RESEARCH ARTICLE

Inhibition of toll-like receptor 2 (TLR-2)-mediated response in human alveolar epithelial cells by mycolic acids and Mycobacterium tuberculosis mce1 operon mutant

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The early events involved in the host response to M. tuberculosis infection in the lung are still poorly understood. This paper sheds light into the initial mechanisms of infection by demonstrating that mycolic acid negatively modulates the alveolar epithelial cell response mediated by TLR2, suggesting that differences in the mycolic acid content in the M. tuberculosis cell wall may contribute to the fate of infection.

Keywords
Mycobacterium tuberculosis; tuberculosis; mycolic acid; A549 cells; mce1 operon; alveolar epithelial cells.

Abstract
In human lungs, the earliest encounter of Mycobacterium tuberculosis, the agent of tuberculosis, involves alveolar epithelial cells. Droplets expectorated by a patient with tuberculosis are likely to contain a mixed population of M. tuberculosis cells in different physiologic and metabolic states from the lung lesions of the patient. Here, we compared the chemokine expression patterns of human epithelial cell line A549 and RAW 264.7 macrophage cells infected with wild-type M. tuberculosis H37Rv against patterns induced by a mutant that accumulates free mycolic acids in its cell wall (Δmce1). We also examined the effect of free mycolic acids on toll-like receptor-2 (TLR-2). Wild-type M. tuberculosis induced significantly higher levels of IL-8, MCP-1, RANTES, and IP-10 in both cell types than did Δmce. Free mycolic acids reduced the ability of the mammalian cells to respond to a TLR-2 agonist in a dose-dependent manner. These observations suggest that differences in mycolic acid abundance in the M. tuberculosis cell wall can affect TLR-2-mediated pro-inflammatory response in both epithelial and macrophage cells. The final fate of a new infection may be ultimately determined by the proportion of M. tuberculosis cells expressing free mycolates in the infecting inoculum population.

Introduction
Mycobacterium tuberculosis is a leading infectious cause of death in adults worldwide (WHO, 2009). Most often, the initial site of infection in lungs of someone exposed to M. tuberculosis inhaled in droplets released from an index case of tuberculosis is the alveolar air space. Most studies that examine host innate immune response to M. tuberculosis have focused on the effector functions of alveolar macrophages and dendritic cells (Tailleux et al., 2003; Herrmann & Lagrange, 2005; Wolf et al., 2007; Ehlers, 2010). Mycobacterium tuberculosis has been shown to activate alveolar macrophages in a TLR-2- and TLR-4-dependent manner (Means et al., 2001), and several lines of evidence suggest the innate immunity-mediated protective role of TLR-2 in mycobacterial infections (Fulton et al., 2004; Pai et al., 2004; Scanga et al., 2004; Harding & Boom, 2010). However, these events that involve alveolar professional phagocytes most likely occur many days or weeks after the initial entry M. tuberculosis into the alveolar space.

The alveolar space is mostly composed of type I and type II pneumocytes. The former comprises about 96% of the

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alveolar surface area, while type II cells cover about 4% of the surface area but comprise 60% of all the alveolar epithelial cells (Castranova et al., 1988). Thus, the inhaled tubercle bacilli most likely encounter these epithelial cells first before they can be taken up by alveolar macrophages and dendritic cells. A number of groups have recently begun to address the active involvement of alveolar epithelial cells in the early innate host defense against M. tuberculosis (Bermudez et al., 2002; Roy et al., 2004; Kohiwattanagun et al., 2007; Rivas-Santiago et al., 2008a, b; Torrelles et al., 2008; Lee et al., 2009).

In the 1950s, Segal and Bloch showed that M. tuberculosis isolated from mouse lungs use fatty acids to stimulate respiration instead of carbohydrates used by the organism grown in liquid cultures (Segal & Bloch, 1957). Thus, during transmission, the tubercle bacilli in droplets released from a patient with tuberculosis are likely to be comprised of a population of cells in different metabolic states representing those in the lung lesions. Thus, another important factor that could determine the outcome of a new infection is the relative proportions of bacterial cells in different metabolic states at the time of transmission.

We previously demonstrated that an M. tuberculosis strain disrupted in a 13-gene operon called the mce1 Δmce1 fails to induce a characteristic pro-inflammatory response in mice or ex vivo-infected murine macrophages (Shimono et al., 2003). The mce1 operon is a member of a family of four related operons (mce1-4) that have been suggested by phylogenomic analyses to encode ABC transporters (Casali & Riley, 2007). Pandey and Sassetti have proposed that the mce4 operon encodes a possible cholesterol importer (Pandey & Sassetti, 2008). de la Paz Santangelo et al. (2009) have suggested that the mce3 operon is a regulon involved in lipid metabolism of M. tuberculosis. Dunphy et al. (2010) showed that the mce1 operon may be involved in recycling mycolic acids released from dying bacteria during latent infection. They found that a functional disruption of fatty acyl-CoA synthetase encoded by fadDS in the mce1 operon caused the mutant to be diminished in its growth in minimum medium supplied only with mycolic acid as a carbon source (Dunphy et al., 2010).

The mce1 operon mutant is hypervirulent in BABL/c and C57BL/6 mice (Shimono et al., 2003; Lima et al., 2007). It causes early death and induces poorly formed granulomas in mouse lungs with aberrant migration of inflammatory cells, resembling a Th2-type immune response (Shimono et al., 2003). Mouse peritoneal macrophages or RAW cells infected ex vivo with the mutant expressed significantly lower levels of TNF-α, IL-6, and MCP-1, compared with the cells infected with the wild-type strain (Shimono et al., 2003). Interestingly, the cell wall of M. tuberculosis disrupted in the mce1 operon contains more than 10-fold greater amounts of mycolic acids than that of wild-type M. tuberculosis (Cantrell et al., 2013). Moreover, the excess mycolic acids in the mutant are not covalently linked to these fatty acids’ canonical substrates arabinogalactan or trehalose (Barry et al., 1998; Cantrell et al., 2013).

We have shown that the mce1 operon is repressed in wild-type H37Rv M. tuberculosis during the first 4 weeks of infection in mice (Uchida et al., 2007). That is, the wild-type strain behaves like the mce1 operon mutant at one phase of infection in vivo and that in lung lesions, a proportion of the bacterial population may indeed be comprised of cells that express free mycolic acids in their cell wall. This mixed population of cells in expectorated droplets is what is likely to be transmitted to a new host. Here, we propose that the ultimate fate of a new infection in the lungs is determined by the relative proportion of bacterial cells expressing and not expressing free mycolic acids during their initial encounter with alveolar epithelial cells. To address this hypothesis, we compared the interaction of M. tuberculosis and its mce1 operon mutant with A549 epithelial and RAW 264.7 macrophage cells.

Materials and methods

Bacterial strains and growth conditions

Wild-type M. tuberculosis H37Rv, its derivative mce1 operon mutant strain (Δmce1), and the complemented mce1 strain were grown in Middlebrook 7H9 broth (Difco Laboratories, MI) containing 10% albumin–dextrase–catalase (ADC) enrichment (Beckton-Dickinson, MD), supplemented with 0.2% glycerol (Fisher Scientific, NJ), and 0.05% Tween 80 (Fischer Scientific; 7H9-ADCT) in sterile, vented tissue culture flasks at 37 °C in 5% CO2. We also examined an M. tuberculosis strain disrupted in the negative transcriptional regulator (mce1R) of the mce1 operon (Casali et al., 2006). The construction of Δmce1, Δmce1R and their phenotype is previously reported (Shimono et al., 2003; Casali et al., 2006). Bacterial strains were grown until the optical density (OD580 nm) of the cultures reached 1 (unless otherwise stated). When necessary, the above bacterial strains were also grown on solid agar medium Middlebrook 7H11 containing OADC (Beckton-Dickinson), 0.5% glycerol, and 100 μg mL−1 of antifungal agent cycloheximide (Sigma, MO).

Single-cell suspensions

Single-cell suspensions of each M. tuberculosis strain were prepared according to a previously described method (Grover et al., 1967) and quantified by enumeration of their colony-forming units (CFUs) on solid agar medium. Briefly, a 1.0 mL aliquot of a frozen mycobacterial culture (frozen at OD580 nm = 1.0) was used to inoculate 30 mL of sterile Middlebrook 7H9-ADCT in a 75 cm² vented tissue culture flask. The 30 mL culture was incubated at 37 °C in the presence of 5% CO2. After the culture was grown to OD580 nm = 1.0, 10 mL of the culture was placed in a sterile 50 mL polypropylene tube and centrifuged at 3337 g for 15 min. The supernatant was discarded, and the pellet was suspended in 5 mL of RPMI 1640 cell culture media containing 5% fetal bovine serum (FBS; Omega Scientific, CA). The suspension was mixed thoroughly by pipetting and vortexing to dissociate clumped bacteria. The 5 mL suspension was then passed through a sterile 5.0 μm pore Acrodisc syringe filter (Pall Corp., MI) with a sterile 5 mL
After adding 10 TLR-2 blocking antibody (clone T2.1; eBioscience, CA). One set of A549 cells was treated with a mouse anti-human alveolar epithelial cells Anti-TLR-2 antibody pretreatment and infection of triplicate. Cytokine quantification experiments were performed in each experiment, 2 of antibodies, cells were incubated for 72 h at 37 °C in 5% CO2 atmosphere. Uninfected cells served as negative controls for both set of cells. As a positive control, cells from each set were incubated for 72 h with 1 μg mL−1 of synthetic TLR-2 agonist Pam3Cys-Ser-(Lys)4 (Pam3Cys; Sigma, CO). All experiments were performed in triplicate.

**Pretreatment of alveolar epithelial cell line with mycolic acids and stimulation with TLR-2 agonist**

Mycolic acids extracted from a clinical strain of *M. tuberculosis* were purchased from Sigma-Aldrich, CO. Lypoilized mycolic acids were resuspended in sterile DMSO (Sigma-Aldrich) to a concentration of 5 mg mL−1. Cells were pre-incubated with either varying amounts of mycolic acids (125, 50, 25 ng mL−1) or without any mycolic acids at room temperature for 30 min. After the pre-incubation, cells were washed three times with prewarmed RPMI 1640 medium to remove excess mycolic acids. Washed cells were incubated with 1 μg mL−1 of TLR-2 agonist Pam3Cys (Sigma) for 72 h. These doses of mycolic acids and Pam3Cys were established in pilot dose–response studies. The ability of mycolic acids to block TLR-2-induced IL-8 and MCP-1 up-regulation in a dose-dependent manner was determined by ELISA.

**Enumeration of intracellular bacteria**

At different time points after infection, the supernatants were removed, and A549 cells were lysed to enumerate the intracellular bacterial load. The infected cells were lysed by incubation with 0.5 mL of 0.1% Triton X-100 (Sigma) in sterile water for 20 min at room temperature. The resulting cell lysates were serially diluted and plated in triplicate on solid medium. Three weeks after plating, CFUs were enumerated.

**Enzyme-linked immunosorbent assays (ELISA)**

Infected (with *M. tuberculosis* or its derivative strains) or uninfected (negative control) A549 cells were incubated for up to 72 h. Supernatants were harvested at 6, 24, 48, and 72 h postinfection and sterilized by passing through a 0.2 μm Acrodisc PF filter (Gelman Sciences, MI). Chemo- kine concentrations in supernatants were measured by ELISA, according to the manufacturer’s protocol (R&D Systems, MN). OD readings were obtained with a microplate reader model 450. Cytokines in the supernatants were quantified against the OD values obtained for the cytokine standards provided by the kit (R&D Systems). All cytokine quantification experiments were performed in triplicate.

**Anti-TLR-2 antibody pretreatment and infection of alveolar epithelial cells**

One set of A549 cells was treated with a mouse anti-human TLR-2 blocking antibody (clone T2.1; eBioscience, CA). After adding 10 μg mL−1 of antibodies, cells were incubated at room temperature for 30 min, as previously described (Asai *et al.*, 2003). The second set of A549 cells was exposed to the same conditions, except for the addition of the TLR-2 blocking antibody. Following incubation, cells were washed three times with prewarmed RPMI 1640 medium and infected with the bacterial strains at an MOI of 1 : 50 (cells/bacteria). Six hours after the infection, extracellular bacteria were removed by washing cells three times with warm RPMI 1640 5% FBS. After the third wash, RPMI 1640 5% FBS used to wash the cells was plated on solid medium to assess the presence or absence of extracellular bacteria. Infected monolayers were then incubated for 72 h at 37 °C in a 5% CO2 atmosphere. Uninfected cells served as negative controls for each experiment.
additional experiment was performed in triplicate with RAW macrophage cells with mycolic acids alone to assess immunogenicity.

**Statistical analysis**

Mean values of triplicate measurements for different cell groups were compared by the Student’s t-test.

**Results**

**Production of chemokines by A549 cells infected with M. tuberculosis H37Rv or mce1 mutant**

The amounts of chemokines expressed by A549 cells infected with Δmce1 were significantly lower than those in cells infected with the WT or mce1 complemented M. tuberculosis strains. IL-8 and MCP-1 measured in the supernatants of A549 cells infected with the Δmce1 strain were approximately threefold lower than those measured in the supernatants of A549 cells infected with WT strain or complemented strain at 72 h postinfection (Fig. 1a and b). Also, the mutant-infected A549 cells produced 1.8 times less IP-10 than the cells infected with WT or the complemented strain ($P < 0.04$; Fig. 1c). Similar differences were observed in the amounts of RANTES produced by the A549 cells; mutant-infected cells produced 2.09 times less RANTES than cells infected with the WT strain or mce1 complemented strain at 72 h postinfection ($P < 0.02$; Fig. 1d). None of the above M. tuberculosis strains (as high as 50 organisms per cell) induced MIP-1α in these experiments (data not shown).

In a different experiment, the induction of chemokines by Δmce1R and Δmce1 mutants in A549 cells was compared. mce1R is the negative regulator of the mce1 operon; M. tuberculosis disrupted in this gene constitutively expresses the mce1 operon genes (Casali et al., 2006). Like the mce1 operon mutant, Δmce1R is hypervirulent in mice; it causes accelerated death in mice by inducing massive pro-inflammatory response (Uchida et al., 2007). WT or Δmce1R-infected A549 cells produced the same amounts of IL-8, while the IL-8 production in Δmce1-infected cells was fourfold lower (Fig. 2a). Similarly, less MCP-1 was produced in Δmce1-infected A549 cells compared with WT or Δmce1R-infected A549 cells (Fig. 2b).

**Growth of mce1 mutant and control strains in A549 cells**

Similar numbers of CFUs were recovered from WT, Δmce1 mutant, or mce1 complemented strain-infected A549 cells from day 0 to the final time point of the experiment, at 72 h postinfection (Fig. 3). Also, the measurement of live A549 cells with trypan blue exclusion assay indicated that the cell counts in each well were similar. Therefore, these data indicated that the difference in above chemokine production by A549 cells infected with WT or Δmce1 was not due to differences in the number of bacteria in these cells or cell viability.

**Involvement of A549 cell TLR-2 in innate immune response to M. tuberculosis infection**

The production of IL-8 and MCP-1 was equally diminished in A549 cells that had their TLR-2 blocked before exposure to the WT M. tuberculosis. There was a threefold decrease in IL-8 production when cells were pretreated with anti-TLR-2 antibody prior to the infection with the WT (Fig. 4a). Similarly, there was a threefold decrease in MCP-1 production when cells were pretreated with anti-TLR-2 antibody prior to the infection with the WT (Fig. 4b). The treatment of cells with anti-TLR-2 antibody prior to the infection with the Δmce1 mutant did not significantly decrease the expression of IL-8 or MCP-1 in A549 cells. At 72 h postinfection, anti-TLR-2

![Fig. 1](image-url) Analysis of production of IL-8 (a), MCP-1 (b), IP-10 (c), and RANTES (d) by A549 cells infected with either WT (H37Rv), mce1 mutant (Δmce1), or mce1 complemented strain (mce1 Ki). Production of IL-8 (a), MCP-1 (b), IP-10 (c), and RANTES (d) by A549 cells infected at an MOI of 1 : 50. The scale is different for each cytokine and chemokine. Error bars represent standard error of the mean calculated from the results of three independent assays.
antibody pretreated cells and infected with the Δmce1 produced low concentrations of IL-8 and MCP-1 similar to the cells that were not pretreated with the antibody but were infected with the Δmce1 (Fig. 4a and b). The production of IL-8 and MCP-1 in A549 cells (pretreated or not pretreated with anti-TLR-2 antibodies) infected with the mce1 complemented strain was comparable to WT-infected A549 cells (Fig. 4a and b). The ability of anti-TLR-2 antibody to block the TLR-2 binding was confirmed by the low amounts of IL-8 (295 pg mL\(^{-1}\)) and MCP-1 (176 pg mL\(^{-1}\)) produced by anti-TLR-2 antibody-treated A549 cells in response to the TLR-2 agonist Pam3CysSerLys\(_3\) (Pam3Cys; Fig. 4c). Recovery of CFUs from A549 cells either pretreated or not treated with anti-TLR-2 antibodies was similar (Figs 3 and 4d).

**Effects of interaction between mycolic acids and TLR-2 in A549 cells on innate immune response**

Because of the previously reported effects of *M. tuberculosis* products on macrophage TLR-2-modulated pro-inflammatory response, the macrophage anti-Th1-type immune response that we observed with the mce1 operon mutant (Shimono *et al.*, 2003), and the excess amounts of unbound mycolic acids in the cell wall of Δmce1 (Cantrell *et al.*, 2013), we studied the effect of purified mycolic acids on A549 cells. Free mycolic acids diminished the ability of TLR-2 agonist Pam3Cys to induce production of IL-8 and MCP-1 in A549 cells in a dose-dependent manner. A549 cells pretreated with 25, 50, and 125 ng mL\(^{-1}\) of mycolic acids produced 1.3, 5.8, and 8.3 times less IL-8 than nontreated cells. Similarly, A549 cells pretreated with 25, 50, and 125 ng mL\(^{-1}\) of mycolic acids produced 2.03, 6.7, and 7.8 times less MCP-1 than nontreated cells (Fig. 5a and b).

Similar to A549 cells, the response of RAW macrophages to the TLR-2 agonist Pam3Cys was also affected by the mycolic acids pretreatment. The effect of pretreatment of RAW macrophages with 25 ng mL\(^{-1}\) of mycolic acids on IL-12, TNF-\(\alpha\), and MCP-1 production was small; that is, the decrease in cytokine or chemokine production between pretreated and nonpretreated was 0.4%, 17.5%, and 3.9%, respectively. However, RAW cells pretreated with 50 ng mL\(^{-1}\) of mycolic acids produced 4.5 times (75.9%), 4.8 times (81.4%), and 4 times (73.3%) less IL-12, TNF-\(\alpha\), and MCP-1, respectively, than untreated cells. RAW macrophages pretreated with 125 ng mL\(^{-1}\) of mycolic acids produced 9.2 times (89.1%), 7.4 times (86.5%), and 14 times (93%) less IL-12, TNF-\(\alpha\), and MCP-1, respectively, than nontreated cells (Fig. 6a–c).

**Discussion**

We previously showed that *M. tuberculosis* disrupted in the mce1 operon proliferates unchecked in BALB/c mouse lungs, but the same pattern of bacterial proliferation was not observed in less susceptible C57BL/6 mice (Lima *et al.*, 2007). Nevertheless, C57BL/6 mice infected with the Δmce1 succumbed to the infection faster than the WT-infected mice (Lima *et al.*, 2007). These observations suggested that rather than the bacterial burden, products of the mce1 operon may directly or indirectly modulate the host immune response.

The mce1 operon of *M. tuberculosis* belongs to a family of four related operons (mce1-4) that have been suggested by phylogenomic analyses to comprise ABC transporters involved in lipid importation (Dassa & Bouige,
Fig. 4 Effect of anti-TLR-2 antibodies on the production of IL-8, MCP-1, and the growth of bacteria in A549 cells. Production of IL-8 (a) and MCP-1 (b) by A549 cells after 72 h of Mycobacterium tuberculosis infection, in the presence or absence of 10 μg mL⁻¹ of anti-TLR-2 blocking antibody. Cells were infected with either WT (H37Rv), mce1 mutant (Δmce1), or mce1 complemented strains (mce1 KI). (c) Production of IL-8 and MCP-1 was stimulated by incubating A549 cells for 72 h with 1 μg mL⁻¹ of the TLR-2 agonist Pam3Cys, in the presence or absence of 10 μg mL⁻¹ of anti-TLR-2 blocking antibody. (d) Intracellular growth of WT (H37Rv), mce1 mutant (Δmce1), and mce1 complemented strains (mce1 KI) in anti-TLR-2 blocking antibody-treated A549 cells. Asterisks indicate differences that are statistically significant between H37Rv and Δmce1 (*) and H37Rv and mce1KI (**). Error bars represent standard error of the mean calculated from the results of three independent assays.

Fig. 5 Effect of mycolic acid on production of IL-8 and MCP-1 in A549 cells. Stimulation of IL-8 (a) and MCP-1 (b) with 1 μg mL⁻¹ of the TLR-2 agonist Pam3Cys in A549 cells pretreated or nonpretreated with Mycobacterium tuberculosis-derived mycolic acids. IL-8 and MCP-1 were measured 72 h after the infection. Error bars represent standard error of the mean calculated from the results of three independent assays.
Dunphy et al. (2010) showed that the mce1 operon may serve to recycle mycolic acids released from dying M. tuberculosis inside granulomas. The operon contains a gene fadD5 that encodes fatty acyl-CoA synthetase. They showed that a mutant functionally disrupted in fadD5 is diminished in growth in minimum medium supplied only with mycolic acid as a carbon source; the mutant grew normally in minimum medium supplied with glycerol or other fatty acids as a carbon source (Dunphy et al., 2010). More recently, we showed that the mce1 operon mutant contained nearly 11-fold more mycolic acids in the cell wall compared with the wild-type H37Rv strain (Cantrell et al., 2013). The excess mycolic acids were not bound to arabinogalactan or trehalose. The mutant and wild-type strains contained similar amounts of bound mycolic acids. These observations led us to examine the possible role of mycolic acids in the interaction of M. tuberculosis with epithelial cells.

Uchida et al. (2007) showed that during the first 4 weeks of infection in mice, the mce1 operon of wild-type M. tuberculosis is not expressed. Indeed, Casali et al. (2006) have shown that the operon is negatively regulated by mce1R, which is active only when M. tuberculosis is intracellular. Thus, during a natural course of infection, wild-type M. tuberculosis may undergo a physiologic state exhibited by the mce1 operon mutant – that is, it may make excess amounts of mycolic acids. If a new transmission occurs with a population of bacterial cells mostly in this physiologic state, they could down-modulate the innate immune response of a contact host’s alveolar epithelial cells as well as dendritic and macrophage cells, which may allow M. tuberculosis to establish persistence. If transmission occurs when most of the cells are not expressing free
mycolic acids, the infection may be eliminated by the contact host at this initial encounter.

In this study, we chose A549 cells as the primary cell line to represent early cellular responses to mycolic acids and Δmce1. A549 cells are an immortalized cell line derived from human alveolar epithelial cells, which are represented by type I and II pneumocytes. Much of the surface area of the alveolar space is covered by type I pneumocytes (Castranova et al., 1988). Type II pneumocytes are more numerous and they synthesize and secrete a variety of surface active products, including surfactant proteins A and D (known as collectins), which play an important role in innate immune protection against pathogens, including against M. tuberculosis (Ferguson et al., 1999; Sano & Kuroki, 2005; Hall-Stoodley et al., 2006; Torrelles et al., 2008). Thus, alveolar epithelial cells are more likely to serve as the first line of innate defense against inhaled pathogens, even before alveolar macrophages and dendritic cells set in.

Here, we showed that Δmce1 M. tuberculosis and purified mycolic acids can inhibit chemokine and cytokine response in A549 cells as well as in a macrophage-like cell line in a TLR-2-dependent manner. Free mycolates blocked the effect of the TLR-2 agonist Pam3Cys. Birchler et al. (2001) have demonstrated that the A549 alveolar epithelial cell line constitutively expresses TLR-2 on its apical surface. Armstrong et al. (2004) detected TLR-2 in situ on alveolar epithelial cells by immunohistochemistry. Thus, it is conceivable that, in vivo, mycolic acids exert an inhibitory effect on alveolar pneumocytes via TLR-2.

The above observations demonstrate the need to better understand the interaction of M. tuberculosis with host cells at a stage much earlier than what is traditionally studied. We also need to better understand the effect on outcomes of infection due to physiologic differences in bacterial populations that serve as the infectious inoculum, rather than assuming that all the bacterial cells exert a similar effect on their target cells once they enter the lungs.

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