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Consequence of enhanced LC3-trafficking for a live, attenuated *M. tuberculosis* vaccine

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Introduction¹

Mycobacterium tuberculosis kills more people than any other infectious agent.

Mycobacterium bovis-bacillus Calmette-Guérin (BCG) is a century-old vaccine that fails to protect adults from pulmonary disease, the most common and communicable manifestation of tuberculosis (TB). Since BCG was derived from *M. bovis* through a series of deletions that removed more than one hundred genes, one thought is that BCG would be more effective if it contained these missing epitopes. Thus, one approach has been to attenuate *M. tuberculosis* by introducing auxotrophic mutations. These auxotrophic strains have generally proven safer than BCG, and equivalent, but not better, in protective efficacy. There are ongoing efforts to improve on BCG and the auxotrophic strains, principally by altering how they impact host pathways, including phagosome maturation, apoptosis, and autophagy [1, 2]. By disrupting immune evasion strategies of *M. tuberculosis* that are also present in the vaccine strains, it might be possible to improve their efficacy.

One approach that has been suggested to improve upon existing *M. tuberculosis* vaccines is to enhance the degree to which they activate macroautophagy (hereafter autophagy).

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Conflict of interest statement

The authors declare no conflicting interests.

Authorship

SK and JAP designed the experiments, analyzed the data, performed the statistical analysis, and wrote the paper. TK and CPC designed, performed, and analyzed the antigen presentation experiments. KP and CS provided material.

¹Abbreviations: LAP- LC3-associated phagocytosis

Autophagy involves the capture of cytoplasmic components by a double-membrane, LC3-decorated compartment called the autophagosome. When microorganisms are sequestered by an autophagosome, it is referred to as xenophagy. Autophagy can promote antigen presentation for MHC Class I and II presentation [3] and is critical for the efficacy of the successful yellow fever vaccine [4]. As part of its virulence strategy, *M. tuberculosis* inhibits autophagy, so only a portion of the invading bacilli are cleared through xenophagy. The xenophagy that does occur depends on the bacterial ESX-1 Type VII secretion system damaging the phagosomal membrane [5]. In BCG, *ESX-1* is disrupted, so the vaccine strain does not efficiently damage the phagosome, and, hence, it induces even less xenophagy than *M. tuberculosis* [6]. Thus one idea to improve upon *M. tuberculosis* vaccines is to enhance their ability to activate xenophagy. In support of this idea, when rapamycin is used to chemically activate autophagy at the time of BCG administration, BCG vaccine efficacy is enhanced [7]. In addition, a BCG strain engineered to damage the phagosome shows enhanced protection [8].

LC3-associated phagocytosis (LAP) is related to, but distinct from, xenophagy [9, 10]. In both xenophagy and LAP, LC3-decorated organelles fuse with lysosomes, resulting in cargo degradation. LAP relies upon many of the same autophagy-related (ATG) proteins that are required for autophagy, but the two processes also have unique requirements. LAP might also promote presentation of extracellular antigen to MHC Class II as well as cross presentation for MHC Class I presentation [3, 11], although its role in *M. tuberculosis* vaccine efficacy is unclear. We recently showed that the *M. tuberculosis* LytR-CpsA-Psr family protein, CpsA, inhibits LAP [12]. An *M. tuberculosis* strain lacking *cpsA* (*cpsA*) exhibits dramatically enhanced co-localization with LC3, and it is cleared in macrophages and mice by the LAP pathway [12]. Identification of CpsA as a LAP inhibitor provided a way for us to test whether enhancing LAP could improve TB vaccine efficacy.

Material and methods

Media and strains

M. tuberculosis strains H37Rv, *cpsA*, mc²6206 (*leuCD panCD*), mc²6230 (*RD1 panCD*), mc²6206 *cpsA* (*leuCD panCD cpsA*), mc²6230 *cpsA* (*RD1 panCD cpsA*), and *M. bovis* BCG Danish were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 (Sigma), BBL Middlebrook OADC Enrichment, and 0.2% glycerol (Sigma). Media was supplemented with: pantothenic acid (24 µg/ml) for strains lacking *panCD*, leucine (50 µg/ml) for strains lacking *leuCD*, and hygromycin (50 µg/ml) for *cpsA* strains. mc²6230 is genotypically identical to the published strain mc²6030 except that it does not contain a hygromycin resistance marker [13]. Mc²6230 has the same *panCD* deletion as mc²6020 [14] and is deleted of *leuC* and *leuD*.

Construction of *cpsA* deletion strains

cpsA was deleted from mc²6206 and mc²6230 using the *Che9c*-phage mediated recombination, replacing the *cpsA* locus by a hygromycin resistance (*hyg^R*) marker as described [12]. The deletions were verified by PCR of genomic DNA and Western blotting using a CpsA antibody described elsewhere [12].

Macrophage infections

Stem cells isolated from tibia and femurs of 6–12 weeks old C57BL/6 mice and those expressing LC3-GFP were differentiated into macrophages as previously described [12]. For microscopy, 10^5 LC3-GFP BMDMs were seeded in 8 wells chamber slides and infected the following day with a MOI of 5 as previously described [12]. Four hours post infection (**hpi**) macrophages were washed and fixed with 1% paraformaldehyde/PBS overnight. Ubiquitinated proteins were stained using the FK2 antibody (Millipore, #04-263) and Alexa Fluor 647 anti-mouse secondary antibody. *M. tuberculosis* strains were visualized based upon their autofluorescence using a Nikon DAPI filter cube. Images were acquired and analyzed as described previously [12]. Contrast was not altered prior to automated image analysis; for reproduced images, alterations were applied equally to all samples. To measure intracellular bacterial survival, BMDMs from C57BL/6 mice were infected at an MOI of 5, and intracellular bacteria were enumerated by serial dilution on 7H11 agar plates as previously described [12].

Vaccination-challenge studies

Groups of six- to eight-week-old C57BL/6 mice (8–10 per group) purchased from The Jackson Laboratories were vaccinated subcutaneously with saline or 10^6 CFU of *M. bovis* BCG Danish, mc²6206, or mc²6206 *cpsA*. Two months later, mice were challenged by aerosol with 400 CFUs of *M. tuberculosis* H37Rv using the inhalation exposure system from Glas-Col as previously described [12]. The challenge dose was confirmed from lung homogenates from 3 mice sacrificed 24 hours after infection. Twenty-eight and sixty days post challenge, mice were euthanized and the right lung and the spleen were homogenized in PBS containing 0.5% Tween 80 through a 70 µm cell strainer (BD Falcon). Bacterial growth was assessed by plating serial dilutions of lung and spleen homogenates on 7H11 Middlebrook agar. The New York University School of Medicine Institutional Animal Care and Use Committee approved all work with mice.

Antigen presentation assays

The antigen presentation assay was performed as previously described [15]. Briefly, bone marrow-derived dendritic cells (BMDCs) were isolated from C57BL/6 mice and infected with a single cell suspension of the indicated mycobacterial strains at an MOI of 3. After 4 h, extracellular bacteria were removed by washing 3 times with PBS. Infected BMDCs were incubated at 37°C for an additional 20 h, after which T-helper 1 (T_H1) polarized CD4⁺ effector cells specific for peptide 25 of *M. tuberculosis* Ag85B protein were added. 24h after co-culture of infected BMDCs with CD4⁺ T cells, culture supernatants were collected, filtered, and assayed for IFN- γ by ELISA (BD Biosciences).

Results and Discussion

CpsA inhibits LC3-trafficking in mc²6206

To test whether increased LC3-trafficking enhances vaccine efficacy, we deleted *cpsA* from the *leuCD panCD* (mc²6206) vaccine strain (Fig. 1a), generating *cpsA leuCD panCD* (mc²6206 *cpsA*). We used mc²6206 rather than BCG as the parent strain, reasoning that to

maximally augment LC3-trafficking, we should use a strain that has *ESX-1*, which is missing in BCG and required for xenophagy. We verified deletion of *cpsA* in mc²6206 by PCR analysis and the absence of detectable CpsA protein by Western blotting (Fig. 1b, c). Using fluorescence microscopy, we compared LC3-trafficking of mc²6206 *cpsA* to the parent strain by infecting BMDMs expressing GFP-LC3. We quantified the mean fluorescence intensity (**MFI**) of LC-GFP associated with the bacilli using automated image analysis, as previously described [16]. As with virulent *M. tuberculosis*, the auxotrophic parent strain failed to efficiently recruit LC3 to the phagosome, whereas as the *cpsA* mutant strain exhibited dramatically enhanced co-localization with LC3-GFP (Fig. 2a, b) [12]. This demonstrated that the immune evasion mechanism that enables virulent *M. tuberculosis* to inhibit LC3-trafficking also functions in the auxotrophic vaccine strain. The enhanced LC3-trafficking was similar to what we observed when macrophages were treated with IFN- γ prior to infection, which induces autophagy [17]. Along with increased co-localization with LC3, mc²6206 *cpsA* also exhibited increased association with ubiquitinated proteins (Fig. 2a, c). For both LC3 and FK2 there was no further increase associated with the *cpsA* mutation in IFN- γ -treated macrophages, concordant with our findings in virulent *M. tuberculosis* [12]. Whereas deletion of *cpsA* in the H37Rv background attenuated growth in macrophages [12], in the already attenuated auxotrophic strain, there was no further decrease in intracellular survival associated with the *cpsA* deletion (Fig. 2d).

To determine whether the enhanced LC3-trafficking depends upon *ESX-1*, we deleted *cpsA* in mc²6230 (*RD1 panCD*), which carries a deletion similar to the one disrupting *ESX-1* in BCG. Upon visual inspection, there was not a dramatic difference in LC3-colocalization in mc²6230 *cpsA* as compared to the parent, although there was a significant increase based upon automated quantification (Fig. 2e). Notably, there was dramatically less LC3-association with the strain lacking *ESX-1* (mc²6230 *cpsA*) as compared to the strain containing *ESX-1* (mc²6206 *cpsA*), demonstrating that maximal LC3-trafficking is achieved in strains lacking *cpsA* when *ESX-1* is functional in these auxotrophic strains (Fig. 2d). Combined with our previous findings [12], this suggests that in the context of *M. tuberculosis* infection, LAP is partially dependent upon *ESX-1*. Since LAP can be initiated by inert particles such as fungal zymosan, it must not be strictly dependent upon phagosomal damage, so some other aspect of ESX-1 function may potentiate LAP during *M. tuberculosis* infection. Alternatively, CpsA may antagonize both LAP and xenophagy in mc²6206.

Contribution of CpsA to vaccine efficacy

We compared the ability of mc²6206, mc²6206 *cpsA*, and BCG to protect mice from subsequent challenge with virulent *M. tuberculosis* as compared to unvaccinated controls. C57BL/6 mice (8–10 per group) were vaccinated subcutaneously with saline or with 10⁶ CFU of *M. bovis* BCG Danish, *M. tuberculosis* mc²6206, or mc²6206 *cpsA*. Two months later, the mice were challenged with ~400 CFUs of *M. tuberculosis* H37Rv by aerosol (Fig. 3a). Compared to unvaccinated controls, mice vaccinated with mc²6206 showed the expected reduction in *M. tuberculosis* load after challenge in the lungs at 28 and 60 days post-challenge (Fig. 3b, c), similar to what was seen with BCG. The *cpsA* deletion did not confer any increased protection. Plating of lung homogenates on media containing

hygromycin did not result in any bacterial growth, demonstrating that mc²6206 *cpsA* did not disseminate to the lung. In the spleens, immunization with the mc²6206 parent strain failed to provide any protection. In contrast, the mc²6206 *cpsA* strain conferred persistent protection, as did BCG. Thus, deleting *cpsA* did not improve protection in the lung, but modestly limited dissemination to the spleen, resulting in similar overall protection as BCG. These data demonstrate that enhanced LC3-trafficking, at least within the context of the auxotrophic strain, is not a route to a significantly better vaccine.

To further investigate why deleting *cpsA* did not improve protection, we examined the ability of bone marrow derived dendritic cells (BMDCs) infected with mc²6206 *cpsA* to activate CD4⁺ T cells *in vitro*, and we found no difference compared to mc²6206 infection (Fig. 3d). Thus, within the context of this auxotrophic strain, enhanced LC3 trafficking did not appear to increase MHC Class II presentation or CD4⁺ T cell activation. One possibility is that the extreme attenuation of this strain limits its ability to be improved upon. To see whether CpsA influenced antigen presentation in the context of fully virulent *M. tuberculosis*, we compared dendritic cells infected with the *cpsA* mutant to those infected with wild type bacilli in the H37Rv background. Despite a dramatic effect of CpsA on LC3-trafficking in the H37Rv background [12], there was no difference in the ability of these strains to promote CD4⁺ T cell activation (Fig. 3e). Thus, enhancing LAP does not appear to promote the ability of *M. tuberculosis*-infected antigen presenting cells to activate CD4⁺ T cells. Interestingly, deletion of *nuoG*, which encodes a subunit of the NADH dehydrogenase 1, dramatically increases LC3-colocalization [18], similar to what we observe with the *cpsA* deletion. NuoG, like CpsA, antagonizes NOX2-dependent reactive oxygen species, suggesting that they both inhibit a related LAP pathway [12, 18, 19]. When *nuoG* was deleted from a BCG vaccine strain that was also engineered to damage the phagosome, the *nuoG* mutant provided better protection and enhanced immune responses upon *M. tuberculosis* challenge compared to the parent [18]. In further optimizing live, attenuated vaccines of *M. tuberculosis*, it will be important to determine whether the difference observed between *nuoG* and *cpsA* are due to their different strain background or the functions of *NuoG* and *CpsA* with respect to antigen presentation or other immune parameters.

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Highlights

- Auxotrophic *M. tuberculosis* vaccine, mc²6206, inhibits LC3-trafficking
- CpsA, a LytR-CpsA-Psr protein, inhibits LC3-trafficking in mc²6206
- ESX-1 is required for LC3-trafficking in the vaccine strain
- Enhanced LC3-trafficking enhances protection from disseminated infection

Fig. 1. Deletion of *cpsA* from mc²6206

(a) Genomic organization of the *cpsA* (Rv3484) region. *cpsA* was replaced by a hygromycin resistance gene. The green (5' junction), blue (3' junction) and purple (*cpsA* internal) arrows indicate the positions and directions of oligonucleotide binding sites used for confirmation of the *cpsA* deletion.

(b) PCR from genomic DNA confirmed the deletion of *cpsA* from mc²6206 (mc²6206-*cpsA*). * indicates a nonspecific product. For the parent strain (mc²6206+*cpsA*) a product was only seen in the control reaction.

(c) Western blotting of cellular lysate from mc²6206 mc²6206-*cpsA* confirmed the absence of CpsA. * indicates a nonspecific band.

Fig. 2. Deletion of *cpsA* from mc²6206 enhances LC3-associated trafficking

(a) Immunofluorescence imaging of naïve (non-IFN- γ treated) BMDMs infected with mc²6206 and mc²6206 Δ cpsA for 4 hours. LC3-GFP is in green, ubiquitinated proteins are red, and autofluorescent *M. tuberculosis* is blue. The co-localization of LC3-GFP (b) or ubiquitin (c) with bacilli was quantified using automated image analysis in naïve macrophages and those that were pre-treated with 200 units of IFN- γ as indicated. (d) Intracellular bacterial survival was evaluated in BMDMs over three days from five replicates. (e) The co-localization of LC3-GFP was quantified 4 hpi in the indicated strains. (b–e) Data show mean + s.e.m. from one representative experiment from at least two

independent experiments. (b, c, e) At least 100 bacilli were analyzed per sample. Data were analyzed using one-way ANOVA with Tukey's multiple comparison test, ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns- not significant.

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Fig. 3. Vaccination of mice with mc²6206, mc²6206 *cpsA*, and BCG Danish result in similar protective efficacy against *M. tuberculosis* challenge

(a) Scheme of vaccination experiment. Mice were vaccinated subcutaneously with 10⁶ CFUs of mc²6206, mc²6206 *cpsA*, BCG Danish, or PBS control. After 8 weeks, mice were challenged by aerosol with 400 CFU *M. tuberculosis* (H37Rv). After 28 and 60 days, bacterial survival was determined in (b) lung and (c) spleen homogenates. (d–e) The ability of infected BMDCs to activate CD4⁺ T cells was assessed as previously described [15]. BMDCs were infected with (d) mc²6206 and mc²6206 *cpsA* or (e) wild type and *cpsA* in the H37Rv strain background for 24 h prior to co-culture with T-helper 1 (T_H1) polarized CD4⁺ effector cells specific for peptide 25 of *M. tuberculosis* Ag85B protein. (b–c) Data

show mean $\pm$ s.e.m. and were analyzed using one-way ANOVA with Krushal-Wallis test or (d–e) Student's *t*-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns- not significant.