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Survival of Mycobacteria in Macrophages Is Mediated by Coronin 1-Dependent Activation of Calcineurin

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SUMMARY

Pathogenic mycobacteria survive within macrophages by avoiding lysosomal delivery, instead residing in mycobacterial phagosomes. Upon infection, the leukocyte-specific protein coronin 1 is actively recruited to mycobacterial phagosomes, where it blocks lysosomal delivery by an unknown mechanism. Analysis of macrophages from coronin 1-deficient mice showed that coronin 1 is dispensable for F-actin-dependent processes such as phagocytosis, motility, and membrane ruffling. However, upon mycobacterial infection, coronin 1 was required for activation of the Ca^{2+}-dependent phosphatase calcineurin, thereby blocking lysosomal delivery of mycobacteria. In the absence of coronin 1, calcineurin activity did not occur, resulting in lysosomal delivery and killing of mycobacteria. Furthermore, blocking calcineurin activation with cyclosporin A or FK506 led to lysosomal delivery and intracellular mycobacterial killing. These results demonstrate a role for coronin 1 in activating Ca^{2+} dependent signaling processes in macrophages and reveal a function for calcineurin in the regulation of phagosome-lysosome fusion upon mycobacterial infection.

INTRODUCTION

Mycobacterium spp. include highly successful pathogens that evade innate immunity by manipulating the host to ensure long-term survival. One of the strategies employed by pathogenic mycobacteria relies on their capacity to block phagosomal maturation; while, normally, all microbes that are internalized by macrophages are transferred to lysosomal organelles followed by their degradation, mycobacteria have evolved strategies to block delivery to lysosomes (Armstrong and Hart, 1971; Russell, 2001), thereby ensuring their survival within phagosomes.

The capacity of mycobacteria to block phagosome-lysosome fusion relies on the expression of several virulence factors that ensure their retention within mycobacterial phagosomes (Vergne et al., 2005; Walburger et al., 2004). At the host side, a number of factors have been characterized that modulate the outcome of the entry of mycobacteria into macrophages. For example, the small GTPase LRG-47 appears to be involved in the regulation of phagosome maturation (MacMicking, 2005; Singh et al., 2006). Furthermore, the plasma-membrane component cholesterol is involved in allowing mycobacteria to enter macrophages, possibly by modulating signal transduction following engagement of surface receptors, or via rearrangement of the membrane-cytoskeleton network during phagocytosis (Gatfield and Pieters, 2000; Peyron et al., 2000). Finally, mycobacteria actively recruit coronin 1 (also known as P57 or TACO, for tryptophan aspartate containing coat protein), a host cytoskeleton-associated molecule that links the cytoskeleton with the plasma membrane in leukocytes (Gatfield et al., 2005). Coronin 1 transiently accumulates on phagosomal membranes (Ferrari et al., 1999; Itoh et al., 2002) and is actively retained by living mycobacteria residing inside phagosomes, suggesting that the retention of coronin 1 on mycobacterial phagosomes is responsible for blocking phagosome-lysosome fusion (Ferrari et al., 1999; Nguyen and Pieters, 2005).

Coronin 1 shows ~30% homology with *Dictyostelium discoideum* coronin, where it localizes with F-actin filaments at crown-shaped phagocytic cups and macropinosomes (de Hostos et al., 1991, 1993; Fukui et al., 1999; Maniak et al., 1995). A *Dictyostelium* coronin-null mutant is affected in cell locomotion, phagocytosis, macropinocytosis, and cytokinesis, suggesting that in *Dictyostelium* coronin is functionally involved in F-actin-based motility-related processes (de Hostos et al., 1993). In the yeast

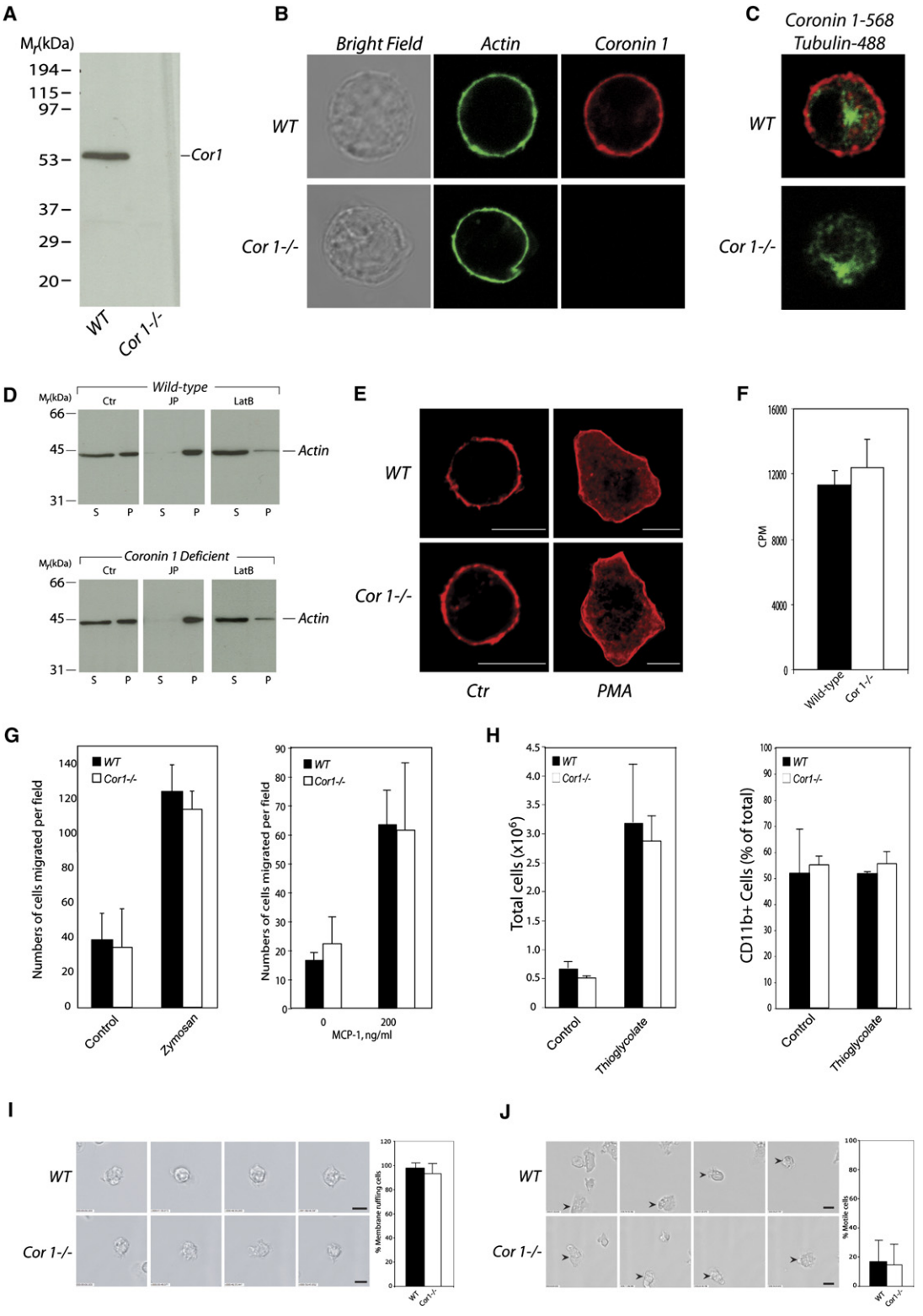


Figure 1. Phenotype of Macrophages in the Presence and Absence of Coronin 1

(A) Lysates from WT or coronin 1 deficient (Cor $1^{-/-}$) macrophages were analyzed for coronin 1 by immunoblotting.

(B) WT or coronin 1-deficient macrophages were stained for actin and coronin 1.

(C) WT or coronin 1-deficient macrophages were methanol fixed and stained for coronin 1 and tubulin.

S. cerevisiae, the single coronin isoform Crn1p also localizes to cortical F-actin patches and in yeast as well as mammalian T cells coronin 1 has been implicated in regulating Arp2/3 complex activity (Foger et al., 2006; Heil-Chapdelaine et al., 1998; Humphries et al., 2002; Rodal et al., 2004). However, in yeast, upon deletion of the *crn* gene, actin dynamics as well as F-actin-dependent processes remain unaltered (Heil-Chapdelaine et al., 1998).

In mammalian macrophages no biological activity has been assigned to any of the coronin isoforms expressed. To analyze a role for coronin 1 in vivo, we generated mice containing a targeted deletion of the *coronin 1* gene. In this work, we show that in macrophages, coronin 1 was dispensable for F-actin-dependent processes such as cell motility, phagocytosis, and macropinocytosis. However, upon mycobacterial infection of wild-type macrophages, coronin 1 was essential for the activation of the Ca^{2+}-dependent phosphatase calcineurin, thereby blocking lysosomal delivery of mycobacteria. In the absence of coronin 1, calcineurin was not activated, resulting in lysosomal transfer and death of internalized mycobacteria. Coronin 1 is therefore an essential survival factor for mycobacteria within macrophages through activation of calcineurin.

RESULTS

Macrophage F-Actin Cytoskeleton in the Absence of Coronin 1

To analyze a function for coronin 1 in macrophages, coronin 1 expression was ablated using gene targeting in mice (Figure S1). The resulting coronin 1-deficient mice were fertile, reproduced normally, and appeared healthy. Bone marrow-derived macrophages isolated from coronin 1-deficient mice showed the absence of coronin 1 by immunoblotting as well as immunostaining on fixed cells, whereas the F-actin and tubulin cytoskeleton was not affected (Figures 1A–1C). Since in *Dictyostelium* coronin is an F-actin-interacting protein (de Hostos et al., 1991), we analyzed whether coronin 1 is involved in the redistribution of F- versus G-actin using a biochemical assay to analyze in situ F-actin levels. To that end, macrophages from either wild-type or coronin 1-deficient animals were lysed under conditions that preserve F-actin, and the F-actin cytoskeleton was isolated as described in Experimental Procedures. As a control, macrophages were treated either with the F-actin-stabilizing compound Jasplakinolide (Bubb et al., 1994) or the F-actin-depolymerizing reagent Latrunculin B (Spector et al., 1989) prior to cell lysis. Whereas Jasplakinolide and Latrunculin B resulted in significant changes in the ratio of G- versus F-actin, the presence or absence of coronin 1 did not alter the ratio of F- versus G-actin (Figure 1D). To analyze the capacity of macrophages to spread on a surface upon activation, macrophages were incubated with the phorbol ester PMA for 30 min, fixed, and stained for actin. Both wild-type as well as coronin 1-deficient macrophages showed similar spreading and reorganization of the actin cytoskeleton (Figure 1E). Also, in vitro proliferation of macrophages was unaltered in the absence of coronin 1 (Figure 1F).

To analyze whether in macrophages the absence of coronin 1 affects migration, as is the case in *Dictyostelium* (de Hostos et al., 1993), macrophages from wild-type or coronin 1-deficient macrophages were analyzed by Transwell assay. Both upon stimulation of the cells by zymosan, as well as by the macrophage-specific migration factor monocyte chemoattractant protein-1 (MCP-1), similar numbers of macrophages migrated into the lower chamber (Figure 1G). To analyze migration of wild-type and coronin 1-deficient macrophages in vivo, recruitment of macrophages to the peritoneum upon stimulation was analyzed. Both wild-type as well as coronin 1-deficient macrophages were recruited in similar numbers to the peritoneal cavity (Figure 1H). Finally, macrophages derived from wild-type or coronin 1-deficient mice were analyzed by video microscopy to asses membrane dynamics and random cell movements in the presence and absence of coronin 1. As shown in Figures 1I and 1J and the accompanying videos, membrane dynamics and ruffling as well as cell motility was similar in wild-type and coronin 1-deficient macrophages. Together, these results suggest that coronin 1 is dispensable for these F-actin-mediated processes in macrophages.

(D) WT and coronin 1-deficient (Cor $1^{-/-}$) macrophages were left untreated or treated with the indicated compounds, lysed, and separated into membrane and cytosolic fractions and analyzed for the distribution of F-actin (in the pelletable fraction, *'P'*) and G-actin (in the supernatant fraction *'S'*) as described in the Experimental Procedures.

(E) WT or coronin 1-deficient (Cor $1^{-/-}$) macrophages were allowed to adhere on glass slides and activated with 10 nM PMA for 30 min followed by fixation and staining with anti-actin antibodies. Bar: 10 μM.

(F) WT or coronin 1-deficient macrophages were analyzed for the capacity to proliferate by incorporation of tritiated thymidine. Shown are mean values ± standard deviation (SD) from triplicate samples.

(G) Macrophages from WT or coronin 1-deficient (Cor $1^{-/-}$) mice were laid on the top of the migration chamber and allowed to migrate toward the bottom half containing human serum activated zymosan (left) or MCP-1 (right), and migration was determined as described in the Experimental Procedures. For quantitation, ~125 cells were counted in triplicates. Results are expressed as mean ± SD and are a representative of at least two independent experiments.

(H) In vivo chemotaxis. WT or Cor $1^{-/-}$ mice were either left untreated or treated with thioglycolate for 96 hr to recruit macrophages. Cells were collected from the peritoneum and the number of CD11b+ cells determined. The results are expressed as total cells and % of CD11b+. Results show the mean ± SD (n = 3).

(I and J) Membrane ruffling (I) and random motility (J) of *WT* or coronin 1-deficient (*Cor* $1^{-/-}$) macrophages and imaged at 30 s intervals. Bar: 10 μM. For quantitation, results are shown as mean ± SD. At least 100 cells from six different sequences were analyzed. Motility was scored as cells having moved a distance of ~50 μm over a 30 min time point. Video sequences are available in the Supplemental Data.

Phagocytosis and Macropinocytosis in Coronin $1^{-/-}$ Macrophages

In *Dictyostelium*, coronin is involved in phagocytosis and macropinocytosis, and several reports have suggested that coronin 1 may perform a similar function in macrophages (Schuller et al., 2001; Yan et al., 2005). To directly analyze a role for coronin 1 in phagocytosis, macrophages derived from either coronin $1^{-/-}$ mice or wild-type littermates were incubated with human serum treated *Lactobacillus casei* expressing green fluorescent protein (GFP) or IgG-coated fluorescent polystyrene beads for 30 min at 37°C. Both wild-type as well as coronin $1^{-/-}$ macrophages were found to have a similar capacity to take up cargo by either complement receptor or Fc receptor-mediated phagocytosis (Figures 2A and 2B). Similarly, uptake of latex beads, *E. coli*, as well as *Salmonella* was identical in macrophages derived from wild-type or coronin 1-deficient mice (Figures 2C–2E). To analyze a potential role for coronin 1 in phagocytosis in more detail, wild-type or coronin $1^{-/-}$ macrophages were incubated with beads ranging in size from 1 to 6 μm, and the amount of beads phagocytosed as well as the number of beads internalized per cell was quantified. No qualitative or quantitative differences were apparent between wild-type and coronin 1-deficient macrophages (Figure S2). Finally, phagocytosis of latex beads by wild-type or coronin $1^{-/-}$ macrophages was analyzed by video microscopy, showing no qualitative differences (Figure 2F and the accompanying videos).

Analysis of macropinocytosis showed that both the number of macropinosomes as well as the percentage of cells undergoing macropinocytosis was identical in the absence or presence of coronin 1 (Figures 3A and 3B). To analyze qualitative or temporal differences in macropinocytic activity between wild-type and coronin $1^{-/-}$ cells, macrophages were stimulated with PMA (Albrecht et al., 2006; Steinman and Swanson, 1995) and followed by video microscopy. No differences in macropinosome formation were apparent in wild-type or coronin 1-deficient cells (Figure 3C and the accompanying video sequences). Together these data establish that coronin 1 is not essential for phagocytosis or macropinocytosis in macrophages.

Entry, Localization, and Survival of Mycobacteria in Coronin $1^{-/-}$ Macrophages

Although coronin 1 does not participate directly in phagocytosis (see above), upon infection with mycobacteria, coronin 1 is recruited around nascent phagosomes and is actively retained by living bacilli on mycobacterial phagosomes (Ferrari et al., 1999; Tailleux et al., 2003). To assess whether coronin 1 plays a role in the internalization of mycobacteria, wild-type or coronin 1-deficient macrophages were stimulated as indicated in Figures 4A and 4B prior to infection with *M. bovis* BCG expressing the GFP (Dhandayuthapani et al., 1995; Walburger et al., 2004). Both in wild-type as well as in coronin $1^{-/-}$ macrophages similar numbers of mycobacteria were internalized (Figures 4A and 4B). Furthermore, video microscopy revealed no qualitative differences in the uptake process between wild-type and coronin 1-deficient macrophages (Figure 4C). However, analysis of the intracellular localization of mycobacteria showed that while in wild-type macrophages, as expected, mycobacteria were predominantly retained in nonlysosomal phagosomes, in macrophages lacking coronin 1, mycobacteria were largely present in lysosomal organelles as judged by colocalization of mycobacteria and lysosomal-associated membrane protein-1 (LAMP-1) or the acidotrophic compound LysoTracker (Figures 4D and 4E). In addition, subcellular fractionation by organelle electrophoresis (Hasan et al., 1997; Tulp et al., 1994) independently confirmed lysosomal transfer of mycobacteria in coronin 1-deficient macrophages: while in wild-type macrophages, mycobacteria were largely retained in nonlysosomal fractions, in coronin 1-deficient macrophages the majority of internalized mycobacteria colocalized with lysosomal organelles (Figure 4F), showing a role for coronin 1 in the modulation of phagosome-lysosome fusion upon mycobacterial infection.

The lysosomal delivery of mycobacteria could result in their killing through mycobacteriocidal activities residing within lysosomes. To directly analyze bacterial viability, wild-type or coronin $1^{-/-}$ macrophages were incubated with mycobacteria for the times indicated in Figures 4G and 4H, and mycobacterial proliferation and survival were analyzed by incorporation of tritiated uracil and colony-forming unit enumeration, respectively. While in wild-type macrophages, as expected, mycobacteria were able to proliferate, in coronin 1-deficient macrophages bacteria could not proliferate and failed to survive (Figures 4G and 4H). Together, these results demonstrate that coronin 1 prevented lysosomal delivery and mediated intracellular survival of mycobacteria.

Coronin 1 and Calcineurin Activation

Since in T cells, coronin 1 regulates Ca^{2+} entry (P.M., J.M., R.J., I.A., C. Blum, R. Ceredig, H.-R. Rodewald, A.G., J.P., unpublished data), we analyzed whether in macrophages, the role for coronin 1 in sustaining mycobacterial viability was due to its role in modulating intracellular Ca^{2+} concentration. To analyze intracellular Ca^{2+} fluxes, macrophages from wild-type or coronin 1-deficient animals were loaded with the fluorescent Ca^{2+} indicator Rhod-2 AM. Upon addition of mycobacteria to Rhod-2-loaded macrophages, the cells responded with an immediate raise in cytosolic Ca^{2+} (Figure 5A, blue line). In contrast, when coronin 1-deficient macrophages were incubated with mycobacteria, no increase in cytosolic Ca^{2+} was observed (Figure 5A, red line). The difference in cytosolic calcium release between wild-type and coronin 1-deficient macrophages was not due to an intrinsic problem with the intracellular Ca^{2+} stores, since treatment of the cells with thapsigargin, a specific inhibitor of the sarco-endoplasmic Ca^{2+} ATPase (Thastrup et al., 1990), induced store release in both wild-type as well as coronin 1-deficient macrophages, whereas

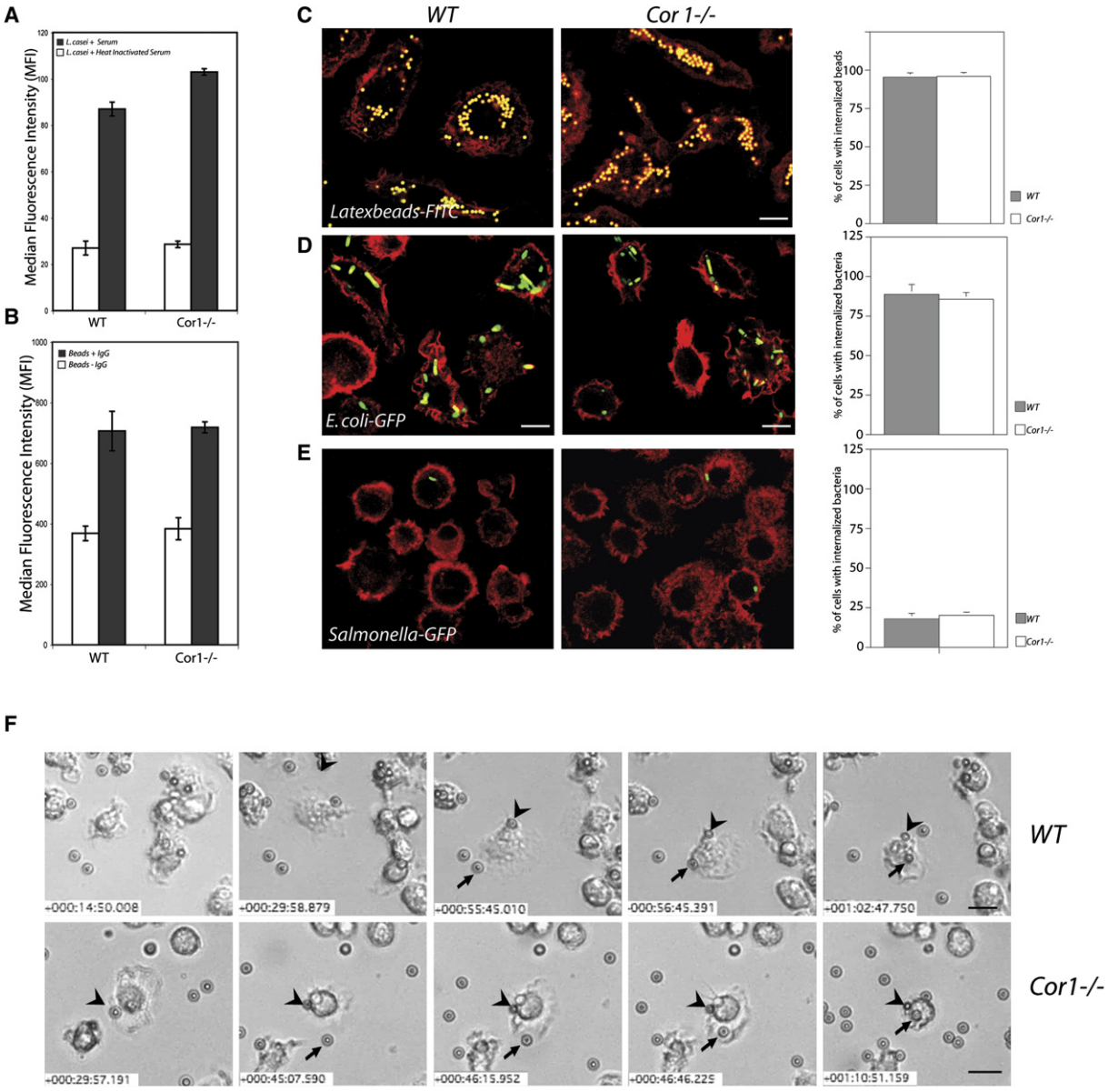


Figure 2. Phagocytosis in the Presence and Absence of Coronin 1

(A and B) Flow cytometric analysis of receptor mediated phagocytosis (A) or complement receptor 3 (CR3)-mediated uptake coronin 1-knockout (Cor $1^{-/-}$) mice or WT macrophages.

(C–E) WT (left panels) or coronin 1-deficient (Cor $1^{-/-}$, middle panels) macrophages were incubated with either FITC-labeled latex beads (Ø 1 μm, C), GFP expressing *E. coli* (D) or GFP expressing *Salmonella* (Salmonella-GFP, E) for 40 min at 37°C. After washing, cells were fixed and stained for F-actin using phalloidin-568. Bar: 10 μm. For quantitation (right panels), cells (n = 200–500) were scored for the presence of internalized beads or bacteria. Shown are the mean values (± SD) from one representative experiment.

(F) For video microscopy, 3 μm latex beads were added and images collected at 30 s intervals. Frames selected show two independent phagocytosis events (indicated by arrowhead and arrow, respectively) in both wild-type (top panels) and coronin $1^{-/-}$ (bottom panels) macrophages. Bar: 10 μm. Full sequences can be accessed in the Supplemental Data.

subsequent Ca^{2+} entry was drastically depressed in the absence of coronin 1 (Figure S3).

To analyze whether the coronin 1-mediated Ca^{2+} influx was responsible for the block in lysosomal delivery and survival of mycobacteria, calcium was chelated using either the cell permeable chelator BAPTA-AM or cell impermeable reagent BAPTA before and during the internalization of mycobacteria. Subsequently, the degree of lysosomal localization as well as intracellular survival was analyzed as judged by colocalization with LAMP or LysoTracker as well as incorporation of ^{3}H-uracil and colony-forming unit enumeration. As shown in Figures

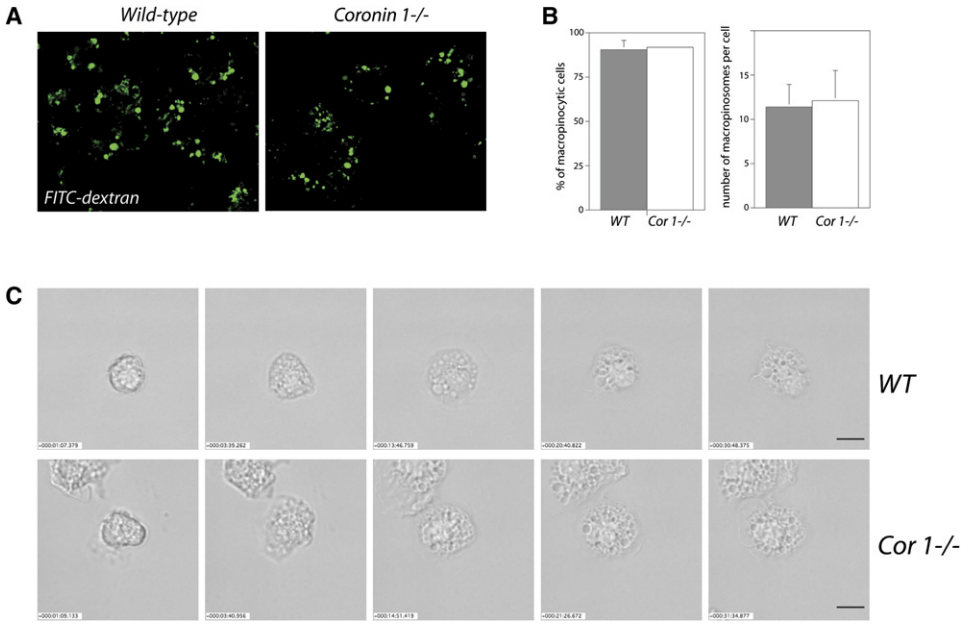


Figure 3. Macropinocytosis in the Presence and Absence of Coronin 1

(A) WT (left panels) or coronin 1-deficient (Cor $1^{-/-}$, right panels) macrophages stimulated with PMA (10^{-7} M for 30 min) were incubated with FITC-Dextran (MW 70,000) for 20 min at 37°C. Bar: 10 μm.

(B) For quantitation, cells (n = 500) were scored for the presence of internalized FITC-Dextran (left) and for the number of macropinosomes per cell (n = 500) (right). Shown are the mean values (± SD) from one representative experiment.

(C) For video microscopy, cells were imaged for 15 min, after which PMA was added (10 nM). Images were collected every 30 s. Selected frames show formation of macropinosomes in WT (top panels) and coronin 1-deficient (bottom panels) macrophages. Bar: 10 μm. Video sequences are available in the Supplemental Data.

5B–5F, in the presence of BAPTA-AM as well as BAPTA, virtually all mycobacteria were transported to lysosomes and killed, to a similar degree as in coronin 1-deficient macrophages.

To directly analyze a role for coronin 1 in mediating Ca^{2+} release that results in blocking lysosomal delivery of mycobacteria, coronin 1-deficient macrophages were incubated in the presence of a low dose of the Ca^{2+} ionophore calcimycin to increase cytosolic Ca^{2+} levels. Subsequently, the intracellular residence of the internalized mycobacteria was analyzed by colocalization with LysoTracker. As shown in Figure 5D, the presence of calcimycin prevented the lysosomal delivery of mycobacteria in coronin 1-deficient macrophages to a similar degree as wild-type macrophages. The block of lysosomal transfer by calcimycin treatment of coronin 1-deficient macrophages was confirmed by organelle electrophoresis (Figure S4). Furthermore, mycobacterial proliferation and survival in coronin $1^{-/-}$ macrophages was drastically enhanced in the presence of calcimycin (Figures 5E and 5F).

An immediate result of an increase in cytosolic calcium is the activation of calcineurin (Winslow et al., 2003). To assess whether calcineurin was differentially activated upon mycobacterial infection in macrophages, wild-type or coronin 1-deficient macrophages were infected with mycobacteria followed by the determination of calcineurin activity. While calcineurin phosphatase activity was close to background levels in lysates from both wild-type and coronin 1-deficient cells, infection of mycobacteria resulted in a drastic increase in calcineurin activity in wild-type lysates, whereas no increase was observed in lysates from coronin 1-deficient macrophages (Figure 5G). Incubation of macrophages with the Ca^{2+} ionophore calcimycin resulted in calcineurin activation in both wild-type as well as coronin 1-deficient macrophages (Figure 5H), showing no intrinsic defect with calcineurin activation in the absence of coronin 1. Also, coronin 1 was not involved in altering the subcellular distribution of calcineurin following mycobacterial uptake, since similar amounts of calcineurin were recovered from cytosol or membrane fractions in infected wild-type or coronin 1-deficient macrophages (Figure S5). Together these data indicate that while in wild-type macrophages, infection of mycobacteria induced calcineurin activation, in the absence of coronin 1 calcineurin was not activated.

To investigate whether the coronin 1-dependent phagosomal retention and survival of mycobacteria was dependent on calcineurin-induced gene transcription (Gallo et al., 2006), mycobacteria were internalized in the presence of the protein synthesis inhibitor cycloheximide. While cycloheximide efficiently blocked protein synthesis without compromising macrophage viability, the presence of cycloheximide did not result in alteration of the intracellular routing or survival of mycobacteria (Figure S6),

indicating that the coronin 1-mediated block of phagosome-lysosome fusion did not occur as a result of induction of translation due to the activation of transcription factors.

To further substantiate that activation of calcineurin was responsible for prevention of lysosomal transfer and death of internalized mycobacteria we made use of the specific calcineurin inhibitors cyclosporin A and FK506. Cyclosporin A and FK506 are chemically unrelated natural products that are highly specific inhibitors of calcineurin activity (Liu et al., 1991). To analyze the consequence of calcineurin inhibition on intracellular mycobacterial trafficking and survival, macrophages were incubated with either cyclosporin A or FK506, followed by infection with *M. bovis* BCG. Staining of fixed cells for mycobacteria as well as lysosomes showed that both inhibitors induced a complete relocation of mycobacteria to lysosomes (Figures 6A and 6B). Similarly, when murine J774 macrophages were infected with *M. tuberculosis* H37Rv in the presence of cyclosporin A or FK506, the majority of the internalized mycobacteria were found within lysosomes (Figure 6C). Furthermore, mycobacteria were unable to survive and proliferate within macrophages when calcineurin was blocked by either cyclosporin A or FK506, as analyzed by mycobacterial proliferation (Figure 6D) or colony-forming units enumeration (Figure 6E). Together these results show that coronin 1-dependent calcineurin activation is essential to allow intracellular survival of mycobacteria within macrophages.

In Vivo Localization of *M. bovis* BCG and *M. tuberculosis* in the Presence and Absence of Coronin 1

To reveal whether the differential trafficking in wild-type versus coronin 1-deficient macrophages could be demonstrated in vivo, coronin 1-deficient mice or wild-type littermates were infected with either *M. bovis* BCG or *M. tuberculosis* H37Rv. To analyze the subcellular localization of mycobacteria, mice were sacrificed after 1 week, and thin sections of the liver containing infiltrated mycobacteria-positive macrophages (Ferrari et al., 1999) were prepared for immunostaining. While in sections from wild-type mice mycobacteria largely failed to colocalize with LAMP, in coronin 1-deficient mice the majority of the bacilli colocalized with LAMP (Figures 7A and 7B). These results therefore strongly suggest that also in vivo, coronin 1 is important to block transfer of pathogenic mycobacteria to lysosomes.

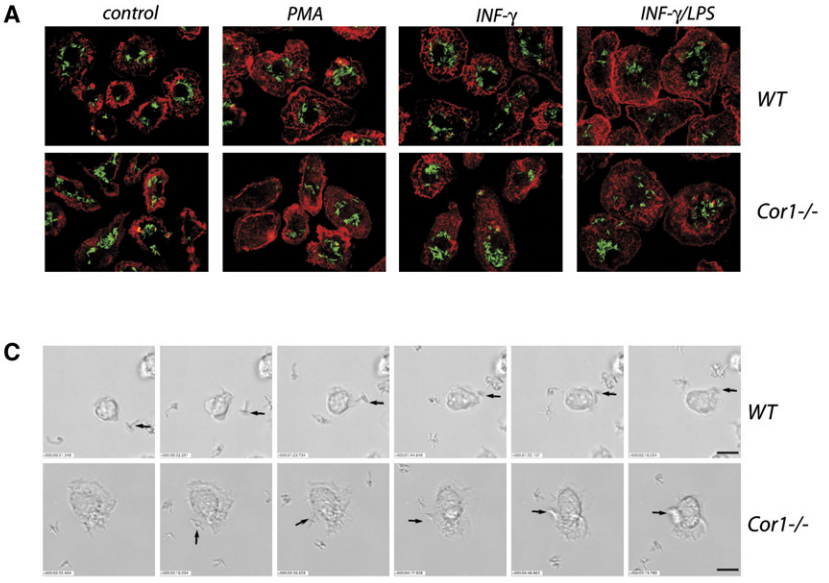
Finally, to analyze the effect of calcineurin inhibition in vivo, DBA/2 mice were infected with *M. tuberculosis* H37Rv and subsequently treated daily with FK506 or saline. After 3 days, mice were sacrificed, and thin sections of liver were prepared for immunostaining using antibodies against mycobacteria and the lysosomal protein LAMP. Whereas in sections from control animals, *M. tuberculosis* was largely found to localize in regions that were not labeled for LAMP in sections obtained from FK506-treated animals *M. tuberculosis* largely colocalized with LAMP (Figures 7C and 7D). These data therefore indicate that, in vivo, calcineurin activity is important to prevent lysosomal delivery of *M. tuberculosis*.

DISCUSSION

While in the unicellular organism *Dictyostelium*, the single coronin gene is involved phagocytosis, macropinocytosis, and cell motility, a role for coronins in macrophages has not been described. We here demonstrate that in the absence of coronin 1, mammalian macrophages are fully functional; phagocytosis, macropinocytosis, as well as cell motility occurred normally in coronin $1^{-/-}$ macrophages, suggesting that coronin 1 is dispensable for these actin-dependent processes. However, in accordance with a role for coronin 1 in the intracellular survival of mycobacteria (Ferrari et al., 1999), internalized mycobacteria were rapidly transferred to lysosomes and killed in coronin 1-deficient macrophages. We found that upon mycobacterial infection, coronin 1 is responsible for the activation of the Ca^{2+}-dependent phosphatase calcineurin, which in turn blocks lysosomal delivery of mycobacteria.

The lack of an F-actin-dependent phenotype in coronin 1-deficient macrophages is consistent with the finding that yeast lacking the single coronin gene has no obvious defect in any actin-related function (Heil-Chapdelaine et al., 1998). In mammalian cells, up to seven coronin isoforms are expressed, several ubiquitously (Rybakin and Clemen, 2005). While it is possible that in mammalian cells isoforms other than coronin 1 are responsible for the modulation of F-actin, experimental evidence from in vivo analyses is currently not available. Importantly, in mammals, the expression of coronin 1 is restricted to leukocytes, and therefore the here defined role for coronin 1 in calcineurin activation suggest that coronin 1 has evolved to specifically modulate this process in leukocytes. Whether or not other coronin isoforms perform a similar function in nonleukocytes remains to be analyzed.

Importantly, the here observed coronin 1-dependent Ca^{2+} mobilization occurred upon infection of nonopsonized mycobacteria, which is in contrast with the reported suppression of Ca^{2+} signaling by opsonized mycobacteria (Malik et al., 2000). It should be noted, however, that the mechanisms of internalization of opsonized versus nonopsonized mycobacteria are quite distinct, resulting in the activation of different downstream signaling pathways (Le Cabec et al., 2002; Velasco-Velazquez et al., 2003). In accordance with differential signaling pathways involved in these distinct modes of entry, while following infection with opsonized mycobacteria, Ca^{2+} entry occurs through a pathway that depends on sphingosine kinase (Malik et al., 2003), the coronin 1-mediated Ca^{2+} mobilization stimulated by mycobacterial entry was independent of sphingosine kinase (data not shown). Importantly, Ca^{2+} is a highly versatile signaling molecule that can be used in a wide array of physiological responses, with the precise signaling pathways employed dependent on the spatial and temporal organization of the Ca^{2+} fluxes (Berridge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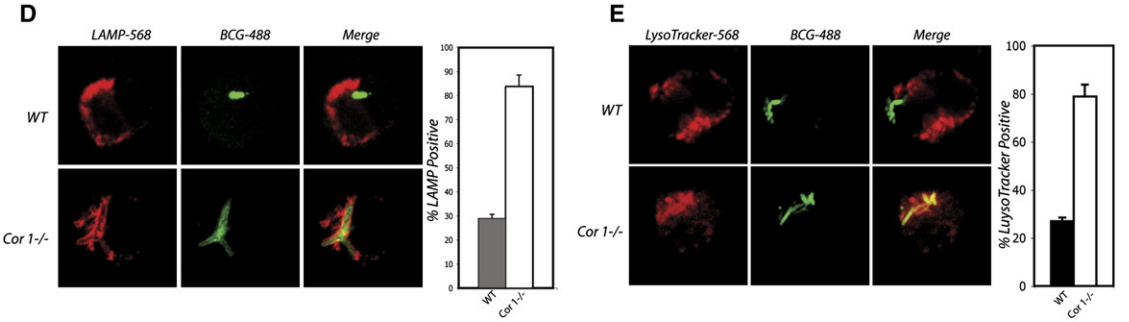
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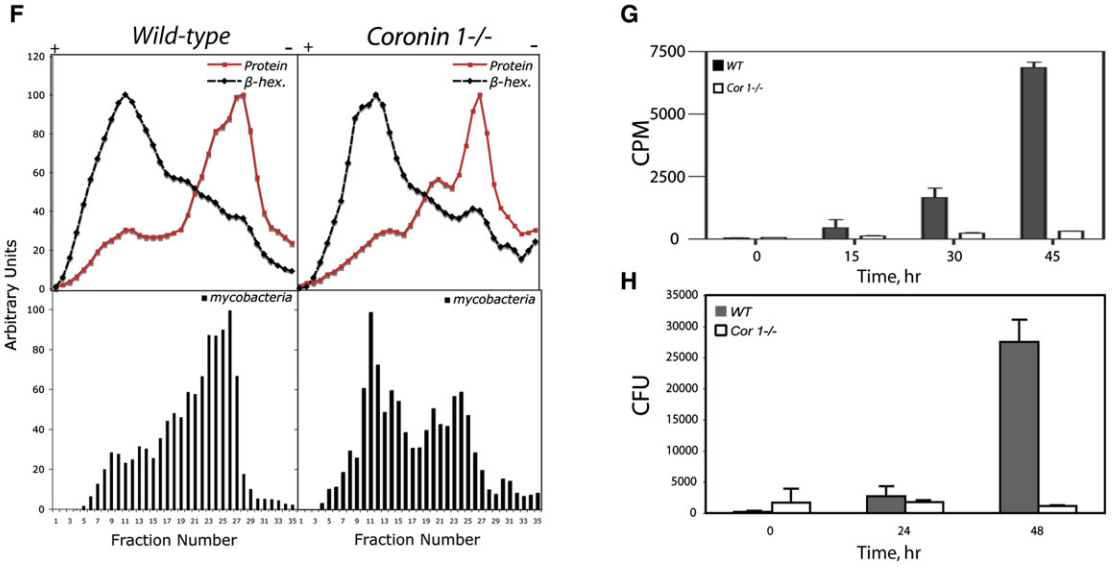
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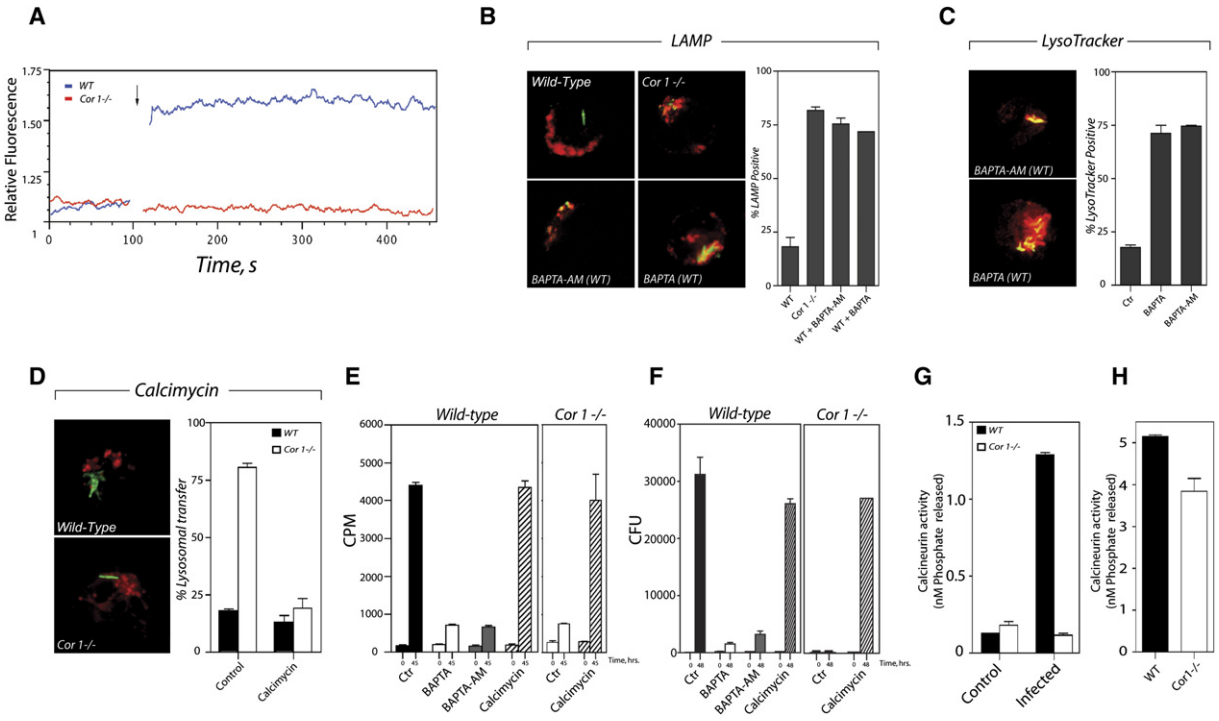

Figure 5. Ca^{2+}-Dependent Signaling in the Presence or Absence of Coronin 1

(A) Macrophages from WT (blue) or coronin 1 deficient (red) were loaded with Rhod-2 a.m. as described in the Experimental Procedures and stimulated with mycobacteria (arrow). Shown are representative profiles from at least five independent experiments.

(B and C) WT or coronin 1-deficient (Cor $1^{-/-}$) macrophages were allowed to adhere to slides in the presence of BAPTA-AM (1 μM) or BAPTA (3 mM) and infected with *M. bovis* BCG for 1 hr. The cells were chased for 3 hr, at the end of which they were fixed and stained for mycobacteria and LAMP-1 (B). Alternatively, cells were infected with GFP expressing mycobacteria and labeled with LysoTracker red (C). For quantitation, cells (n = 50) were scored for the colocalization of bacteria with LAMP-1 (B) or LysoTracker (C) and represented as percentage colocalization with SD ± values from three independent experiments.

(D) WT or coronin 1-deficient macrophages were seeded in the presence of callcimycin (50 nM) and incubated with GFP expressing mycobacteria as above along with LysoTracker red. Quantitation was performed as mentioned above.

(E and F) Proliferation (E) and survival (F) of mycobacteria within WT or coronin 1-deficient (Cor $1^{-/-}$) macrophages in the presence of the indicated reagents. The data show representative results obtained from at least three independent experiments.

(G) Wild-type (black bars) or coronin 1-deficient (white bars) macrophages were left untreated (control) or infected with mycobacteria for 1 hr followed by a 3 hr chase. Cells were lysed, and calcineurin activity was analyzed as detailed in the Experimental Procedures.

(H) Calcineurin activity following stimulation with calcimycin (2 μM).

et al., 2003), possibly explaining why triggering macrophages with distinct ligands may result in different downstream signaling processes. For example, mycobacteria may exploit physiological Ca^{2+} fluxes for their survival, while, at the same time, they can prevent elevation in Ca^{2+} that triggers macrophage microbicidal effects

Figure 4. Internalization, Trafficking, and Survival of Mycobacteria in WT and Coronin 1-Deficient Macrophages

(A) WT (upper panels) or coronin 1-deficient (Cor $1^{-/-}$, bottom panels) macrophages were left untreated (*control*), or stimulated either with PMA, interferon-γ (*INF-γ*) or interferon-γ in the presence of lipopolysaccharide (*INF-γ/LPS*) and incubated with BCG-GFP for 40 min at 37°C, washed, fixed,and stained for F-actin using phalloidin 568. Bar: 10 μm.

(B) For quantitation, cells were scored for the presence of internalized bacteria. Shown are the mean values (n = 500, ± SD).

(C) Internalization of mycobacteria imaged by video microscopy. WT or coronin $1^{-/-}$ macrophages were incubated with mycobacteria and imaged at 10 s intervals (WT, top panels; coronin 1 deficient, bottom panels). Scale bar: 10 μm. Full video sequences are available in the Supplemental Data.

(D and E) Intracellular trafficking of mycobacteria. WT or coronin 1-deficient macrophages were incubated with mycobacteria for 1 hr followed by a 3 hr chase, fixed and stained for *M. bovis* BCG and LAMP-1 (D) or LysoTracker (E). For quantitation, cells (n = 150) were scored for the colocalization of bacteria with the lysosomal marker and represented as percentage colocalization with SD ± values from at least three independent experiments.

(F) Macrophages from wild-type or coronin 1-deficient mice were incubated for 3 hr with mycobacteria, homogenized, and subjected to organelle electrophoresis. The distribution of organelle-specific markers and the amount of bacteria per fraction were determined as described in the Experimental Procedures.

(G and H) Mycobacterial proliferation (G) and survival (H) determined as described in the Experimental Procedures. In (G), error bars are from triplicates and the data shown representative of at least three independent experiments. In (H), the results shown are mean ± SD, n = 2, and are representative of at least three independent determinations.

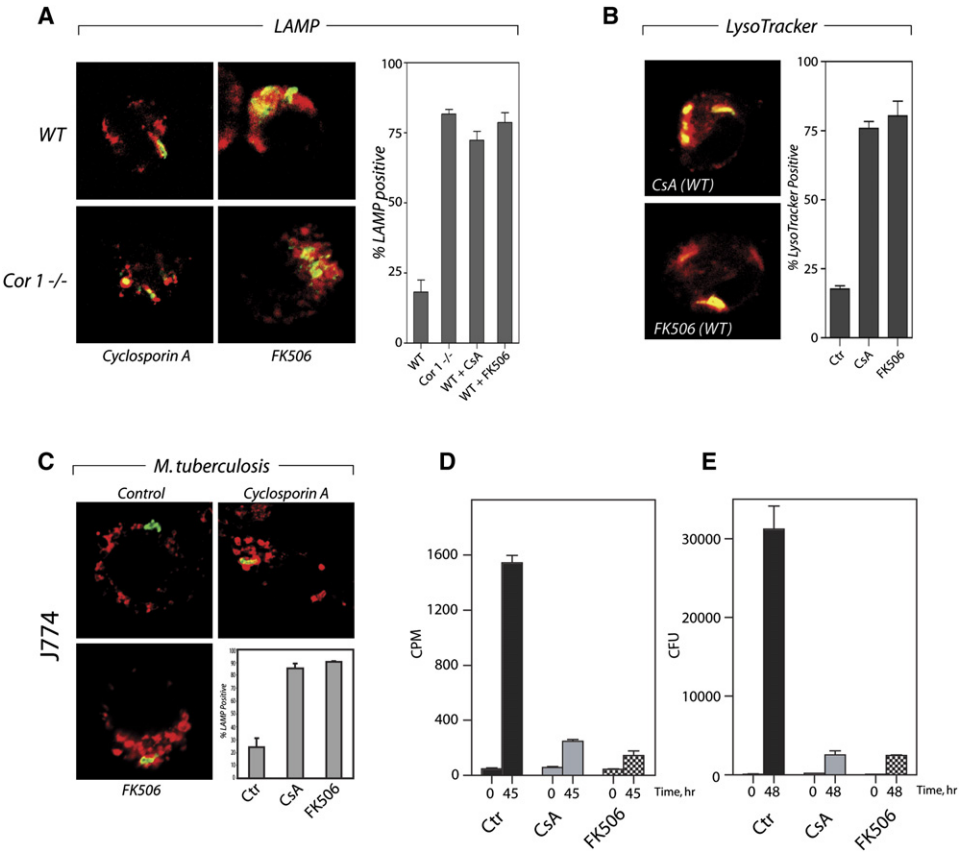

Figure 6. Intracellular Transport and Survival of Mycobacteria in the Presence and Absence of Coronin 1 and Calcineurin Inhibitors

(A and B) WT or coronin 1 deficient (Cor $1^{-/-}$) macrophages were infected with *M. bovis* BCG in the presence of cyclosporin A (0.1 μM) or FK506 (0.5 μM) and chased for 3 hr, at the end of which the cells were methanol fixed and stained for mycobacteria and LAMP-1 (A) or LysoTracker (B). For quantitation, cells (n = 50) were scored for the colocalization of bacteria with the lysosomal markers and represented as percentage colocalization with SD ± values from three independent experiments.

(C) Murine macrophages (J774A1) were infected with *M. tuberculosis* H37Rv and processed for immunofluorescence analysis as described in (A). For quantitation, 50 infected cells from each condition were counted. Results expressed as mean ± SD (n = 3).

(D and E) Viability of mycobacteria within WT or coronin 1-deficient (Cor $1^{-/-}$) macrophages in the presence of cyclosporin A and FK506. Macrophages were infected as described in the legend to Figure 4 and chased for the times indicated. Proliferation (D) and survival (E) of the bacteria were analyzed as described in the Experimental Procedures.

when internalized following opsonization. When such physiological Ca^{2+} fluxes cannot take place, as in the case of coronin 1-deficient macrophages, mycobacteria are routed to lysosomes and killed.

The here defined function for coronin 1 in activating Ca^{2+} influx upon mycobacterial entry may be related to its capacity to link the F-actin cytoskeleton to the membrane in leukocytes (Gatfield et al., 2005). Recent work has revealed an increasingly important role for the cytoskeleton in activating cytosolic Ca^{2+} influx (Gallo et al., 2006; Parekh and Putney, 2005). These results suggest a scenario in which the cytoskeleton functions to locally assemble the signaling machinery involved in Ca^{2+} release, such as mediating the interaction of the endoplasmic reticulum stores with the plasma membrane (Zhang et al., 2005). One intriguing possibility is that coronin 1 may be involved in the local recruitment of intracellular Ca^{2+} stores to the phagosomal membrane, thereby spatially coordinating Ca^{2+} efflux from the stores with local activation of calcineurin, resulting in the modulation of membrane-fusion events. Indeed, the endoplasmic reticulum has been suggested to be recruited to nascent phagosomes (Gagnon et al., 2002; Muller-Taubenberger et al., 2001) and may be utilized by mycobacteria via coronin 1 retention to allow Ca^{2+} signaling to activate calcineurin. Finally, the definition of calcineurin as a survival factor for mycobacteria within macrophages might allow the development of strategies to interfere with mycobacterial survival.

EXPERIMENTAL PROCEDURES

Construction of Coronin 1-Deficient Mice

A detailed description of the generation of the coronin 1-deficient mice is provided in the Supplemental Data. In brief, mouse embryonic stem cell (129/Ola, TMCF, Biozentrum Basel) genomic DNA was used to

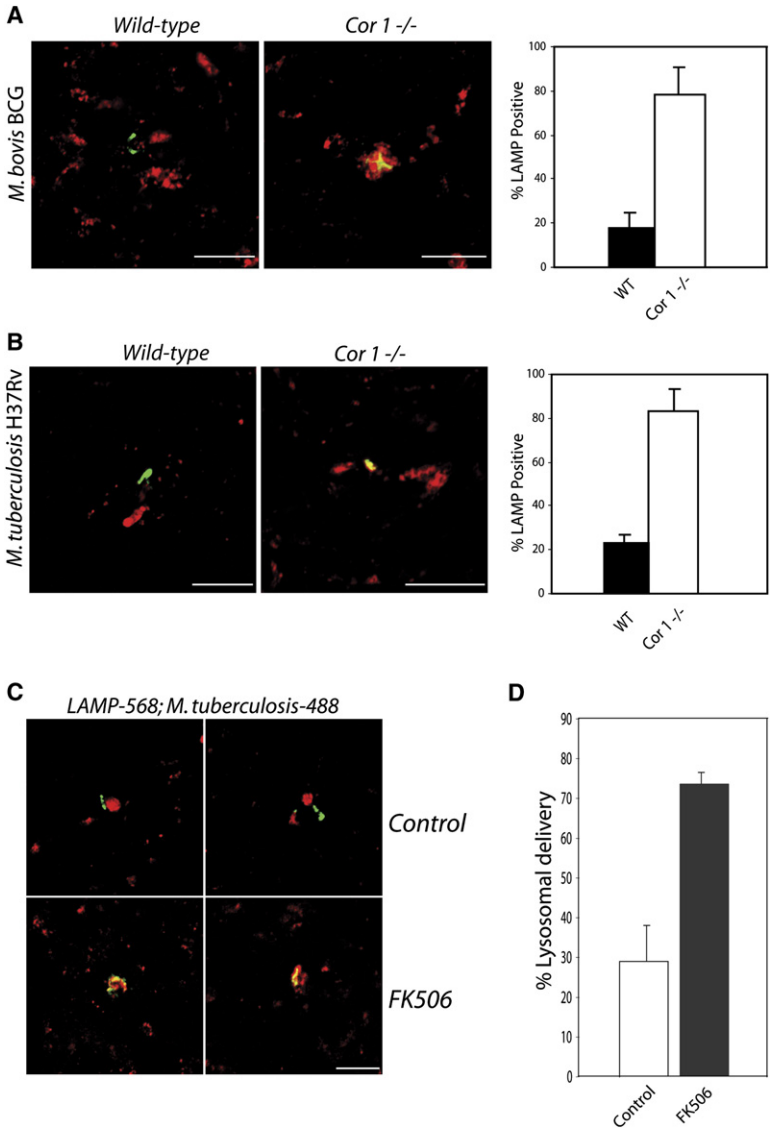

Figure 7. In Vivo Localization of *M. bovis* BCG and *M. tuberculosis* in the Presence and Absence of Coronin 1 and FK506

(A and B) WT or coronin $1^{-/-}$ mice were infected with 10^6 CFU of either *M. bovis* BCG (A) or *M. tuberculosis* H37Rv (B). One week later, mice were sacrificed and cryosections (8 μm) were prepared from the liver and immunostained for mycobacteria and LAMP-1 using Alexa-fluor goat anti-rabbit-488 and Alexa-fluor goat anti-rat-568 labeled secondary antibodies, respectively. Bar: 10 μm. For quantitation of lysosomal delivery of *M. bovis* BCG-infected or *M. tuberculosis*-infected animals, 100 events were scored per animal (n = 3). In the case of *M. bovis* BCG infected coronin $1^{-/-}$ animals, the number of events scored was between 15 and 30 due to a paucity of events. Results are expressed as mean ± SD.

(C) Mice were infected with 10^6 CFU of *M. tuberculosis* H37Rv and were either untreated or treated with a daily dose of FK506 (5 mg/kg body weight). Mice were sacrificed 3 days after infection. Cryosections (8 μm) were prepared from the liver and immunostained for mycobacteria and LAMP-1 using Alexa-fluor goat anti-rabbit-488 and Alexa-fluor goat anti-rat-568 labeled secondary antibodies, respectively. Bar: 10 μm.

(D) Quantitation of lysosomal delivery of *M. tuberculosis* in control and FK506-treated animals. Fifty to one hundred events were scored per animal (n = 3). Results are expressed as mean ± SD.

amplify 5′- and 3′-flanking homology regions. The coding region for enhanced green fluorescent protein (EGFP) was similarly amplified, and the targeting vector constructed by subcloning the 5′-flanking homology region into pBluescript SK+ (Stratagene). The EGFP cassette was inserted 3′ of the 5′-flanking homology region. The 3′-flanking homology region was then added 3′ of EGFP. A SV40polyA signal and neomycin resistance cassette (Hippenmeyer et al., 2005) was inserted between EGFP and the 3′-flanking homology region. The linearized targeting construct was electroporated into mouse embryonic stem cells (strain 129/Sv), and clones were selected, identified by Southern blot, and microinjected into C57/Bl6xBDF1 blastocysts (RCC, Fullinsdorf, Switzerland). Male chimeras obtained were crossed with C57BL/6 females. Transgenic offspring was genotyped by Southern blotting following digestion of genomic DNA with EcoRI.

Biochemical Methods

Protein analysis and organelle electrophoresis was essentially done as described (Ferrari et al., 1997, 1999; Tulp et al., 1994). Bacteria were detected after sedimentation of the different fractions followed by fixation (PFA) and staining with propidium iodide or using acid fast staining (Becton Dickinson).

F- Versus G-Actin Analysis

F- versus G-actin was quantitated using an in vivo assay kit (Cytoskeleton, Inc). Briefly, 10^6 bone marrow macrophages were treated with Latrunculin B (4 μM, Calbiochem) or Jasplakinolide (1 μM, Calbiochem) for 45 min at 37°C in culture medium. Cells were pelleted (850 *xg*, 5 min, 37°C) and washed with prewarmed Tris-buffered saline and lysed in 0.5 ml of "Lysis and F-actin stabilization buffer" (50 mM PIPES at pH 6.9, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% Glycerol, 0.1% NP-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercapto-ethanol, 0.001% AntifoamC) in the presence of 1 mM ATP, phosphatase inhibitors (Sigma), and protease inhibitors (Calbiochem) at 37°C for 10 min. Lysates were centrifuged as before to discard incompletely lysed cells. Supernatants were centrifuged at 100,000 *xg* for 2 hr at 37°C and transferred to a fresh tube, and the pellet was resuspended to the same volume as that of the supernatant with ice-cold Milli-Q water containing 10 μM Cytochalasin D. The sample was incubated on ice for 1 hr to allow dissociation and resuspension of F-actin. Equal

volumes were loaded from supernatant and pellet fractions after denaturing with SDS-sample buffer onto a 10% SDS-PAGE gel and electrophoresed. The gel was transferred onto a nitrocellulose membrane and probed with mouse anti-actin antibody (Chemicon).

Microscopy

Infection experiments were performed as described (Ferrari et al., 1999; Walburger et al., 2004). In experiments involving calcineurin inhibitors (cyclosporin A [0.1 μM] and FK506 [0.5 μM]), calcium chelators (BAPTA [3 mM]; BAPTA-AM [1 μM]), Calcimycin (50 nM), or cycloheximide (75 μg/ml), the reagents were added at the time of seeding the macrophages. Analysis was performed on a Zeiss LSM510 Meta confocal laser scanning microscope. Video microscopy was performed as described before (Gatfield and Pieters, 2000).

Cell Migration

Migration assays were performed in Costar24 well migration chambers with pore size of 8 μm. Chemoattractants were human serum activated by 50 mg/ml Zymosan A or Monocyte chemoattractant protein-1 (MCP-1) at 200 ng/ml. After 4 hr of incubation (37°C, 5% CO_2) filters were excised and stained with propidium iodide. Cells were counted using fluorescence microscopy and expressed as number of cells migrated per field. Cells present in 10–15 fields were counted.

In Vivo Chemotaxis

Mice were either injected with 0.5 ml of 3.85% thioglycolate intraperitoneally or left untreated. Ninety six hours after injection, mice were sacrificed and cells were recovered by flushing the peritoneal cavity with 5 ml of DMEM containing 10% fetal calf serum (FCS) and 2 mM glutamine. Live cells were counted using a Neubauer chamber, and the number of macrophages determined by flow cytometer after staining for CD11b (Beckton Dickinson) conjugated to PE (dilution 1:200).

Mycobacterial Proliferation and Survival

Mycobacterial proliferation was performed as described earlier (Walburger et. al, 2004). For experiments involving calcineurin inhibitors or calcium chelators, the reagents were added at the time of seeding of the cells and subsequent steps carried out in the presence of the reagents. Enumeration of colony-forming units was performed after cell lysis in 0.05% SDS for 4 min at room temperature. The lysate was centrifuged at 4000 *xg* (20 min), and the resulting pellet was resuspended in 100 μl of 7H9 medium, plated into 7H11 agar plates at different dilutions, and incubated at 37°C for 2–3 weeks prior to counting.

Macrophage Proliferation

Macrophages (5×10^4) from either wild-type or coronin 1-deficient mice were seeded in 200 μl of BMM medium per well in a 96-well plate and incubated in the presence of tritiated thymidine 0.5 μCi for 24 hr. Cells were lysed by hypotonic shock and the DNA harvested using Packard FilterMate Harvester with Unifilter-96 and GF/C filter, and incorporated radioactivity was measured using the TopCount (Perkin Elmer) microplate scintillation counter.

Receptor-Mediated Phagocytosis

Fc-receptor-mediated phagocytosis was analyzed using fluorescent polystyrene beads (10 μl, 2% suspension; Molecular Probes FluoSpheres beads) incubated with 50 μg/ml rabbit IgG (Cappel/ICN) in PBS for 1 hr. Beads were washed and resuspended in DMEM/10%FCS/2 mM glutamine. For complement receptor-mediated uptake *Lactobacillus casei* expressing GFP was treated at an optical density or OD_{600} of 0.2 with 1:10 diluted fresh or heat-inactivated (56°C, 30 min) human serum in PBS for 30 min at 37°C, washed, and resuspended in the same medium. Cells were incubated at 4°C, followed by addition of fluorescent beads or serum-treated Lactobacilli and by a 30 min incubation at either 37°C or 4°C (cold control). Phagocytosis was determined by flow cytometry (FACSCalibur, Becton Dickinson).

Calcium Measurements

Cells were loaded with calcium sensitive fluorophore Rhod-2 AM. (Molecular Probes; 3 μM) for 45 min at 37°C and 5% CO_2 in the presence of 2.5 mM probenecid and washed with Ringer's solution (155 mM NaCl, 4.5 mM KCl, 10 mM D-glucose, 5 mM HEPES at pH 7.4, 1 mM $MgCl_2$, 2 mM $CaCl_2$). *M bovis* BCG, washed in Ringer's solution prior to addition was added at 0.2 OD. Analysis was carried out in BD FACS flow cytometric systems with excitation at 488 and emission measured in Fl-2 (590 nM). Rhod2 was used to avoid interference from the minimal EGFP signal in the coronin 1 knockout macrophages (Bolsover et al., 2001; Rubart et al., 2004).

Calcineurin Activity

Macrophages (10^7) from wild-type or coronin 1-deficient mice were either untreated or infected with *M bovis* BCG at an OD of 0.01 and incubated for an additional 3 hr, at the end of which the cells were sedimented at 850 *xg* for 5 min at 4°C and washed three times with ice-cold Tris-buffered saline (150 mM NaCl, 20 mM Tris at pH 7.2), and the resulting pellet was lysed in lysis buffer (50 mM Tris, 1 mM DTT, 100 μM EDTA, 100 μM EGTA, 0.2% NP 40 at pH 7.4) containing protease inhibitors. To analyze calcineurin activity, cytosol (isolated by centrifugation: 100,000 *xg* for 1 hr at 4°C) was passed through a P6 DG desalting column to remove free phosphates/nucleotides from the extract. Calcineurin dephosphatase activity (okadaic acid resistant and EGTA sensitive) was assessed on a 96-well plate using equal volumes of cytosol for the different conditions with 150 μM RII phosphopeptide, a well-characterized substrate for calcineurin. Calcineurin-specific phosphate release was measured in the following buffer: 100 mM NaCl, 50 mM Tris, 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM DTT, 0.025% NP-40 at pH 7.5 including 2.5 μM okadaic acid in a total volume of 50 μl. Background was determined in the same buffer excluding the RII phosphopeptide. Plates were incubated at 30°C for 30 min, and free phosphate released was detected using malachite green by monitoring the absorption at OD 620 nm. The values were converted to nanomoles of phosphate released using the phosphate standards generated in parallel during the assay (background was ~0.35 nM of free phosphate).

Lysosomal Delivery In Vivo after Treatment with FK506

DBA/2 mice (male, 8–10 weeks old) were infected with 10^6 CFU *M. tuberculosis* H37Rv intravenously (Tanghe et al., 2001). One group of animals was treated with PBS, while another group received daily dose of FK506 (5 mg/kg body weight in PBS) intraperitoneally (Butcher et al., 1997). After 3 days animals were sacrificed and 8 μm thin sections of liver were prepared using a Leica Cryostat CM1100 set to −17°C. Slides were air dried and fixed with ice-cold methanol at −20°C for 4 min and immunostained for *M. tuberculosis* and LAMP-1, followed by Alexa-fluor goat anti-rabbit 488 and Alexa-fluor goat anti-rat 568 labeled secondary antibodies, respectively, and mounted using antifade (Biorad). Slides were analyzed using the confocal laser scanning microscope LSM510 Meta (Zeiss) and the corresponding software.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, six figures, and ten movies and can be found with this article online at http://www.cell.com/cgi/content/full/130/1/37/DC1/.

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