**Mycobacterium tuberculosis** Sulfolipid-1 Activates Nociceptive Neurons and Induces Cough

**Highlights**

- An *Mtb* organic extract activates nociceptive neurons and induces cough in guinea pigs
- *Mtb* sulfolipid-1 is necessary and sufficient to trigger neuronal activation and cough
- Guinea pigs infected with an SL-1-deficient Mtb mutant do not cough

**In Brief**

*Mycobacterium tuberculosis* produces a glycolipid called sulfolipid-1 (SL-1) that triggers cough by activating nociceptive neurons.

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**Mycobacterium tuberculosis** Sulfolipid-1 Activates Nociceptive Neurons and Induces Cough

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**SUMMARY**

Pulmonary tuberculosis, a disease caused by Mycobacterium tuberculosis (Mtb), manifests with a persistent cough as both a primary symptom and mechanism of transmission. The cough reflex can be triggered by nociceptive neurons innervating the lungs, and some bacteria produce neuron-targeting molecules. However, how pulmonary Mtb infection causes cough remains undefined, and whether Mtb produces a neuron-activating, cough-inducing molecule is unknown. Here, we show that an Mtb organic extract activates nociceptive neurons in vitro and identify the Mtb glycolipid sulfolipid-1 (SL-1) as the nociceptive molecule. Mtb organic extracts from mutants lacking SL-1 synthesis cannot activate neurons in vitro or induce cough in a guinea pig model. Finally, Mtb-infected guinea pigs cough in a manner dependent on SL-1 synthesis. Thus, we demonstrate a heretofore unknown molecular mechanism for cough induction by a virulent human pathogen via its production of a complex lipid.

**INTRODUCTION**

A hallmark symptom of active pulmonary tuberculosis and a major mechanism of disease transmission is a persistent, sometimes bloody cough. The vast majority of de novo Mycobacterium tuberculosis (Mtb) infections are acquired from contact with actively infected, coughing individuals (Fenelley et al., 2004; Jones-López et al., 2015). Previous hypotheses regarding the mechanism of cough in pulmonary tuberculosis emphasized the role of infection-induced lung irritation and production of inflammatory mediators (Turner, 2019; Turner and Bothamley, 2015). However, empirical evidence for these hypotheses is lacking. We generated an alternative hypothesis that Mtb produces a cough-triggering molecule, thereby enhancing its own transmission.

The cough reflex, which may have evolved as a defense against aspiration of food and gastric contents or inhalation of irritants and infectious particles, is a complex, highly organized, neuromuscular response conserved across mammalian species (Brooks, 2011; Canning, 2008, 2010). Chemosensory afferent neurons innervating the upper airway and lungs respond to a variety of chemicals, including exogenous noxious molecules like capsaicin, particulates, and cigarette smoke (Mazzone, 2005; Mazzone and Undem, 2016). Such neurons have been directly visualized in whole mount biopsies of mouse, rat, guinea pig, and human lungs (De Proost et al., 2007a, 2007b; Pintelon et al., 2007), where their peripheral terminals encode both ion channel and G-protein-coupled chemosensory receptors (Mazzone, 2005; Mazzone and Undem, 2016). While pulmonary defense against and response to respiratory infection is considered a major function of the cough reflex, whether virulence molecules from cough-inducing pathogens directly stimulate airway afferent neurons is unknown (Cherry, 2013; Hewlett et al., 2014; Turner and Bothamley, 2015).

Recently, diverse species of bacteria have been shown to directly stimulate peripheral nerves. For example, by producing molecules that act on nerve receptors, Staphylococcus aureus causes pain (Chiu et al., 2013) while Mycobacterium ulcerans, a species related to Mtb, causes peripheral anesthesia through interaction of the polyketide mycolactone with neurons (Marion et al., 2014).

Because Mtb is mainly a respiratory pathogen that is predominantly transmitted via cough, cough is initiated by nociceptive neurons (Canning, 2007), and bacteria are capable of altering neuronal activity, we hypothesized that Mtb produces a molecule to stimulate cough through activation of respiratory nociceptive neurons thereby facilitating its spread from infected to uninfected individuals. To test this hypothesis, we first demonstrated that guinea pigs with pulmonary tuberculosis cough, and that an organic-phase extract of Mtb consisting primarily of lipid components of its cell wall and fat-soluble molecules is sufficient to...
induce cough. Exposing nociceptive neurons in vitro to the Mtb extract provokes a rapid increase in intracellular calcium, similar to the nociceptive molecule capsaicin. We determined that the active molecule in the Mtb extract is the mycobacterial lipid sulfo-lipid-1 (SL-1), an important component of the Mtb cell wall (Goren, 1970a, 1970b; Goren et al., 1971; Schelle and Bertozzi, 2006). Genetic disruption of SL-1 biosynthesis results in an Mtb extract that cannot trigger neurons in vitro or induce cough in guinea pigs. Finally, we find that guinea pigs infected with Mtb mutants lacking SL-1 synthesis fail to cough. This work represents the identification of a cough-inducing molecule from a virulent human pathogen, with major implications for the pathogenesis and transmission of tuberculosis.

**RESULTS**

**Mtb Infection Induces Cough in Guinea Pigs**

To determine if Mtb produces molecules that induce cough, we first established animal models of coughing, both during Mtb infection and with isolated organic Mtb extracts. Guinea pigs have long been used as a model for Mtb infection (Koch, 1912; Lurie, 1930d; Orme and Ordway, 2016; Perla, 1927) and as an established experimental model to test isolated compounds for cough induction (Laude et al., 1993; Tanaka and Maruyama, 2005; Zaccone et al., 2016). Although it was demonstrated 100 years ago that guinea pigs can transmit Mtb from infected to naive animals (Koch, 1912; Lurie, 1930a, 1930b; Perla, 1927), to date, no studies have reported whether guinea pigs cough in the context of Mtb infection. Thus, to first assess if guinea pigs with pulmonary tuberculosis cough, we infected outbred Hartley guinea pigs with the Mtb Erdman strain via aerosol and measured cough over 24 h using whole-body plethysmography (WBP) (Morice et al., 2007; Zaccone et al., 2016). We infected guinea pigs with ~200 colony-forming units (CFU) of Mtb, placed individual animals in WBP chambers for 24 h every 2 weeks and monitored cough events by plethysmography (Figures 1A and 1B). Coughs were quantified using a pressure transducer attached to the WBP chamber (Figure 1A). Each cough was automatically identified by a predetermined algorithm based on the box flow pattern (sharp increase and decrease of box flow) that measures the sharp inspiration and expiration of air during a cough (Figure 1B). Uninfected control and Mtb-infected guinea pigs showed no statistically significant difference in coughs at 2- or 4-weeks post infection. However, at 6 weeks post-infection, Mtb-infected guinea pigs had twice as many coughs (mean 12 versus 6) compared to control guinea pigs (Figure 1C). Thus, Mtb-infected guinea pigs have more coughs than uninfected animals 6 weeks after low-dose infection, at a time of maximal lung CFU (Basaraba et al., 2006; Orme and Ordway, 2016; Turner et al., 2003).

**An Organic Extract of Mtb Is Sufficient to Induce Cough in Guinea Pigs**

To determine if Mtb produces a mediator of neuronal activation and cough, we performed a Folch extraction (Folch et al., 1957) from Mtb grown in liquid culture to extract polar and non-polar lipids and tested if the organic phase of the extract (hereafter called “Mtb extract”) alone was sufficient to induce cough. We chose the organic fraction because the polyketide mycolactone of M. ulcerans is also isolated via chloroform: methanol extraction (George et al., 1998) and because Mtb has a lipid and organic metabolite rich cell wall with many active molecules (Jankute et al., 2015). We then used an established model of cough induction, namely, transient exposure of unrestrained, naive, healthy guinea pigs to noxious molecules (Laude et al., 1993; Zaccone et al., 2016).
1993; Tanaka and Maruyama, 2005) via a nebulizer and WBP
analysis to test if the Mtb extract could trigger cough. As an inde
pendent control, we exposed guinea pigs to 0.4 M citric acid, a
known cough agonist (Laude et al., 1993; Morice et al., 2007). Af
fter compound nebulization, we quantified coughs over 20 min as
previously reported (Laude et al., 1993; Tanaka and Maruyama,
2005; Zacccone et al., 2016). Each animal was exposed to vehicle
control, Mtb extract, or citric acid on alternating days separated
by a rest day to avoid tachyphylaxis. Neubulization of the vehicle
control for the Mtb extract did not trigger coughing, while expo
sure to the Mtb extract (20 mg/mL final) produced more coughs
(mean 8 versus 0.5 coughs in vehicle controls) (Figure 1D). As ex
pected, nebulization of 0.4 M citric acid resulted in a mean of 12
coughs in 20 min (Figure 1D). Thus, an Mtb organic-phase
extract induces cough in guinea pigs.

**Mtb Organic Extract Activates Nociceptive Neurons
In Vitro**

Having established that the Mtb extract alone is sufficient to
induce cough, we next investigated if the Mtb extract activates
nociceptive neurons in cell culture using live cell imaging of intra
cellular calcium dynamics. When nociceptive neurons are trig
gered by nociceptive agonists, they demonstrate an increase in
intracellular [Ca\(^{2+}\)] (Glaser et al., 2016) that can be quantified
using Ca\(^{2+}\) responsive dyes (Gryniewicz et al., 1985; Iatridou
et al., 1994; Kao et al., 2010). To first determine if the Mtb extract
can trigger neurons, we used the immortalized mouse embryonic
dorsal root ganglion (DRG) cell line, MED17.11, which demon
strates properties of nociceptive neurons (Doran et al., 2015).
We loaded MED17.11 cells with the fluorescent semiquantitative
Ca\(^{2+}\) dye Fluo-4 and monitored fluorescence using live cell imag
ing after exposure to either vehicle control, Mtb extract, or
capsaicin, a known transient receptor potential cation channel
subfamily V member 1 (TRPV1) agonist (Caterina et al., 1997). Af
fter treatment with either the positive control capsaicin or the Mtb
extract, we observed maximum intracellular [Ca\(^{2+}\)] responses at
~45 s, with the fluorescence returning to baseline by 90 s (Fig
ure 2A; Video S1). We quantified the maximum change in fluores
cence for each individual cell in the field of view using ImageJ
and then averaged the response (Figure 2B). Not every cell re
sponded to the Mtb extract or capsaicin, suggesting that the dif ferentiated MED17.11 cells may not be a uniform population.
However, in contrast to the vehicle control (DMSO), both Mtb
extract and capsaicin triggered a significant increase in intracel
lular [Ca\(^{2+}\)] (Figures 2A and 2B).

While Fluo-4 allows for a semiquantitative determination of intracellular [Ca\(^{2+}\)], the ratiometric dye Fura-2 allows quantification
of intracellular [Ca\(^{2+}\)] by determining the ratio of Fura-2 excitation
at 340 nm and 380 nM (Gryniewicz et al., 1985; Iatridou et al.,
1994; Kao et al., 2010). Thus, to confirm our results with Fluo-4,
we exposed Fura-2-loaded MED17.11 neurons to DMSO, the
Mtb extract, and capsaicin and monitored intracellular [Ca\(^{2+}\)]
over time for individual cells. Each compound was perfused
onto the neurons and then removed by washing before the follow
ing compound was added (Figure 2C). While DMSO perfu
sion did not alter intracellular [Ca\(^{2+}\)], Mtb extract triggered a rise
in intracellular [Ca\(^{2+}\)] at ~45 s after exposure as can be seen in in
dividual cell traces (Figure 2C), the average response (Figure 2D),
and the quantified response for all cells (Figure 2E). The response
of MED17.11 cells to Mtb extract was limited to capsaicin-responsive
neurons, as capsaicin non-responsive cells did not demon
strate a significant increase in intracellular [Ca\(^{2+}\)] (Figure S1).
Furthermore, the Ca\(^{2+}\) source was predominantly from intracel
lular Ca\(^{2+}\) stores as there was no difference in the change in intra
cellular [Ca\(^{2+}\)] if MED17.11 neurons were bathed in Ca\(^{2+}\)-contain
ing or Ca\(^{2+}\)-free media during activation by Mtb extract (Figure S2).
To determine whether the specificity of neuronal activation was
restricted to the organic compartment, we tested if other Mtb
compartment, including cell membrane extracts, cytosol frac
tions, soluble proteins from the cell wall, Triton X-114 soluble pro
teins, or secreted proteins can activate MED17.11 neurons. Only
the total lipid compartment activated neurons (Table 1).

To determine if the response to the Mtb extract could be
observed in primary mouse neurons, we first isolated and cul
tured murine DRG neurons, loaded them with Fura-2, and
exposed them to the Mtb extract. Similar to the MED17.11 cells,
primary mouse DRG neurons exposed to the Mtb extract had a
large increase in intracellular [Ca\(^{2+}\)] at ~45 s after perfusion, in
contrast to the DMSO control (Figures 2F–2H), and this response
was predominantly in capsaicin-responsive (TRPV1\(^{+}\)) neurons
(Figure S1). Because the cough reflex is mediated by vagal afferent
nerves whose cell bodies are found within the nodose
and jugular ganglia (Mazzzone and Undem, 2016), we also tested
if primary nociceptive neurons isolated from murine nodose and
jugular ganglia and loaded with Fura-2 could respond to Mtb
extract. As with the primary mouse DRG neurons, exposure of
mouse nodose/jugular ganglia neurons to the Mtb extract
demonstrated a significant increase in intracellular [Ca\(^{2+}\)]
entirely in capsaicin-responsive (TRPV1\(^{+}\)) cells, in contrast to the DMSO
control (Figures 2I–2K and S1). Thus, an organic Mtb extract is
sufficient to activate a mouse nociceptive neuronal cell line, pri
mary mouse DRG neurons, and primary mouse nodose/jugular
ganglia neurons in vitro.

We next assessed if the nociceptive neuron response to the Mtb
extract is evolutionarily conserved in humans by measuring the
change in intracellular [Ca\(^{2+}\)] of primary human DRG neurons ob
tained from deceased donors (n = 2, one male, one female) after
exposure to DMSO, Mtb extract, and capsaicin. Human nocicep
tive neurons were loaded with fluorescent Fluo-8AM dye and intra
cellular [Ca\(^{2+}\)] measured over time using live cell imaging (Figures
2L–2N). As with the mouse neurons, DMSO-treated human neu
rons had a minimal response both with respect to intracellular
[Ca\(^{2+}\)] and number of cells responding. Exposure to Mtb extract
generated a substantial increase in intracellular [Ca\(^{2+}\)] in multiple
cells that were both capsaicin-responsive and non-responsive
(Figures 2L–2N and S1). Of the total cell population cultured from
the human DRG, 56% in one donor and 100% in another donor
of capsaicin-responsive (TRPV1\(^{+}\)) neurons were activated by the
Mtb extract (Table S1). Thus, an organic Mtb extract activates
both mouse and human nociceptive neurons in vitro.

**Identification of the Nociceptive-Neuron Activating
Molecule as Sulfolipid-1**

Because we performed all of the preliminary experiments
with the Mtb Erdman strain, we next determined the mycobacte
rial species specificity of the neuron-activating activity of
mycobacterial organic extracts. We prepared organic extracts from a variety of mycobacterial species including virulent and non-virulent Mtb species, along with non-tuberculous mycobacteria (NTM), and tested their ability to trigger increased intracellular \([\text{Ca}^{2+}]\) in MED17.11 neurons. Of the extracts tested, neuronal activation overlapped with virulent bacteria in the Mtb complex (Figure S3; Table 2). We noted that while the virulent Mtb H37Rv strain activated neurons, its attenuated derivative H37Ra (Steenken and Gardner, 1946; Steenken et al., 1934) did not (Table 2). Because H37Ra has a mutation in the PhoP transcription factor that prevents it from producing the cell wall glycolipid SL-1 (Chesne-Seck et al., 2008), we obtained pure SL-1 and tested if it was sufficient to activate MED17.11 cells. Consistent with the lack of SL-1 in the H37Ra strain compared to H37Rv, pure SL-1-activated MED17.11 cells (Figure 3A).

SL-1 is the most abundant sulfated glycolipid located in the outer membrane and cell wall of mycobacteria (Middlebrook et al., 1959), and its presence is unique to pathogenic mycobacteria (Goren et al., 1974). The structure of SL-1 includes a disaccharide trehalose-2-sulfate (T2S) core that is modified with four fatty acyl units (typically 2 hydroxyphthioceranic acids, 1 phthioceranic acid, and 1 palmitic acid) (Layar et al., 2011) (Figure 3B, left). In addition to SL-1, Mtb produces several similarly complex cell wall lipids such as trehalose monomycolate (TMM), trehalose dimycolate (TDM; also known as cord factor), phthiocerol dimycocerosate (PDIM), and others (Figure 3B, center; Table 1). Thus, to assess the chemical specificity of the neuronal response to SL-1, we first determined if the response to SL-1 was saturable by performing a dose response experiment using MED17.11 neurons, yielding an EC_{50} for SL-1.
of 33 nM (Figure 3C). We next determined the ability of similarly complex molecules to activate nociceptive neurons by exposing MED17.11 cells to purified Mtb lipids. Of the tested lipids, only SL-1 activated neurons (Figure 3A; Table 1). In addition, only extracts from mycobacterial species that produce complex sulfated glycolipids were able to activate MED17.11 neurons including species of the Mtb complex (producers of SL-1) and M. avium (producer of a sulfated glycopeptidolipid but not SL-1) (Mougous et al., 2002a) (Figure S4; Table 2). Although M. smegmatis has been reported to produce small sulfated compounds (Mougous et al., 2002b; Rivera-Marrero et al., 2002), it does not produce SL-1 (Mougous et al., 2002b; Rivera-Marrero et al., 2002), and an organic extract from M. smegmatis did not activate MED17.11 neurons (Figure S4; Table 2). Finally, mammalian cells including oligodendrocytes and Schwann cells produce a sulfated sphingolipid called sulfatide (3-O-sulfogalactosylceramide) (Figure 3B, right) that comprises 4% of myelin lipids (Takahashi and Suzuki, 2012). To determine if mammalian sulfatide could also activate nociceptive neurons and to further test the chemical specificity of neuronal activation, we exposed MED17.11 neurons to sulfatide and observed that neither sulfatide nor its non-sulfated analog galactocerebroside (Figure 3B) were able to trigger increased intracellular [Ca^{2+}] (Figure 3D). Thus, SL-1 alone can activate neurons and the neuronal response to SL-1 does not represent a generic response to non-sulfated mycobacterial cell wall glycolipids or sulfated monosaccharide lipids.

SL-1 is synthesized in mycobacteria through an enzymatic pathway initiated by sulfation of the symmetric disaccharide trehalose to trehalose-2-sulfate (T2S) by the enzyme sulfotransferase 0 (Stf0), followed by a series of acylation reactions (Figure 3E) (Seeliger et al., 2012). Importantly, genetic deletion of stf0 in Mtb results in a failure to produce SL-1 (Mougous et al., 2004). To determine if SL-1 synthesis was necessary for neuronal activation, we tested organic extracts from Mtb Erdman wild-type, Mtb ErdmanΔstf0, and the complemented strain Mtb ErdmanΔstf0::stf0 for their ability to activate MED17.11 neurons. While the organic extract from the Mtb ErdmanΔstf0 strain failed to activate neurons, both the Mtb wild-type and Mtb ErdmanΔstf0::stf0 extracts triggered neuronal activation (Figure 3F). We also verified that SL-1 was present in the wild-type and Mtb ErdmanΔstf0::stf0 extracts, but not in the Mtb ErdmanΔstf0 extract (Figure S5). To confirm that the Mtb ErdmanΔstf0 extract did not suppress the response of MED17.11 cells to another ligand, we exposed MED17.11 cells first to the Mtb ErdmanΔstf0 extract followed by pure SL-1 and observed that MED17.11 retained their ability to respond (Figure 3G).

In order to characterize the minimal SL-1 precursor capable of activating nociceptive neurons, we prepared extracts from Mtb mutants at each step of the SL-1 biosynthetic pathway and tested their ability to activate MED17.11 cells. As expected from prior studies (Converse et al., 2003; Kumar et al., 2007; Seeliger et al., 2012), pathway mutants accumulated precursor molecules through mass action (Figure S5). Interestingly, while an extract from the Mtb ErdmanΔstf0 again failed to activate neurons, extracts from mutants in all subsequent steps retained a modest ability to activate neurons (Figure 3H). Thus, our genetic analysis demonstrated that while SL-1 precursors such as SL-659 and SL-1278 potentially activate neurons, T2S is the minimal molecule capable of neuronal activation in Mtb extracts. To confirm this result, we chemically synthesized T2S (Figure S6) and tested if it could also activate neurons. Consistent with the genetic results (Figure 3H), while the non-sulfated precursor trehalose did not activate neurons, chemically synthesized T2S activated neurons (Figure 3I). Thus, synthesis of SL1 is both necessary and sufficient to activate MED17.11 neurons in vitro, and the organically synthesized precursor T2S also activates MED17.11 neurons.

### Table 1. Mycobacterial Compartments or Compounds and Ca^{2+} Response

<table>
<thead>
<tr>
<th>Compound</th>
<th>Triggers increased [Ca^{2+}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>–</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>+</td>
</tr>
<tr>
<td>M. canettii total lipids</td>
<td>+</td>
</tr>
<tr>
<td>H37Rv total lipids (normoxic)</td>
<td>+</td>
</tr>
<tr>
<td>H37Rv total lipids (hypoxic)</td>
<td>+</td>
</tr>
<tr>
<td>CDC1551 cell membrane extract</td>
<td>–</td>
</tr>
<tr>
<td>HN878 cell membrane extract</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv cell membrane extract</td>
<td>–</td>
</tr>
<tr>
<td>CDC1551 cytosol fraction</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv cytosol fraction</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv soluble cell wall proteins</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv TX-114 soluble proteins</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv purified liporabinomannan (LAM)</td>
<td>–</td>
</tr>
<tr>
<td>CDC1551 culture filtrate proteins</td>
<td>–</td>
</tr>
<tr>
<td>HN878 culture filtrate proteins</td>
<td>–</td>
</tr>
<tr>
<td>Sulfolipid-1</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose monomycolate</td>
<td>–</td>
</tr>
<tr>
<td>Trehalose dimycocerolate</td>
<td>–</td>
</tr>
<tr>
<td>C24:1 mono-sulfo-galactosyl(1) ceramide (d18:1/24:1)</td>
<td>–</td>
</tr>
<tr>
<td>Galactocerebroside</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv phosphatidylinositol mannosides 1 &amp; 2 (PIM1,2)</td>
<td>–</td>
</tr>
<tr>
<td>H37Rv phthiocerol dimycolerate (PDIM)</td>
<td>–</td>
</tr>
<tr>
<td>Trehalose</td>
<td>–</td>
</tr>
<tr>
<td>Trehalose-2-sulfate</td>
<td>+</td>
</tr>
</tbody>
</table>

Various mycobacterial compartments including cell wall, lipid and protein mixtures, or isolated compounds were tested for their ability to induce increased [Ca^{2+}] in MED17.11 neurons.
Table 2. Bacterial Strain Extract, Production of SL-1, and Ca²⁺ Response

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Produces SL-1</th>
<th>Triggers Increased [Ca²⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> Erdman</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> HN878</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> CDC1551</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Ra</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>M. marinum</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. smegmatis</em> (mc²²/¹⁵⁵)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. canettii</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em> (MACH1)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Extracts from various mycobacterial species were prepared and tested for their ability to induce increased [Ca²⁺] in MED17.11 neurons. Production of sulfolipid by each bacterial species is also shown.

DISCUSSION

In this study, we determined that both pulmonary Mtb infection and an organic extract from Mtb induce cough in guinea pigs, and an Mtb organic extract activates a neuronal cell line and primary neurons from both mice and humans. Through a combination of intracellular Ca²⁺ imaging assays with mycobacterial extracts and isolated Mtb lipids, we identified SL-1 as the mycobacterial product responsible for neuronal activation. Genetic ablation of SL-1 synthesis in Mtb prevented neuronal activation and cough induction by Mtb extracts, and SL-1 alone was sufficient to induce cough in naive guinea pigs. Finally, guinea pigs infected with Mtb lacking SL-1 synthesis had a blunted cough response despite having a high bacterial burden and pulmonary pathology consistent with active tuberculosis. Together, our data demonstrate a previously undescribed role for Mtb SL-1 in nociceptive neuron activation and cough induction.

SL-1 was identified as a major component of the Mtb cell wall more than 60 years ago (Middlebrook et al., 1959), but its activity has remained elusive despite the fact that SL-1 represents 1% of the dry weight of Mtb (Goren, 1970a). Because virulence of Mtb strains correlated with the abundance of SL-1 (Goren et al., 1974), it was proposed nearly 5 decades ago that SL-1 is an Mtb virulence factor. However, Mtb mutants that fail to produce SL-1 are not attenuated in mice (Gilmore et al., 2012; Rousseau et al., 2003) or guinea pigs (Rousseau et al., 2003) although SL-1 deficiency enhances Mtb survival within human macrophages potentially through increased resistance to cationic peptides (Gilmore et al., 2012). Our findings are consistent with the in vivo evidence that SL-1 is not a classic virulence factor that enhances Mtb survival. Based on our cell culture and in vivo data, and the observation that only pathogenic mycobacteria produce SL-1 (this report and reviewed in Daffe et al., 2014; Minnikin et al., 2002), we uncover a heretofore unrecognized function of SL-1 in nociceptive neuron activation and cough induction. Because cough is a major mechanism of Mtb transmission, we propose...
Figure 3. Identification and Characterization of SL-1 as the Nociceptive-Neuron Activating Molecule

(A) Max ΔF of Fluo-4 loaded MED17.11 cells after treatment with DMSO, Mtb Erdman organic extract (Mtb), sulfolipid-1 (SL-1), trehalose (Tre), trehalose monomycolate (TMM), and trehalose dimycolate (TDM).

(B) Structures of SL-1, TMM, TDM, sulfatide, and galactocerebroside.

(C) Dose response of SL-1 using Fura-2 loaded MED17.11 cells. EC_{50} calculated by nonlinear regression analysis.

(D) Max ΔF/F₀ of Fluo-4 loaded MED17.11 cells after treatment with DMSO, Mtb organic extract, SL-1, sulfatide (Sulf) or galactocerebroside (Gal).

(E) Biosynthetic pathway of SL-1, Stf0 (trehalose 2-sulfotransferase; Rv0295c), PapA2 (polyketide synthase-associated protein A2; acyltransferase; Rv3820c), PapA1 (polyketide synthase-associated protein A1; acyltransferase; Rv3824c), Sap (sulfolipid-1-addressing protein; sulfolipid exporter; Rv3821), Chp1 (cutinase-like hydrolase protein; SL1278 acyltransferase; Rv3822), and MmpL8 (sulfolipid-1 exporter, Rv3823c).

(F) Max ΔF/F₀ of Fluo-4 loaded MED17.11 cells after treatment with wild-type Mtb, MtbΔstf0 (Δstf0), and MtbΔstf0::stf0 complemented (stf0::stf0) extracts.

(legend continued on next page)
that SL-1 is a major contributor to the spread of Mtb from infected to naive individuals.

In addition to Mtb, other mycobacterial species interact with the nervous system through production of complex molecules. For example, *M. ulcerans*, the etiologic agent of Buruli ulcer, causes peripheral anesthesia through the direct activity of a polyketide known as mycolactone (George et al., 1999) on peripheral nerves (Marion et al., 2014). To date, mycolactone has not been isolated from Mtb. Conversely, *M. ulcerans* does not produce SL-1 owing to genomic loss of the pks2 locus (Stinear et al., 2007), suggesting that each species has evolved unique neuromodulatory molecules to facilitate its own lifestyle. Indeed, neuromodulatory molecules have also been observed in gram-negative and gram-positive organisms. Both lipopolysaccharide from *E. coli* (Ochoa-Cortes et al., 2010) and formyl peptides and alpha-hemolysin from *Staphylococcus aureus* (Chiu et al., 2013) activate nociceptive neurons to trigger a pain response and inhibit innate immunity (Baral et al., 2018; Pinho-Ribeiro et al., 2018). Thus, direct neuronal engagement by bacterial molecules to either enhance or suppress cellular responses reflects a common virulence strategy of pathogens.

We tested a variety of Mtb isolates and mycobacterial strains for their ability to produce SL-1 and increase intracellular [Ca²⁺] in neurons. We observed a direct relationship between the presence of a complex sulfolipid and neuronal activation. Notably, in the matched pair of virulent (H37Rv) and attenuated (H37Ra) strains, SL-1 synthesis is lacking due to a mutation in PhoP (Chesne-Seck et al., 2008), a phenotype that is corroborated in Mtb mutants of the PhoPR two-component system (Gonzalo-Asensio et al., 2006; Walters et al., 2006). Likewise, an extract from *M. bovis* did not increase neuronal [Ca²⁺] and did not have SL-1, in accordance with the observations that *M. bovis*, like H37Ra, is also mutated in the PhoP/PhoR two component system (Gonzalo-Asensio et al., 2014). While children typically develop either cervical lymphadenopathy or gastrointestinal disease when infected with *M. bovis* owing to ingestion of contaminated dairy products (Dankner et al., 1993), primary pulmonary disease occurs in ~50% of adults, in line with probable airborne transmission.

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Figure 4. SL-1 Activates Both Mouse and Human DRGs In Vitro

(A–C) Intracellular [Ca²⁺] measurement using MED17.11 cells loaded with Fura-2.

(D–F) Intracellular [Ca²⁺] measurement using primary mouse DRG neurons loaded with Fura-2.

(G–I) Intracellular [Ca²⁺] measurement using primary mouse nodose/jugular ganglia neurons loaded with Fura-2.

(J–L) Intracellular [Ca²⁺] measurement using primary human DRG neurons loaded with Fura-8AM.

Dashed lines represent the addition of SL-1. For each, the ΔF/F₀ trace of representative neurons from a single dish (A, D, G, and J), the average ΔF/F₀ trace for all neurons in a single dish (B, E, H, and K), and the maximum change in ΔF/F₀ fluorescence ratio combining the data from individual neurons in 2 or more experiments (minimum 50 cells) (C, F, I, and L) are shown. For experiments with mouse neurons (MED17.11, DRG and nodose/jugular) representative experiments of at least 3 are shown. For human DRG neurons, shown is the combined data from two donors. Error bars in (C), (F), (I), and (L) are SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001 by Student’s t test.

(G) Max ΔF/F₀ of Fluo-4 loaded MED17.11 cells after treatment with Mtb extract, pure SL-1, MtbΔstf0 (Δstf0), or reactivation of MtbΔstf0 extract treated cells by the addition of SL-1. *p < 0.05 by paired Student’s t test for the MtbΔstf0 versus restimulation experiment.

(H) Max ΔF/F₀ of Flu-4 loaded MED17.11 cells after treatment with extracts from Mtb mutants in the SL-1 synthesis pathway. **p < 0.01, ***p < 0.005, ****p < 0.0001 by Kruskal-Wallace (A, D, F, G, H) or Friedman’s test (I).
acquisition either from infected livestock or humans (Dankner et al., 1993; Vayr et al., 2018). Because cough is a feature of pulmonary tuberculosis disease caused by M. bovis in both livestock and humans, it is likely that molecules other than sulfolipids are involved in M. bovis-mediated cough and transmission. In contrast to M. bovis and Mtb H37Ra, an organic extract from the NTM species M. avium also activated neurons, consistent with its known production of a complex sulfated glycopeptidolipid (Mougous et al., 2002a). Chronic cough is a feature of M. avium infection in immunocompromised individuals such as those with human immunodeficiency virus (HIV) (Daley, 2017), cystic fibrosis (Martiniano et al., 2016), andLady Windermere syndrome (Reich and Johnson, 1992). While the general consensus is that most primary pulmonary NTM infections are due to exposure to environmental reservoirs (Daley and Griffith, 2010; Griffith et al., 2007; Jeon, 2019), there are conflicting data on the possibility of human-to-human transmission of NTM (Bryant et al., 2013, 2016; Doyle et al., 2019). Thus, as with M. bovis, M. avium induced cough and possible airborne transmission occur in an SL-1 independent manner, although for M. avium its sulfated glycopeptidolipid may have an analogous activity.

In addition to Mtb, many viral and bacterial organisms such as measles virus, rhinovirus, coronavirus, influenza virus, Bordetella pertussis, and Mycoplasma pneumonia have cough as a primary symptom and are transmitted via airborne or droplet particles. In the case of airway viruses, it has been proposed that infections can sensitize cough-evoking sensory nerves through upregulation of receptors involved in cough hypersensitivity (Abdullah et al., 2014; Omar et al., 2017), induction of inflammatory cytokines, leukotrienes, and neuropeptides (Dicpinigaitis, 2014; Footitt and Johnston, 2009), and excess production of airway...
mucous (Nadel, 2013). Some infections can also cause cough after pathogen clearance. Such post-infectious cough states are similar to pain responses in other tissues that are mediated by nociceptive sensory neurons such that pain signaling commonly outlasts the presence of the original stimulus (Price and Inyang, 2015; Reichling and Levine, 2009). Thus, post-infectious cough might be similar to a chronic pain state where there is ongoing afferent nociceptive input for weeks or months after the injury has healed. Like viral pathogens, Mtb also induces production of a variety of eicosanoids and leukotrienes such as leukotriene B(4) (Tobin et al., 2010) that can function as an agonist of the TRPV1 cough receptor (Koskela et al., 2012). In addition, extracellular ATP (eATP), a damage-associated molecular pattern (Schmid and Evans, 2019) that can modulate host immunity to Mtb infection (Lammas et al., 1997; Molloy et al., 1994) is a ligand for the purinergic ion channel family (P2X). As the P2X3 receptor has been identified as a cough receptor and possible drug target for chronic cough (Abdulqawi et al., 2015; Bonvini and Belvisi, 2017; Garceau and Chauret, 2019), it is also possible that eATP could activate neuronal P2X3 receptors during Mtb infection. In this study, animals infected with an Mtb strain deficient only in SL-1 synthesis had significantly reduced cough. While we did not measure eicosanoids, leukotrienes or eATP in the lungs of infected animals, overall lung inflammation was equal between wild-type and mutant Mtb infected guinea pigs. Furthermore, our observation that pure SL-1 and wild-type Mtb, but not MtbΔslf0, organic extract rapidly induced cough in naive guinea pigs suggests that the mechanism of cough is through direct activation of a neuronal receptor by SL-1 rather than indirectly through production of a secondary metabolite. Thus, we identify SL-1 as a pathogenic bacterial product with cough-inducing activity.

In this work, we demonstrate that SL-1 is necessary and sufficient for cough induction in guinea pigs. Because guinea pigs and humans demonstrate a similar cough reflex to noxious agonists (Canning, 2008; Laude et al., 1993), SL-1 may also induce cough in humans. Although a link between Mtb virulence and the production of sulfolipids has previously been suggested (Goren et al., 1974), whether SL-1 is required for Mtb transmission remains unknown (Shiloh, 2016). Among the Mtb strains we tested, the HN878 W-Beijing strain is considered to be the most transmissible based on epidemiologic data (Karmakar et al., 2019; Kato-Maeda et al., 2010), and in our hands it produces SL-1 like the virulent laboratory strains Mtb Erdman, CDC1551, and H37Rv. Because our mass spectrometry analysis was qualitative, we are unable to correlate epidemiologic transmission data with SL-1 production. We are currently developing methods to quantify SL-1 in mycobacterial extracts using mass spectrometry as well as methods to safely test Mtb transmission between experimental animals in large scale. Future work will test the impact of SL-1 and cough in the transmission of Mtb as well as the role of putative SL-1 receptors in these processes.

Our results reveal a previously undescribed role for a mycobacterial glycolipid, SL-1, in neuronal activation and cough induction. These findings provide new directions not only in the study of Mtb pathogenesis and transmission, but also a framework for studies on other respiratory pathogens spread by the airborne route. Identification of unique, cough-inducing, pathogen-derived molecules and their host receptors could lead to the development of novel therapeutics that mitigates the spread of disease. For Mtb, in the absence of an effective vaccine to prevent disease acquisition by naive humans, a cough-inhibiting adjuvant therapy has the potential to significantly reduce transmission with major global health implications.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cell.2020.02.026.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


### STAR★METHODS

#### KEY RESOURCES TABLE

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LEAD CONTACT AND MATERIALS AVAILABILITY

Please direct requests for resources and reagents to Lead Contact, Michael Shiloh (Michael.Shiloh@utsouthwestern.edu). Distribution of T2S will require signing a Material Transfer Agreement (MTA) in accordance with the policies of the University of Texas Southwestern Medical Center.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Guinea Pig Studies
Male Hartley outbred guinea pigs aged 4-6 weeks (weight 200-250 g) were purchased from Charles River Laboratories. Guinea pig health status was monitored daily. For cough studies with Mtb extracts or compounds, animals were naive at the outset of the study. Each experimental animal was treated with vehicle control and extract or compound separated by a day to avoid tachyphylaxis. Guinea pigs were housed in standard cages with Alpha-dri bedding (Shepherd Specialty Papers, Kalamazoo, MI) and provided food and water ad libitum. Autoclaved pressed timothy hay cubes (Bioserv, Flemington, NJ) were also provided. Guinea pig experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center and followed the eighth edition of the Guide for the Care and Use of Laboratory Animals. The University of Texas Southwestern is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Mouse Studies
Institute for Cancer Research (ICR) outbred mice originally purchased from Envigo were bred at the University of Texas at Dallas and used for DRG or nodose/jugular ganglion neuron preparation at 4 weeks of age (weight 18-20 g). Mice were housed in standard cages with aspen wood bedding (Lab Supply, Fort Worth, TX) placed on ventilated rack systems with food and water ad libitum. Cotton squares were also provided for nesting. Mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center (protocol #14-04) and followed the eighth edition of the Guide for the Care and Use of Laboratory Animals.

Human DRG Studies
Primary human nociceptive neurons were isolated from DRGs obtained from two anonymous donors. Donor 1 was a 33-year-old female and Donor 2 was a 20-year-old male. All human DRGs used for this study were obtained from brain-dead organ donors in the United States after obtaining informed consent in accordance to Federal and State regulations, and United Network for Organ Sharing (UNOS) policies (Anabios, San Diego, CA).
Bacterial strains and culture conditions

*M. tuberculosis* Erdman was the predominant lab strain used in the study. Wild-type and mutant strains of *M. tuberculosis* were grown in Middlebrook 7H9 medium or on Middlebrook 7H11 plates supplemented with 10% oleic acid-albumin-dextrose-catalase. Freshly prepared tween 80 (Fisher T164-500) was added to liquid medium to a final concentration of 0.05%. All other mycobacterial species (Table 2, Bacterial strains and Key Resources Table) were grown under similar conditions.

Cell lines

MED17.11 cells (Doran et al., 2015) were provided by M. Nassar and were grown using DMEM F12/ Glutamax (Thermo) with pen/strep/glutamine (100 U/mL penicillin, 100 μg/mL streptomycin, 292 μg/mL L-glutamine, Corning), 50 units/mL interferon gamma (R&D Systems), 0.5% chick embryonic extract (US Biological). The MED17.11 cell line was not authenticated as mouse short tandem repeat testing was not available at the time the studies were performed. Undifferentiated cells were maintained at 33°C and 5% CO2 in a humidified incubator. For differentiation and microscopy, 5 × 10⁴ cells were plated onto 35 mm dishes (MatTek) and supplied with differentiation media consisting of DMEM/F12, pen/strep/glutamine, 10% FBS, 10 ng/mL b-FGF (R&D Systems), 0.5 mM di-butyryl cAMP (Sigma), 25 μM forskolin (R&D Systems), 5 μg/mL rock inhibitor Y-27632 (R&D Systems), 100 ng/mL NGF (R&D Systems) and 20 ng/mL GDNF (Sigma). Cells were then maintained at 37°C and 5% CO2 in a humidified incubator for 7 days, changing the media every 2-3 days.

METHOD DETAILS

Extraction of mycobacterial lipids

Mycobacteria were grown to an OD₆₀₀ of 0.8 in Middlebrook 7H9 supplemented with 0.01% tween 80. Then, 200 mL of bacteria were collected by centrifugation at 3500 RPM (Allegra X-14R) for 10 min, resuspended in 2 mL of PBS, and transferred to a glass tube containing 45 mL chloroform-methanol (2:1, vol/vol), and placed in a 58°C water bath for 16 hr. Samples were centrifuged at 2000 RPM (Allegra X-14R) for 5 min, and the upper-phase and cell debris was removed. The remaining organic phase was washed with 8 mL of water followed by a final wash with 2 mL methanol-water (1:1, vol/vol) to remove any remaining polar components. Extracts were then dried down using a RotoVap (IKA HB10) or under nitrogen.

Mass spectrometry

Extracts from mycobacteria were first dried down. Dried extracts were then resuspended in 1:1 MeOH:IPA 16 mM NH₄F at a 4 mg/mL final concentration. Samples were manually infused with a syringe on a quadrupole TOF TripleTOF 6600+ mass spectrometer (SCIEX, Framingham, MA). Electrospray ionization source parameters were as follows: ion source gas 1 (GS1) and gas 2 (GS2) set to 25 and 55 psi, respectively; curtain (Cur) gas set to 25 psi; source temperature of 300°C; and ion-spray voltage of −4,500 V in the negative ionization mode. GS1 and GS2 were zero-grade air, while Cur gas was nitrogen. TOF scans from 100-3000 m/z were collected over 60 s with a flow rate of 10 μL/min. Data were analyzed in PeakView (SCIEX) to identify SL659 at 659-661 m/z, SL1278 at 1277-1279 m/z, and SL-1 at 2000-3000 m/z.

Mouse DRG neuron isolation and culture

Primary mouse DRGs from male ICR mice were extracted, digested and cultured following an established protocol (Burton et al., 2017). Briefly, once dissected, DRGs were placed on ice in Hank’s Balanced Salt Solution (HBSS) (Invitrogen). Ganglia were enzymatically dissociated by serial addition of collagenase A (1 mg/ml, Roche Diagnostics, Basel, Switzerland), collagenase D (1 mg/ml; Roche Diagnostics, Basel, Switzerland) and papain (30 μg/ml; Roche Diagnostics, Basel, Switzerland) at 37°C in HBSS, followed by addition of a trypsin inhibitor (1 mg/ml; Roche) that contained bovine serum albumin (bovine serum albumin, 1 mg/ml; Fisher), and the ganglia were further mixed with a polished Pasteur pipette. Disaggregated tissue was then filtered through a 70 μm nylon cell strainer (Falcon; Corning, NY) and resuspended in Dulbecco’s modified Eagle’s medium F-12 GlutaMax media (Invitrogen) containing 10% fetal bovine serum (Hyclone Laboratories, Inc., South Logan, UT) and 1 μg/mL streptomycin (Invitrogen). The media also contained nerve growth factor (10 ng/ml; Millipore, Billerica, MA) and 5-fluoro-2'-deoxyuridine + uridine (3.0 μg/ml + 7.0 μg/ml; Sigma) to reduce proliferation of glia and fibroblasts. Neurons were cultured on 35 mm dishes (MatTek) coated with poly-D-lysine at 37°C with 95% air and 5% CO2 for 3-5 days. All DRGs from 4-6 mice were combined to generate approximately 4-6 dishes of primary cells per experiment.

Mouse nodose/jugular ganglia neuron isolation and culture

Primary mouse nodose/jugular ganglia from male ICR mice were extracted, digested and cultured following an established protocol similar to the DRG neuron isolation with slight modifications (Burton et al., 2017). Both sides of the nodose/jugular ganglia from 8-9 mice were combined to generate 2 dishes of primary cells. Neurons were cultured on 35 mm dishes (MatTek) coated with poly-D-lysine (Sigma) and laminin (Sigma) at 37°C with 95% air and 5% CO₂ overnight.
Human DRG neuron isolation and culture
DRGs from 2 human donors from the first thoracic vertebra (T1) through the first sacral vertebra (S1) were used in the present study. The DRGs were stripped of connective tissue and enzymatically digested at 37°C for 2 hr using the methods described by Davidson et al. (2014). Dissociated cells were seeded on 96-well plastic bottom plates (Corning) that had been pre-coated with poly-D-lysine. Cells were maintained in culture at 37°C with 5% CO2 in DMEM/F12 supplemented with 10% horse serum (Thermo Fisher Scientific), 2 mM glutamine, 10 ng/ml hNGF (Cell Signaling Technology), 10 ng/ml GDNF (Peprotech), and penicillin/streptomycin (Thermo Fisher Scientific). Half of the culture media was replaced with fresh media every 3 days.

Live-cell intracellular calcium imaging of MED17.11 neurons and primary mouse DRG and nodose/jugular ganglion neurons
Cells on 35 mm dishes (MatTek) were loaded with Fluo-4 (Thermo, F10471) or Fura-2 (Thermo, F1221). The dye was removed and 1 mL of HBSS with 20 mM HEPES, pH 7.4 (Thermo, 14170112) was added to each dish for 10 min prior to microscopy. A brightfield image was taken prior to cell stimulation and imaging. Fluo-4 microscopy was performed using a spinning disk confocal microscopy at 40X (PerkinElmer) with Velocity imaging software (Perkin Elmer). Cells were imaged at 488 nm. After a 10 s baseline recording, cells were treated with controls or extracts for 30 s and images were obtained every 1 s. A waiting period of 2-5 min was performed between treatment of cells with another molecule to minimize neurons being desensitized.

For Fura-2 experiments, after dye loading, cells were placed in 1 mL of HBSS with 20 mM HEPES for 10 minutes prior to recording. Using an Olympus IX73 inverted microscope, data was acquired with MetaFluor Fluorescence Ratio imaging software (Olympus) at excitation wavelengths of 340/380 nm and emission wavelength of 510 nm with image acquisition every 1 s. A 100 s baseline was first recorded after which DMSO was added and recording was obtained for 300 s. Next, compounds or extracts were added with continuous recording for 300 s. Finally, 200 nM was capsaicin added with continuous recording for 300 s.

Live cell calcium imaging of human DRG neurons
Experiments were conducted on isolated human DRG neurons from post-mortem donors by Anabios, Inc., San Diego, CA. Cells were loaded with 3 μM Fluo-8-AM (AAT Bioquest) containing 0.1% Pluronic F-127 (Sigma) for 30 min at room temperature. Extracellular solution contained in (mM): 145 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose adjusted to pH 7.4 with NaOH. Fluo-8-loaded cells were excited at 480 nm and emission was collected at 520 nm with a pcoEDGE sCMOS camera (PCO) mounted on an inverted microscope (Olympus IX71). Images were acquired at 0.2 Hz for 5 min, adding the compound of interest or the vehicle (1% DMSO) after 30 s for a total of 2 min, then washing with the external solution. At the end of the response profiling, an additional application of 200 nM capsaicin (Sigma) was performed to identify the nociceptor human DRG neurons. Image acquisition and data analysis were performed using MetaMorph software (Molecular Devices).

Whole-Body Plethysmography
Guinea pigs were placed inside a specialized chamber (Data Sciences International) fitted with a sensitive pressure transducer and an opening for a nebulization device to aerosolize compounds into the chamber. Using the Buxco FinePointe software, the primary cough data recorded were the Bias Flow of the system, the slope of the Bias flow, and the Delta Half Peak Crossing (DHPC) which is the time it takes to transition from the compression phase to the expulsive phase during respiration (Lomask and Larson, 2004). While not used in the cough analysis, other respiratory data, such as respiration rate, were recorded. When cough paroxysms occurred, each individual cough was counted separately.

Nebulization experiments were performed by placing one Hartley guinea pig into a chamber fitted with the nebulization device. All extracts and compounds were resuspended in 10% Methanol in PBS (Vehicle). The first day of the experiment consisted of nebulizing the vehicle for 10 min and recording coughs for 20 min. After a 2-day rest period, each guinea pig received 1 mL of each extract (1 mL at 4 or 20 mg/mL) or 1 mL of sulfolipid-1 (250 μg/ml final). Once all treatment groups were delivered over the course of the experiment, a 2-day waiting period occurred, followed by a treatment of 0.4 M citric acid (positive control). Coughs recorded by the instrument were simultaneously verified by direct observation.

Aerosol infection and Guinea Pig Plethysmography
Mtb cultures in 7H9 broth were centrifuged and the pellet was washed 3 times with PBS. The pellet was then resuspended in PBS and centrifuged (Sorvall Legend X1) at 500 rpm to remove clumps. To generate a single cell suspension, the bacteria-containing supernatant was then sonicated in a water bath using an ultrasonic processor with cup horn attachment (Cole Palmer) at 90% intensity for 15 s. Bacteria were then diluted in PBS to yield an OD600 of 0.1 (~3 x 107 CFU/ml), the bacterial suspension placed into the nebulization chamber of a GlasCol aerosolization unit and guinea pigs were infected with ~100-200 CFU. At day 0, the right lung from 3 guinea pigs of each treatment group were homogenized and plated to determine initial CFU. For whole body plethysmography, guinea pigs were placed individually into the nebulization chamber for 24 hr. Depending on the experiment, this was done at either 2, 4 and 6 weeks, or 3- and 6-weeks post infection. For the comparison of wild-type, MtbΔstf0 and MtbΔstf0::stf0, infections were staggered to allow for exact comparison of time points. At the completion of the study, the right lung was homogenized to determine CFU and the left lung was fixed in 10% formalin for 16 hr for histology.

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Histology
Lungs were first fixed in formalin for 16 hr. The formalin was replaced by PBS, lung tissue was embedded in paraffin and sectioned. Sections (6 μm) were then placed on glass slides and stained with hematoxylin and eosin. The sections in their entirety were scanned at 40x magnification using a NanoZoomer S80 digital slide scanner. For quantification of inflammation, an individual blinded to the samples used ImageJ to identify regions of inflammation and calculate percent inflammation by dividing the inflamed area by the total lung area.

Synthesis of trehalose-2-sulfate (T2S)
A 5 mL flame-dried reaction vial was purged under argon. To a solution of 52 mg 4,6,4′,6′-dibenzylidene α,α-D-trehalose (0.1 mmol) (Guiard et al., 2008) in pyridine (0.06M) was added 3 equivalents 25 mg SO₃-pyridine in dimethylformamide (0.5M) dropwise. The reaction was stirred at room temperature for 24 hr. The solvent was then removed under reduced pressure and left to dry overnight under high vacuum. The desired product was separated from starting material and its regiosomer via high performance liquid chromatography (H₂O (0.1% trifluoroacetic acid (TFA):acetonitrile). The resulting fractions were combined and neutralized with 5% NaHCO₃ and solvent was removed under reduced pressure. The dimethoxytrityl (DMT) protecting group was cleaved during concentration of the fractions. The remaining water was removed via lyophilization and the resulting mixture contained the product and TFA salt. The TFA salt was removed via a double resin exchange procedure. The obtained mixture was dissolved in water, and added to the Dowex 50WX8 resin (H⁺ form, 10 equiv) in a scintillation vial. After stirring for 2 hr, the resin was filtered off and dried via lyophilization overnight. The product was again dissolved in water along with the Dowex 50WX8 resin (Na⁺ form, 10 equiv). The mixture was stirred for 2 hr. After filtration off the resin, the desired product trehalose-2-sulfate (T2S) was dried via lyophilization overnight as a white crystalline solid in 8.4 mg, 19% overall yield. ¹H NMR (500 MHz, CD₃OD): 5.50 (d, J = 3.6 Hz, 1H), 5.10 (d, J = 3.7 Hz, 1H), 4.18 (dd, J = 9.8, 3.6 Hz, 1H), 4.04 (ddd, J = 10.2, 4.9, 2.5 Hz, 1H), 3.94 (t, J = 9.3 Hz, 1H), 3.90 - 3.74 (m, 4H), 3.72 - 3.65 (m, 2H), 3.48 (dd, J = 9.7, 3.8 Hz, 1H), 3.46 - 3.40 (m, 1H), 3.34 (dd, J = 8.6, 1.8 Hz, 1H). HPLC-MSD: cal’d for [C₁₂H₂₂O₁₄S-H⁺] 421.07, found: 421.10.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was performed using GraphPad Prism. For in vitro intracellular Ca²⁺ assays, either Student’s t test for pairwise comparisons, or analysis of variance with Kruskal-Wallace (Fluo-4) or Friedman’s test (Fura-2) with correction for multiple comparisons were used. For the restimulation experiment, the MtbΔstf0 and restimulated cells were compared using the paired Student’s t test. For the SL-1 dose response, the normalized average maximum fluorescence change at each dose was plotted and the EC₅₀ was determined using a nonlinear curve fit. For guinea pig experiments with nebulization of Mtb extracts or pure SL-1, Friedman’s test for matched, non-parametric data with correction for multiple comparisons was used. For Mtb infected guinea pig cough experiments, the non-parametric Mann-Whitney U test or the Kruskal-Wallis test with correction for multiple comparisons was performed. To identify outliers in animal studies both Dixon’s Q test (Q₉₉%) and Grubb’s outlier test (alpha 0.01) were used, and an outlier was only removed from the statistical analysis if it was identified by both methods.

DATA AND CODE AVAILABILITY
This study did not generate any unique datasets or code. All other data supporting the findings of this study are available in the manuscript or the supplementary materials and available from the authors upon reasonable request.
Figure S1. Mtb Extract and SL-1 Increases Intracellular \([\text{Ca}^{2+}]\) in Nociceptive Neuron, Related to Figures 2 and 4

(A-D). Fura-2 intracellular calcium assays (Ex 340/380, Em 510 nm) using MED17.11 cells (A), primary mouse DRG neurons (B), primary mouse nodose/jugular ganglia neurons (C) and primary human DRG neurons (D) exposed to Mtb extract. The maximum change in Fura-2 fluorescence ratio for capsaicin responsive (TRPV1+) neurons, capsaicin unresponsive (TRPV1-) and all cells in an experiment (minimum 50 cells) (A-C) are shown. (E-H) Fura-2 intracellular calcium assays using MED17.11 cells (E), primary mouse DRG neurons (F), primary mouse nodose/jugular ganglia neurons (G) and primary human DRG neurons (H) exposed to SL-1. For experiments with mouse (MED17.11, DRG and nodose/jugular) neurons, representative experiments of at least 3 are shown. For human DRG neurons, shown is the combined data from two donors. Error bars represent SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001 by paired Student’s t test.
Figure S2. Effect of Extracellular Ca²⁺ on Intracellular [Ca²⁺] Responses of MED17.11 Neurons to Mtb Extract, Related to Figure 2

(A) Quantification of the average ΔF/ΔF₀ MED17.11 cells loaded with Fura-2 and treated with vehicle (DMSO) or WT Mtb extract (0.4 mg/mL final). Cells were placed in HHBSS with (DMSO and Mtb extract) or without Ca²⁺ (DMSO NO Ca²⁺ and Mtb NO Ca²⁺). ***p < 0.0005, ****p < 0.0001 by Kruskal-Wallace test.
Figure S3. Intracellular Ca²⁺ Changes of MED17.11 Neurons in Response to Various Bacterial Organic Extracts, Related to Table 2

(A) Quantification of the average max ∆F/F₀ MED17.11 cells loaded with Fluo-4 and treated with vehicle (DMSO) or organic extracts from a variety of bacterial species (0.4 mg/mL final). ***p < 0.001, ****p < 0.0001 by Kruskal-Wallis test.
Figure S4. Mass Spectrometry of Mycobacterial Extracts, Related to Table 2
(A) Mass spectra from 2000 – 3000 m/z of pure SL-1 and mycobacterial extracts.
Figure S5. Mass Spectrometry of SL-1 Pathway Mutants, Related to Figure 3
(A) Mass spectra from 2000 – 3000 m/z of Mtb WT, Mtb Δstf0 and Mtb Δstf0::stf0. (B) Mass spectra of Mtb mutants in the SL-1 synthesis pathway in the regions of 659-661 m/z (for SL659), 1277-1279 m/z (for SL1278) and 2000-3000 m/z (for SL-1). Stf0 (Trehalose 2-sulfotransferase; Rv0295c), PapA2 (Polyketide synthase-associated protein A2; acyltransferase; Rv3820c), PapA1 (Polyketide synthase-associated protein A1; acyltransferase; Rv3824c), Sap (Sulfolipid-1-addressing protein; sulfolipid exporter; Rv3821), Chp1 (Cutinase-like hydrolase protein; SL1278 acyltransferase; Rv3822), MmpL8 (Sulfolipid-1 exporter, Rv3823c). Arrows identify SL659 and SL1278.
Figure S6. Synthesis of Trehalose-2-Sulfate and Corresponding NMR Spectra, Related to Figure 3
(A) Synthesis of trehalose-2-sulfate from trehalose. (B) $^1$H NMR spectrum. (C) COSY NMR spectrum.