Overview

- Reading a paper
- ESX/ESAT-6 background
- Presentation of figures
- Discussion/questions on methods
- Critical appraisal

Introduction:
• Topic?
• Relevance to field?
• Purpose: New methodology? Settle controversy? Test hypothesis?

Methods:
• Measurements appropriate to questions?
• If models used, do they accurately recapitulate actual system?

(Results)

Discussion
• Do results support conclusions?
• Are there other possible explanations for results?
• What should the next steps be?

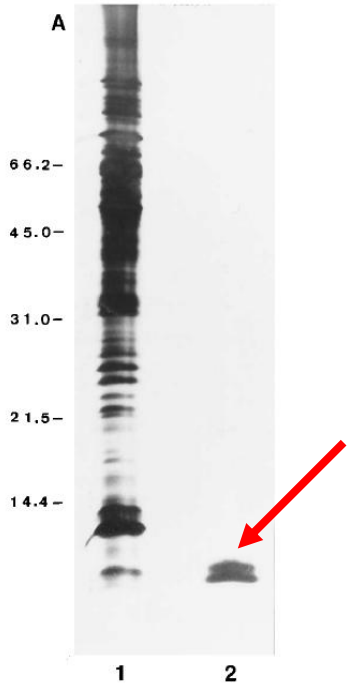
Key questions/hypotheses

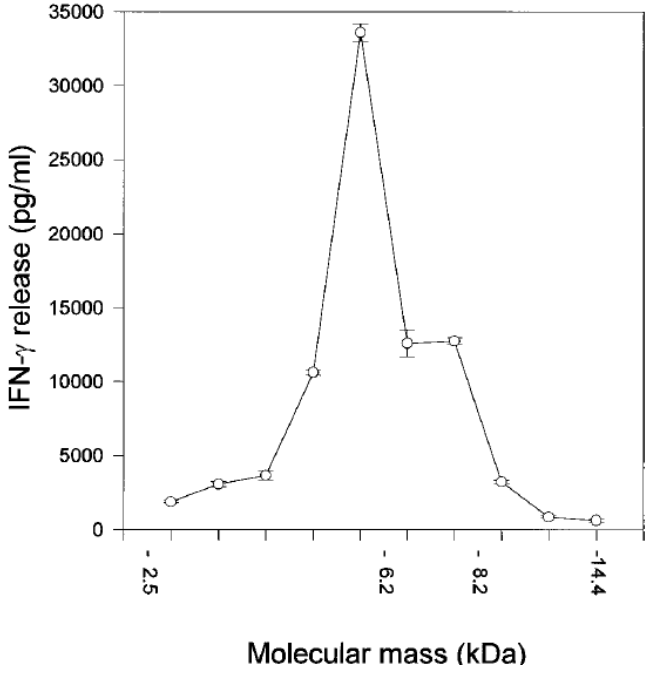
- The ability to translocate to the cytosol is linked to mycobacterial virulence
- Translocation is ESX-dependent

Discovery: ESAT-6

- Antigen mining/immunomics: identification of ESAT-6

ESAT-6 = Early secreted antigenic target 6 kDa = EsxA

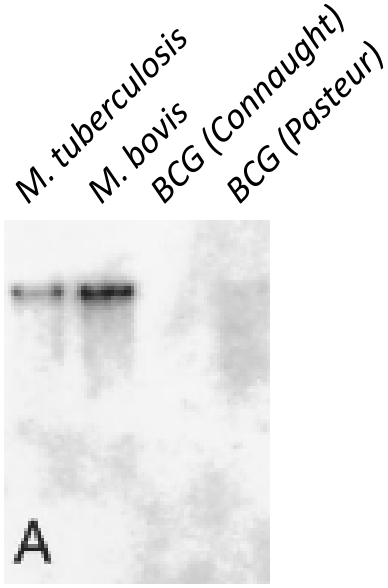


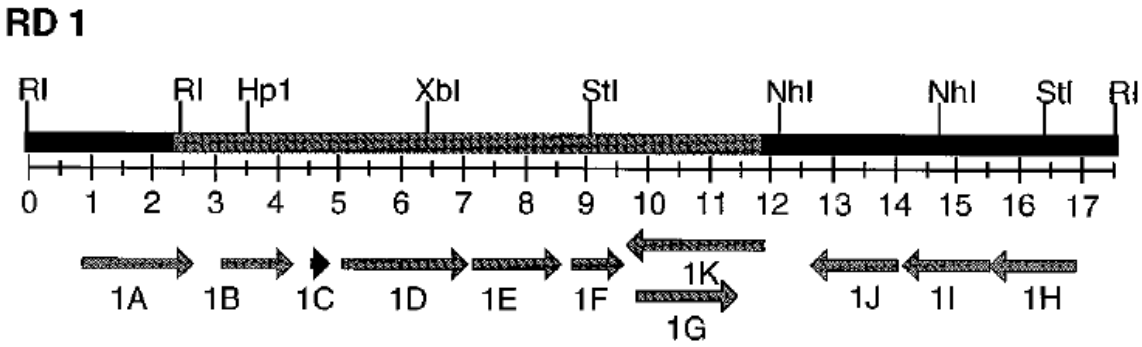


Sørensen *et al* (1995) *Infect. Immun.*

Discovery: RD1

- Genomics: whole genome comparison between *M. bovis* BCG and *M. tuberculosis* H37Rv
- Identified RD1 = region of difference 1

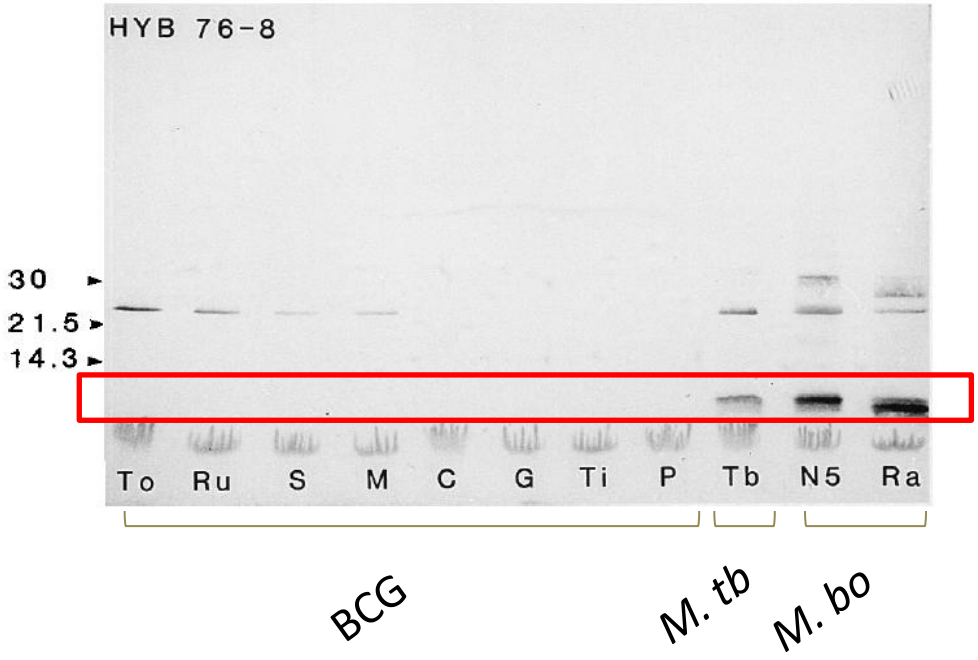


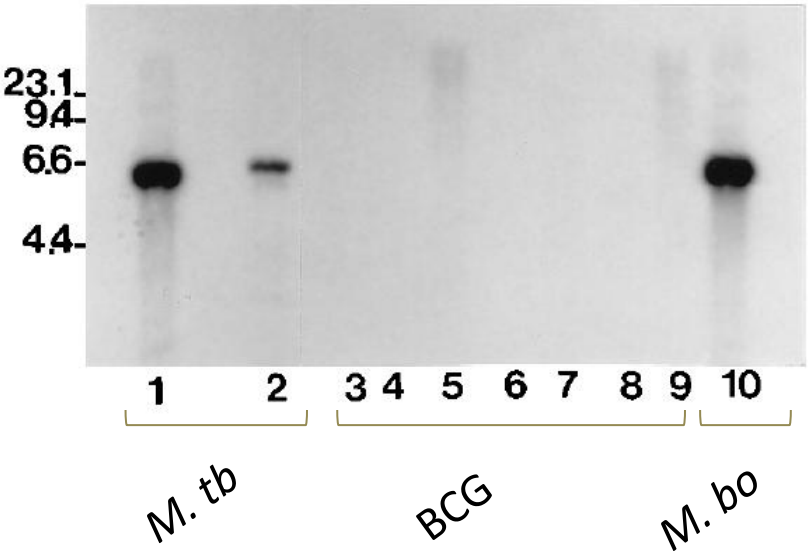


Mahairas *et al* (1996) *J. Bacteriol.*

Discovery: ESAT-6

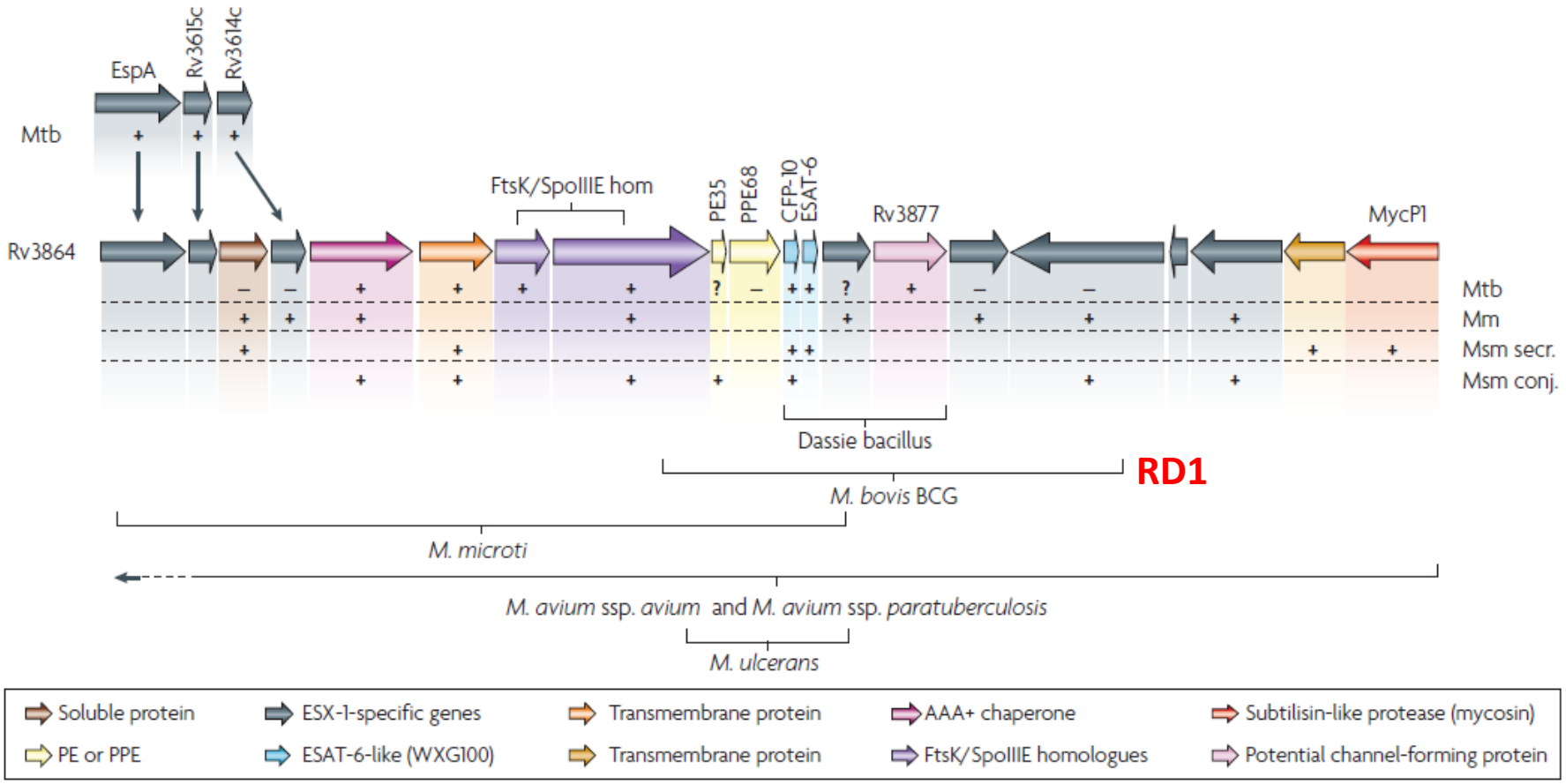
- Antigen mining/immunomics: ESAT-6 is not expressed/present in BCG





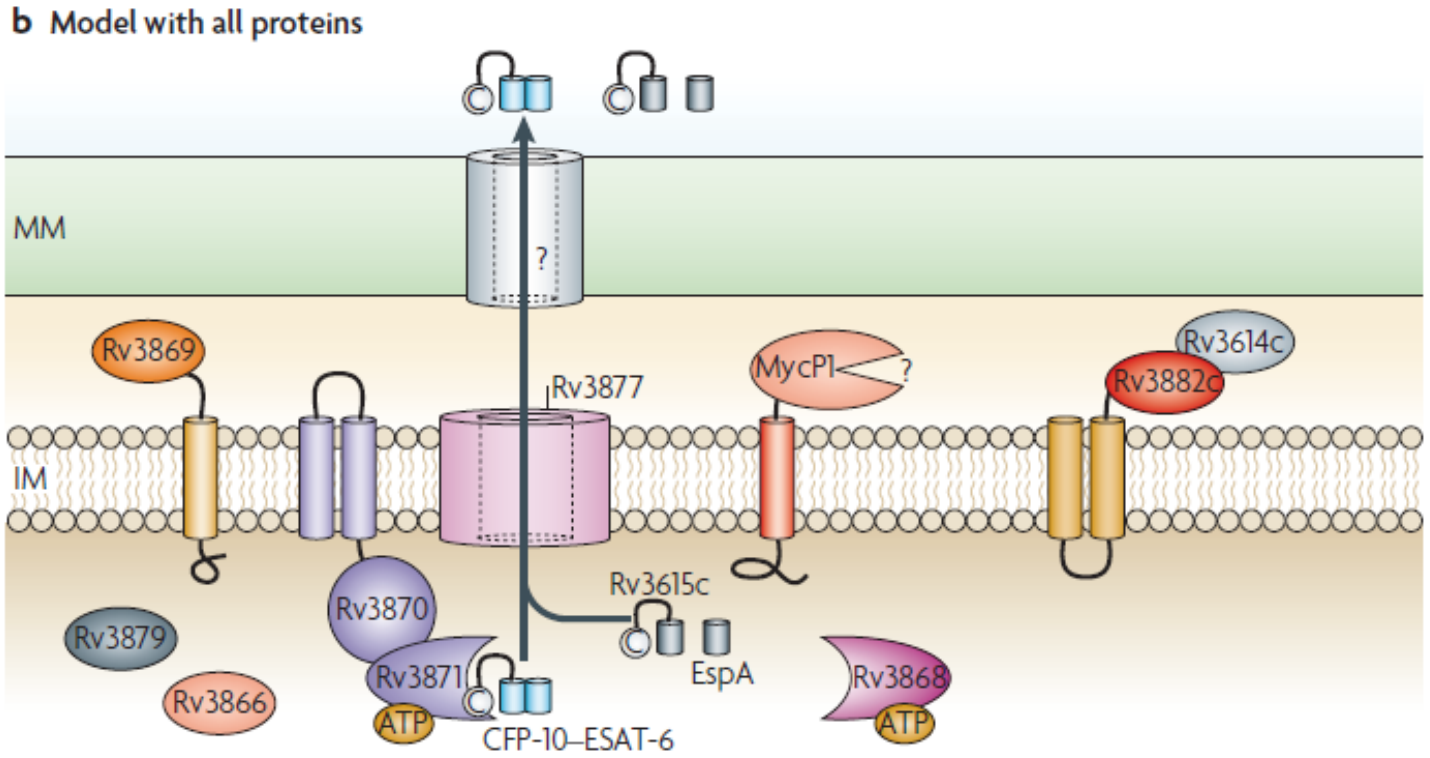
Harboe *et al* (1996) *Infect. Immun.*

ESX region: genomic arrangement



Abdallah *et al* (2007) *Nature reviews*

ESX-1 secretion machinery



Abdallah *et al* (2007) *Nature reviews*

The intracellular lifestyle of *M. tuberculosis*

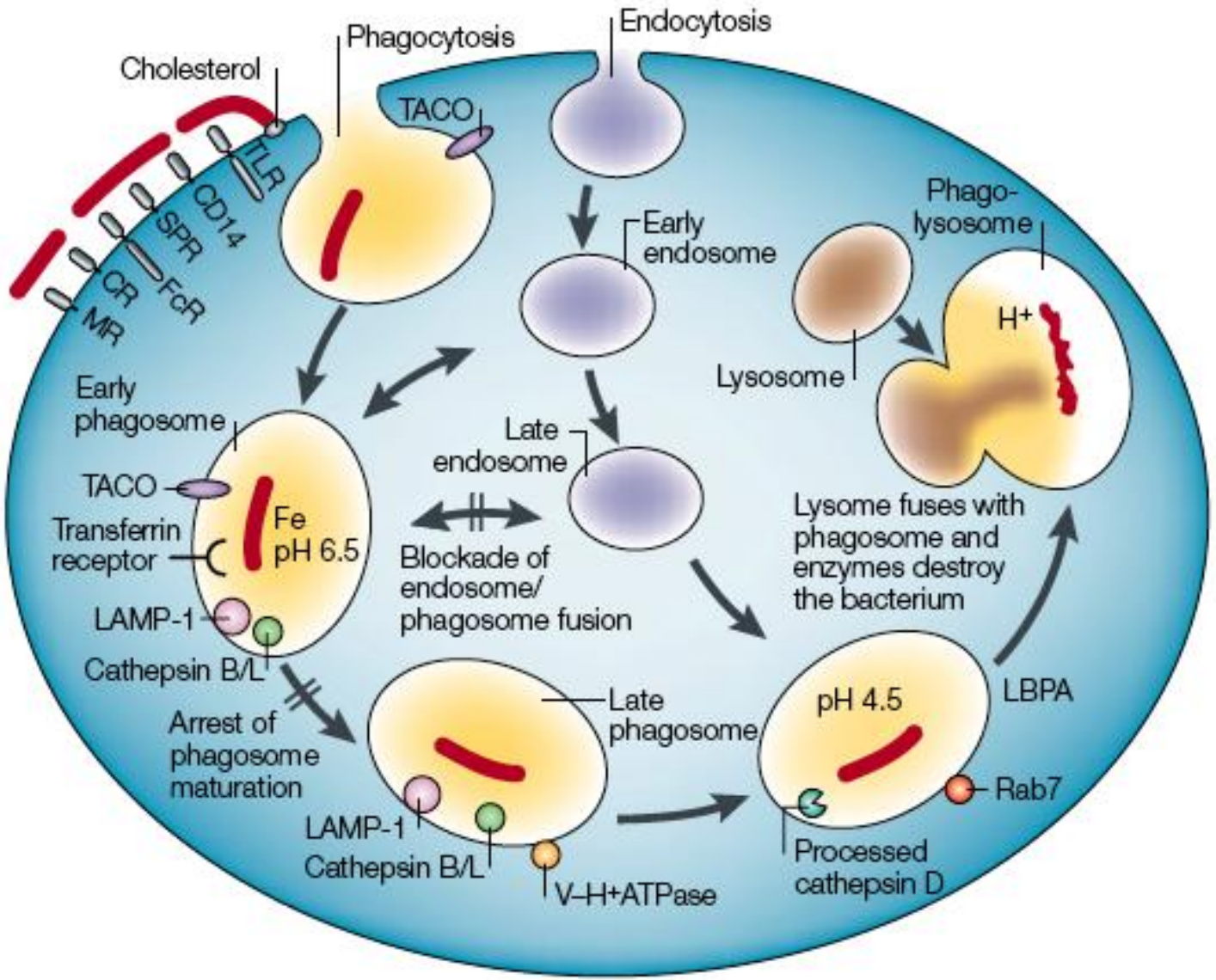


Table 1. Characteristics of various mycobacterial species and their pathogenicity.

Mycobacterium strains	Pathogenicity in humans	ESX-1 genes	ESAT-6 secretion	Transloc in cells
M. tuberculosis H37Rv	Pathogenic	+	+†	+
M. tuberculosis ancient Beijing	Pathogenic	+	+*	+
M. tuberculosis Beijing	Pathogenic	+	+*	+
M. tuberculosis Harlingen	Pathogenic	+	+*	+
M. tuberculosis 1243	Pathogenic	+	+*	+
M. leprae	Pathogenic	+	+**	+
M. bovis	Pathogenic	+	+	+
M. marinum	Pathogenic	+	+‡	+
M. szulgai	Opportunistic	+	+†	+/–
M. kansasii type I	Opportunistic	+	+	+/–
M. kansasii type V	Non-pathogenic	+	+	–
M. smegmatis	Non-pathogenic	+	+***	–
M. avium	Opportunistic	–	–†	–
M. fortuitum	Opportunistic	–	–†	–
M. bovis BCG	Non-pathogenic	–	–	–
M. gilvum	Non-pathogenic	–	–*	–

Supplemental Figure 1: ESAT-6 secretion of different mycobacteria.
M. marinum E11, *M. szulgai* and *M. kansasii* show expression and secretion of ESAT-6 into the supernatant fraction, detected with the monoclonal a-ESAT-6 antibody Hyb 76-8. *M. fortuitum*, which has no RD1 region, is negative for ESAT-6.

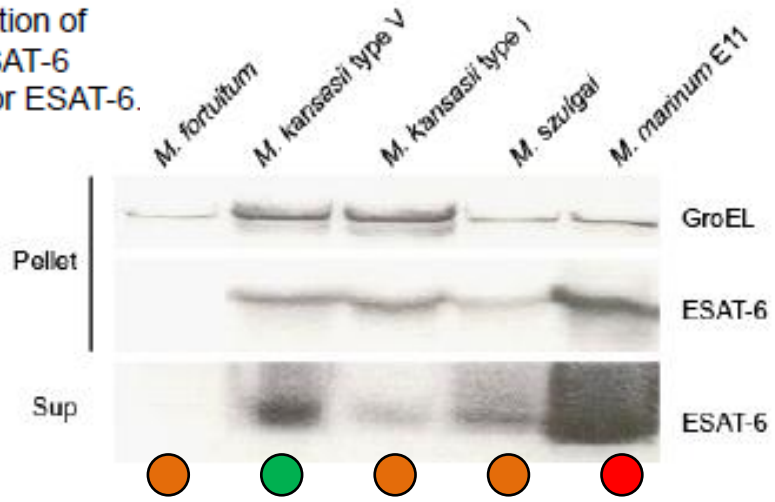
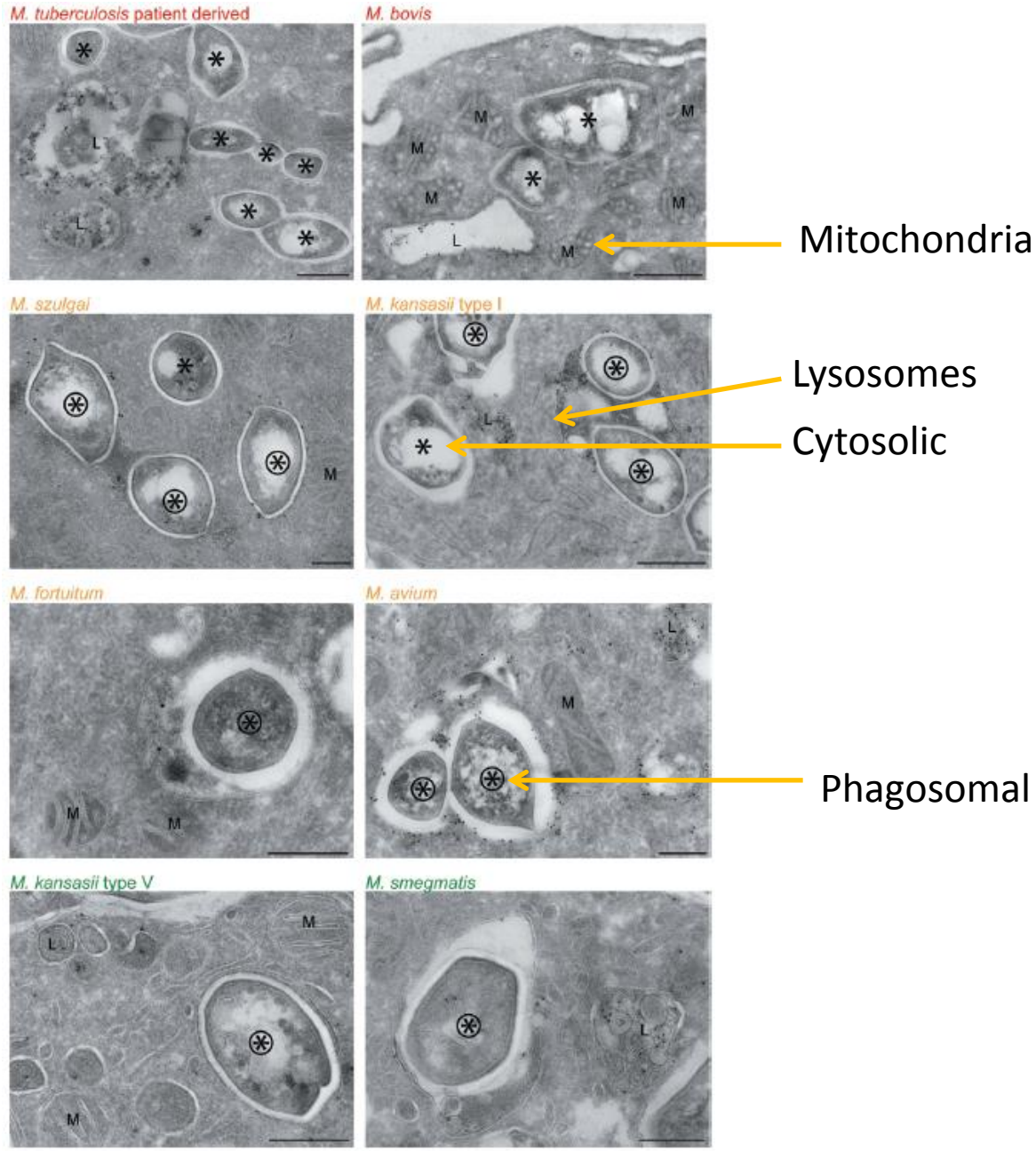


Figure 1


Pathogenic
Opportunistic
Non-pathogenic
M. tuberculosis patient derived
M. bovis
M. szulgai
M. kansasii type I
M. fortuitum
M. avium
M. kansasii type V
M. smegmatis
Mitochondria
Lysosomes
Cytosolic
Phagosomal

Supplemental Figure 4: Overview and details of THP-1cell infected for 3 days with *M. szulgai*.

The electron micrograph of the *M. szulgai* infection presented in Figure 1 is used to visualize phagosomal (A) and cytosolic (B) bacteria at a high magnification and at low magnification (C). At a high magnification the host cytosol, presence (A) or absence (B) of the phagosomal membrane, capsular layer, bacterial plasma membrane and bacterial cytosol can be discerned. The low magnification (C) of the infected cell demonstrates that the CD63 labeling is specifically labeling the phagosomal membrane, and small vesicles. Bar represents 100 nm; black boxes in C represents enlarged area A and B; asterisk, cytosolic bacterium; encircled asterisk, phagosomal bacterium; m represents mitochondria; v vesicles and red circles represent CD63 labeling.

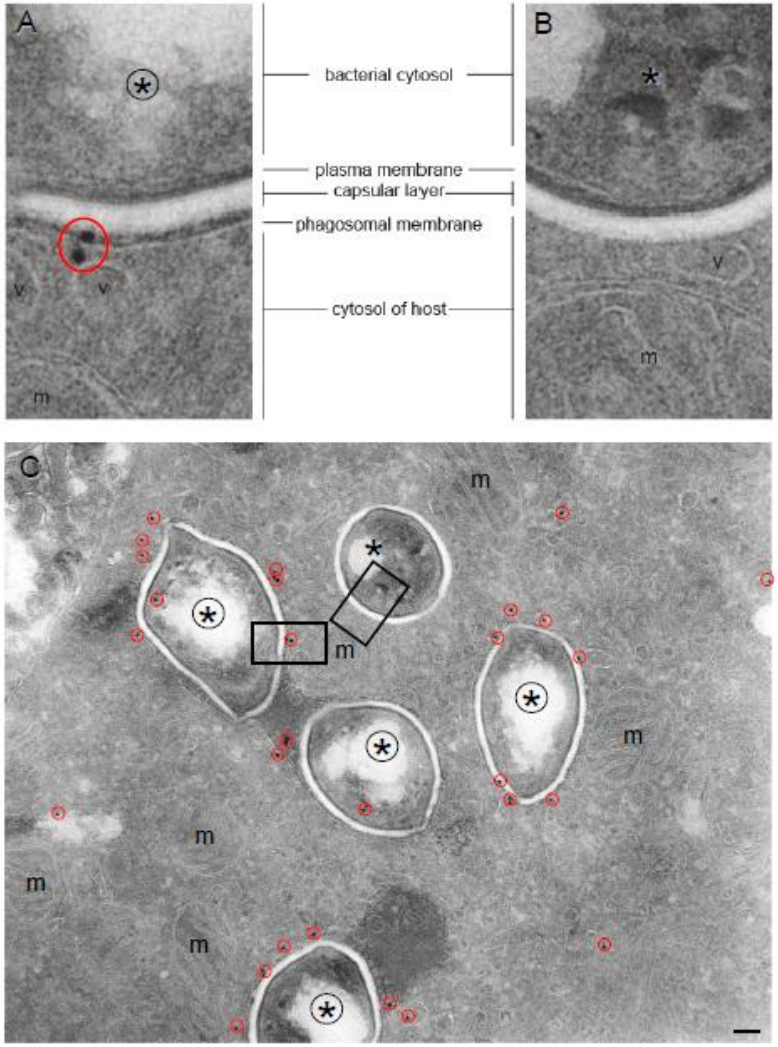


Figure 2

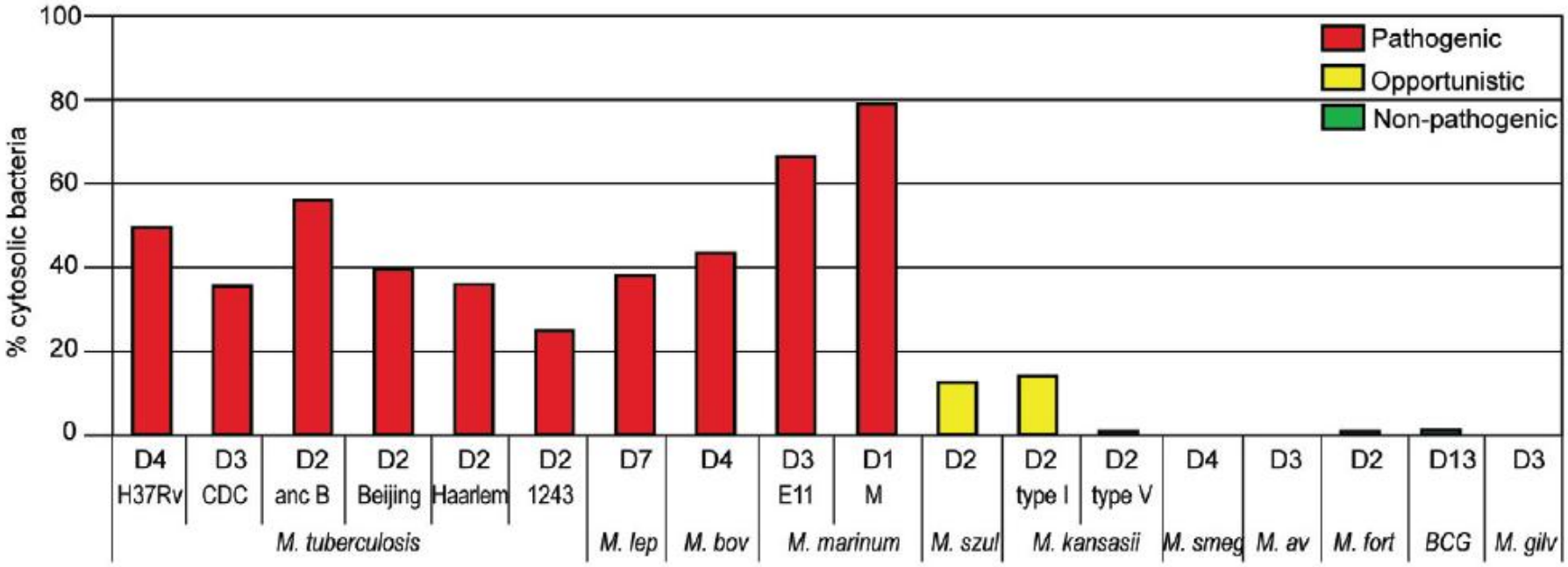


Fig. 2. Translocation is specific for pathogenic mycobacteria. Percentage cytosolic bacteria in adherent, non-apoptotic THP-1 cells infected with different mycobacterial species (see Table 1 and Fig. S3). Infections were performed with moi 10 and kept as long as cells survived, or until day 10 in *M. bovis* BCG infections. The day (D) of infection on which the percentage of cytosolic bacteria was highest is indicated under each bar. The pathogenic species are represented as red, opportunistic species as yellow and non-pathogenic as green.

Figure 3

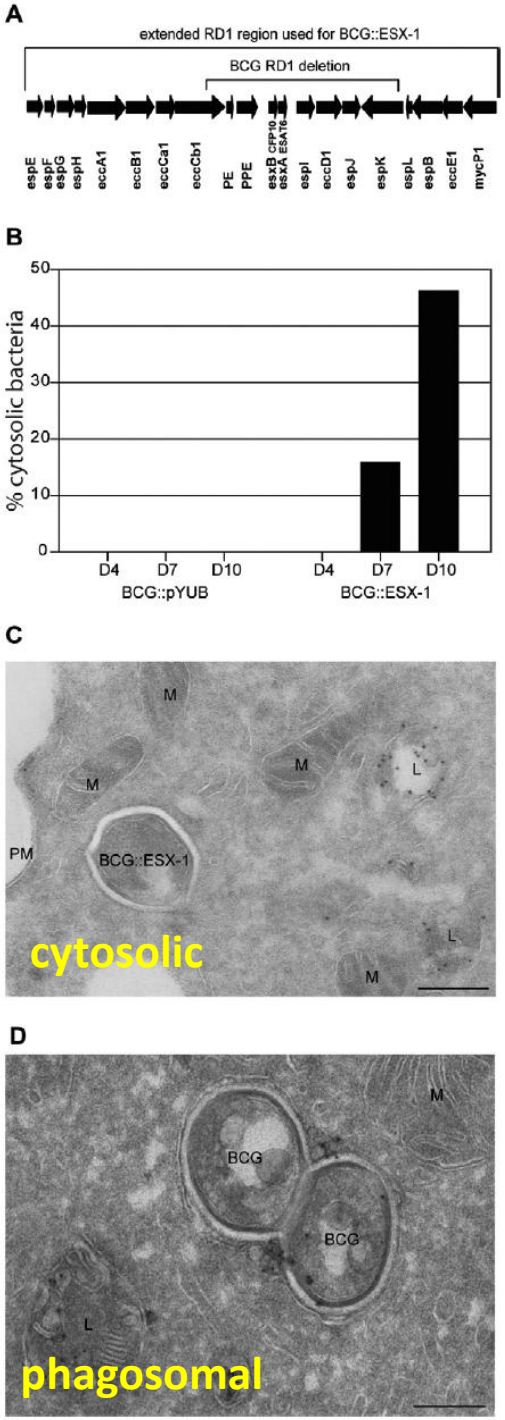


FIg. 3. RD1 knock-in is sufficient for translocation of BCG.
A. Schematic representation of the RD1 deletion region naturally occurring in *M. bovis* BCG and the extended RD1 region naturally occurring in *M. tuberculosis* and reintroduced into *M. bovis* BCG (BCG::ESX-1) using the RD1-2F9 construct containing a 32 kb segment encoding Rv3861–Rv3885.
B. Percentage cytosolic bacteria at days 4, 7 and 10 post infection in stimulated THP-1 cells. In the absence of RD1 (BCG::pYUB vector control) no bacteria were found in the cytosol, but in BCG with ESX-1 knocked-in (BCG::ESX-1), translocation was detected at 7 and 10 days.
C. Representative electron micrograph of cytosolic BCG::ESX-1 at day 10 post infection in a stimulated THP-1 cell. Immunogold (10 nm) labelling of LAMP-2 marks lysosomal structures (L). Bar represents 300 nm; M, mitochondria; PM, plasma membrane.
D. Representative electron micrograph of phagosomal *M. bovis* BCG at day 1 post infection, immunolabelled (10 nm gold) for CD63. Bar represents 200 nm; M, mitochondrium; L, lysosome.

Figure 4

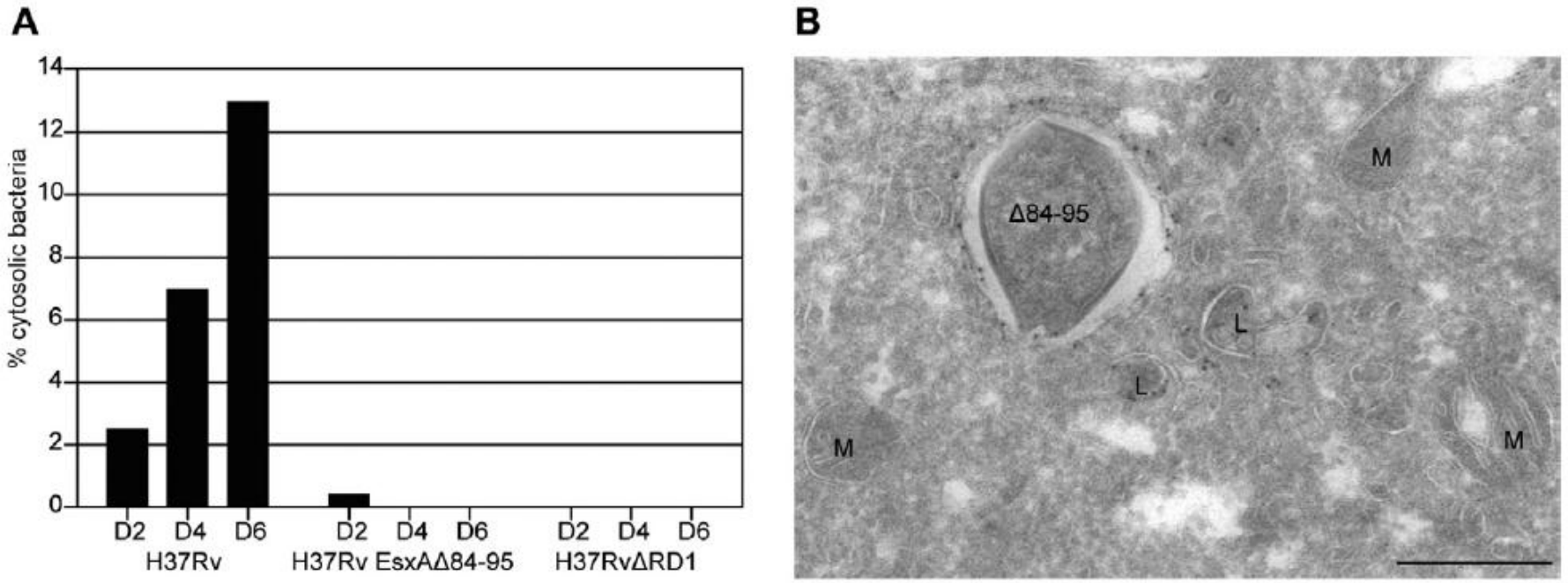


Fig. 4. C-terminus ESAT-6 crucial for translocation.
A. Percentage cytosolic H37Rv (moi 2), H37Rv EsxAΔ84-95 (moi 10) or H37RvΔRD1 (moi 10) at day 2, 4 or 6 post infection in stimulated THP-1 cells. The translocating *M. tuberculosis* strain, H37Rv, and non-translocating RD1 deletion mutant of *M. tuberculosis* H37Rv (H37RvΔRD1) acted as positive and negative controls.
B. Representative electron micrograph of phagolysosomal ESAT-6 mutant H37Rv EsxAΔ84-95 at day 2 post infection in THP-1 cells. Immunogold (10 nm) labelling of LAMP-2 marks the lysosomal structure (L). Bar represents 300 nm; M, mitochondria.

Supplementary Figure 5: Amino acid sequence alignment of the *esat-6* gene in RD1-positive mycobacteria.

All *Mycobacterium tuberculosis* isolates used here (H37Rv, Beijing and ancient Beijing, Harlingen, and reactivated nontransmitting isolate 1243) have identical sequences. GenBank accession numbers are FJ014499 (*M. tuberculosis*), BX248347 (*M. bovis*), CP000854 (*M. marinum M*), EU826486 (*M. szulgai*), X90946 (*M. leprae*), EU888292 (*M. kansasii* type I), EU888297 (*M. kansasii* type V), and CP000480 (*M. smegmatis*).

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M. tuberculosis        MTEQQWNFAG IEAAASAIQG NVTSIHSLLD EGKQSLTKLA AAWGGSGSEA
M. bovis AF2122/97     .......... .......... .......... .......... ..........
M. marinum M           .......... ....S.S.G. ..G....... ......H... ..........
M. szulgai 07-1790     .......... ......S... .......... .........Q S..A.A.QG.
M. leprae TN           EMI.A.H.PA LQG.VNEL.. SQSR.DA..E QCQE.....Q SS.H...N.S
M. kansasii type I     .......... .......... .......... .......... ..........
M. kansasii type V     .......... .......... .......... .......... ..........
M. smegmatis MC2 155   ....V..... ..GG..E.H. A.STTAG... ...A...T.. S....T....

M. tuberculosis        YQGVQQKWDA TATELNNALQ NLARTISEAG QAMASTEGNV TGMFA*-
M. bovis AF2122/97     .......... .......... .......... .......... ......-
M. marinum M           .........S ..Q....... .......... ...S...... A...G.-
M. szulgai 07-1790     .EQ....... ..Q...SS.. .......... .H.Q...... ......-
M. leprae TN           .SS...RFNQ NTEGI.H..G D.VQA.NHSA ET.QQ..AG. .S..TG*
M. kansasii type I     .......... ..Q....... ..S....... .......... ......-
M. kansasii type V     .......... ..Q...S... ..S....... .......... ......-
M. smegmatis MC2 155   ..A..AR..S .SN...L... ...Q...... .T..Q..AG. ......-
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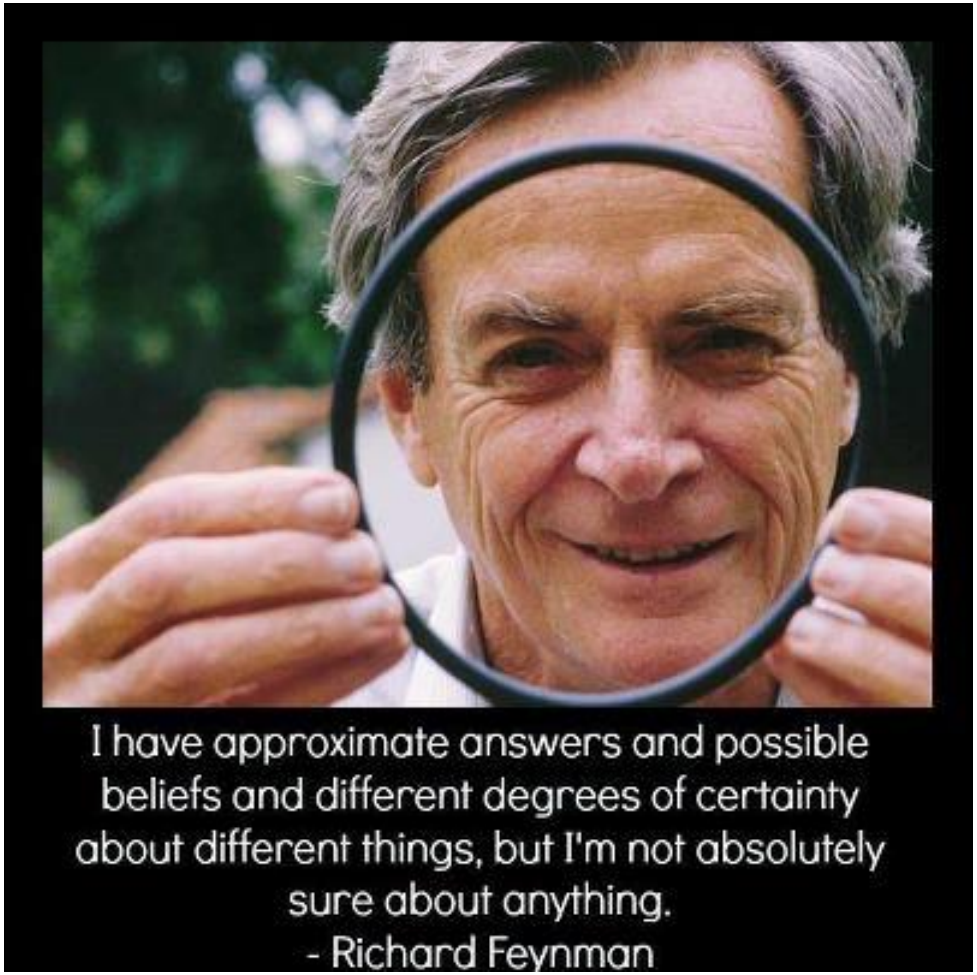
Methods

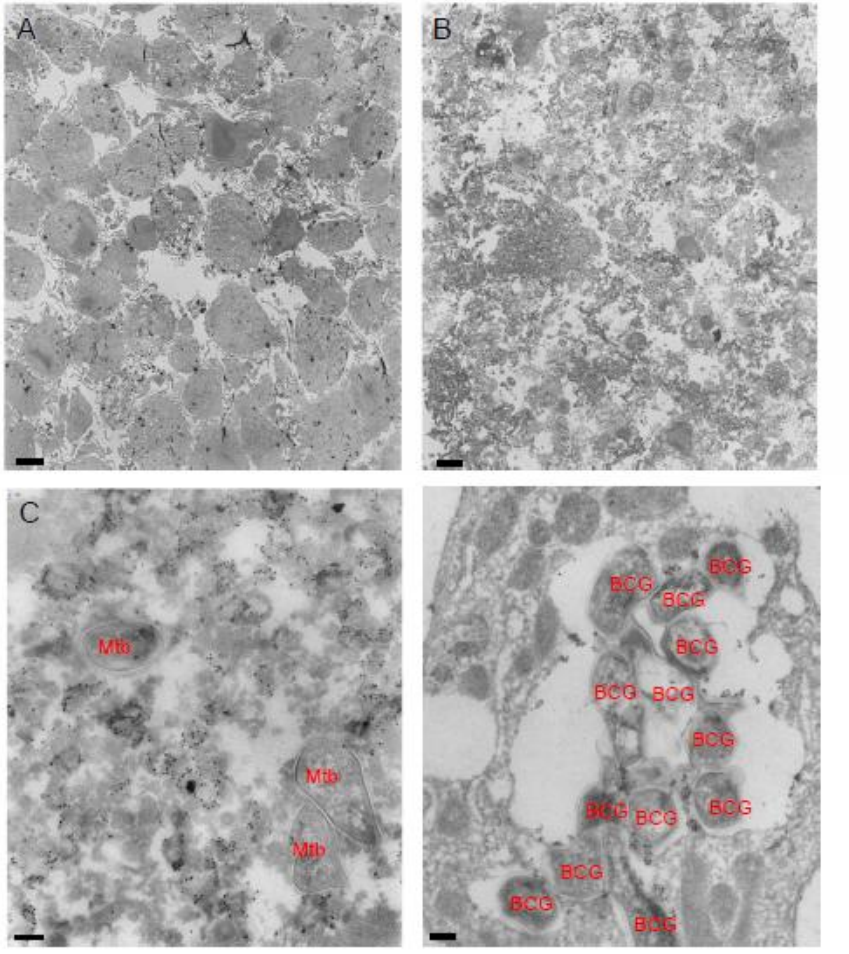
- Macrophage infections
- EM
- Western blotting

Learning Outcomes

- Critically appraise the paper
- Briefly describe the role of the ESX secretion system in mycobacterial virulence, with specific reference to translocation
- Evaluate the evidence provided in favour of cytosolic translocation of *M. tuberculosis*
- Highlight selected methodological shortcomings


I have approximate answers and possible beliefs and different degrees of certainty about different things, but I'm not absolutely sure about anything.
- Richard Feynman

Supplemental Figure 2. Difference between morphology cell cultures without or with nonadherent, dying cells.
After the infection period only the adherent (mostly viable) cells were fixed and processed (A; *M. tuberculosis* H37Rv, 4 days) or all cells, adherent and nonadherent were fixed, collected together and processed for electron microscopy (B; *M. tuberculosis* patient derived, 5 days). In a high magnification an example of a nonviable cell infected with *M. tuberculosis* (H37Rv, day 3 of infection) and immunogold labeled for LAMP. In (D) a nonviable cell infected by *M. bovis* BCG for 9 days and immunogold labeled for CD63. Bars in A and B represents 5 µm and in C and D 200 nm.



Supplemental Figure 3: Percentage cytosolic bacteria in THP-1 in time.
Percentage cytosolic bacteria in adherent, non-apoptotic THP-1 cells infected with different mycobacterial species (see Figure 1, 2, Table 1 and Table S1). Infections were performed with MOI 10 and kept as long as cells survived, or until day 10 in *M. bovis* BCG infections. Then cells were fixed and processed for immunogold labeling with lysosomal marker (CD63 or LAMP-1). Bacteria were scored as cytosolic when no immunogold label was present and no membranes were detected surrounding the bacteria. All infections were performed without antibiotics, except for the *M. smegmatis* infection in which Amikacin was used to kill extracellular bacteria. The THP-1 cell survived the infection for 2 days without and 4 days with antibiotic. The day (D) of infection is indicated under each bar. The pathogenic species are represented as red, opportunistic species as yellow and non-pathogenic as green.

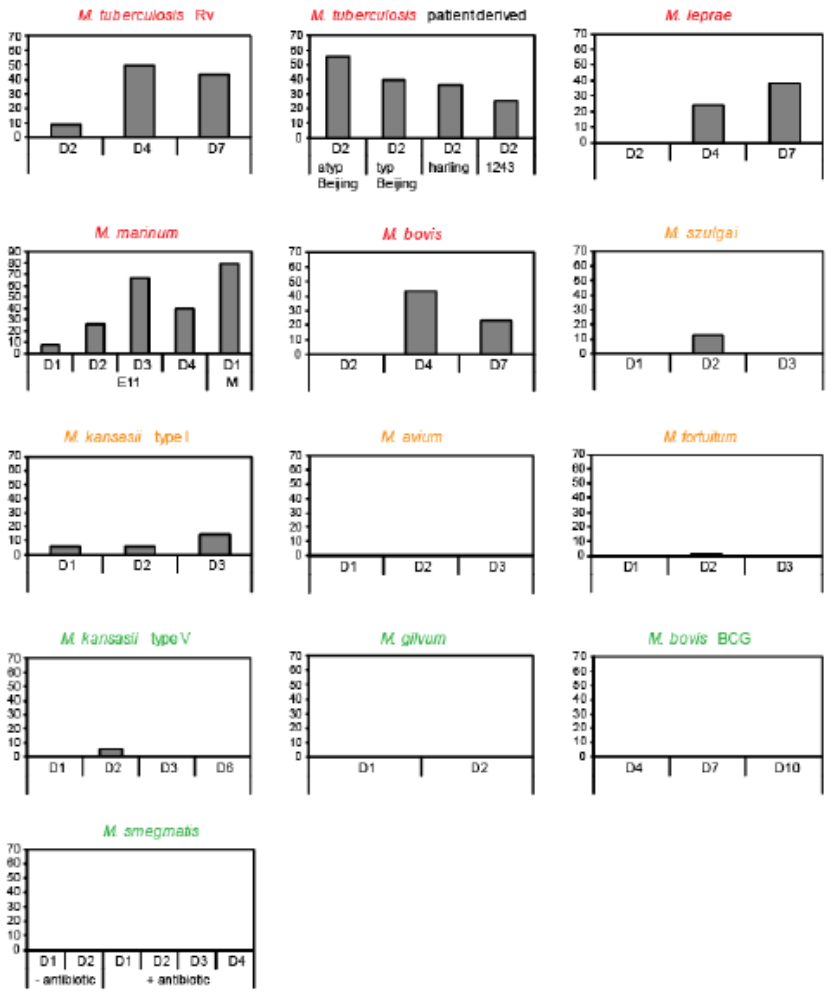


Figure 5

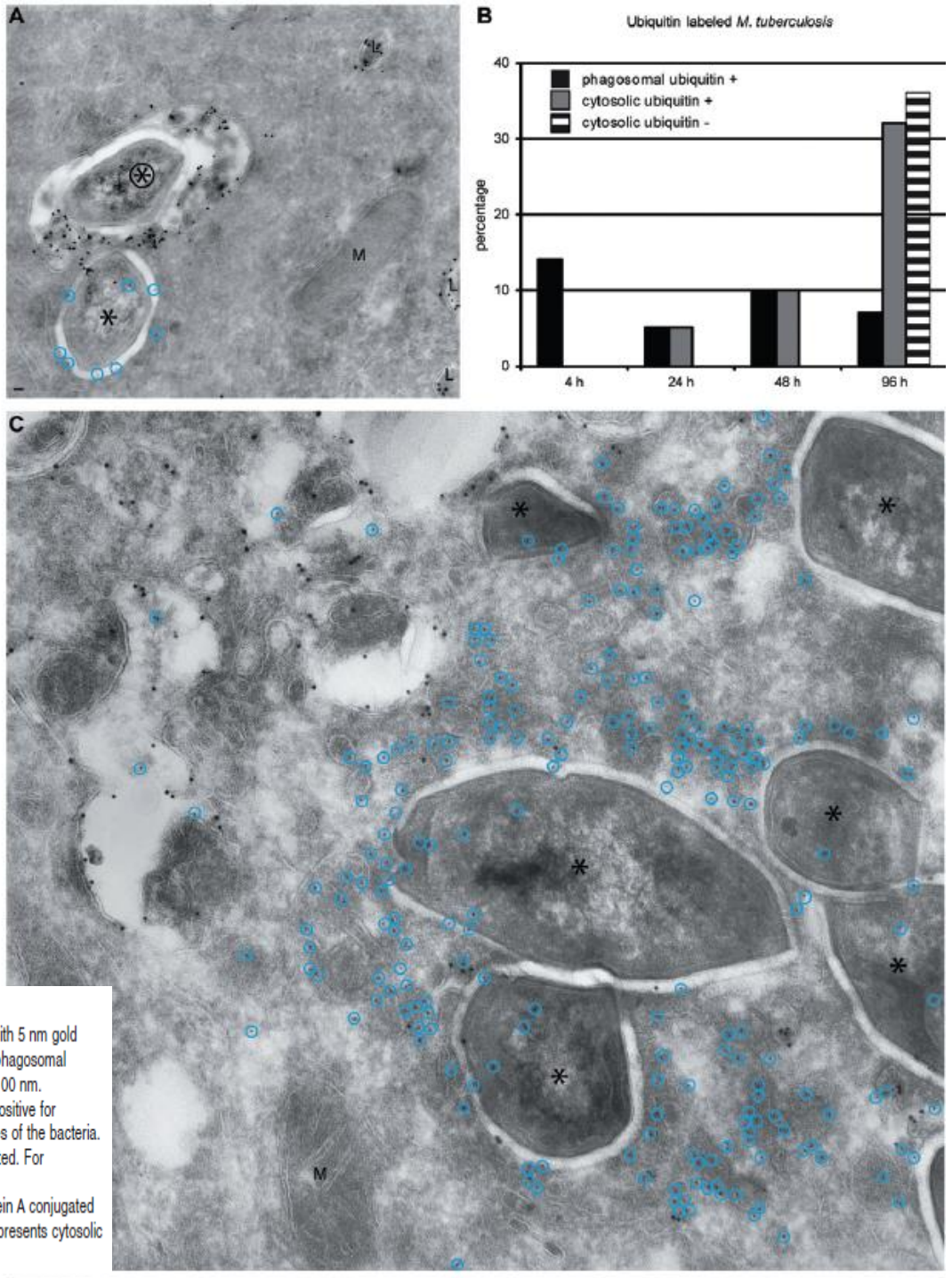
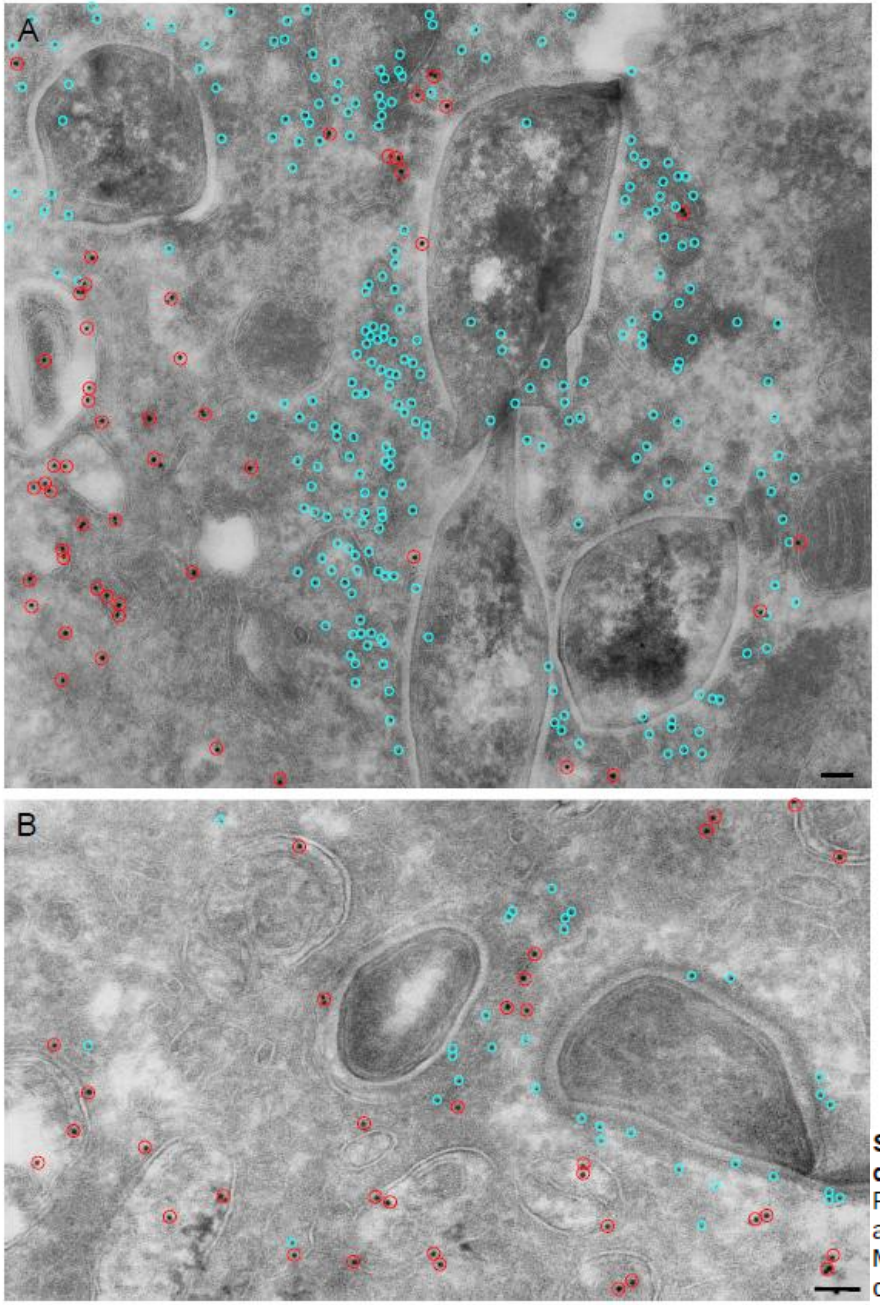


Fig. 5. Ubiquitin on perturbed phagolysosomes.
A. Representative electron micrograph of *M. tuberculosis* H37Rv at 3 days of infection in DCs, immunolabelled for ubiquitin with 5 nm gold coupled to protein A and subsequently LAMP-1 with 10 nm gold. Asterisk represents cytosolic bacterium; Encircled asterisk, phagosomal bacterium; M, mitochondrium; L, lysosome; blue circles, ubiquitin label surrounding cytosolic *M. tuberculosis*; bar represents 100 nm.
B. Quantification of ubiquitin labelling associated with clusters of *M. tuberculosis*. *M. tuberculosis* clusters were regarded as positive for ubiquitin when 7 or more gold particles per bacterium were detected in the area extending out to 300 nm from the outer edges of the bacteria. Percentages of phagosomal bacteria ubiquitin labelled (+), cytosolic ubiquitin labelled (+) and cytosolic unlabelled (–) are plotted. For simplicity, the unlabelled phagosomal bacteria are left out of the graph.
C. Primary human DCs infected with H37Rv for 3 days, immunogold labelled for ubiquitin using a ubiquitin antibody and protein A conjugated to 5 nm gold and subsequently LAMP-1 with 10 nm gold. Labelling was present on small membranous structures. Asterisk represents cytosolic bacterium; M, mitochondrium; blue circles, ubiquitin label; bar represents 100 nm.



Supplementary Figure 6: Limited co-localisation ubiquitin and LAMP-1 on disrupted phagolysosomes.
Primary human DCs infected with H37Rv for 96 hours, immunogold labelled for ubiquitin and protein A conjugated to 5 nm gold and subsequently labeled for LAMP-1, Rabbit anti Mouse bridging and protein A conjugated to 10 nm. Bar represents 100 nm and blue circles ubiquitin (5 nm), red circles LAMP-1 (10 nm) gold particles.