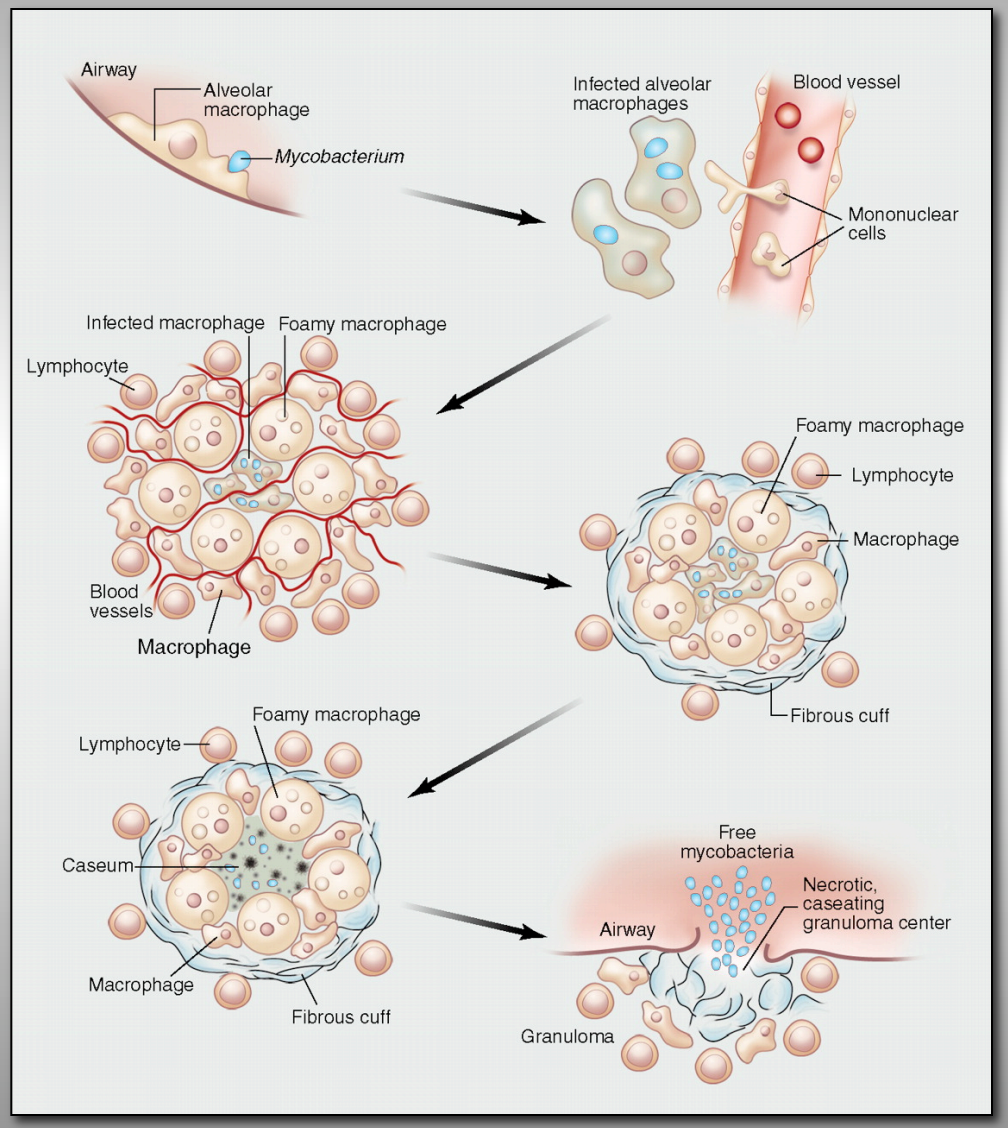
What Environments does Mtb Experience During Infection?



The natural history of the *M. tuberculosis* infection.

1.The bacterium is inhaled and phagocytosed by an alveolar macrophage.

2.The infected macrophage invades the tissue and recruits fresh phagocytes from the bloodstream.

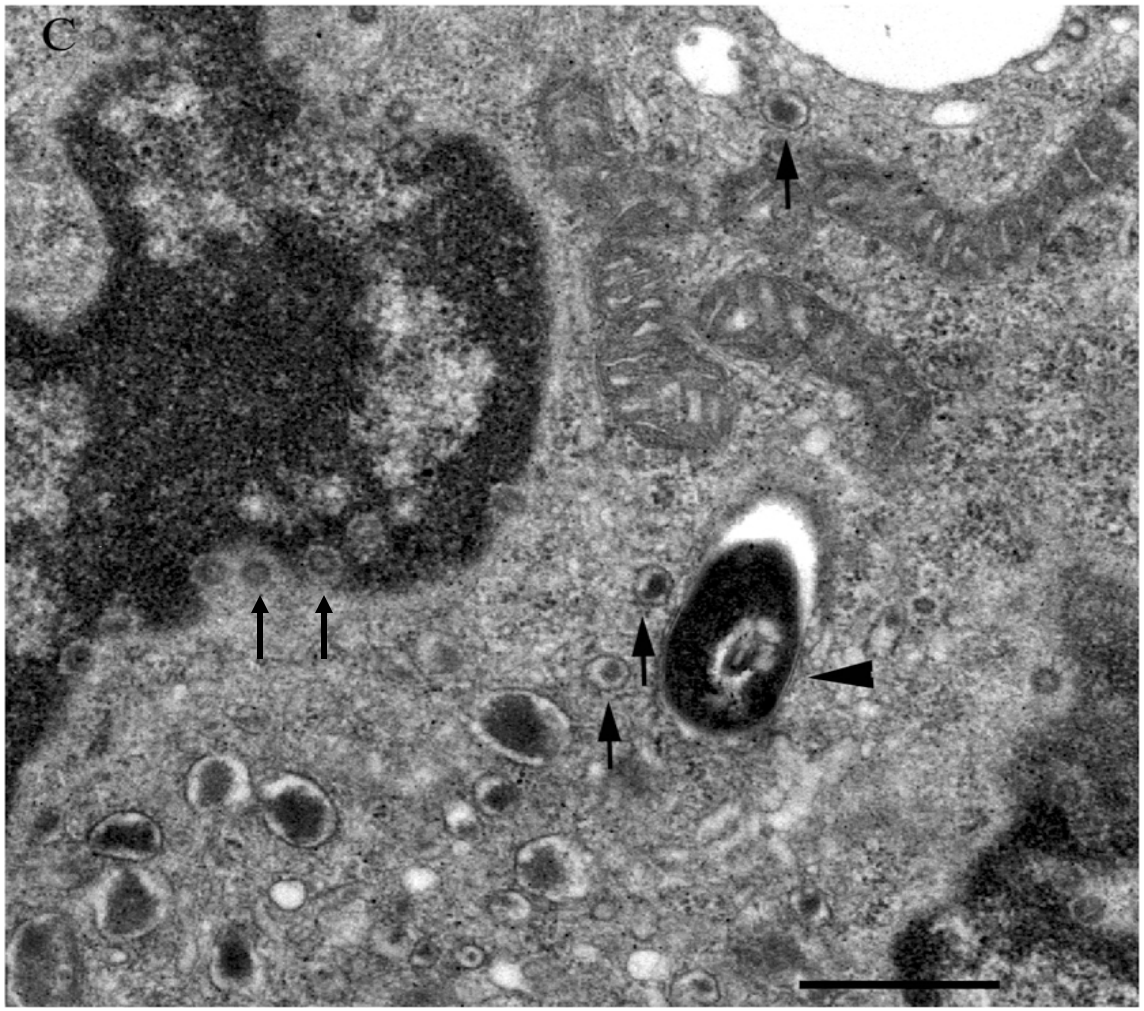
3.The phagocytes are joined by lymphocytes and form a granuloma.

4.The macrophages differentiate into foam cells, giant cells and epithelioid cells and a fibrous cuff emerges.

5.The center of the granuloma caseates, becomes necrotic, and releases live, infectious bacteria into the airways.

6.A cough ensues!

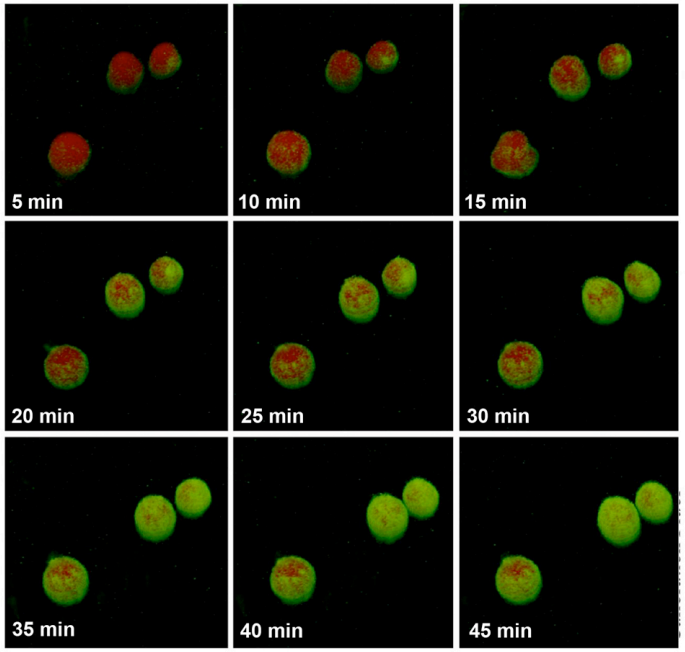
Intramacrophage Mtb are predominantly intravacuolar in human alveolar macrophages



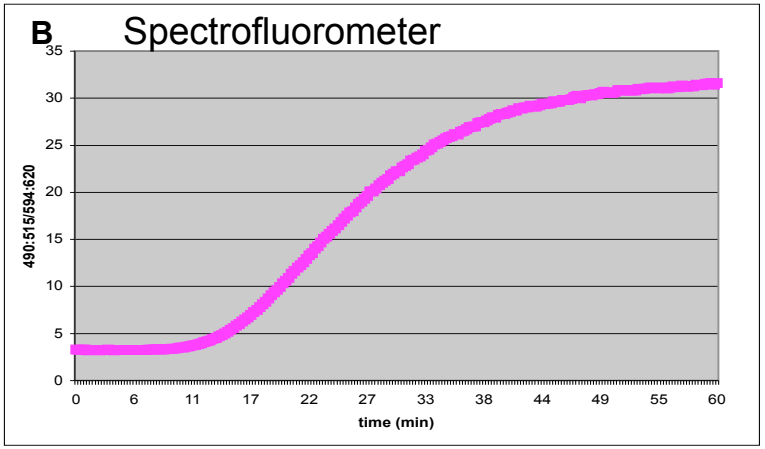
An alveolar macrophages from an HIV-infected, TB patient in Malawi.

Measurement of Intraphagosomal Physiology

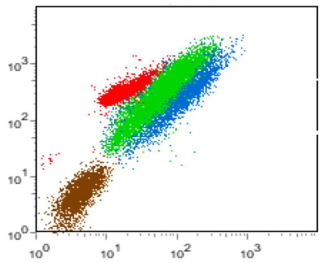
A Confocal microscope

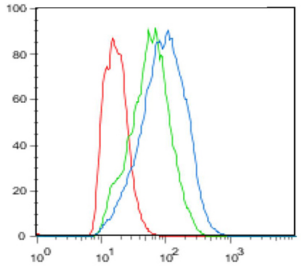


B Spectrofluorometer



C FACS





Brown: Mø w/o beads

Red: beads w/o Mø

Green: 30 min post uptake

Blue: 240 min post uptake

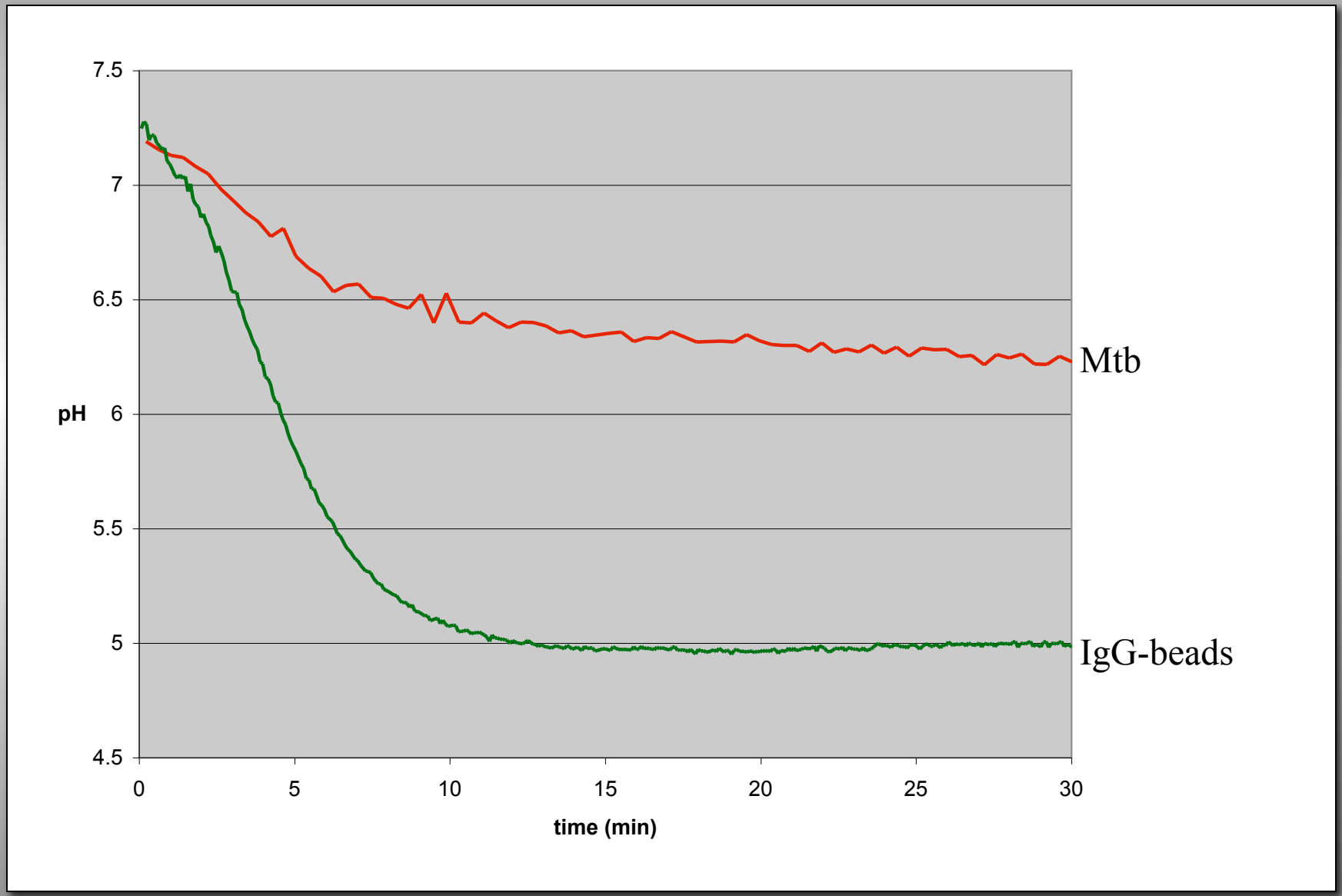
DQ BSA protease reporter beads beads analyzed by three different methods. A. Confocal imaging of beads inside phagosomes in macrophages. B. Spectrofluorometric analysis of the increase in substrate (green) fluorescence as a ratio over the calibration fluor (red) against time. C. FACS analysis of the substrate fluorescence following uptake by human alveolar macrophages.

We have assays for the following intraphagosomal parameters:

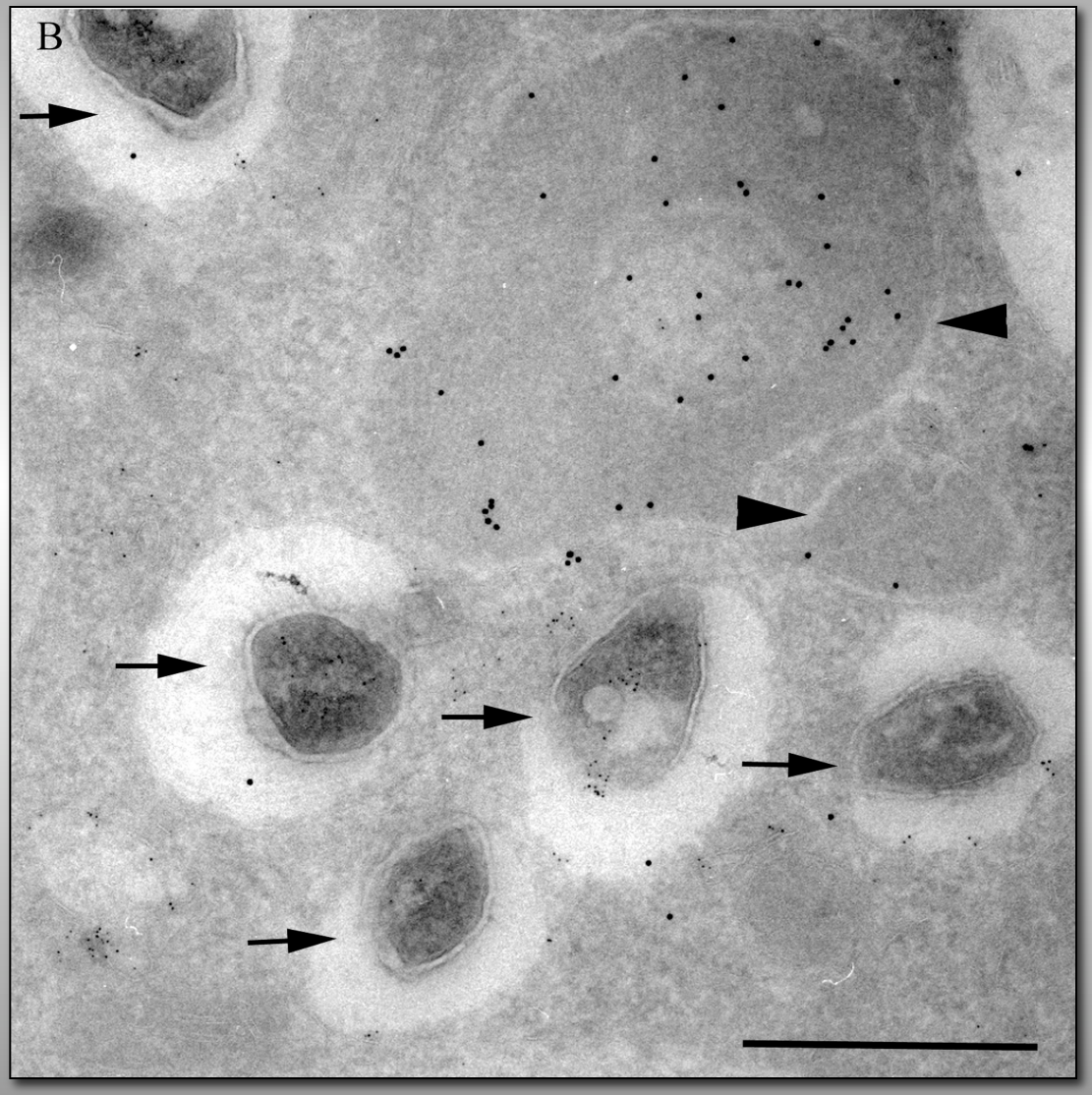
pH ***Lysosome fusion*** ***Cysteine Proteinase*** ***Bulk proteinase*** ***Lipolysis*** ***β-galactosidase***

Superoxide radicals ***Lipid peroxidation*** ***Reducing capacity***

Intra-Phagosomal pH in Mtb Phagosomes

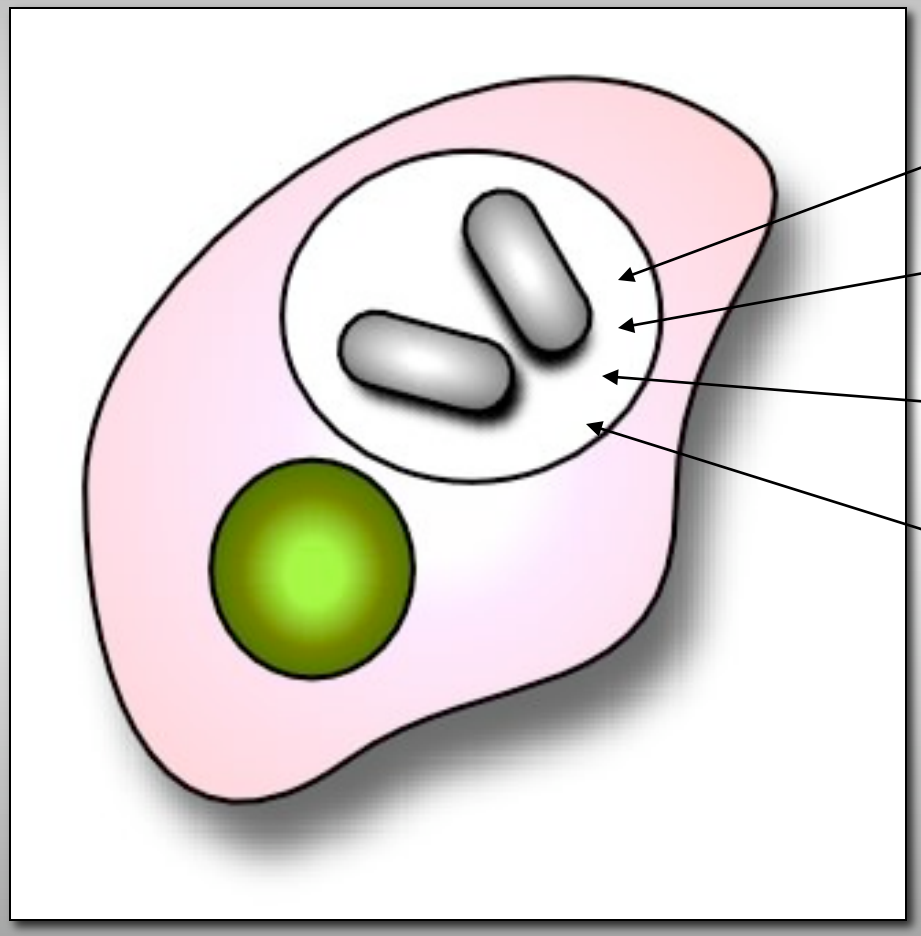


M. tuberculosis-containing phagosomes fail to acidify, unlike neighboring lysosomes (human alveolar macrophages from TB patient).



Macrophages labeled with the pH indicator DAMP for 30 minutes prior to fixation

Summarizing the properties of the Mtb-containing phagosome in a resting Mø



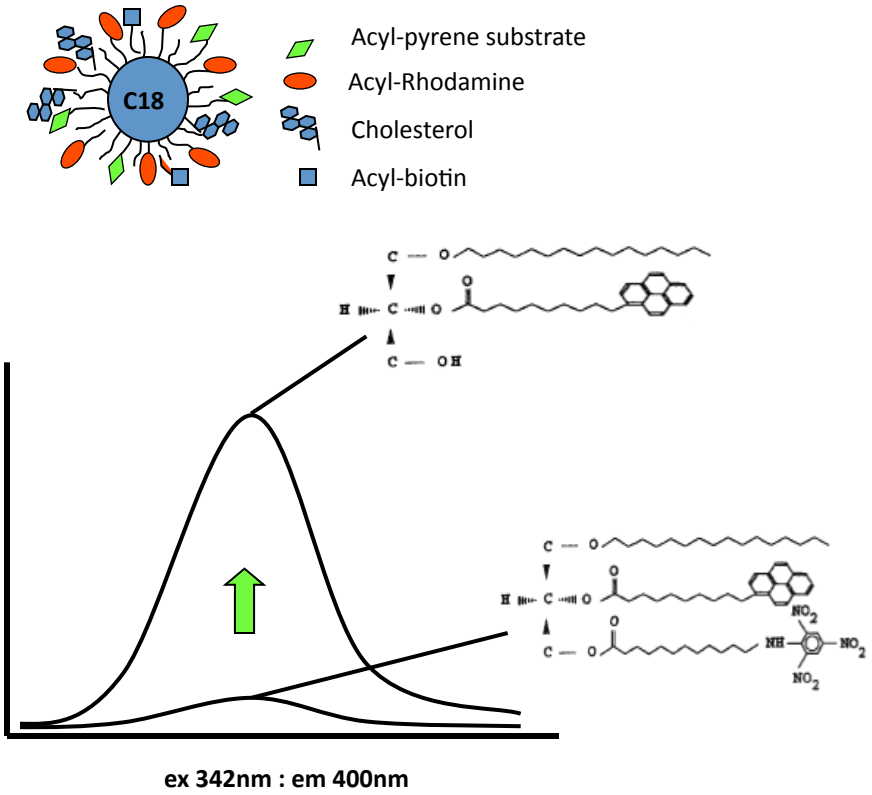
Relatively high pH, 6.4.
(activated Mø pH 5.2)

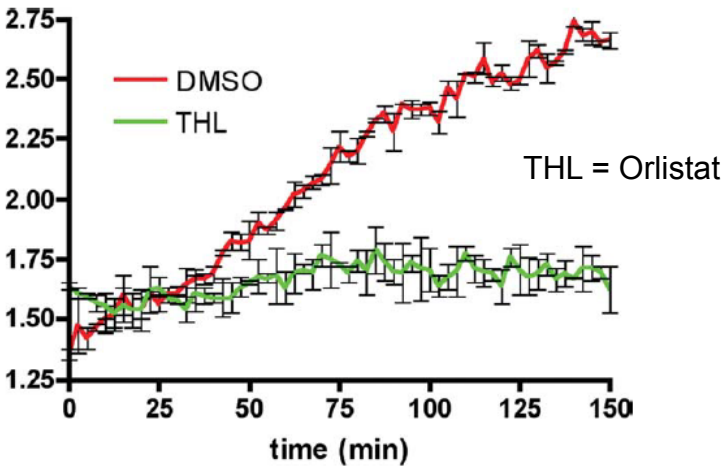
Transient production of ROIs (20 min)

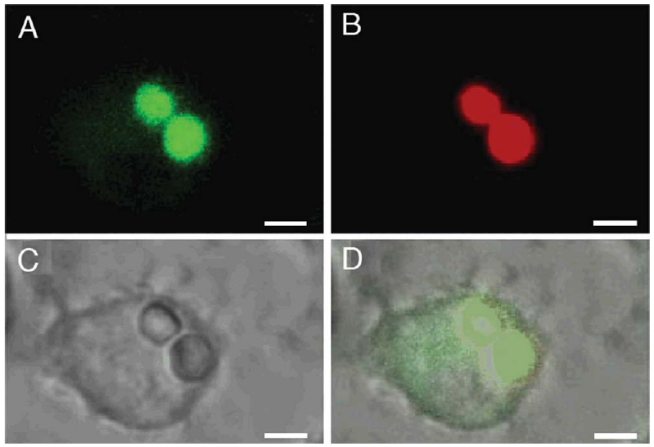
Low proteolytic activity (cysteine proteases)

High lipolytic activity (LPL/Hep lipase)

High-throughput screen for inhibitors of phagosomal lipolysis

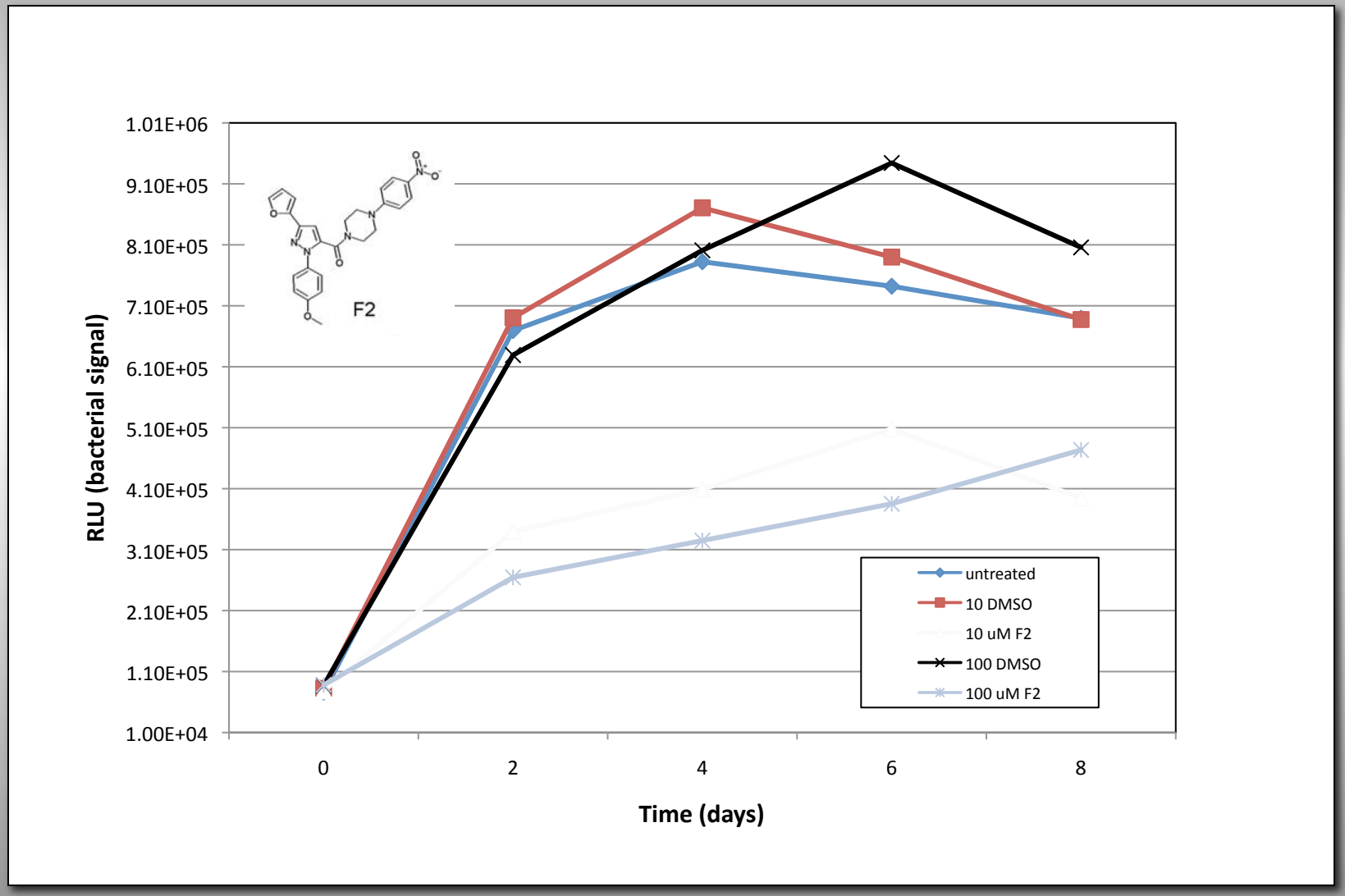




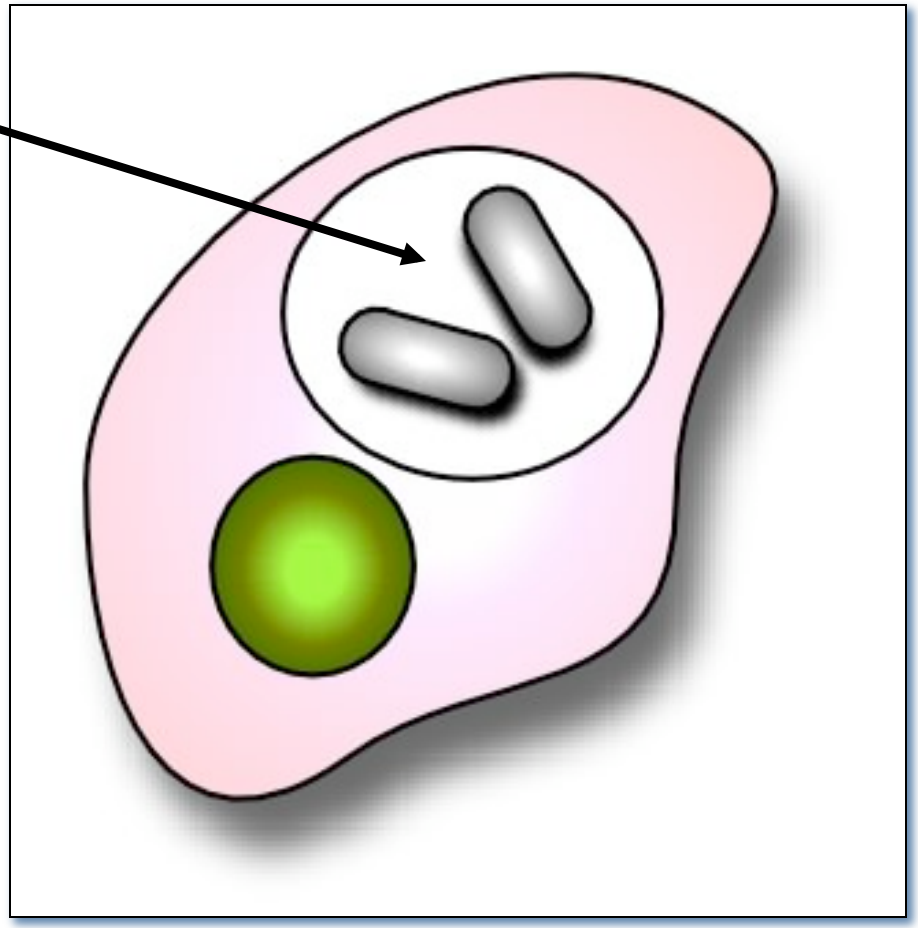


We screened a library of 90,000 compounds and isolated 32 inhibitory compounds of which 25 shared a common pyrazole-methanone backbone.

The pyrazole-methanone compound F2 restricts Mtb proliferation in J774 macrophages (but not in broth culture)



LDL (cholesterol, triacylglycerol, phospholipids)

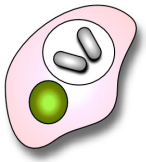
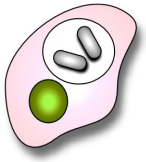


The lipase inhibitors are blocking host enzymes required to render host lipids useable by Mtb.

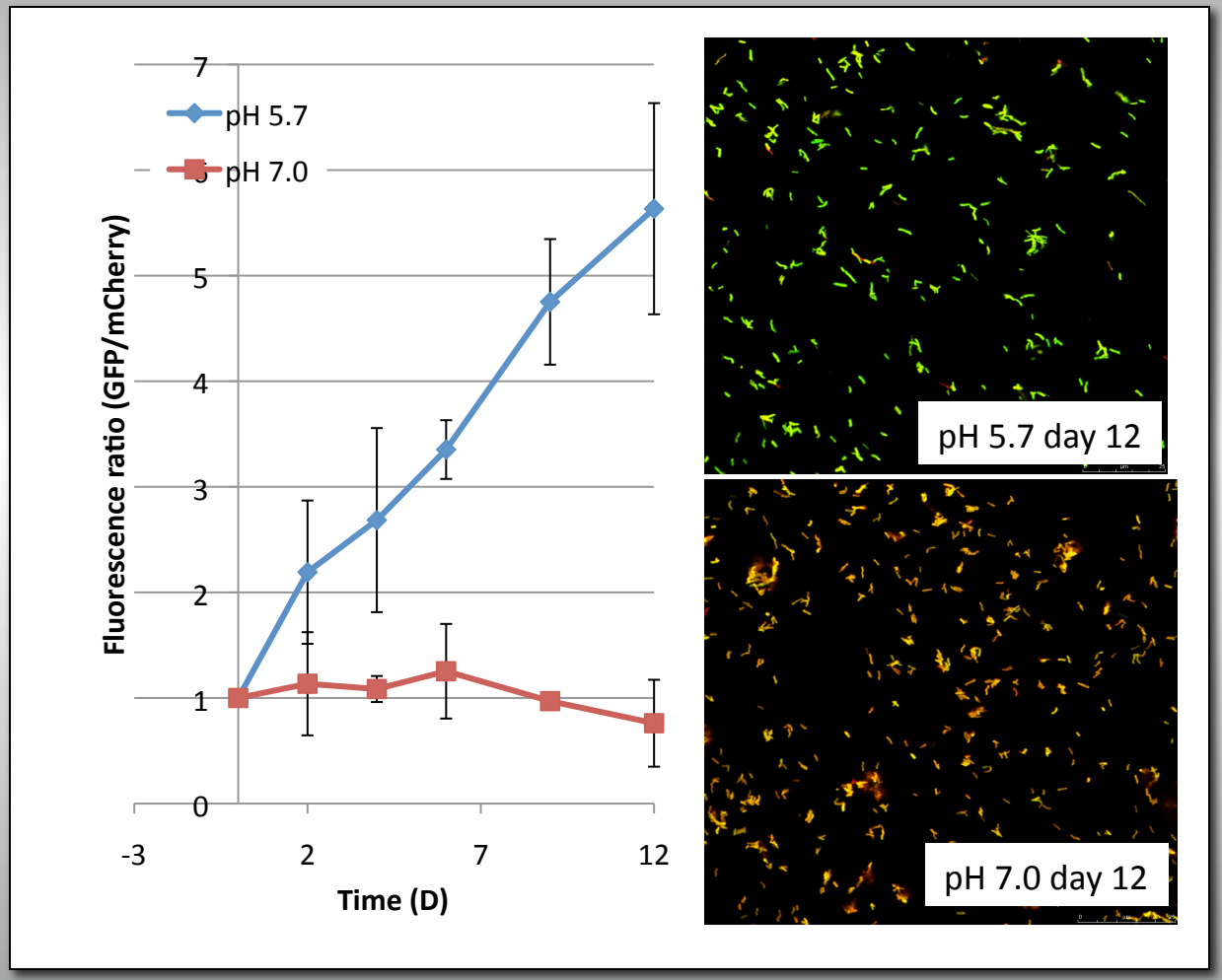
Or

The lipase inhibitors inhibit a Mtb enzyme that is not expressed or required for growth in broth or minimal medium with TAG

Pipeline for generation of pH "reporter" bugs

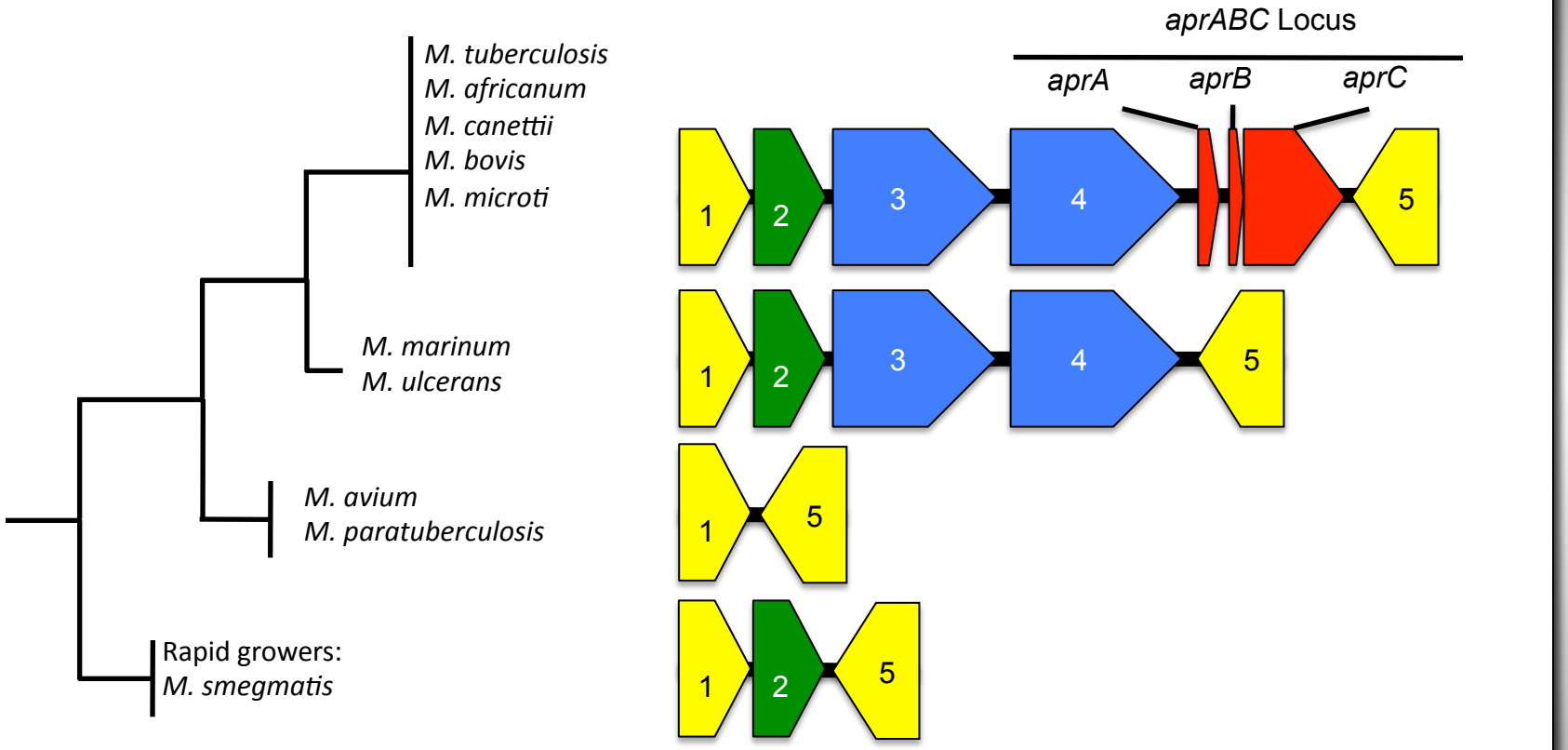
		Number of candidates
	Microarray analysis of *M. tuberculosis* in macrophages 60 minutes post infection	68 genes
	Microarray analysis of *M. tuberculosis* in macrophages (+ Concanamycin A) 60 minutes post infection (subtract from above)	24 genes
	Microarray analysis of *M. tuberculosis* exposed to low pH in vitro	18 genes
	Analysis of candidate genes to identify individual transcriptional units/promoters	17 genes
	Clone the promoters upstream of GFP, transform into *M. tuberculosis*, and check for induction in vitro	17 genes
	Run controls for specificity of induction to the stress of interest by exposing bacteria to a range of different stresses	4 genes

Dual Fluorescent Protein reporters enable ratiometric measurements of CDC1551 (aprA'::GFP, smyc'::mCherry) fluorescence



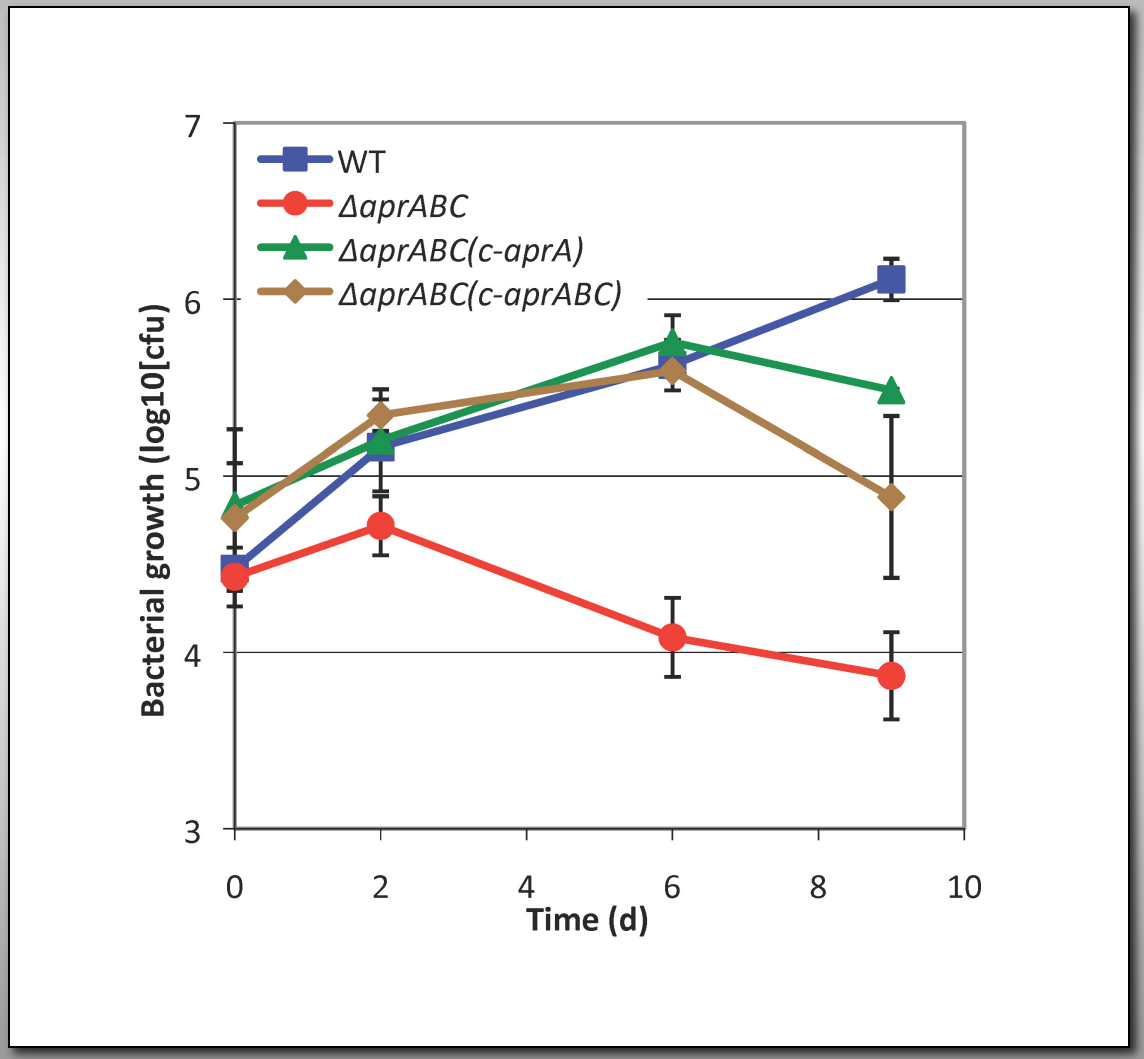
Fluorescence ratio quantitated on a Perkin Elmer Envision Plate reader (3 biological reps)

The *aprABC* locus is specific to the *M. tuberculosis* complex.

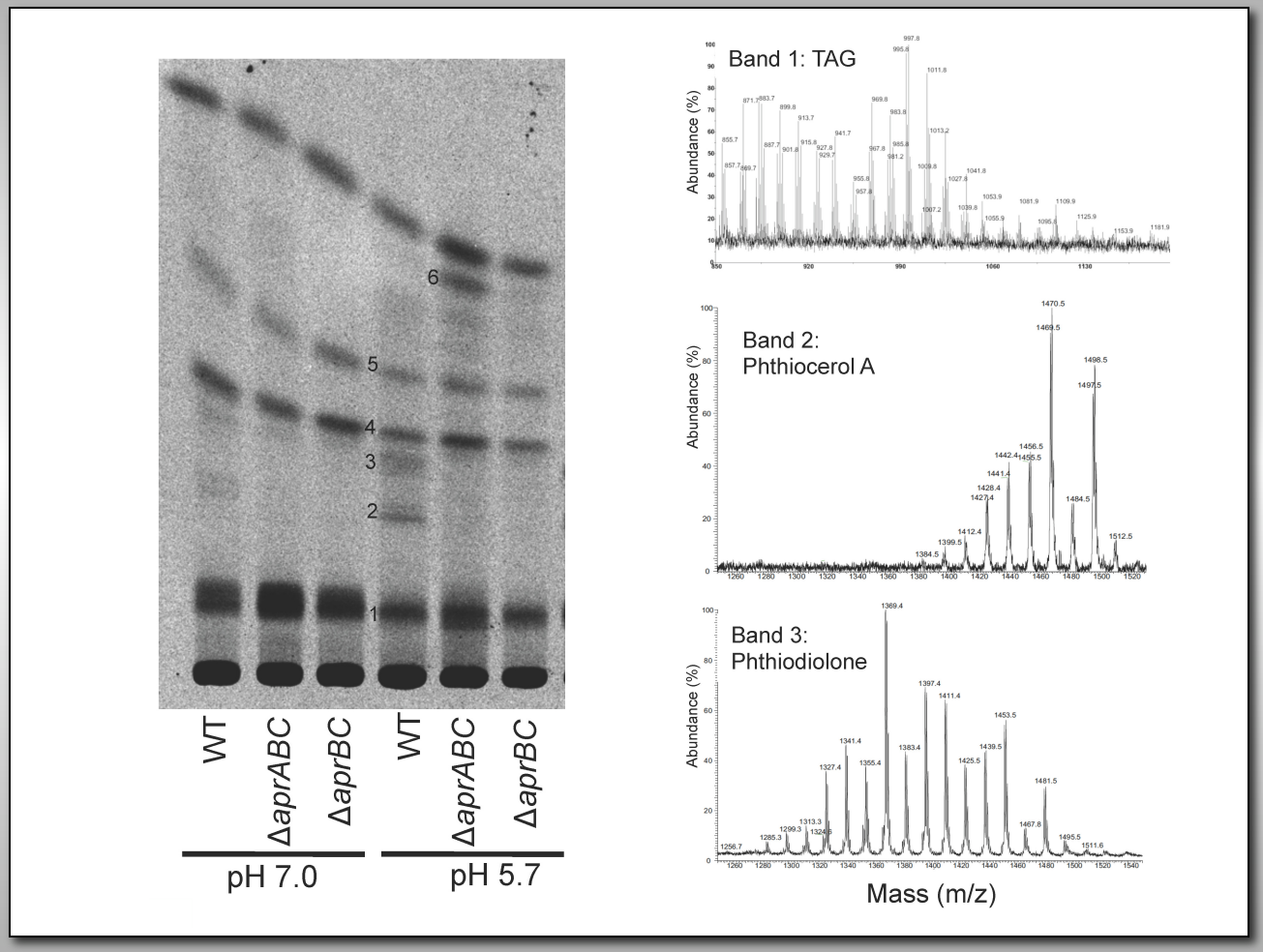


1. Rv2392, 2. Rv2393, 3. Rv2394, 4. Rv2395, 5. Rv2397

Mutants defective in *aprA* show impaired survival in macrophages



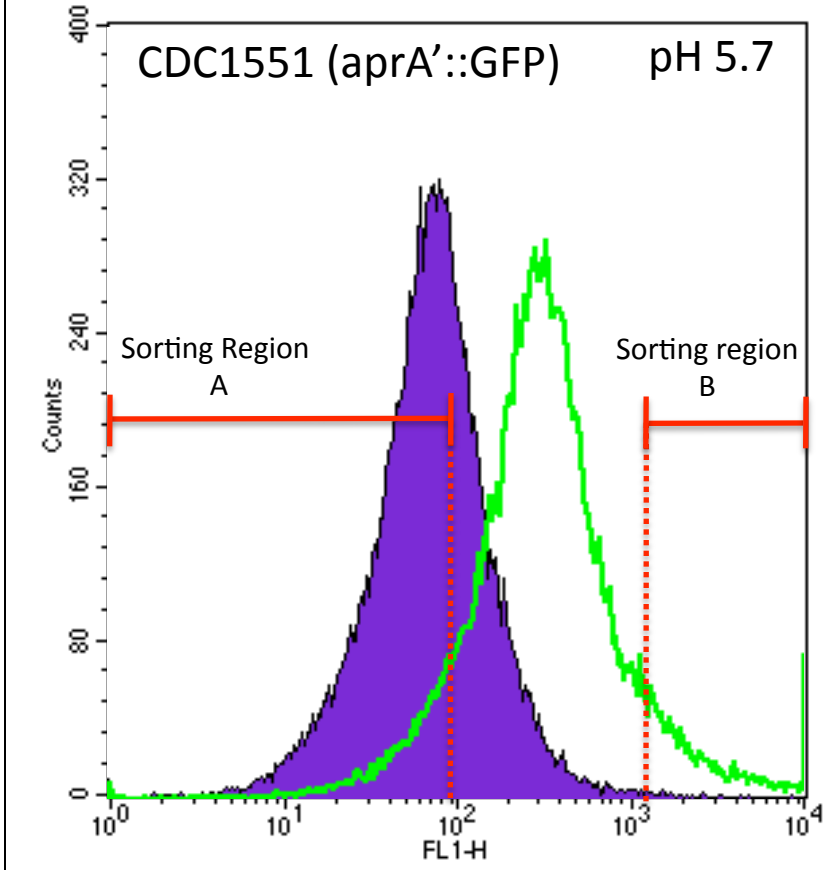
aprA-mediates pH-dependent alterations in the cell wall lipids



The pH-dependent increased synthesis of pthiocerol mycocerates is lost in the *aprA*-deficient mutant, which shows increased TAG levels

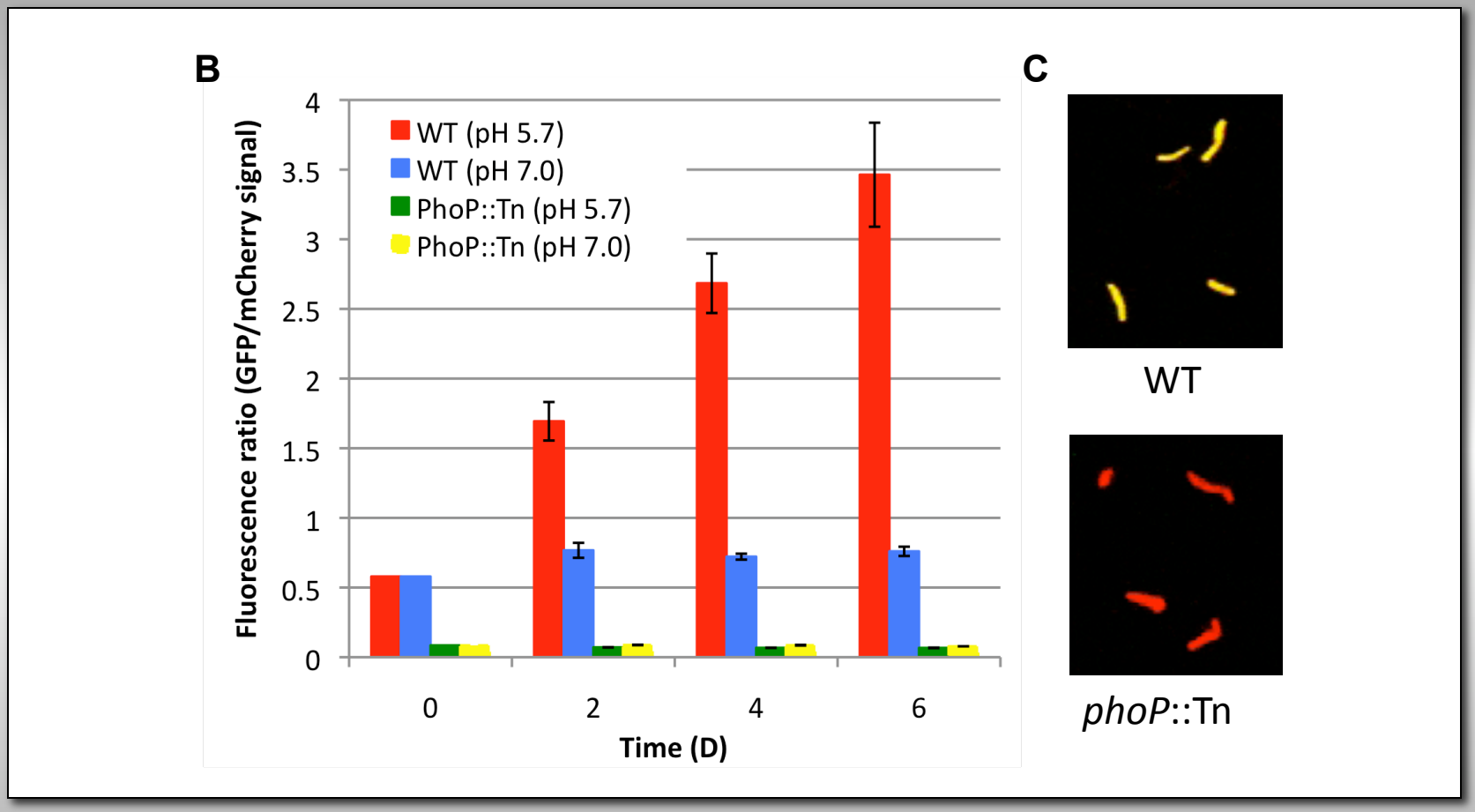
FACS sorting of hypo- and hyper-expressers of *aprA'::GFP*.

The reporter construct was transformed into a transposon-mutagenized library



FACS analysis of the AprA'::GFP synthetic phenotype induced through the transition from pH 7.0 (purple solid) to pH 5.7 (green line). Sorting the shoulders A and B will enrich for mutants that exhibit hypo- and hyper-expression in response to the pH drop.

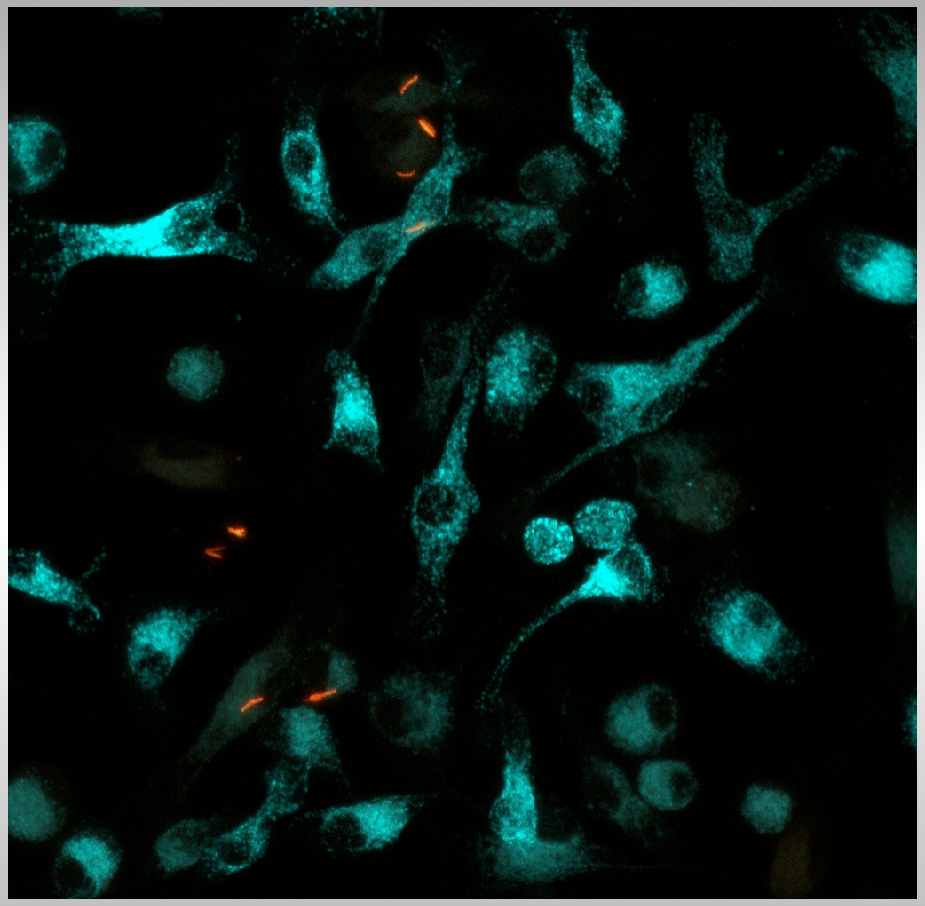
CDC1551(*AprA'*::GFP, *smyc'*::mCherry) is not induced in a *PhoP*::Tn mutant



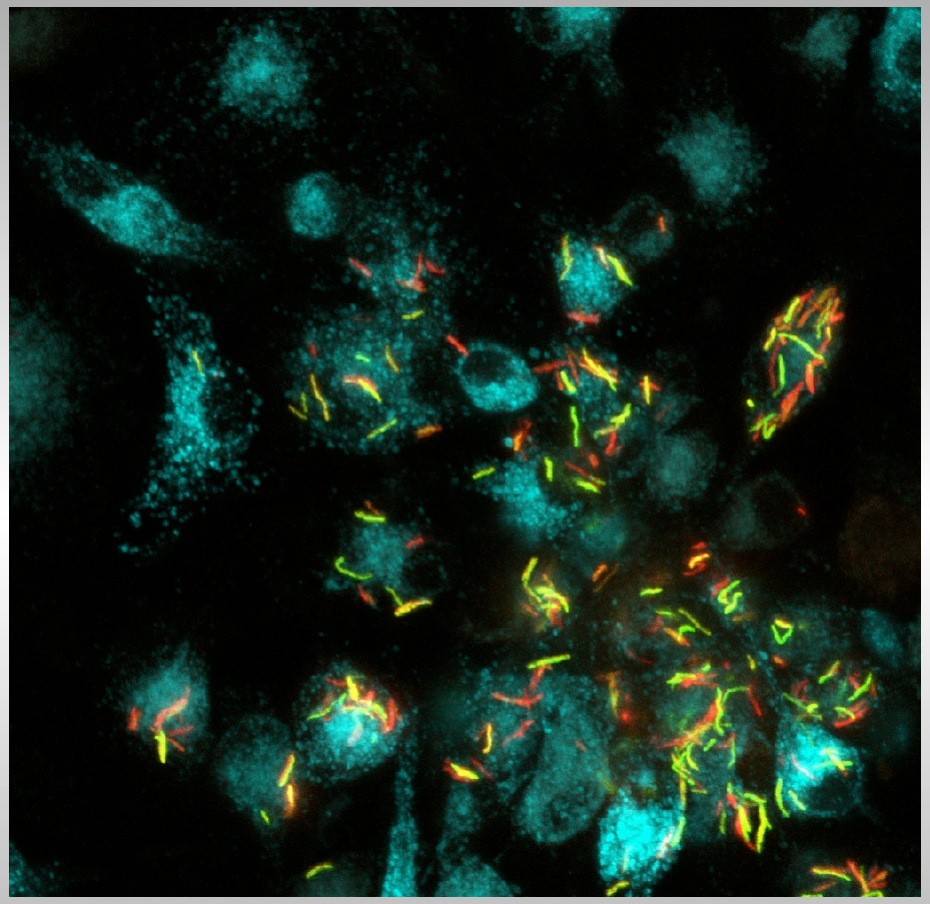
The loss of *AprA'* induced GFP signal suggests that this reporter will be useful to:

1) identify compounds that disrupt PhoPR signaling pathway
2) identify mutants that disrupt *AprABC* locus regulation

Heterogeneity of CDC1551 (aprA'::GFP, smyc:mCherry) induction in macrophages



2 hour post infection



4 days post infection

Activated macrophages

Lysosomes loaded with Alexafluor 647 10000MW dextran (false-color cyan)

Testing the Integrated Erdman Reporter strains in mouse infections

Mouse infections performed by Yao Phuah (Flynn lab)

Mice infected via aerosols with:

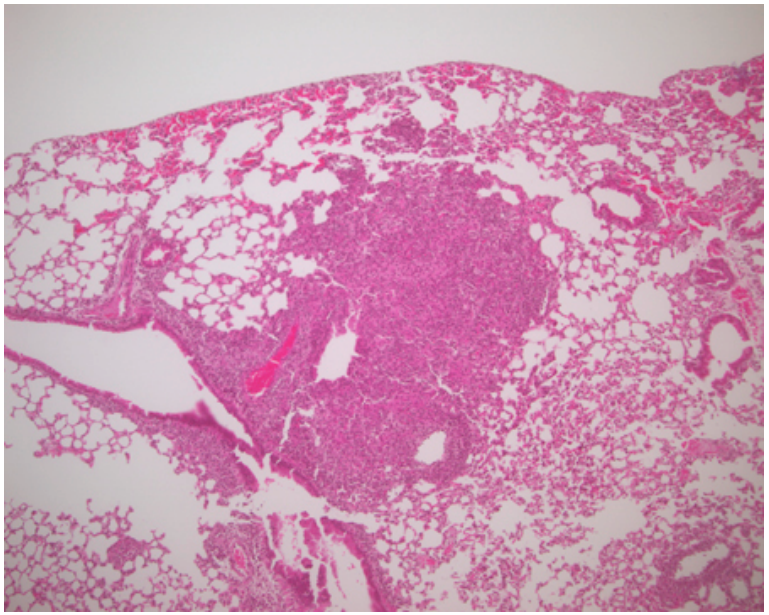
--Erdman (pMV306 aprA'::GFP, smyc::mCherry)

4 weeks post infection mice were euthanized the lungs examined

Presence of Mtb confirmed by acid-fast staining

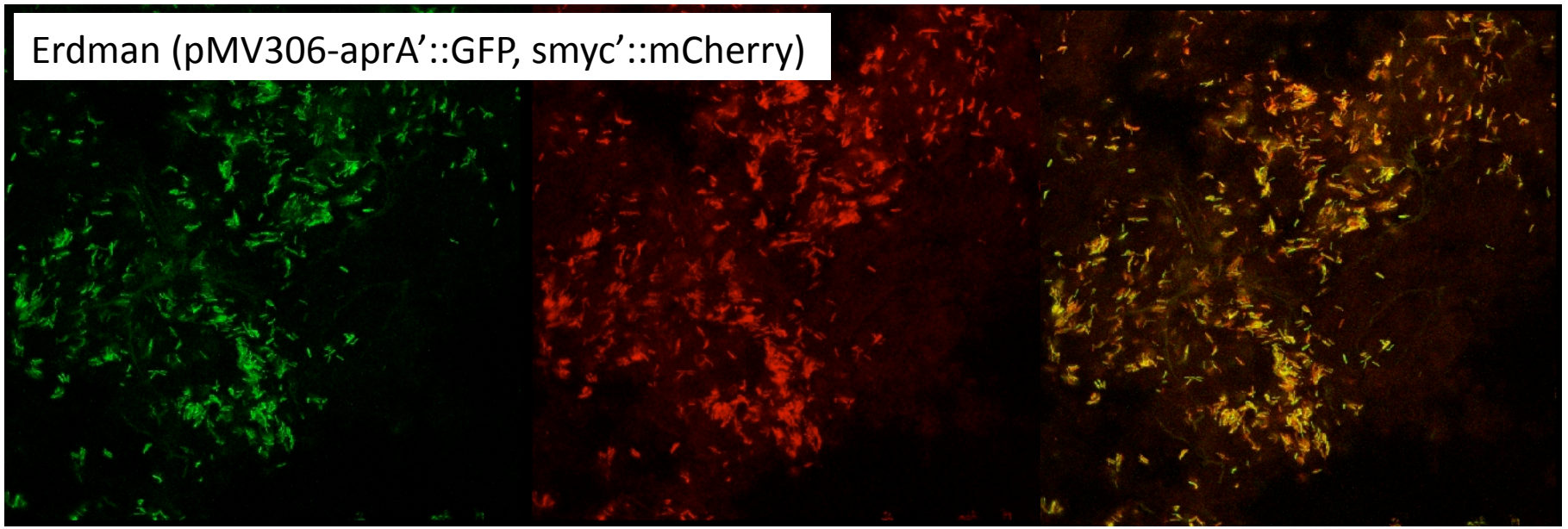
For fluorescence analysis, tissue was prepared by taking thin slices and squash mounting in water.

All imaging done at 63X objective on a Leica SP5 confocal microscope



H&E stain of infected mouse lung (Y. Phuah)

Detection of the integrated Erdman(pMV306-AprA'::GFP, smyc::mCherry) reporter in infected mouse lung



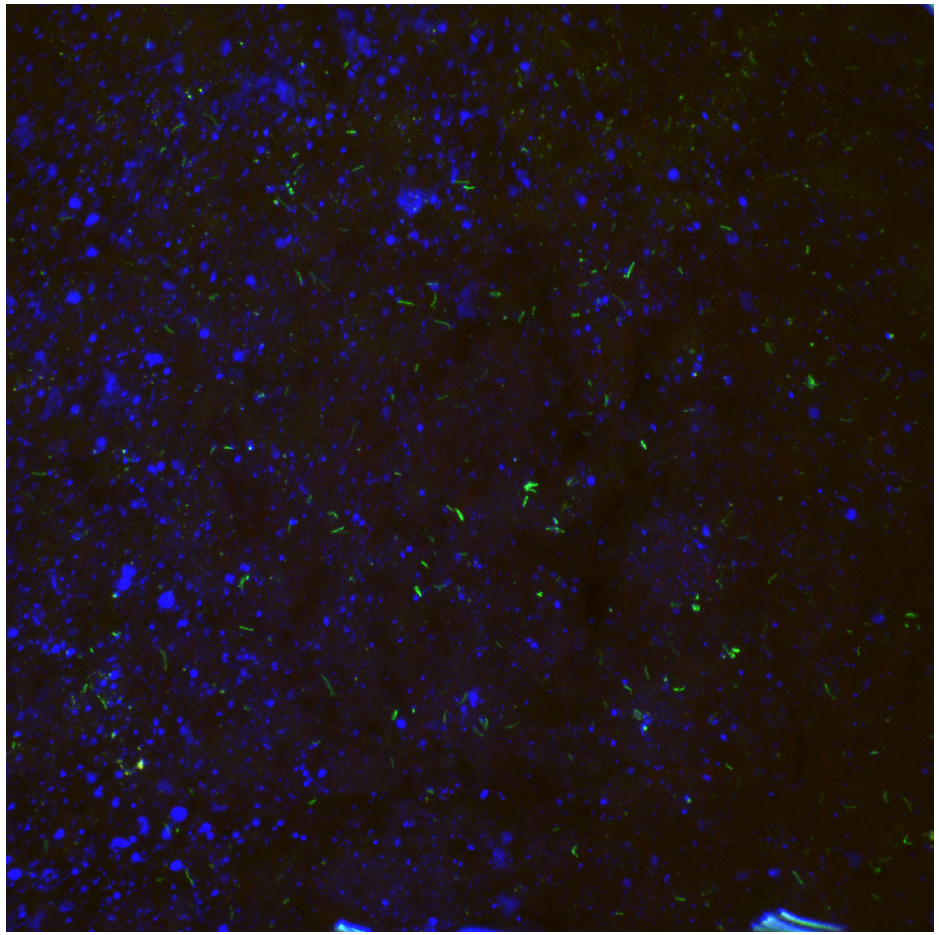
Testing integrated Erdman reporters in monkeys

-- Monkeys were infected with 250 CFU of Erdman (AprA'::GFP, smyc'::mCherry)

-- After 2 months, monkeys exhibited active TB, including pneumonia

-- Green fluorescent bacteria were identified in BAL

Ling Lin & Josh Mattila
(Flynn Lab)

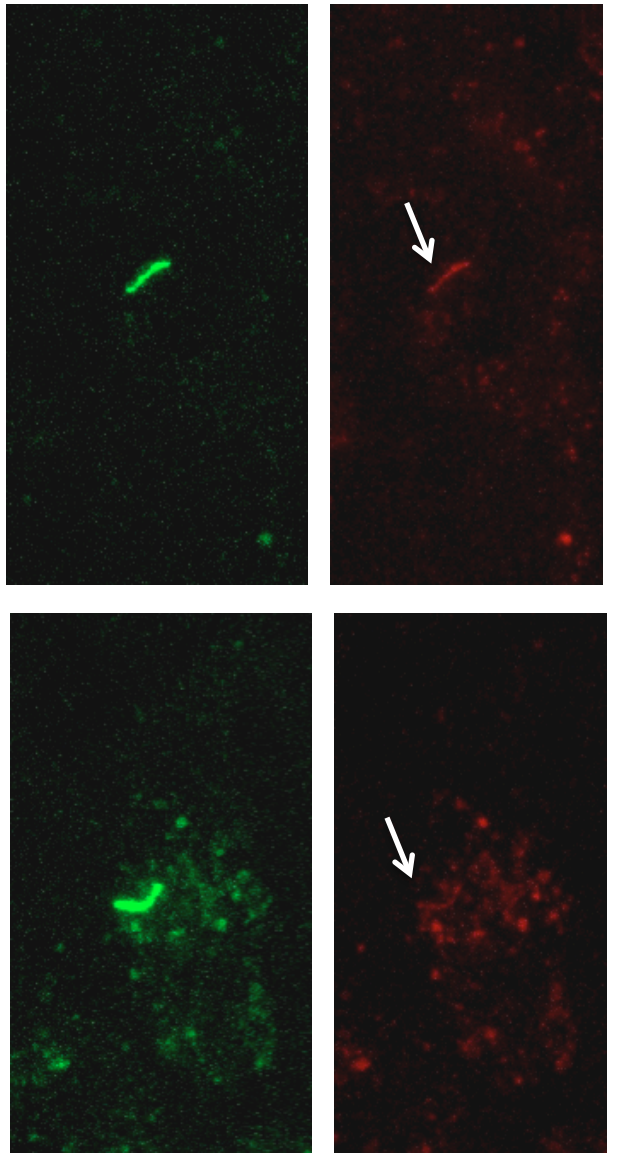


Detection of mCherry signal from the Erdman (*aprA'*::GFP, *smyc'*::mCherry) reporter in primate tissue

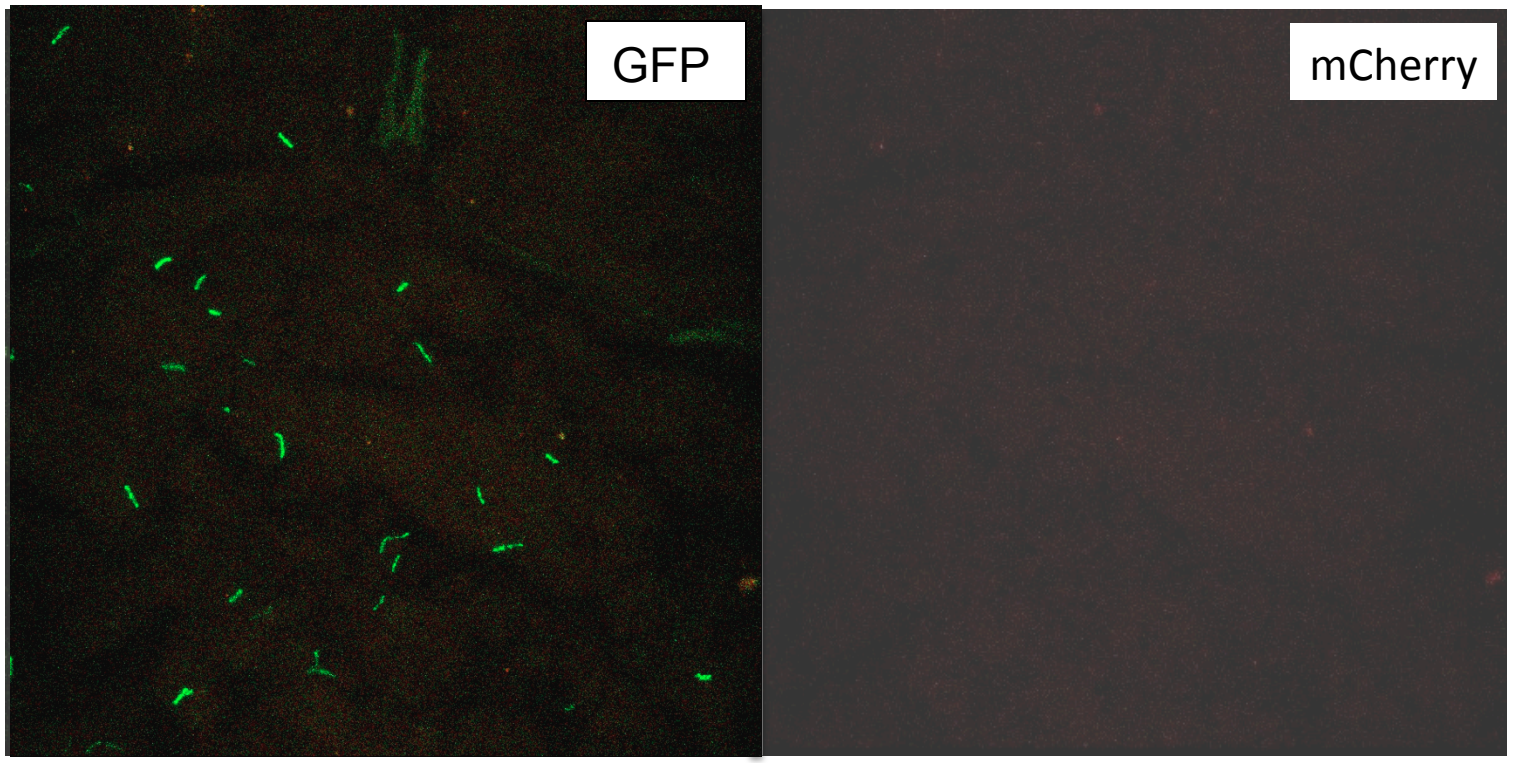
Bacteria were identified with detectable mCherry signal

mCherry signal was typically low compared to background

mCherry signal was often detected in bacteria in non-granulomatous tissue with very few bacteria



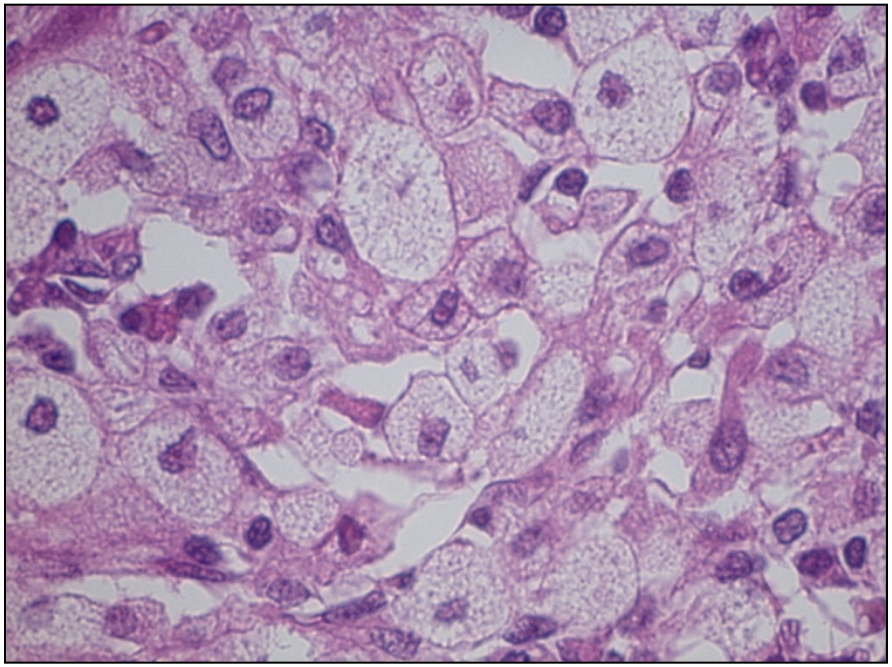
mCherry signal was not detected in GFP expressing bacteria in caseous region

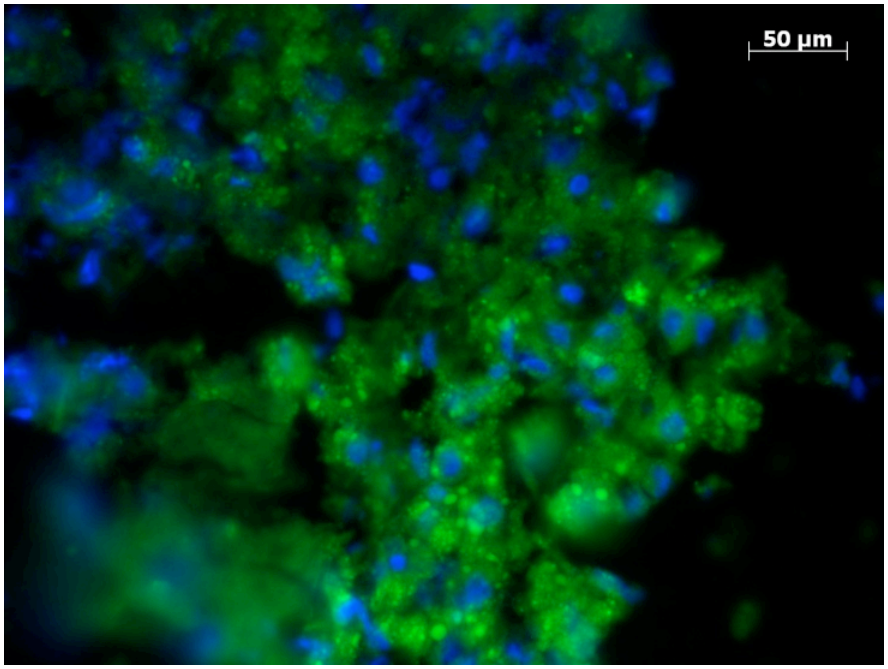


mCherry signal was undetectable in caseous regions of granuloma

Low signal possibly due to dependence of smyc promoter activity on replication

Probing host lipid metabolism in human TB granulomas

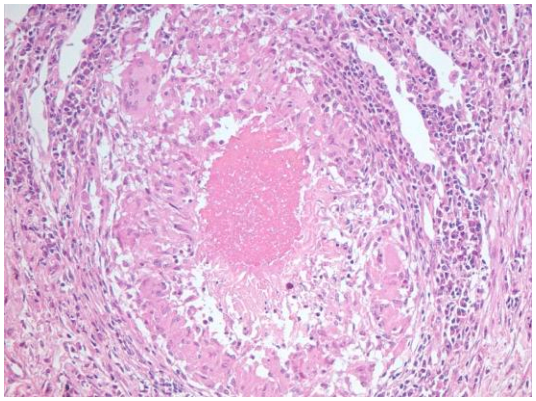




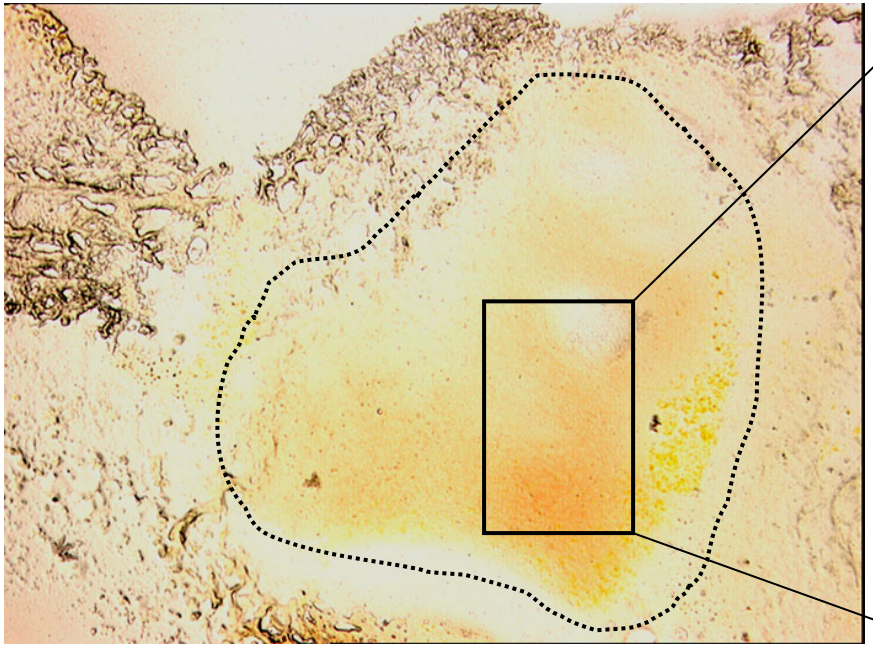
H&E Foamy Macrophages BODIPY493/503

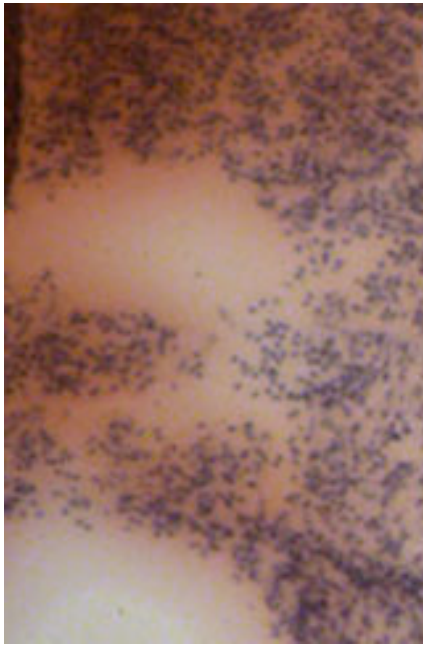
In collaboration with:
Gilla Kaplan, PHRI
Helen Wainwright and Linda-Gail Bekker, University of Cape Town
Fong Hsu, Washington University

LCM was performed on unstained cryosections from caseous human granulomas



We performed laser-capture microdissection on unstained cryosections from caseous granulomas (upper left). These regions were processed for mRNA isolation and microarray hybridization (Human X3P array).





Genes involved in lipid:

Synthesis Processing Sequestration

were highly upregulated in human TB granulomas.

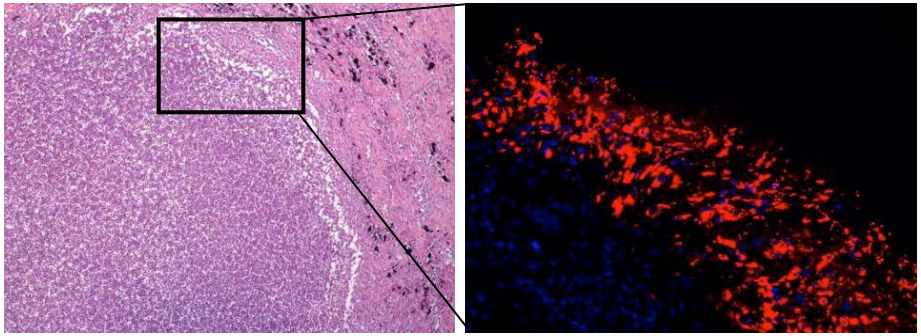
Genes involved in lipid sequestration and metabolism

Gene	Transcript description	Signal Intensity (Ave)	Function
ACSL1	Acyl-CoA synthetase long-chain family member 1	12.72	ACSL1 converts free long-chain fatty acids (C12 to C20) into fatty acyl-CoA esters on plasma membrane, mitochondria, or lipid droplets.
ADFP	Adipose-differentiation related protein	13.08	ADFP sequesters neutral lipids including cholesteryl ester (CE) a nd triacylglycerol (TAG) in lipid droplets
NPC1	Niemann-Pick disease, Type C1	12.47	NPC1 transports intracellular cholesterol and other types of li pids to post-lysosomal destinations via sterol-sensing motifs.

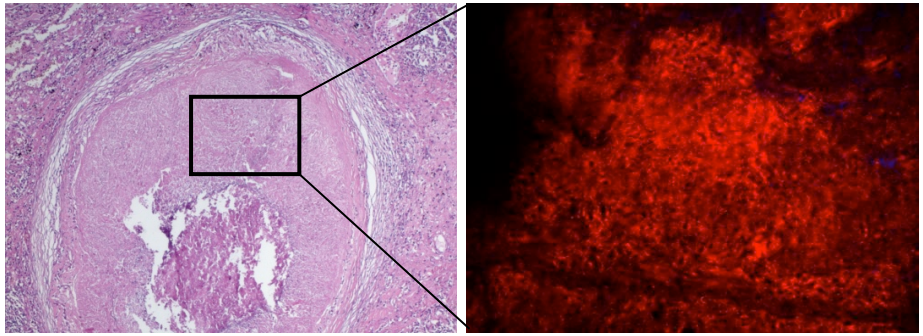
Genes involved in sphingolipid metabolism

Gene	Transcript description	Signal Intensity (Ave)	Function
ASAH1	*N*-acylsphingosine amidohydrolase (acid ceramidase) 1	14.49	ASAH1 catalyzes the synthesis and degradation of ce ramide into sphingosine and fatty acids within lysosomes and/or late endosomes.
GBA	Glucosidase, beta; acid (includes glucosylceramidase)	11.59	GBA breaks down glucocerebroside into glucose and ceramide within lysosomes.
GLA	Galactosidase, alpha	12.99	GLA hydrolyzes the terminal alpha-galactosyl moieties from glycolipids and glycoproteins, mainly ceramide trihexoside, and it can also catalyze the hydrolysis of melibiose into galactose and glucose.
NSMAF	Neutral sphingomyelinase (N-SMase) activation associated factor	11.85	NSMAF is involved in tumor necrosis factor (TNF)-mediated activation of N-SMase by binding the cytoplasmic sphingomyelinase activation domain of the 55kDa TNF receptor (TNF-R55) and may play a role in regulating TNF-induced cellular responses such as inflammation.
PSAP	Prosaposin	14.63	SapC transforms lysosomal membrane, thereby assisting glucosylceramide degradation by glucosylceramide-β-glucosidase.

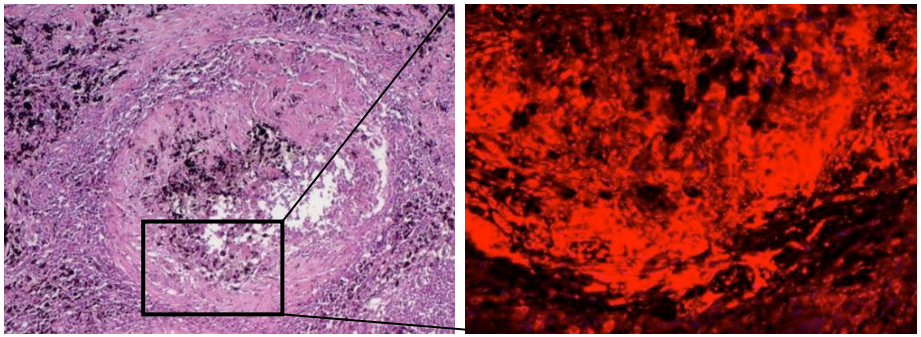
Immunohistology of human TB granuloma



ADFP



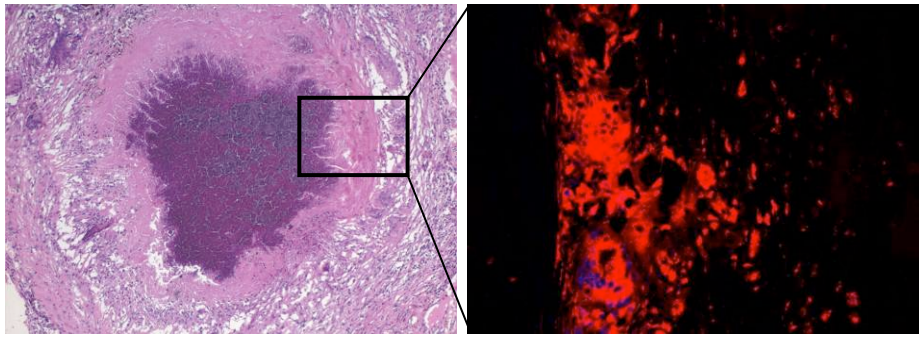
ADFP



ACSL1

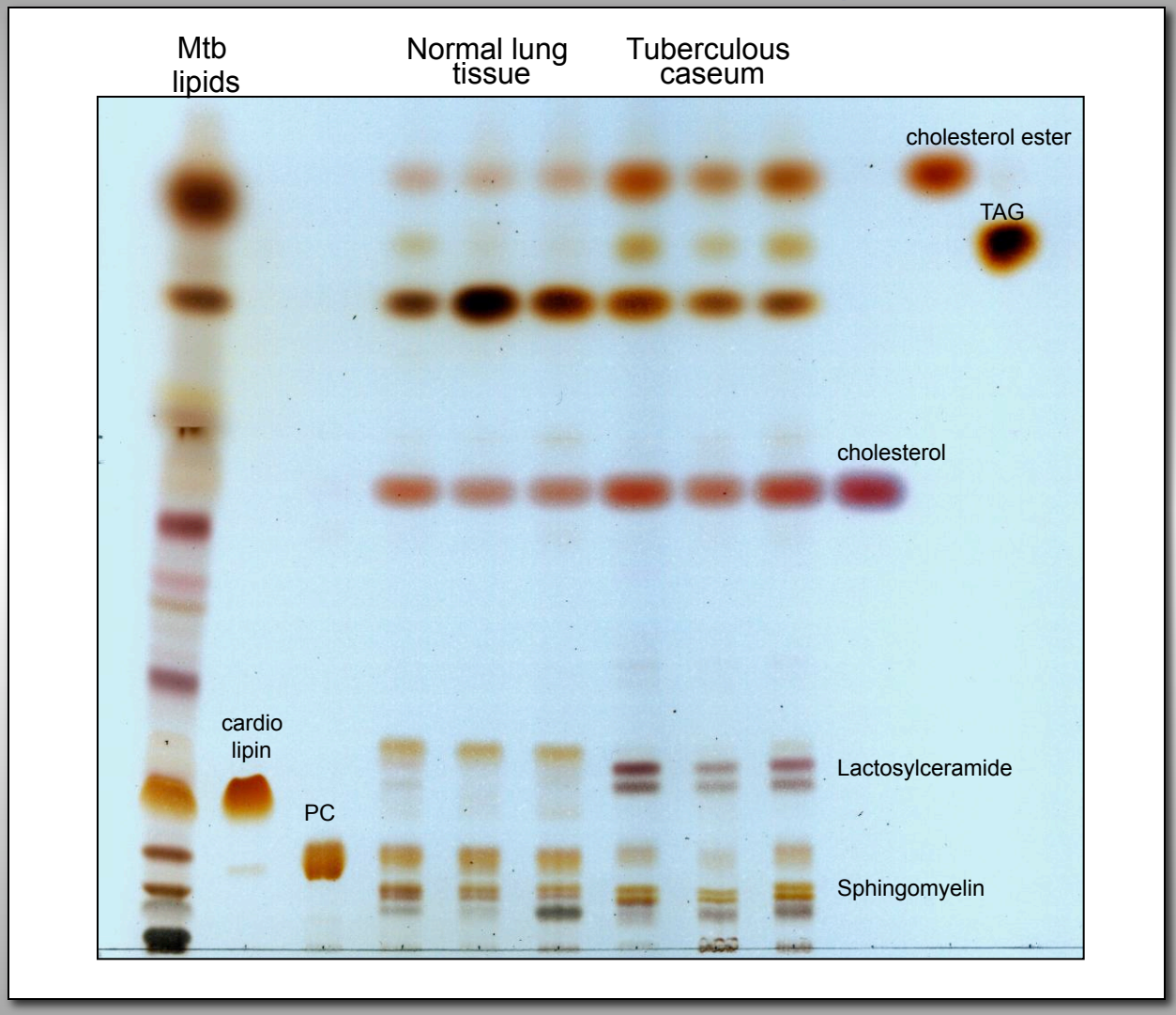
Blue: nucleus

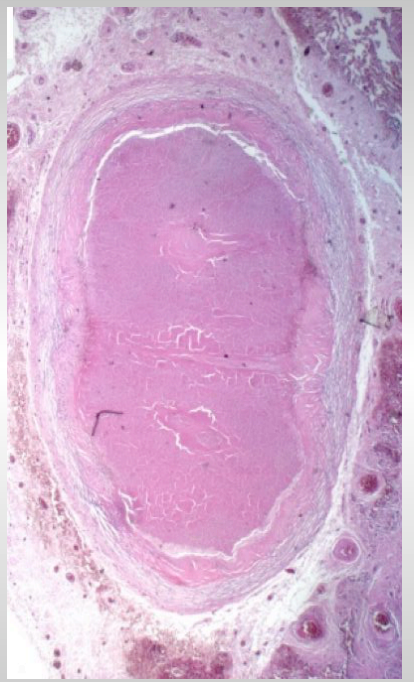
Red: Antigen



SapC

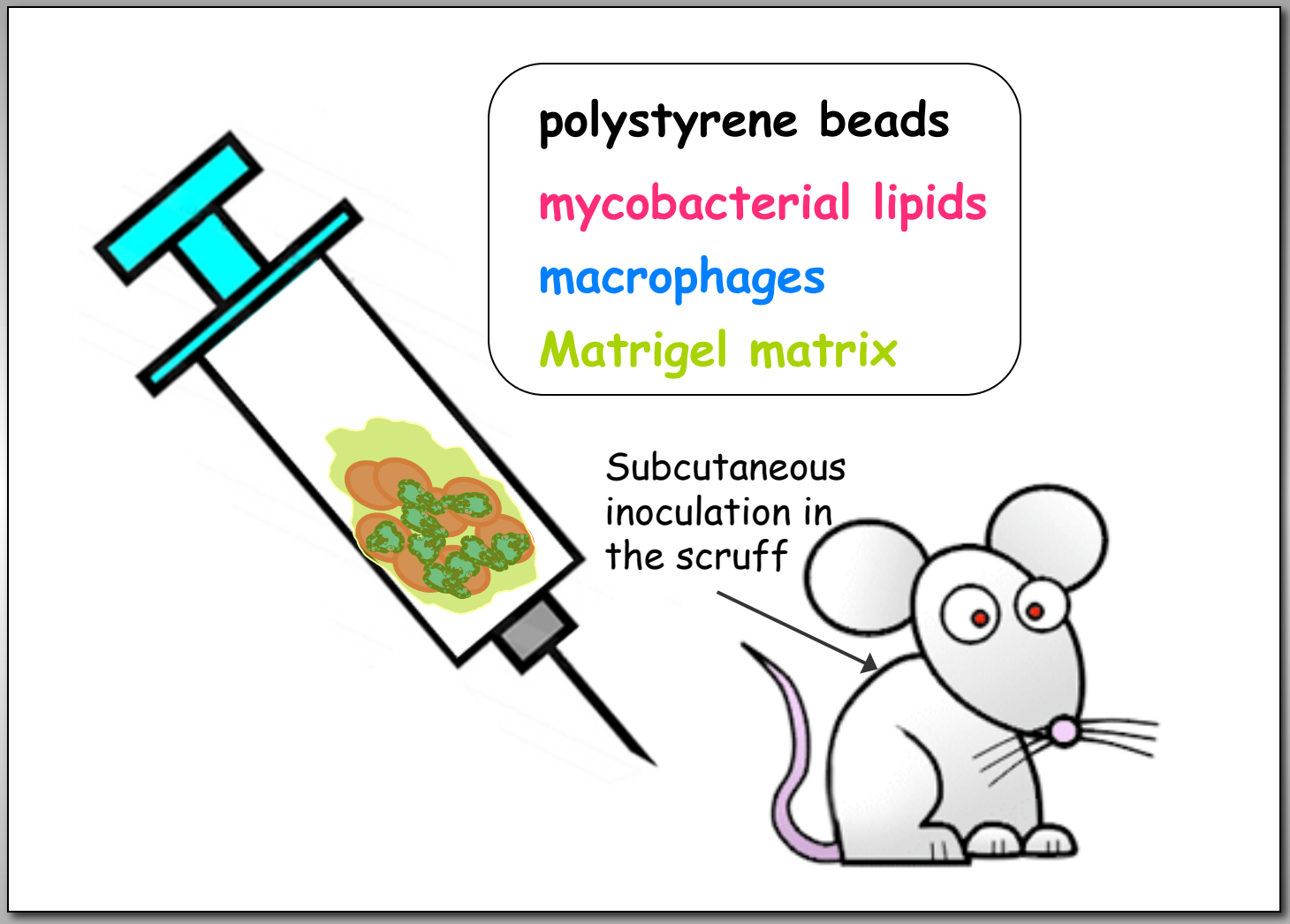
LDL-derived lipids are over-represented in the caseum from human TB granulomas





Lipids were solvent extracted from caseum excised from caseous human tuberculosis granulomas from UCT, South Africa. This was compared to lipids from an uncompromised region of tissue.

Development of a murine granuloma model

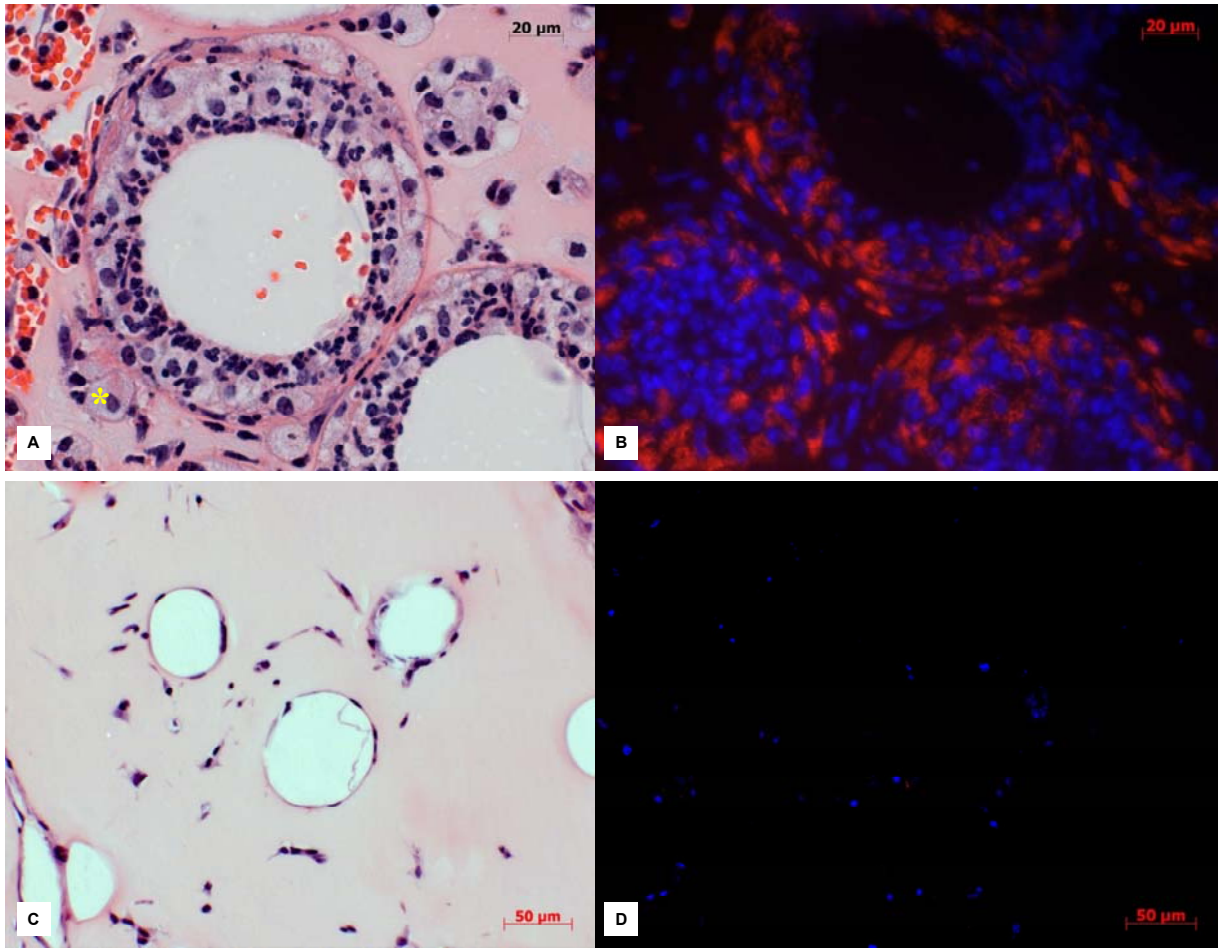


Rhoades, E.R., Geisel, R.E., Butcher, B.A., McDonough, S.M., and Russell, D.G. (2005) A new model of the inflammatory response to Mycobacterium bovis BCG lipids: Elucidation of granuloma formation. **Tuberculosis 85. 159-176.**

Adfp expression in TDM granulomas

TDM

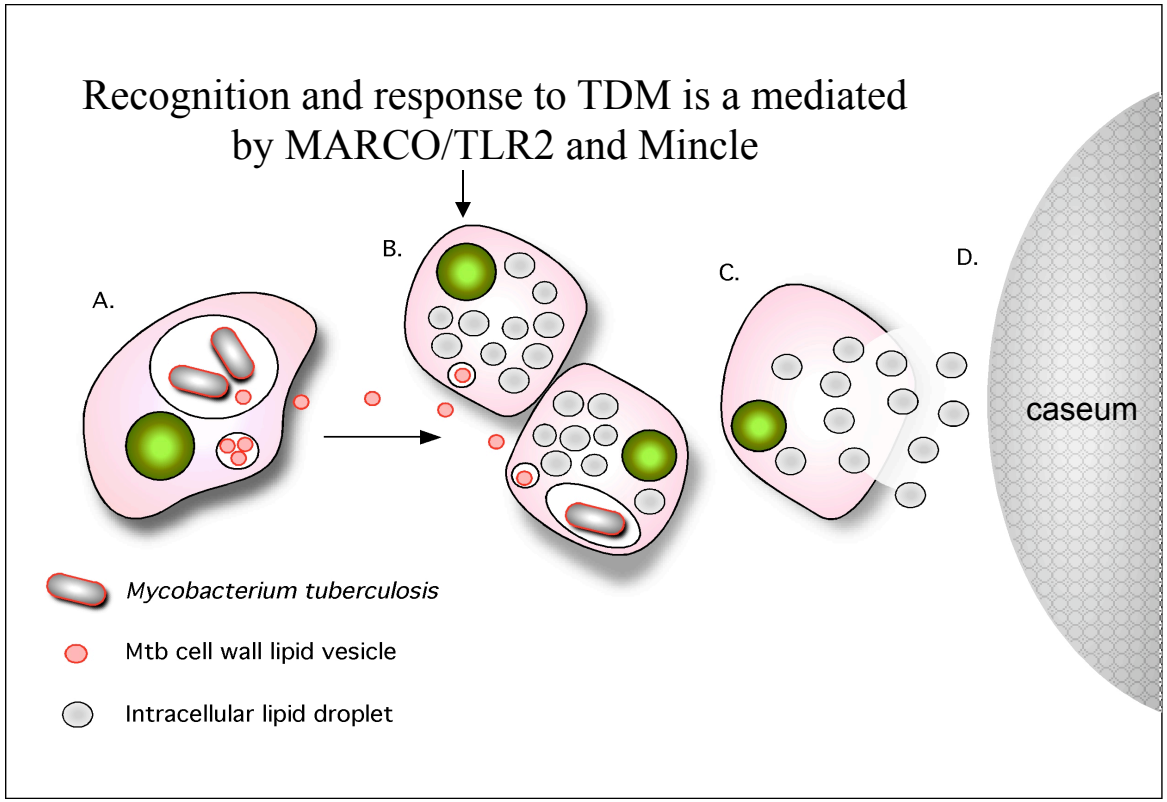
PG



6-day post-inoculation

74% of the genes up-regulated in TDM granulomas are up-regulated in human tuberculosis granulomas.

Pathogen-induced foam cell formation and granuloma progression.



A. Intracellular Mtb release cell wall components, which are exocytosed in vesicular form. B. Both infected and uninfected macrophages are exposed to cell wall mycolates and form foam cells. C. These cells dies via an inflammatory, necrotic process. D. The enclosed nature of the human granuloma leads to accumulation of necrotic debris as caseum. This process is an integral part of the pathology that leads to active disease and transmission.

Kim, M-J., Wainwright, H. Locketz, M.L., Bekker, L-G., Walther, G.B., Maske, C., Dittrich, C., Visser, A., Wang, W., Hsu, F-F., Weichart, U., Tsenova, L., Kaplan, G., and Russell, D.G. (2010) Caseation of Human Tuberculosis Granulomas Correlates with Elevated Host Lipid Metabolism. **EMBO Molecular Medicine. 2: 258-274.**

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