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Cutting Edge: *Mycobacterium tuberculosis* Blocks Ca\(^{2+}\) Signaling and Phagosome Maturation in Human Macrophages Via Specific Inhibition of Sphingosine Kinase

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**One-third of the world’s population is infected with Mycobacterium tuberculosis (Mt b), and three million people die of tuberculosis each year. Following its ingestion by macrophages (MPs), Mt b inhibits the maturation of its phagosome, preventing progression to a bactericidal phagolysosome. Phagocytosis of Mt b is uncoupled from the elevation in MP cytosolic Ca\(^{2+}\) that normally accompanies microbial ingestion, resulting in inhibition of phagosome-lysosome fusion and increased intracellular viability. This study demonstrates that the mechanism responsible for this failure of Ca\(^{2+}\)-dependent phagosome maturation involves mycobacterial inhibition of MP sphingosine kinase. Thus, inhibition of sphingosine kinase directly contributes to survival of Mt b within human MPs and represents a novel molecular mechanism of pathogenesis. The Journal of Immunology, 2003, 170: 2811–2815.**

**Materials and Methods**

**Determination of MP [Ca\(^{2+}\)]\(_i\)**

Human monocyte-derived MPs were isolated from blood of purified protein derivative-negative donors, and loaded with fura 2-AM (Molecular Probes, Eugene, OR) (7). H37Rv Mt b were cultured and opsonized with complement, and levels of MP [Ca\(^{2+}\)]\(_i\) were determined as described (7, 8).

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Measurement of inositol phosphate production

MPs were labeled with 1 μCi/ml [3H]myoinositol (Amersham, Arlington Heights, IL), and incubated with Mtb at a multiplicity of infection (MOI) of 10:1, in the presence of 20 mM LiCl (Sigma-Aldrich, St. Louis, MO) (9). Reactions were terminated with trichloroacetic acid and lysates extracted four times with 10 volumes of water-saturated diethyl ether. Aqueous extracts were neutralized, applied to Dowex AG1X8 columns (Sigma-Aldrich), and total inositol phosphates were eluted and counted by liquid scintillation spectrometry.

Measurement of sphingosine-1-phosphate (S1P) production

MPs were labeled with 10 μCi/ml [3H]serine (Amersham) (10) and infected with Mtb. Reactions were terminated with CH3OH/HCl (200:1), and CHCl3 was added to achieve a final extraction solution of CHCl3/CH3OH/HCl (100:200:1). [3H]S1P was isolated by TLC in n-butanol:acetic acid:water (3:1:1).

Sphingosine kinase (SK) activity

MPs were incubated with Mtb or buffer control for various times, from 0.5 to 10 min. At the end of the incubation, samples were washed with ice-cold PBS and cells scraped into 500 μl of lysis buffer (200 mM Tris (pH 7.4), 15 mM NaF, 40 mM β-glycerolphosphate, 1 mM EDTA, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMFS, 10% glycerol, 0.5 mM deoxyriboxylidine, and 0.01% 2-ME) (10–12). Cells were lysed by freeze-thawing (three cycles) and centrifuged at 100,000 × g for 90 min. Supernatants were incubated with 1 mM sphingosine (Avanti, Pelham, AL) and 20 mM [γ-32P]ATP (5 μCi/sample; Amersham), in a total volume of 200 μl, for 10 min at 37°C. [32P]S1P was isolated by TLC and quantitated with a phosphor imager.

Confocal microscopy

The maturational state of phagosomes was assessed by colocalization of four lysosomal markers: the acidophilic dye Lysotracker red and the lysosomal proteins cathepsin D, CD63, and lysosomal-associated membrane protein-1 (7, 8). The percentage of Mtb phagosomes that colocalized with the lysosomal marker was determined by counting ≥25 phagosomes from at least 10 different fields per condition.

Results and Discussion

To characterize the mechanism of mycobacterial evasion of Ca2+-mediated antimicrobial defenses, we compared the effects of live and killed Mtb (virulent H37Rv strain) on signaling pathways that couple surface receptors to elevations of [Ca2+]i. Incubation of human MPs labeled with the [Ca2+]i indicator fura 2 with serum-opsonized tubercle bacilli that had been killed by gamma irradiation induced a significant increase in MP [Ca2+]i (Fig. 1A). The kinetics of onset, magnitude, and duration of this elevation were indistinguishable from those induced by heat-killed Mtb or other complement-opsonized particles (7). In marked contrast, live, serum-opsonized Mtb induced no change in [Ca2+]i (Fig. 1A), despite being phagocytosed to the same extent as killed organisms (7, 8).

Phagocytosis of both live and killed Mtb by human MPs is primarily mediated by complement receptors (CRs), especially CR3, although additional receptors contribute to ingestion (13, 14). Furthermore, we have recently demonstrated a primary role for CRs in the increase in [Ca2+]i, that occurs upon phagocytosis of killed Mtb (7). Although CR-dependent Ca2+ signaling has not been previously characterized in human MPs, stimulation of neutrophil CRs has been reported to activate phosphatidylinositol-specific phospholipase C (PI-PLC) and initiate an inositol 1,4,5-triphosphate (IP3)-dependent increase in [Ca2+]i (15). To evaluate this mechanism in human MPs, we determined the effects of Mtb on inositol phosphate metabolism in MPs labeled with [3H]myoinositol. Incubation of MPs with either live or killed H37Rv Mtb was not associated with a detectable increase in inositol phosphate generation (Fig. 1B). Platelet-activating factor (PAF; 100 nM), which is known to initiate an inositol 1,4,5-triphosphate (IP3)-dependent increase in [Ca2+]i, produced no change in [Ca2+]i (Fig. 1C). The kinetics of onset, magnitude, and duration of this elevation were indistinguishable from those in MPs incubated with either live or killed H37Rv Mtb, or 100 nM PAF. Thapsigargin (TSG; 1 μM) was added as indicated. Levels of [Ca2+]i, were determined with fura 2, and tracings are representative of triplicate samples from five identical experiments using MPs from different donors. B, [3H]Myoinositol-labeled MPs were incubated with 20 mM LiCl and killed or live Mtb, or 100 nM PAF. [3H]Inositol phosphates were determined by anion-exchange chromatography. Data are expressed as a percentage of inositol phosphate generation by control cells, and represent the mean (±SEM) of duplicate determinations from three identical experiments.

Measurement of calcine-conjugated 1,4,5-triphosphate generation (IP3)

Calcine-conjugated IP3 production was determined by counting 25 phagosomes from at least 10 different fields per condition. Further evidence that Mtb does not activate PI-PLC was obtained with a competitive IP3-binding assay in which MP lysates were incubated with a fixed amount of IP3-binding protein and various concentrations of radiolabeled IP3 (15). No IP3 production was detectable in MPs incubated with live Mtb (0.021 pmol/106 cells ± 0.016, n = 4) or killed Mtb (0.016 ± 0.009), compared with buffer-treated control cells (0.024 ± 0.011), whereas PAF-stimulated cells produced a significant increase in IP3 (3.19 ± 0.21, p < 0.01). Thus, a PI-PLC- and IP3-dependent pathway is not a significant contributor to the elevation of MP [Ca2+]i that is stimulated by killed Mtb.

The SK-catalyzed conversion of sphingosine to S1P has recently been demonstrated to couple stimulation of diverse
plasma membrane receptors to cellular activation, including elevation of $[\text{Ca}^{2+}]_c$ (10, 17). To examine the potential role of SK in $\text{Ca}^{2+}$ signaling induced by killed Mtb, we used dihydroporphosine (DHS), a selective, competitive inhibitor of SK (11). Preincubation of MPs with 25 $\mu$M DHS completely inhibited the increase in $[\text{Ca}^{2+}]_c$ due to killed Mtb (Fig. 1A), without affecting the adherence or phagocytosis of the bacilli (not shown). The integrity of the intracellular $\text{Ca}^{2+}$ stores and the capacitative $\text{Ca}^{2+}$ entry mechanism in DHS-treated MPs were demonstrated by the rapid and sustained increase in $[\text{Ca}^{2+}]_c$, produced by thapsigargin (1 $\mu$M), a specific inhibitor of the endoplasmic reticulum $\text{Ca}^{2+}$-ATPase (Fig. 1A). The MP $[\text{Ca}^{2+}]_c$ response to PAF was unaffected by DHS, further illustrating the specificity of DHS-induced inhibition (Fig. 1A). These data are consistent with the hypothesis that killed, but not live, Mtb stimulates an increase in MP $[\text{Ca}^{2+}]_c$ via activation of SK.

To further test this hypothesis, levels of S1P (the product of SK activity) were directly determined in MPs preincubated with [3H]serine to radiolabel sphingolipids. Addition of killed Mtb produced a rapid increase in [3H]S1P that was maximal at 20–60 s and returned to baseline by 10 min following stimulation (Fig. 2A). The magnitude of the change in S1P levels induced by killed Mtb (123 ± 5% of the control value, $p < 0.01$, $n = 4$) was similar to that previously demonstrated in diverse cell types in response to growth factors, cytokines, and chemotactic factors (17), and was completely blocked by preincubation of MPs with DHS (Fig. 2A). In fact, DHS-treated cells exhibited a time-dependent decrease in levels of S1P (reductions of 37% at 1 min, and 73% at 10 min), despite addition of killed Mtb. This rapid depletion of S1P in DHS-treated cells is consistent with the hypothesis that levels of this bioactive lipid are normally maintained by a tightly regulated balance of synthetic and degradative pathways (17). In contrast to the stimulation of S1P generation by killed tubercle bacilli, infection with live Mtb resulted in no significant change in S1P levels (Fig. 2A).

To distinguish whether the lack of S1P production in response to live Mtb was due to inhibition of SK or to lack of activation of the enzyme, we determined the in vitro activity of SK in lysates from control and infected MPs. Lysates of MPs activation of the enzyme, we determined the in vitro activity of Mtb together, these data demonstrate that live Mtb in lysates from control and infected MPs. Lysates of MPs through the SK-dependent increase in $[\text{Ca}^{2+}]_c$, in CR3-transfected CHO cells. CHO-CR3 cells were incubated with live or killed H37Rv Mtb at an MOI of 10:1, and $[\text{Ca}^{2+}]_c$ was determined via fluorescence of fura 2. In selected experiments, CHO-CR3 were preincubated with 25 $\mu$M DHS for 15 min before addition of killed bacilli. Tracings are representative of triplicate samples from one of three identical experiments.

![FIGURE 2](http://www.jimmunol.org) Live Mtb inhibits MP SK activity. A. [3H]Serine-labeled MPs were incubated with 25 $\mu$M DHS or 0.1% ethanol solvent control before addition of live or killed H37Rv Mtb. At the indicated times, cells were lysed and S1P levels determined by TLC. B, MPs were incubated with either live or killed Mtb for the indicated times. Cells were disrupted by freeze-thaw and SK activity of the supernatant was determined via incorporation of [3P] into purified sphingosine. Data are expressed as a percentage of control values for S1P (A) or SK activity (B), and represent the mean (±SEM) of duplicate determinations from four separate experiments. The mean SK activity in control untreated MPs was 1.26 pmol/min/mg protein (range: 0.88–2.71).

![FIGURE 3](http://www.jimmunol.org) Killed, but not live, Mtb stimulates SK-dependent increase in $[\text{Ca}^{2+}]_c$. Because CRs, particularly CR3, are the primary mediators of phagocytosis of Mtb (13, 14), we used Chinese hamster ovary (CHO) cells transfected with the $\alpha$ (CD11b)- and $\beta$ (CD18)-chains of CR3 (CHO-CR3) (18) to further evaluate the SK-mediated elevations in $[\text{Ca}^{2+}]_c$. Incubation of CHO-CR3 cells with killed Mtb resulted in a significant increase in $[\text{Ca}^{2+}]_c$, accompanied by phagocytosis of the tubercle bacilli (not shown).
killed Mtb. In contrast, addition of live Mtb to control CHO-CR3 cells resulted in no change in [Ca^{2+}]_c (Fig. 3), despite equivalent levels of adherence and phagocytosis (not shown). CHO cells, transfected with empty vector, demonstrated no change in [Ca^{2+}]_c in response to either killed or live Mtb (not shown). These results support the hypothesis that the increase in MP [Ca^{2+}]_c induced by killed Mtb involves CR-dependent stimulation of SK activity.

To gain further information on the mechanism(s) of SK inhibition, lysates were prepared from control untreated MPs or MPs that exhibited elevated levels of SK activity due to prior treatment with killed Mtb. Addition of live Mtb to these lysates resulted in inhibition of both the control and stimulated levels of SK activity (reduction of 38–43%). These data are consistent with the hypothesis that live Mtb inhibits SK by multiple mechanisms, potentially including direct inhibition of the enzyme by mycobacterial components. However, the molecular complexity of the lysates precludes further conclusions at this time. Definitive assessment of this hypothesis will require purification of SK or its components physically interact with SK in infected MPs.

We have previously demonstrated that [Ca^{2+}]_c levels regulate phagosome maturation in Mtb-infected MPs, and that mycobacterial viability is a primary determinant of the level of [Ca^{2+}]_c (7, 8). Because the viability of Mtb is also a critical variable in activation vs inhibition of SK, we tested the hypothesis that stimulation of SK promotes phagosome maturation. Phagosomes containing killed Mtb exhibited several markers of maturation to phagolysosomes, including prominent staining on confocal microscopy for the lysosomal proteins lysosomal-associated membrane protein-1, cathepsin D, and CD63, as well as the acidotropic dye Lysotracker Red (Fig. 4). Preincubation of MPs with DHS significantly inhibited both the acquisition of the lysosomal protein markers and acidification of phagosomes containing killed Mtb, consistent with a requirement for SK activity for phagosome maturation. These data support the hypothesis that inhibition of SK by live virulent Mtb (Fig. 2) results in a block in Ca^{2+}-dependent phagosome maturation.

The mechanism by which live Mtb inhibits SK is unknown, but may involve direct and/or indirect mechanisms. Two mammalian SK isoforms, SK1 and SK2, have been identified (17), but their regulation, subcellular localization, and functions remain incompletely characterized. Because SK1, but not SK2, is inhibited by DHS (17), our results are consistent with the hypothesis that SK1 mediates the increase in [Ca^{2+}]_c induced by killed Mtb.

Multiple biochemical mechanisms regulate levels of [Ca^{2+}]_c. Although our data support a major role for SK in Mtb-induced inhibition of MP Ca^{2+} signaling, it is possible that additional mechanisms, including Ca^{2+} channels, pumps, and exchangers, may also be modulated by live mycobacteria. It is likely that the complexity of TB pathogenesis is due to evasion and/or interruption of multiple host regulatory mechanisms. Inhibition of SK, coupled with our previous demonstration of mycobacterial inhibition of phagosome-specific activation of calmodulin and Ca^{2+}-calmodulin kinase II (8), may be linked to vesicular trafficking defects identified by other investigators,

**FIGURE 4.** Inhibition of SK blocks phagosomal maturation. A, MPs were incubated in the absence (top row; control) or presence (bottom row) of 25 μM DHS, followed by addition of killed H37Rv Mtb. Samples were stained with anti-CD63 Ab (red, left column) and aquareline (green, middle column), and analyzed by confocal microscopy. The merged images (right column) demonstrate that colocalization of the lysosomal marker CD63 and killed Mtb (yellow bacillus, top row) is blocked by DHS (green bacilli, bottom row). B, Cumulative percentage of killed Mtb phagosomes that are positive for each of the lysosomal protein markers in control or DHS-treated MPs. Data are the mean (± range) of duplicate determinations from 25 phagosomes per sample from five different donors.
specifically exclusion of the vacuolar H\(^+\)/ATPase and alterations in Rab5, early endosomal Ag 1, and cellubrevin (2–6).

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**References**