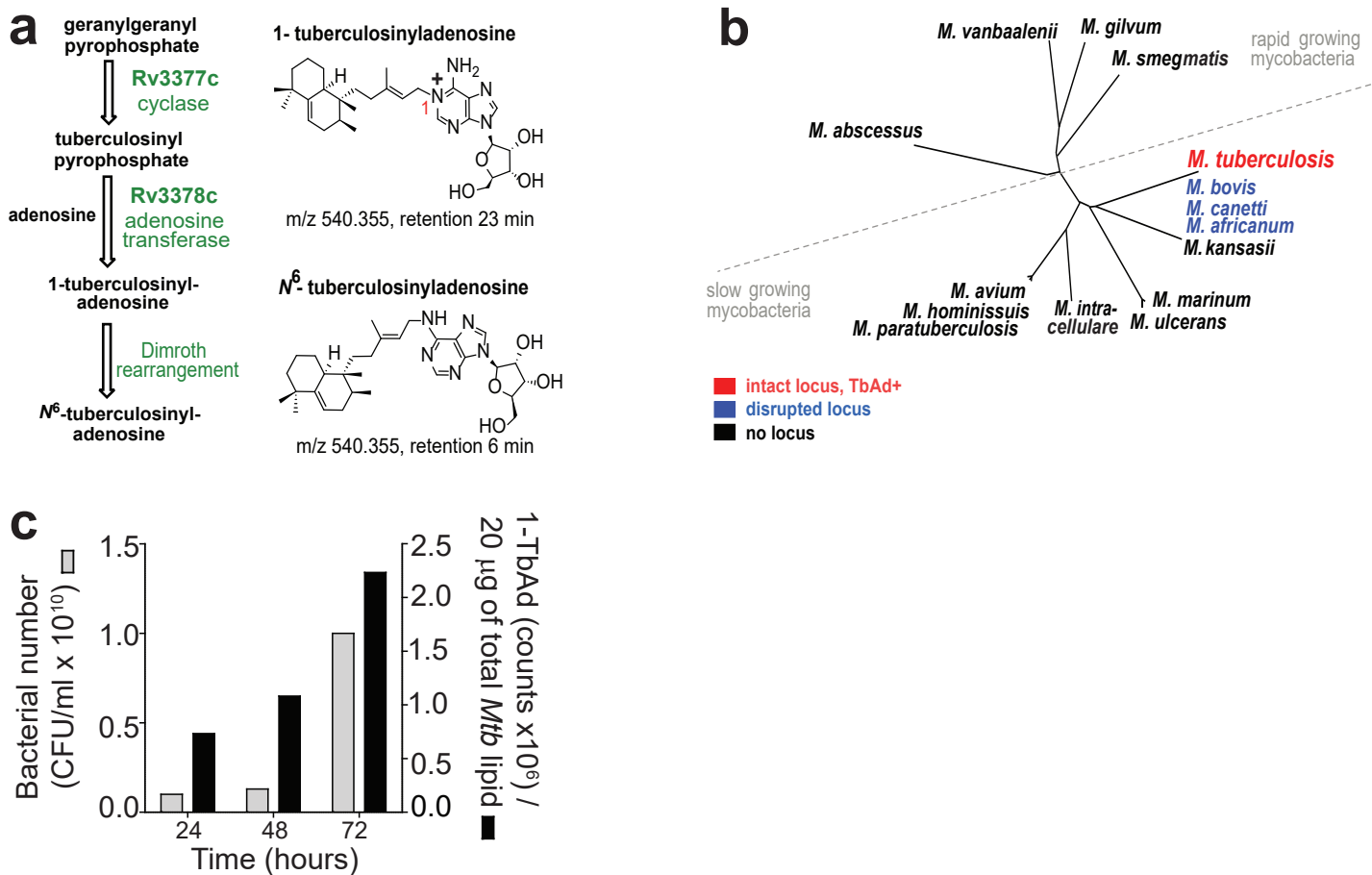


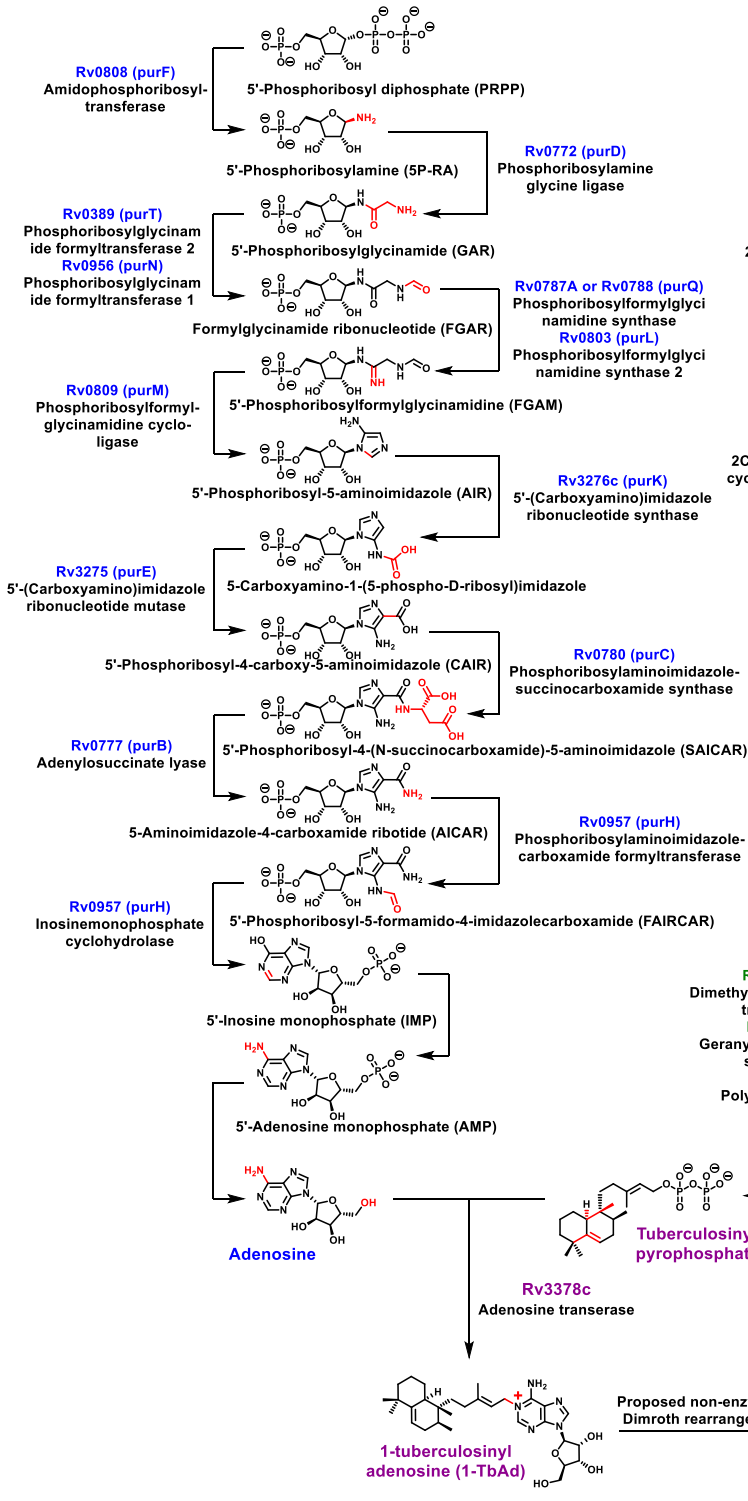
# Buter et al., Supplementary Figure 1



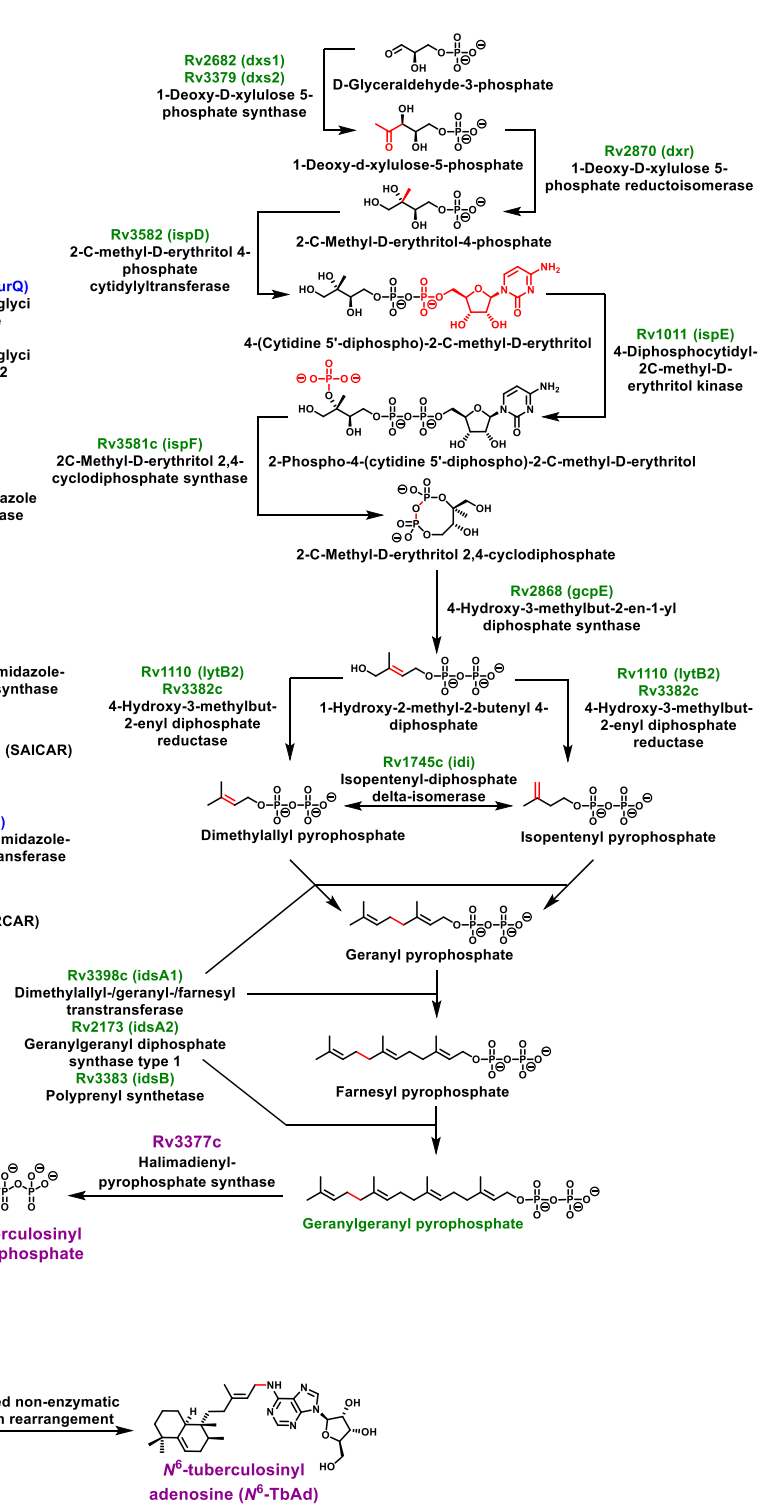
**Supplementary Figure 1. a)** Isoprenoid and adenosine biosynthesis pathways are connected by a two-step enzymatic mechanism to produce 1-TbAd, which can undergo rearrangement to N<sup>6</sup>-TbAd. Molecules are shown with their predominant charge state at neutral pH. **b)** A low stringency BLAST search failed to identify orthologs for *Rv3377c* and *Rv3378c* in any non-mycobacterial species or in the species shown in black. Species shown in blue have orthologs with known frame-shift or point mutations that disrupt this locus. For species in blue, the actual production of 1-TbAd is not known, except for *M. bovis* BCG, which does not produce the molecule. **c)** 1-TbAd intensity was measured using LC-MS with a standard curve, allowing calculation of 1-TbAd production normalized to total Mtb lipid, which was measured using a Mettler XP205 balance. Bacterial counts were determined by colony forming units on agar.

# Buter et al., Supplementary Figure 2

## Purine Bisoyntesis in *Mycobacterium tuberculosis* H37Rv

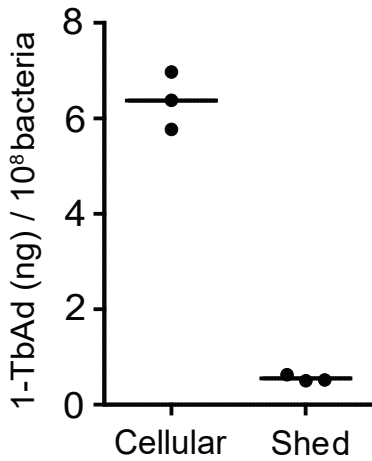


## Non-mevalonate pathway in *Mycobacterium tuberculosis* H37Rv



**Supplementary Figure 2.** Schematic of the known biosynthetic pathways of geranylgeranylpyrophosphate and adenosine, together with identification of two enzymes, *Rv3377c* and *Rv3378c*, which connect these pathways. De novo production of 1-TbAd is predicted to involve 26 steps. Molecules could also be made from recycling mechanisms that are not shown.

Buter et al., Supplementary Figure 3



Calculate 1-TbAd produced in by *Mtb* in log phase growth

**One bacterium can produce:**

cell associated:  $6 \times 10^{-8}$  ng =  $6 \times 10^{-17}$  g

released:  $5.5 \times 10^{-9}$  ng =  $5.5 \times 10^{-18}$  g

Phagosome volume is approximately  $1.2 \times 10^{-15}$  liter

Released 1-TbAd from one bacterium in one phagosome

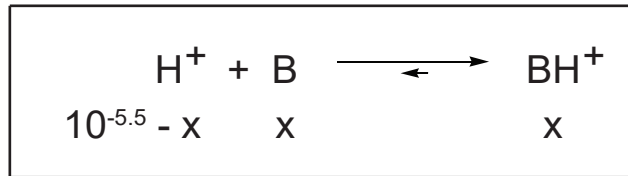
$$\frac{\frac{5.5 \times 10^{-18} \text{ g}}{540 \text{ g/mole}}}{1.2 \times 10^{-15} \text{ liter}} \times 10^6 = 8.5 \text{ } \mu\text{M}$$

**1-TbAd concentration needed for phagosome neutralization:**

pH changes from 5.5 to 6.2

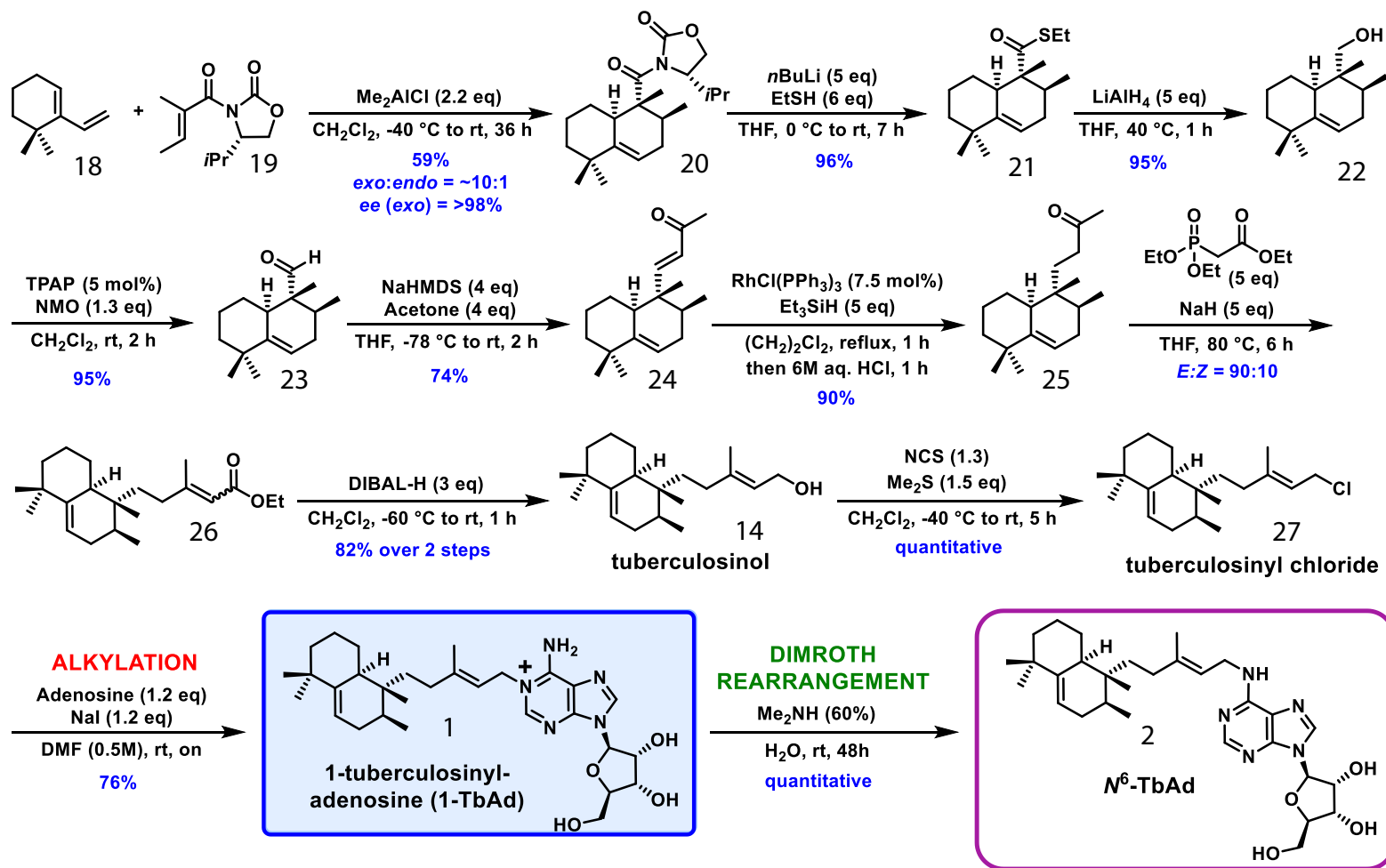
$$10^{-5.5} - x = 10^{-6.2}$$

$$x = 10^{-5.5} - 10^{-6.2} = 2.53 \times 10^{-6} \text{ M} \\ = 2.53 \text{ } \mu\text{M}$$



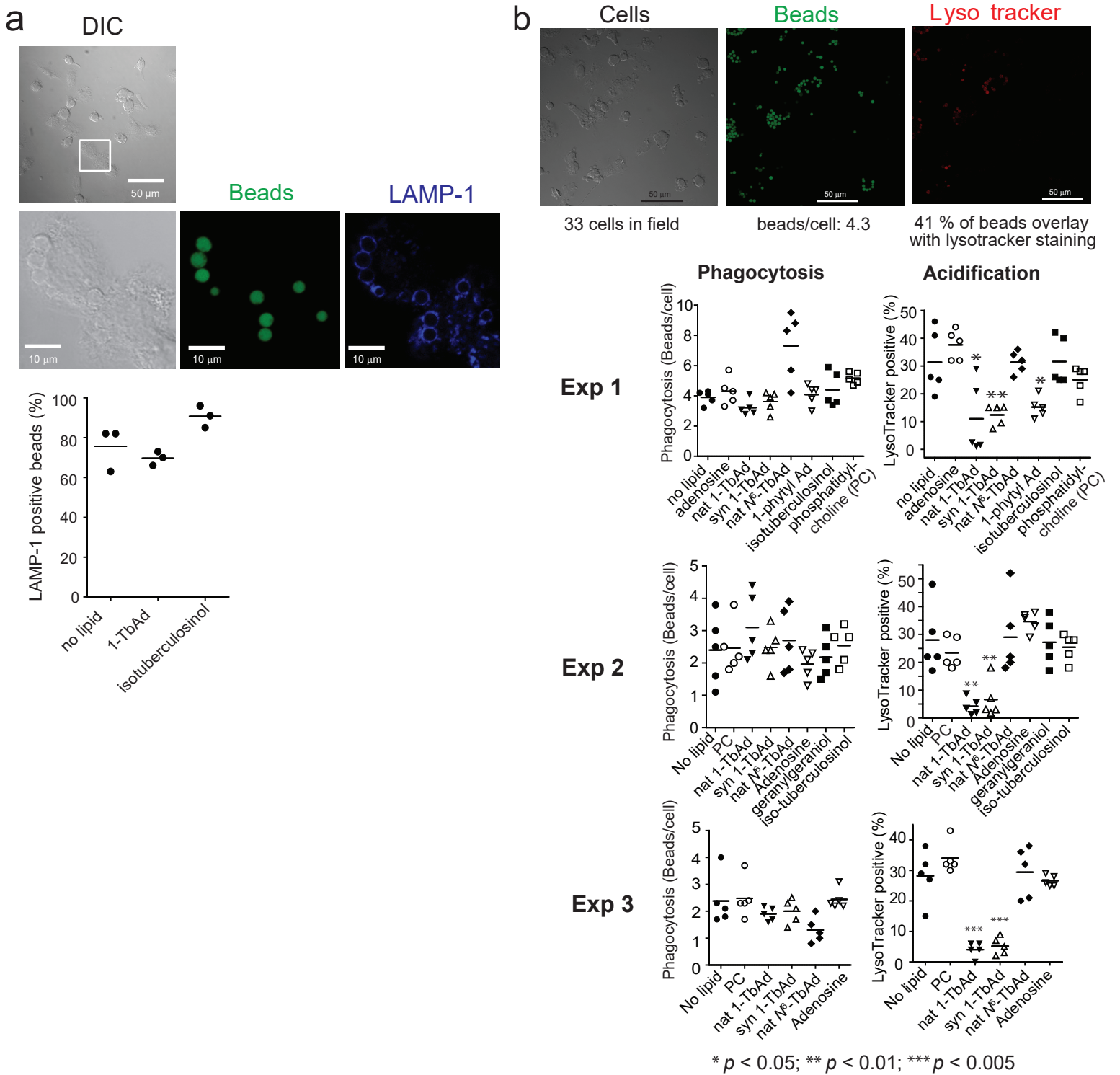
**Supplementary 3.** 1-TbAd biosynthesis on a per bacterium basis. From late log phase *Mtb* culture, we measured bacterial colony forming units on agar, cell-associated 1-TbAd in the *Mtb* pellet and shed 1-TbAd in the culture supernatant using mass spectrometry and an internal standard <sup>13</sup>C<sub>5</sub>-1-TbAd. Calculation of plausible 1-TbAd concentration in phagosomes is based on measured 1-TbAd production on a per bacterium basis, assuming one bacterium per phagosome, low buffering capacity of endosomal fluid and the reported volume of phagosomes (10<sup>-15</sup>L) (Winterbourn et.al., *J. Biol. Chem.* 2006, 281, 39860-39869).

Buter et al., Supplementary Figure 4



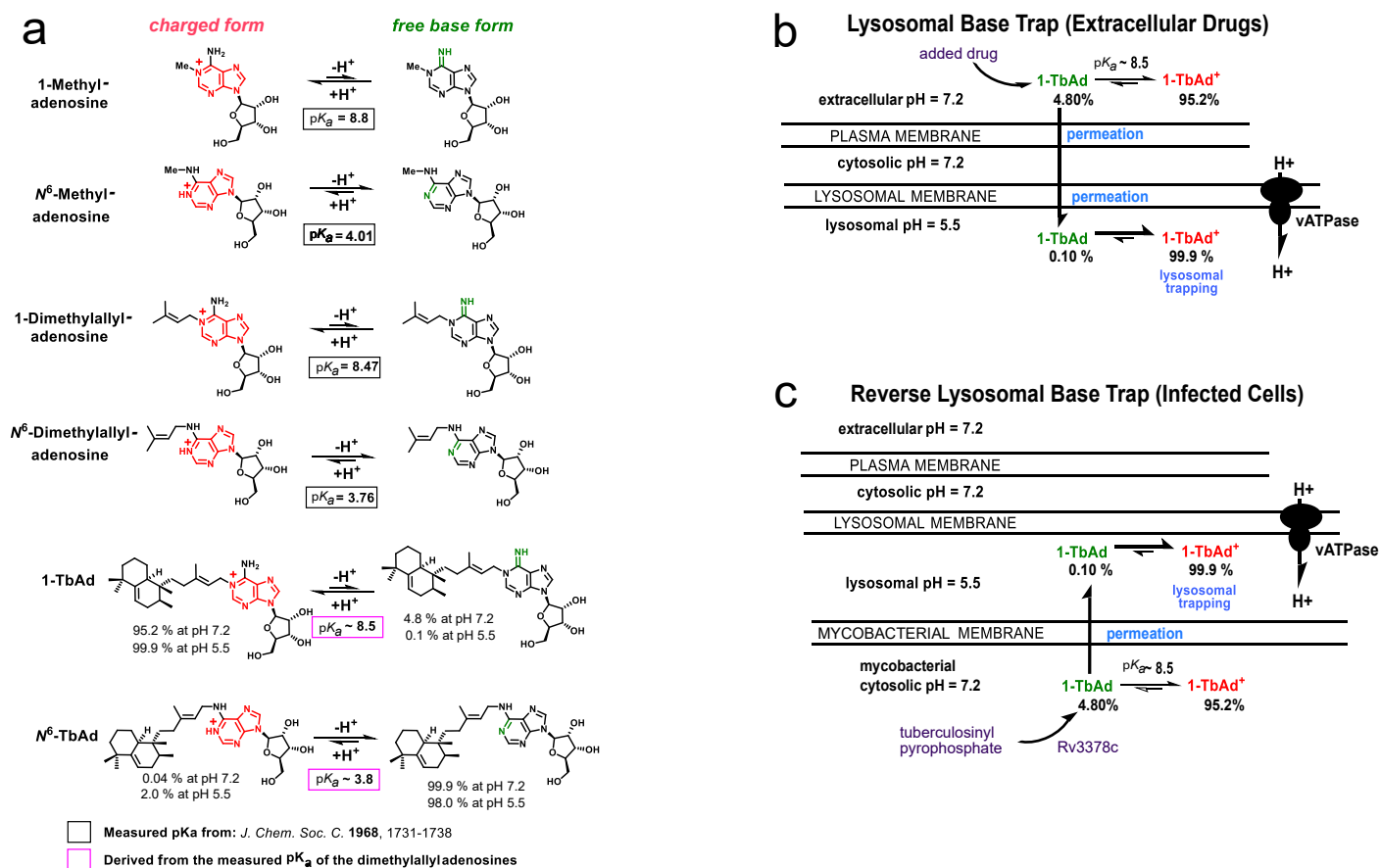
**Supplementary Figure 4.** Chemical synthesis of tuberculosinyladenosines emphasizing the alkylation reactions at the 1-position of adenosine and Dimroth rearrangement to generate *N*<sup>6</sup>-tuberculosinyladenosine. Yields are indicated, and structural analysis of intermediates is detailed in the supplemental methods.



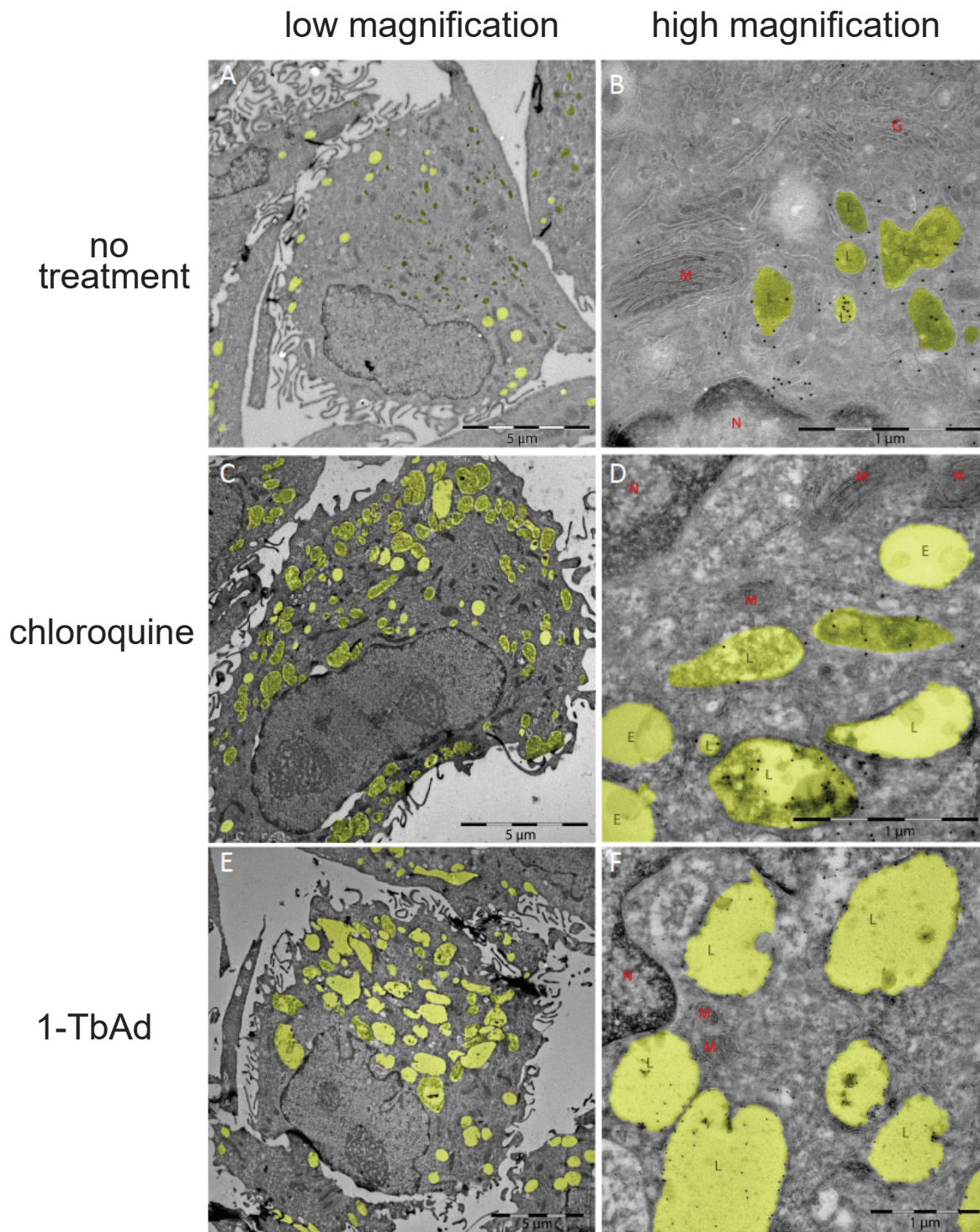


**Supplementary Figure 6. LysoTracker staining of THP-1 cells.** **a)** A pilot study using confocal microscopy of THP-1 cells pretreated with green fluorescent beads and washed three times showed bead localization within lysosomes, as defined by anti-LAMP-1. Quantitation of beads surrounded by LAMP-1 staining as a percentage of all beads seen was carried out on THP-1 cells treated with chloroquine, 1-TbAd (20  $\mu$ M) or no treatment. Three low power fields (>150 beads, > 70 cells) were counted for each condition and reported as a mean of three values with standard deviation. **b)** THP-1 cells (light scatter, left) were counted and analyzed for uptake of phagocytized green fluorescent beads into LysoTracker<sup>+</sup> compartments in the presence of TbAd analogs and controls (20  $\mu$ M, 2 hr). The counting method used is illustrated showing low power images in the upper panel in which 33 cells took up 4.3 beads/cell with 41 percent of compartments showing lysotracker staining. n=5 independent experiments, with more than 150 cells evaluated per condition. This process was repeated for three independent experiments with results shown as mean of quintuplicate values with standard deviations with p values calculated using the two-sided Student's T test.

# Buter et al., Supplementary Figure 7



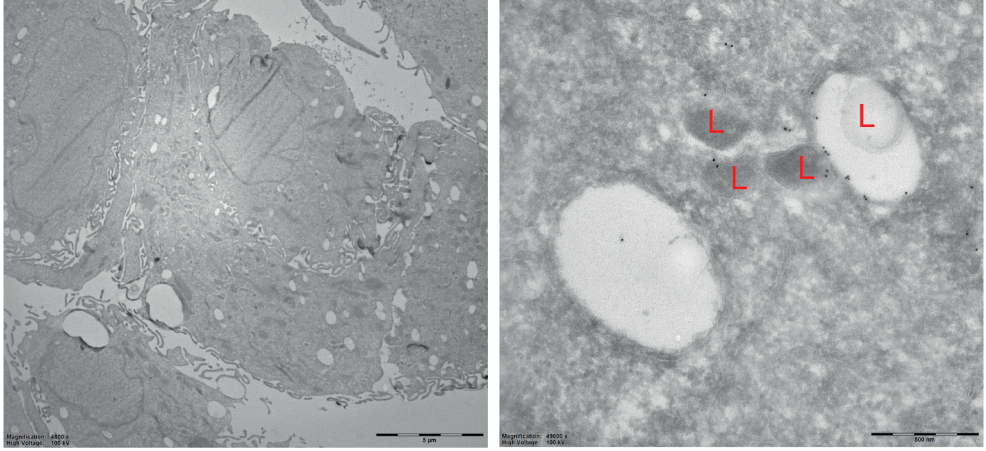
**Supplementary Figure 7. Lysosomal base trapping.** a) The pKa's of 1-linked and *N*<sup>6</sup>-linked tuberculosinyladenosines are estimated by analogy to 1- and *N*<sup>6</sup>-linked dimethylallyladenosines and methyladenosines (Martin and Reese, *J. Chem. Soc. Perkin 1*, 1968, 14, 1731-1738, Kapinos et al., *Chem. Eur. J.* 2011, 17, 8156-8164). b) The Henderson-Hasselbalch equation predicts relative pool sizes of *N*<sup>6</sup>- and 1-linked adenosines at the indicated pH values. c) De Duve outlined that compounds with a pKa ~8 are optimal for lysosomal base trapping in cells (de Duve et al., *Biochem. Pharmacol.* 1974, 23, 2495-2531). When applied as an extracellular drug (Fig. 6), 1-TbAd (pKa ~8.5) has a sufficient fraction of uncharged conjugate base to allow membrane permeation. In the lysosome, the lower pH shifts the equilibrium to the protonated form, which blocks membrane permeation and creates a concentration gradient of uncharged base across the membrane. Compounds with a pKa substantially lower than 8, such as *N*<sup>6</sup>-TbAd (pKa 3.8), can permeate, but are weak bases so fail to generate adequate concentrations of the protonated form needed for trapping in lysosomes. Compounds with a pKa substantially higher than 8 (not shown) are nearly fully protonated so cannot permeate to lysosomes. Low lysosomal pH is maintained by vATPases, which are energy requiring proton pumps. Thus, accumulation of lysosomotropic drugs in lysosomes indirectly requires cellular energy to maintain the pH gradient, but the mechanism of membrane transport does not directly require energy substrates, transmembrane protein transporters or protein pores. The compound pool sizes, trapping and permeation features of the lysosomotropic mechanism originally discovered for extracellular drugs like chloroquine also apply to 1-TbAd compounds made within bacteria. The proposed mechanism of 1-TbAd entry into lysosomes from bacterial cytosol is considered a reverse lysosomotropic trap because compound movement is topologically reversed from that shown for extracellular drugs.



**Supplementary Figure 8.** Electron microscopic analysis of human macrophages treated with no lipid, chloroquine (20  $\mu$ M), or 1-TbAd (20  $\mu$ M) are shown with yellow pseudocoloring of lysosomes. Lysosomes (L) are identified as electron lucent compartments staining with immuno-gold labelled with anti-CD63 gold (10 nm), whereas endosomes lack CD63 staining. Nuclei (N), mitochondria (M), and Golgi stacks (G) are recognized based on morphology. As compared to untreated cells, representative images of lysosomes in cells treated with chloroquine or 1-TbAd show increase in the number of yellow compartments, partial or complete loss of the multi-lamellar pattern as well as increases in lysosomal size with numerous granular intralysosomal inclusions. When lysosomal swelling involves > one-third of the cytosolic area, as judged by two blinded microscopists, the macrophage is designated as swollen, and results are representative of three experiments.

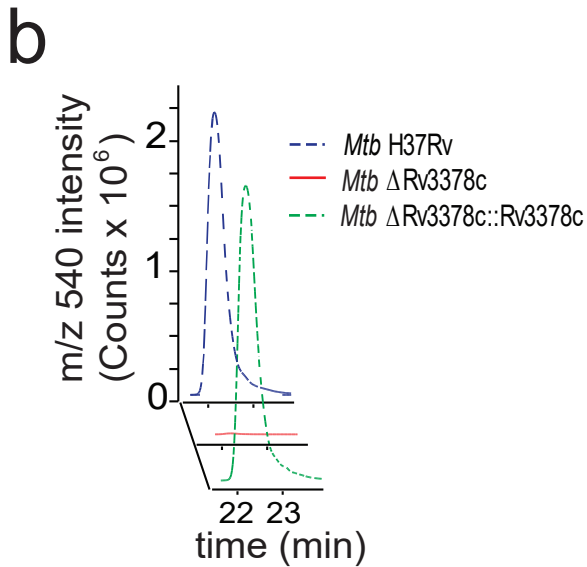
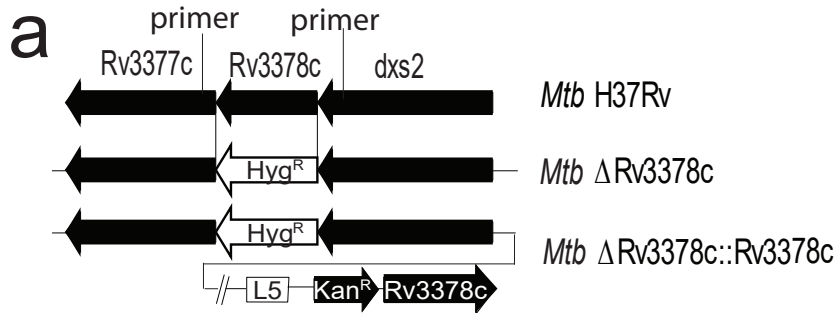


## Buter et al., Supplementary Figure 9



**Supplementary Figure 9.** Human macrophages were treated with tuberculosinol and analyzed as in supplementary figure 8. Unlike chloroquine and 1-TbAd treated macrophages, which had large numbers of distended, CD63+ lysosomes, tuberculosinol-treated cells show predominantly multi-lamellar, electron dense CD63+ compartments. The experiments were performed three times with similar outcome.

Buter et al., Supplementary Figure 10



**Supplementary Figure 10. a)** Map of the TbAd biosynthetic locus illustrates the strategy for replacement of gene *Rv3378c* by homologous recombination with a Hygromycin cassette and complementation with mycobacterial optimized promoter carrying a kanamycin resistance gene. Knockout was confirmed by PCR analysis using primers for the indicated sites, which yield products from the mutant (2.16kb) that matched the expected size. **b)** Knockout and complementation strains were assessed by HPLC-MS analysis in the positive mode tracking ions matching the mass ( $m/z$  540.35) and retention time of 1-TbAd (23 minutes). Complemented clones were tested for TbAd production more than 4 times, with the value for the high expressing clone used in the biological experiment shown here.