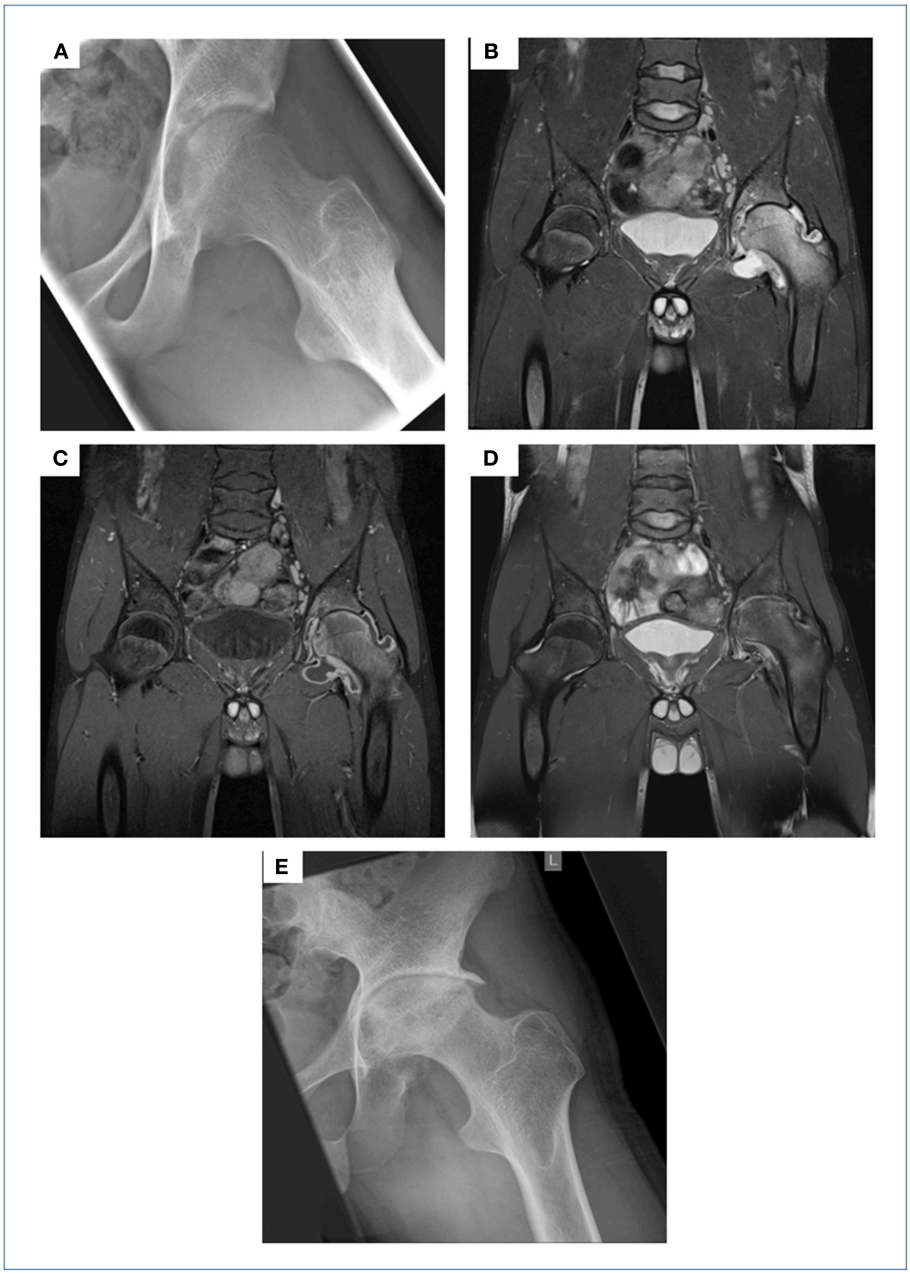

FIGURE 1 | Initial X-ray of the left hip showed blurring of the cortical margins of the femoral head and acetabulum, slight demineralization, and a bulging fat pad surrounding the hip suggesting a joint effusion **(A)**. On the initial MRI scans there was a hyper intensity of the left hip and acetabulum (=oedema of the bone marrow) as well as marked joint effusion on the T2 weighted image with fat suppression **(B)**, synovial thickening and enhancement on the post-contrast T1 weighted image with fat suppression **(C)**. These MRI findings were receding following 13 months of anti-TB treatment (T2 weighted image with fat suppression, **D**) on the one hand, but on the other hand a severe narrowing of the joint space, osteophytes and severe deformity of the femoral head became present. These findings of secondary osteoarthrosis were also demonstrated on the follow-up X-ray few months later **(E)**.

by stimulation of PBMCs with ESAT-6/CFP-10, PPD, and Staphylococcal enterotoxin B (SEB), as a positive control, or no added peptide, as a negative control. PBMCs were surface stained with anti-CD4, anti-CD38, anti-CD27, and anti-HLA-DR, fixed and permeabilized, then stained intracellularly with anti-CD3, anti-IFNy, and anti-Ki67. Data from subjects with active pulmonary TB and LTBI from our previous study to define the cut-offs for each marker to differentiate *aTB* and LTBI were used (14). Remarkably, at T0 all four markers assessed in the TAM-TB assay classified our patient as *aTB*, while at T3 they showed a similar phenotype to the healthy $IGRA^{+}$ group of patients, confirming its potential use in the diagnosis of extra-pulmonary TB (**Figure 2**). For ESAT-6/CFP-10 stimulated cells, the frequency of activated MTB-specific CD4 T cells expressing

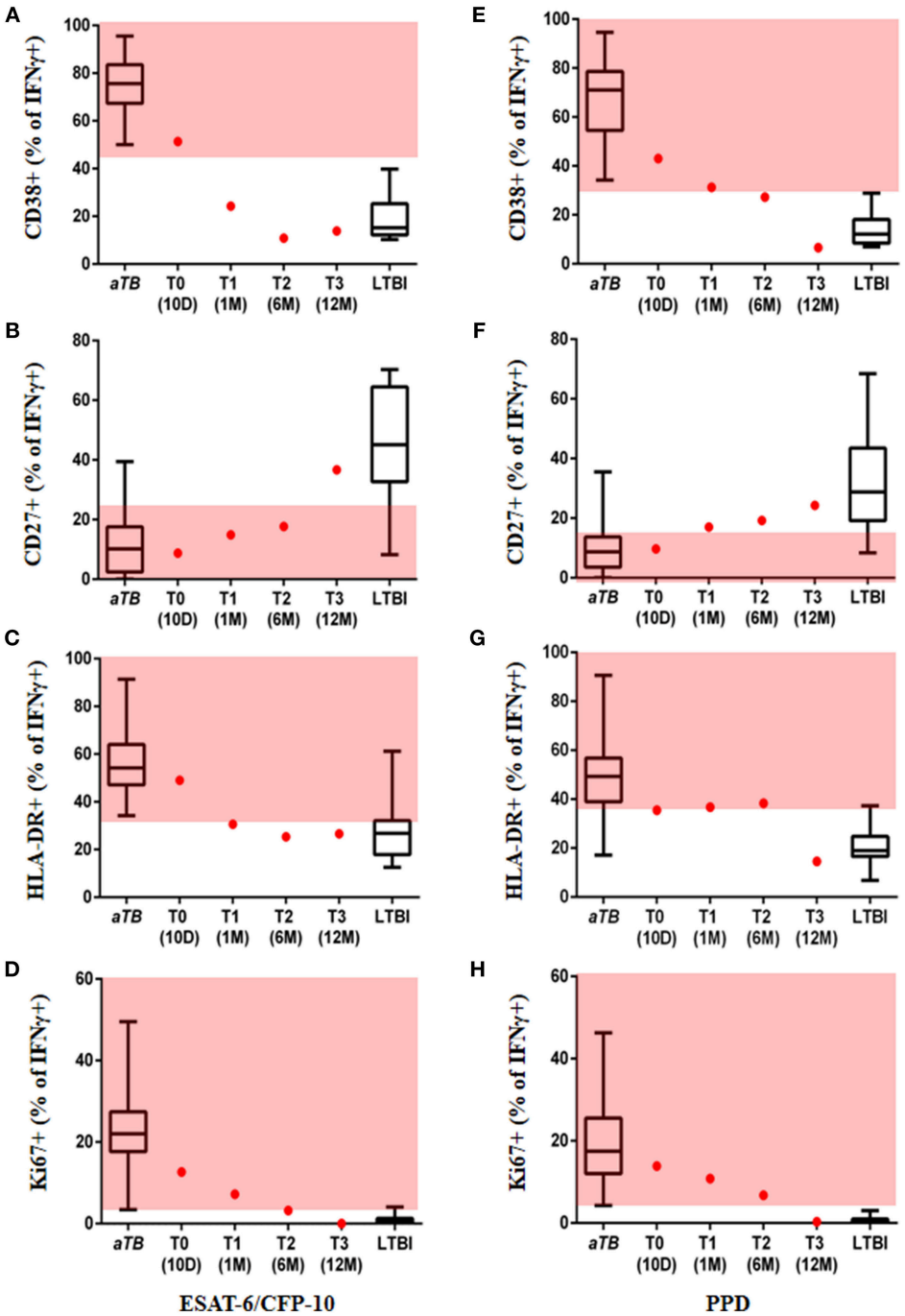

FIGURE 2 | Marker expression on MTB-specific CD4 T cells. Values are provided at the start, during and at the end of treatment. Frequency of ESAT-6/CFP-10 stimulated MTB-specific CD4 T cells expressing the markers CD38 **(A)**, CD27 **(B)**, HLA-DR **(C)**, Ki67 **(D),** and PPD stimulated MTB-specific CD4 T cells expressing CD38 **(E)**, CD27 **(F)**, HLA-DR **(G)**, Ki67 **(H)** are displayed as red dots at 10 days (T0), 1 month (T1), 6 months (T2), and 12 months (T3) after TB treatment initiation; data from subjects with acute pulmonary tuberculosis and healthy/latent TB infection were included as reference (box and whisker plots). Red area represents the region considered to show an active TB profile with cut-offs based on ROC analysis; ROC cut-off values for ESAT-6/CFP-10, CD38 = 44.9, CD27 = 22.1, HLA-DR = 33.15, Ki67 = 2.43 and for PPD, CD38 = 31.55, CD27 = 18.35, HLA-DR = 35.7, Ki67 = 3.67 (14).

CD38 and HLA-DR declined rapidly within the first month after treatment initiation from 51.4% at T0 to 24.3% at T1 and from 49 to 31.6%, respectively (**Figures 2A,C**). Unlike the other two markers (CD27 and Ki67), these markers seem to have provided an LTBI phenotype within a short period (1 month). The frequency of MTB-specific CD4 T cells expressing the cell

cycle marker Ki67 showed a gradual decrease and was still within the range observed for *aTB* at T2 post-treatment, but eventually indicated "*LTBI or cure*" at the end of treatment (T3) (**Figure 2D**). A substantial increase in the frequency of $CD27^+$ MTB-specific CD4 T cells was only observed between T2 and T3 (**Figure 2D**). It is particularly noteworthy that at the end of treatment (T3), the patient showed a TAM-TB assay profile consistent with "*LTBI or cure*" for all four markers. Unlike the ESAT-6/CFP-10 stimulated cells, the frequency of activated PPD stimulated MTB-specific CD4 T cells expressing the activation markers showed the strongest percentage decline between T2 and T3 (**Figures 2E–H**). Two of the markers (CD27 and CD38) seem to show a borderline LTBI phenotype at T2, with all the markers indicating an "*LTBI or cure*" at the end of treatment (T3) (**Figures 2E–H**).

Future studies will be needed to determine whether TAM-TB assay results could have guided earlier discontinuation of anti-TB therapy in patients before the defined 12 months period. Of note, an additional TAM-TB result obtained from the patient 2 months after discontinuation of anti-TB treatment (14M) was still consistent with "*LTBI or cure*" (data not shown).

DISCUSSION/CONCLUSION

Central and Western Europe, Germany in particular, has seen an increase in TB incidence rate in recent years changing from 5.2/100.000 (2012) to 7.2/100.000 (2015) (15). This change in epidemiology has primarily been attributed to an increase in migration activity from low/intermediate-income, high TB burden settings as a consequence of people fleeing areas of war and devastation. Reactivation of latent tuberculosis infection (LTBI) in the high-income, low TB burden countries has thus become a challenge for clinicians both in adult and pediatric care (16). The adolescent case described in this report is a perfect example of how wildly adapted national diagnostics and treatment guidelines are often limited in the way they are tailored to the local population. Almost any youth of German descent presenting with a painful left hip would have most likely been diagnosed and treated sufficiently according to those guidelines. But pediatric or adolescent tuberculosis, especially in its extra-pulmonary form, is such a rare event in the German setting, that this differential diagnosis was missed initially. It is important to highlight the changes in TB epidemiology and to be aware of TB in patients having moved to central Europe from high TB burden settings. Although a good outcome was achieved following hip replacement, this still remains a problematic long-term solution in someone who is 16 years of age and will inevitably be requiring additional revision-interventions in the future. This again underscores the importance of early accurate diagnosis and treatment.

With this perspective we aim to highlight that the TAM-TB assay is a promising new tool with great potential to improve TB diagnostics, particularly in difficult-to-diagnose cases of extra-pulmonary TB or children and adolescents in whom conventional tests are frequently unsuccessful. Immunological biomarkers have already demonstrated to be very promising to indirectly diagnose TB; and they are highly likely to play an even more prominent role in TB diagnostics in the years and decades to come (3, 17, 18). Several studies have shown the benefit of using flow cytometric techniques to distinguish aTB from LTBI, the earliest reporting the CD27 marker (19). This was followed by a plethora of markers such as HLA-DR, CD38, Ki67, or caspase-3 (12, 20). Other methods using cytokine secretion in whole blood may allow diagnosis of aTB (21), however these methods do not seem to be reliable in monitoring TB treatment (22). Another approach makes use of whole blood transcriptional profiling to identify diagnostic RNA signatures for aTB and LTBI, respectively (2, 23, 24). These studies are extremely interesting and yielded very promising results. However, they have not been shown to be of use for treatment monitoring. Furthermore, until the respective diagnostic signatures have been transformed into a simpler diagnostic test that could be used in daily routine, their use appears impractical due to the highly sophisticated resources required (i.e., running microarrays or RNA sequencing).

Sali et al. was able to show that combined use of the QuantiFeronGold® with a heparin-binding hemagglutinin antigen (HBHA)-based IGRA helped to differentiate Quantiferon-positive children with LTBI from those with aTB (25). While further validation on larger cohorts of MTB-infected children will be necessary to describe the potential of this method for treatment monitoring, issues in identifying asymptomatic children with aTB appears to be its main disadvantage.

In flow cytometry-based assays it has been noted that while CD38, HLA-DR, and Ki67 appeared to be useful markers for monitoring of treatment response in a pulmonary TB cohort; CD27 was a marker that converts rather slowly to its "healthy" state (12, 19). Our assay was successful in diagnosing this case of active extra-pulmonary TB and showed an accurate treatment response at the end of the 12 months treatment period by indicating a "healthy/LTBI" state reflected in respective expression values for all four markers. In addition, all markers (with the exception of HLA-DR in the PPD stimulation assay) did indicate a dynamic trend toward the desired treatment outcome. Whether and how such TAM-TB results during anti-TB treatment could potentially inform decisions on treatment discontinuation in individual patients in the future remains to be evaluated in larger cohorts. Since our patient was 16 years of age and because adolescent children essentially behave like adults, this promising test will also have immediate relevance to adults with extra-pulmonary TB.

However, the TAM-TB assay remains an expensive technique requiring specialized equipment and trained personnel to operate, thus its use as a point of care test (POCT, on-site) technique is currently not feasible. However, several studies have shown that flow-cytometry-based assays can be further simplified by using whole blood rather than PBMC, thus reducing the blood volume required and, in consequence, making them more feasible to use, especially in infants and younger children (26, 27)

Nevertheless, our results provide a first important proof-of-concept that the TAM-TB assay might also be a useful and powerful tool to monitor clinical response to treatment

in cases of extra-pulmonary TB. Though the results of this case look promising, they only represent the treatment course of a single patient and one needs to be aware that the respective values of the four TAM-TB markers were referenced to cut-off values obtained from a pulmonary TB cohort. It is likely that since extra-pulmonary TB is a different clinical entity than the pulmonary form of TB, adjusted cut-offs may be required for extra-pulmonary TB cases to allow more accurate interpretation of the results. We do not propose using the TAM-TB assay as a new stand-alone technique for diagnosing tuberculosis, but instead we rather suggest to develop a diagnostic algorithm involving all currently available tools in order to be able to eventually tailor TB treatment duration for individual patients. Still, we are confident that this new assay holds a great potential for the challenging area of TB care and larger prospective clinical trials are currently underway to further validate its performance in the field.

AUTHOR CONTRIBUTIONS

MA, UvB, and CG designed and conducted the diagnostic work-up. MA and UvB wrote the manuscript. ID, CZ, and UvB took clinical care of the patient and collected TAM TB samples. MA, KH, and CG ran and analyzed TAM TB assay. JL-Z reported on imaging results and created respective figure for image display.

FUNDING

This work was supported by the German Center for Infection Research (DZIF).

ACKNOWLEDGMENTS

Written informed consent for conducting TAM TB assay, publication of this article, and respective radiographs has been obtained from the patient.

REFERENCES

1. Marais BJ. Childhood tuberculosis: epidemiology and natural history of disease. *Indian J Pediatr.* (2011) 78:321–7. doi: 10.1007/s12098-010-0353-1
2. Anderson ST, Kaforou M, Brent AJ, Wright VJ, Banwell CM, Chagaluka G, et al. Diagnosis of childhood tuberculosis and host RNA expression in Africa. *N Engl J Med.* (2014) 370:1712–23. doi: 10.1056/NEJMoa1303657
3. von Both U, Kaforou M, Levin M, Newton SM. Understanding immune protection against tuberculosis using RNA expression profiling. *Vaccine* (2015) 33:5289–93. doi: 10.1016/j.vaccine.2015.05.025
4. Sloan DJ, Corbett EL, Butterworth AE, Mwandumba HC, Khoo SH, Mdolo A, et al. Optimizing outpatient serial sputum colony counting for studies of tuberculosis treatment in resource-poor settings. *J Clin Microbiol.* (2012) 50:2315–20. doi: 10.1128/JCM.00043-12
5. Horne DJ, Royce SE, Gooze L, Narita M, Hopewell PC, Nahid P, et al. Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis. *Lancet Infect Dis.* (2010) 10:387–94. doi: 10.1016/S1473-3099(10)70071-2
6. Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, et al. Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. *Lancet Respir Med.* (2013) 1:462–70. doi: 10.1016/S2213-2600(13)70119-X
7. Merle CS, Fielding K, Sow OB, Gninafon M, Lo MB, Mthiyane T, et al. A four-month gatifloxacin-containing regimen for treating tuberculosis. *N Engl J Med.* (2014) 371:1588–98. doi: 10.1056/NEJMoa1315817
8. Jindani A, Harrison TS, Nunn AJ, Phillips PP, Churchyard GJ, Charalambous S, et al. High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. *N Engl J Med.* (2014) 371:1599–608. doi: 10.1056/NEJMoa1314210
9. Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *N Engl J Med.* (2014) 371:1577–87. doi: 10.1056/NEJMoa1407426
10. Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya NE, et al. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. *Lancet Infect Dis.* (2014) 14:931–8. doi: 10.1016/S1473-3099(14)70884-9
11. Rachow A, Heinrich N, Geldmacher C. Early identification of progressive TB disease using host biomarkers. *EBioMedicine* (2015) 2:107–8. doi: 10.1016/j.ebiom.2015.01.009
12. Adekambi T, Ibegbu CC, Cagle S, Kalokhe AS, Wang YF, Hu Y, et al. Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. *J Clin Invest.* (2015) 125:1827–38. doi: 10.1172/JCI77990
13. Nikitina IY, Kondratuk NA, Kosmiadi GA, Amansahedov RB, Vasilyeva IA, Ganusov VV, et al. Mtb-specific CD27low CD4 T cells as markers of lung tissue destruction during pulmonary tuberculosis in humans. *PLoS ONE* (2012) 7:e43733. doi: 10.1371/journal.pone.0043733
14. Ahmed MIM, Ntinginya NE, Kibiki G, Mtafya BA, Semvua H, Mpagama S, et al. Phenotypic changes on mycobacterium tuberculosis-specific CD4 T cells as surrogate markers for tuberculosis treatment efficacy. *Front Immunol.* (2018) 9:2247. doi: 10.3389/fimmu.2018.02247
15. Institute RK. *Bericht zur Epidemiologie der Tuberkulose in Deutschland für (2016)* 2016.
16. Pareek M, Greenaway C, Noori T, Munoz J, Zenner D. The impact of migration on tuberculosis epidemiology and control in high-income countries: a review. *BMC Med.* (2016) 14:48. doi: 10.1186/s12916-016-0595-5
17. Wallis RS, Kim P, Cole S, Hanna D, Andrade BB, Maeurer M, et al. Tuberculosis biomarkers discovery: developments, needs, and challenges. *Lancet Infect Dis.* (2013) 13:362–72. doi: 10.1016/S1473-3099(13)70034-3
18. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. *Nat Rev Immunol.* (2011) 11:343–54. doi: 10.1038/nri2960
19. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. *PLoS ONE* (2007) 2:e735. doi: 10.1371/journal.pone.0000735
20. Adekambi T, Ibegbu CC, Cagle S, Ray SM, Rengarajan J. High frequencies of caspase-3 expressing mycobacterium tuberculosis-specific CD4(+) T cells are associated with active tuberculosis. *Front Immunol.* (2018) 9:1481. doi: 10.3389/fimmu.2018.01481
21. Sun Q, Wei W, Sha W. Potential role for mycobacterium tuberculosis specific IL-2 and IFN-gamma responses in discriminating between latent infection and active disease after long-term stimulation. *PLoS ONE* (2016) 11:e0166501. doi: 10.1371/journal.pone.0166501
22. Leem AY, Song JH, Lee EH, Lee H, Sim B, Kim SY, et al. Changes in cytokine responses to TB antigens ESAT-6, CFP-10 and TB 7.7 and inflammatory markers in peripheral blood during therapy. *Sci Rep.* (2018) 8:1159. doi: 10.1038/s41598-018-19523-7
23. Bloom CI, Graham CM, Berry MP, Wilkinson KA, Oni T, Rozakeas F, et al. Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS ONE* (2012) 7:e46191. doi: 10.1371/journal.pone.0046191
24. Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. *Lancet* (2016) 387:2312–22. doi: 10.1016/S0140-6736(15)01316-1
25. Sali M, Buonsenso D, D'Alfonso P, De Maio F, Ceccarelli M, Battah B, et al. Combined use of Quantiferon and HBHA-based IGRA supports tuberculosis

diagnosis and therapy management in children. *J Infect.* (2018) 77:526–33. doi: 10.1016/j.jinf.2018.09.011

26. Schuetz A, Haule A, Reither K, Ngwenyama N, Rachow A, Meyerhans A, et al. Monitoring CD27 expression to evaluate Mycobacterium tuberculosis activity in HIV-1 infected individuals *in vivo*. *PLoS ONE* (2011) 6:e27284. doi: 10.1371/journal.pone.0027284
27. Musvosvi M, Duffy D, Filander E, Africa H, Mabwe S, Jaxa L, et al. T-cell biomarkers for diagnosis of tuberculosis: candidate evaluation by a simple whole blood assay for clinical translation. *Eur Respir J.* (2018) 51:3. doi: 10.1183/13993003.00153-2018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.