Immunological correlates of vaccine-derived protection: Tuberculosis

Simone A Joosten

Dept. of Infectious Diseases
Leiden University Medical Center, Leiden, The Netherlands
Tuberculosis (Tb)

- Mycobacterium tuberculosis (Mtb) infection can result in TB disease, which still is a major health burden:
  - 1/3 of the world population is infected: 2,000,000,000
  - 8 million people develop TB disease every year, 2 million die
  - Increased incidence of TB disease due to HIV pandemic
  - Multidrug and extensively drug resistant strains spread rapidly

- Infection can be latent for a life-time, active disease can develop upon immune deficiency

- BCG (Bacille Calmette-Guerin): only currently used vaccine
  - Protection is variable (0-80%), poorest around equator
    - Protects children against TB meningitis and miliary TB
    - Does not protect adults against pulmonary TB

- Th1 immunity is induced (antigen specific IFNγ production), but not life-long protection

Source: WHO Stop TB Department, www.who.int/tb
New TB vaccines in development

<table>
<thead>
<tr>
<th>Description</th>
<th>Developmental stage</th>
<th>Sponsor or funder</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVAG85A</td>
<td>Attenuated strain of vaccinia expressing Ag85A</td>
<td>Wellcome Trust, Aeras, Emergent BioSolutions</td>
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<tr>
<td>fMC18cIn</td>
<td>INH overexpressing Ag85B</td>
<td>University of California, Los Angeles, Aeras</td>
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<tr>
<td>AERAS-402</td>
<td>Non-replicating Ad85 expressing Ag85A, Ag85B, and TB10.4</td>
<td>Aeras</td>
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<td>AdAg85A</td>
<td>Non-replicating Ad85 expressing Ag85A</td>
<td>McMaster University</td>
</tr>
<tr>
<td>M72</td>
<td>Recombinant fusion (Mb319 and Mb32) in AS02 and AS01 adjuvant systems</td>
<td>GlaxoSmithKline, Aeras, Tuberculosis Vaccine Initiative</td>
</tr>
<tr>
<td>H1-KC31</td>
<td>Recombinant fusion of Ag85B-ESAT-6 in KC31 adjacent</td>
<td>Statens Serum Institut, Tuberculosis Vaccine Initiative</td>
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<tr>
<td>H1-CAF01</td>
<td>Recombinant fusion of Ag85B-ESAT-6 in CA F01 adjuvant</td>
<td>Statens Serum Institut, Tuberculosis Vaccine Initiative</td>
</tr>
<tr>
<td>H4-KC31 (AERAS-404)</td>
<td>Recombinant fusion of Ag85B-TB10.4 in KC31 adjacent</td>
<td>Statens Serum Institut, Aeras</td>
</tr>
<tr>
<td>rBCG&amp;UreC/hHy (VP10102)</td>
<td>BCG with an endosome escape mechanism</td>
<td>Valzine Projekt Management Tuberculosis Vaccine Initiative, Max Planck Institut</td>
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<tr>
<td>KFUTI</td>
<td>Inactivated M. tuberculosis in liposomes</td>
<td>Achmea Pharma</td>
</tr>
<tr>
<td>M vacuine</td>
<td>Inactivated M vacuine</td>
<td>National Institutes of Health</td>
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</table>

Vaccine efficacy will be hard to study because of the potentially long latency period of Mtb infection > urgent need for surrogate endpoints or correlates that will help in selection of best vaccine candidates

Definitions

- **Biomarker:**
  A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

- **Clinical endpoint:**
  A characteristic or variable that reflects how a patient feels, functions, or survives.

- **Surrogate endpoint (correlate):**
  A biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm) or lack of benefit or harm based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence.
  - most apply to therapeutic intervention trials
  - avoid the confusing term “surrogate markers”

*Biomarkers Definitions Working Group, NIH, CLINICAL PHARMACOLOGY & THERAPEUTICS 69:89-95 (2001)*
Biomarkers in TB: measuring what and in whom?

Surrogate endpoints or correlates of [protection from]:
- infection
- developing latent infection
- developing early disease
- developing late disease (reactivation)
- developing severe disease
- curative response to treatment

Consortia involved in BM identification in TB:
EU Fp6 TBVAC,
BMGF GC6#74,
EU Fp7 NEWTBVAC
and various other
Biomarkers of protection, immunity or disease in TBVAC1.
Status track #1, to be built upon in TBVAC2 and TBVI

**Protection:**
- WBA PPD-induced IFN\(\gamma\) following BCG vaccination
- nHBHA induced IFN\(\gamma\) secretion by PBMCs
- gene expression pattern that allows discrimination between latent MTB infection and TB disease, e.g. Rab33a expression
  - assay development in progress
- granulysin serum levels increase during TB-cure
- mono mycolate glycerol specific T cells releasing IFN\(\gamma\) in latently infected individuals (not TB patients)
- high MTB specific IFN\(\gamma\)/IL-10 ratio
- high IL4\(\delta\)/IL4 ratio
- MIG (CXCL9, monokine induced by IFN-\(\gamma\)) is a new biomarker that can assess IFN-\(\gamma\) induced downstream responses in TB (rather than assessing only IFN-\(\gamma\) production)

**Immune-assays:**
- Elispot assays harmonized & protocols optimized -> SOPs
  - successfully used in both Mtb72F and HYB1 trials, manuscript published in PLoSOne
- 6 day lymphocyte stimulation test for immune profiling (memory & functions)
- Develop ICS protocols to measure correlates / markers in Mtb activated T-cell subsets
- Develop ICS protocols to measure memory T-cell subsets
- Develop MTB killing assays in relation to correlate identification and validation

**Disease:**
- local nHBHA/ESAT-6-CFP-10 specific IFN\(\gamma\) producing T cells
- IL-4-producing TCR\(\gamma\)\(\delta\) cells and CD8 T cells increased in TB patients
- CXCR5+ICOS+CD40L+ Temra Vy9V52 T cells increased in TB patients
- reduced frequency of Teffector and Temra in TB patients
Potential biomarkers of protection or disease: current status track 2
(priorities in blue)

Protection
- MTB specific CD8 T cells identified by HLA tetramers for 5 different epitopes
- Multifunctional MTB reactive T cells identified (IFNγ+IL-2+TNFα+)
- PPD-induced IL-17, IP-10,... following vaccination (multiplex bead array, diluted WBA)
- New Th17 markers identified (CCR6, IL22; other marker profile combinations by ICS)
- Memory T cell subset activation in response to MTB latency antigens (unfinished)
- MTB lipid specific T cells have anti-mycobacterial effector activity
- MTB antigen induced granulysin production
- Ratio latency Ag/ESAT-6 specific T-cells
- nHBHA induced IFNγ/perforin ratio
- nHBHA-specific IFNγ producing CD4 and CD8 T cells frequency
- Chemical genetics identifies targets for MTB phagosome maturation and MTB killing
- TCRγδ T cells have adjuvant activity

Disease
- DC-SIGN expression on alveolar Mfs discriminates TB patients
- CD64 expression
- Lactoferrin expression
- Treg cells among PBMC/local lymphocytes
  - e.g. BCG induced CD8 Tregs suppress via CCL4; HBHA induces CD4 Tregs

Immune assays
- List of genes and sets of probes for multiplex (MLPA, array) genetic profiling
- Large sets of new genome wide expression data from human Mf & DC in TB!
### Potential biomarkers of protection or disease: current status

**track 1 and track 2**

#### Classically restricted T cells (CD4, CD8)

<table>
<thead>
<tr>
<th>Antigens:</th>
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<tbody>
<tr>
<td>1. HBHA reactive IFNγ producing T cells</td>
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<tr>
<td>2. ratio dosR/early antigen specific T cells</td>
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<tr>
<td>3. high frequency of tetramer++ cells</td>
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<table>
<thead>
<tr>
<th>Effector functions:</th>
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<tbody>
<tr>
<td>1. <strong>multifunctional</strong> CD4 T cells identified</td>
</tr>
<tr>
<td>2. new Th17 markers and subsets identified</td>
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<tr>
<td>3. First efforts to detect Tmemory subsets in LTBI</td>
</tr>
<tr>
<td>4. Tregs in TB, and 5. new Treg subset and mechanisms of suppression identified</td>
</tr>
<tr>
<td>6. first multiplex profiles identified</td>
</tr>
<tr>
<td>7. IL4 producing CD8 T cells ass. with disease</td>
</tr>
<tr>
<td>8. IFNγ/IL10 ratios associated with protection / cure</td>
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</table>

<table>
<thead>
<tr>
<th>Cytolytic/killing functions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Genome screens -&gt; Rab33A expression in CD8CTL</td>
</tr>
<tr>
<td>2. perforin expression in various systems</td>
</tr>
<tr>
<td>3. serum granulysin levels</td>
</tr>
<tr>
<td>4. MTB killing</td>
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</table>

#### Non classically restricted T cells

<table>
<thead>
<tr>
<th>Antigens:</th>
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</thead>
<tbody>
<tr>
<td>1. MTB lipid &amp; 2. MMG specific T cells in LTBI, not TB</td>
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</table>

<table>
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<tbody>
<tr>
<td>1. HBHA reactive IFNγ producing T cells</td>
</tr>
<tr>
<td>2. new TCRγδ subset producing IL-4</td>
</tr>
<tr>
<td>3. preliminary MTB killing lipid reactive T cells</td>
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<tr>
<td>4. reduced Teff / Temra in TB</td>
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</table>

#### Assays:

1. PPD-induced IFNγ in WBA and PBMC assay
2. harmonisation & optimisation Elispot assays
3. qPCR and MLPA multiplex assays for expression profiling
4. multifunctional T-cells detected by ICS
Potential biomarkers of protection or disease: current status
track 1 and track 2

Classically restricted T cells (CD4, CD8)

- Antigens:
  1. HBHA
  2. dosR/early Ag
  3. tetramer++ cells

- Effector functions:
  1. multifunctional
  2. new Th17 markers
  3. Tmem subsets
  4-5. new Tregs subsets
  6. multiplex profiles
  7. IL4+ CD8 T cells
  8. IFNγ/IL-10

- Cytolytic/killing functions
  1. Rab33A
  2. perforin
  3. serum granulysin
  4. MTB killing

Non classically restricted T cells

- Antigens:
  1. MTB lipids
  2. MMG

- Effector functions
  1. HBHA induced local response
  2. new TCRγδ subset
  3. MTB killing
  4. red. Teff/Temra in TB

Mf DC

- Antigens:
  1. MTB lipids
  2. MMG

- Effector functions:
  1. MTB killing
  2. apoptosis

- APC function:
  - Tmem formation?

Innate immune compartment, APC function and immune evasion

Markers:
- DC-SIGN on Mf
- PKB/Akt kinases
- array data various sources
- CD64/LF
- MIG/CXCL9

Assays:
- MTB killing
- apoptosis
Multifunctional T cells in TB

- MF T cells simultaneously produce IFNγ, IL-2 and TNFα and are associated with vaccine-mediated protection (Darrah et al Nat Med, 13, 843, 2007)
- BCG elicits multifunctional T cells in mice and men (Darrah et al Nat Med, 13, 843, 2007)

- New TB vaccine, H1 + CAF01 results in higher levels of persisting MF T cells compared to BCG and in enhanced protection (Lindenstrom et al, J Immunol, 182, 8047, 2009)
- Number of systemic MF T cells did not correlate with protection against aerosol challenge (MVA-Ag85A) (Tchilian et al, Infect Immun, 77, 622, 2009)
- TB cases had significantly higher numbers of MF T cells as compared to exposed house-hold contacts (Sutherland et al, Eur J Immunol, 39, 1, 2009)
- MF T cells are much more abundantly present in TB cases as compared to LTBI cases, after treatment the frequency of MF decreased suggesting a link to live bacterial loads (Caccamo et al, Eur J Immunol, 40, 2211, 2010)
Relevance of MF T cells in TB vaccination

- MF T cells can be detected during protection including in vaccination studies with novel TB vaccines
- Patients with active disease also have high numbers of MF T cells, suggesting induction by live bacteria
- MF T cells in TB are not necessarily associated with protection
- More detailed (longitudinal) clinical follow up studies are needed to establish the contribution of MF T cells to the protective immune response in humans.
Results so far…

- Multiparameter signatures are needed, single markers will not fulfill all criteria to serve as surrogate endpoint.

- Biomarker signatures need to be tailor-made dependent on the population to be studied and type of vaccine investigated.

- Different options for multiparameter measurement at protein and expression levels, eg multiplex bead arrays (Luminex) or multiplex gene expression assays (microarray or RT-MLPA).
RT-MLPA for measurement of gene expression levels

<table>
<thead>
<tr>
<th>Microarray</th>
<th>RT - Multiplex Ligation Dependent Probe Amplification (MLPA)</th>
<th>Quantitative PCR (Taqman)</th>
</tr>
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<tbody>
<tr>
<td>thousands of genes</td>
<td>± 60 genes</td>
<td>single genes</td>
</tr>
<tr>
<td>no selection of genes</td>
<td>selection of genes of interest</td>
<td>selection of gene of interest</td>
</tr>
<tr>
<td>± 2 μg RNA required</td>
<td>± 0.2 μg RNA required</td>
<td>± 0.05 μg RNA required</td>
</tr>
<tr>
<td>more expensive</td>
<td>less expensive</td>
<td>more expensive</td>
</tr>
<tr>
<td>enormous amount of data</td>
<td>genes can be changed easily (semi) quantitative</td>
<td>genes can be selected easily quantitative</td>
</tr>
<tr>
<td>not quantitative</td>
<td>96 well format</td>
<td>96 well format</td>
</tr>
<tr>
<td>single sample</td>
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Broad screen for new markers on few samples
Testing multiple candidates in larger groups
Testing single candidates in larger groups

RT-MLPA (Multiplex Ligation Probe Amplification)

Hybridization

Ligation

PCR amplification

Fragment run

Fragment length analysis

PCR primer sequence

Probe 1
Probe 2

Probe 1
Probe 2

cDNA target A
cDNA target B

Fluorescence intensity

Fragment length (bp)
RT-MLPA set 1

MLPA

MAPH
RT-MLPA - Genes in set 1

- Genes differentially expressed between PBMC from TB patients versus healthy contacts (FPR1, BPI, MARCO, SEC14-like 1, RAB24, RAB13, RAB33A, FCGR1A, LTF).

- Genes differentially expressed between tissue located near versus distant from tuberculoma (CCR7, CCL13, IL22RA1, SPP1, BLR1, CCL19, MMP9, TIMP2).

- Immune cell subset markers (CD3, CD4, CD8, CD14, CD19, NCAM1).

- Treg markers (FOXP3, IL-7R, TGFβ1, CTLA4, LAG3, IL-10, CCL4, TNFRSF18, IL-2Rα).

- Effector T cell markers (IFNγ, CXCL10).

- Apoptosis related genes differentially regulated by Mtb (TNFRSF1A, TNFRSF1B, Bcl2, CASP8, TNF, FASLG).

- Literature (IL-4/IL-4Rα2, CCL22, CD163, TGFβ-RII).

- Housekeeping/reference genes (GAPDH, ABR, GUSB, β2M).
Direct ex vivo RNA isolation out of blood: PAXgene

PAXgene tube

- 9 ml tube, contains 6.5 ml fixative and is filled with 2.5 ml blood.
- Can be used with standard vacutainer system.
- Mixing ensures immediate fixation of the RNA profile.
- Fixation is longlasting, stable for more than 24h at room temperature, 1-2 weeks at 4°C, at least 2 months at -20°C, and indefinitely at -80°C.
- RNA isolation is completely standardized using a kit.
- 1 tube contains ± 4 µg RNA, sufficient for > 20 assays.

Direct ex vivo RNA profile
RT-MLPA assay validation - Reproducibility

Pax gene tubes, 12 healthy donors, 5 tubes collected at month 0 and 2 to study variation between donors, tubes, in time and assays.

**CD4**

**CD8α**

**β₂M**

<table>
<thead>
<tr>
<th></th>
<th>Donor:</th>
<th>Collection:</th>
<th>Tube:</th>
<th>RT-MLPA:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donor</strong></td>
<td>27%</td>
<td>22%</td>
<td>30%</td>
<td>21%</td>
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<tr>
<td><strong>Collection</strong></td>
<td>59%</td>
<td>2%</td>
<td>13%</td>
<td>26%</td>
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<tr>
<td><strong>Tube</strong></td>
<td>27%</td>
<td>47%</td>
<td>15%</td>
<td>11%</td>
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</table>

Donor:

Collection:

Tube:

RT-MLPA:
RT-MLPA assay validation - Reproducibility

Relative to total amount of variation in sample

Assay is highly reproducible, most variation is the result of between donor variation.
RT-MLPA assay validation - RT-MLPA versus Taqman

Taqman and MLPA yield similar gene expression patterns
Comparison of MLPA with microarray also showed similar expression profiles

Extensive evaluation of MLPA: robust, reproducible and sensitive assay
TB patients during treatment: The Gambia

Joosten, Haks in preparation; collaboration with MRC The Gambia
TB patients during treatment: Paraguay

Small cohort: trends are the same

Joosten, Haks in preparation; collaboration with Cecile Magis
Combined biomarker signatures

- Lasso statistics: a shrinkage and selection method for linear regression. It minimizes the usual sum of squared errors, with a bound on the sum of the absolute values of the coefficients >> variant of principle component analysis, further reducing the number of variables to obtain best predictive signature.
- Combined analysis of 45 genes in TB patients at diagnosis compared with TST + or - contacts

- Ridge predictor: no selection of genes, include all 45 genes > similar AUC results > Lasso selected optimal set of markers

Joosten, Haks in preparation
Prediction on TB patients on treatment

- Lasso prediction using TB vs TST+ algorithm on samples of TB patients on treatment

- Patients on therapy have a profile in between untreated patients and healthy TST+ individuals

- 6 months of treatment is heterogeneous group, need to re-asses data in combination with clinical information available

Joosten, Haks in preparation
Summary RT-MLPA

- RT-MLPA is easy, fast, sensitive reproducible and reliable
- RT-MLPA is suitable for detection of biomarkers in large cohorts of patients or vaccinees
- RT-MLPA identified biomarker signatures associated with TB disease in Gambian TB patients compared to controls
- Genes can be selected based on particular interest, currently we are developing innate and adaptive immune sets for more general immune profiling
- Applicable on all RNA samples, ex vivo whole blood but also ex vivo stimulated blood, other body fluids or tissue samples
Conclusion correlates of protection in TB vaccination

- Correlates of protection are urgently needed for development of TB vaccines
- Correlates of protection in TB are complex and need to be composed of multicomponent signatures
- Novel multiplex based technologies are useful tools in measuring biomarker signatures in TB
- Value of individual signatures has to be evaluated in large scale clinical studies
LUMC Leiden: Dept of Infectious Diseases
Marielle Haks
Tom Ottenhoff

Dept of Medical Statistics
Jelle Goeman

In collaboration with:
-Jayne Sutherland, Martin Ota
(MRC The Gambia)
-Cecile Magis (UCCZ, Nijmegen)