

## Polarizing the T helper 17 response in *Citrobacter rodentium* infection via expression of resistin-like molecule $\alpha$

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**Abbreviations:** AAMac, alternatively activated macrophage; CAMac, classically activated macrophage; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; RELM, resistin-like molecule; Th, T helper type; WT, wild-type

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*Citrobacter rodentium* infection is a murine model of pathogenic *Escherichia coli* infection that allows investigation of the cellular and molecular mechanisms involved in host-protective immunity and bacterial-induced intestinal inflammation. We recently demonstrated that following *C. rodentium* infection, the absence of Resistin-Like Molecule (RELM)  $\alpha$  resulted in attenuated Th17 cell responses and reduced intestinal inflammation with minimal effects on bacterial clearance. In this addendum, we investigated the cytokine modulatory effects of RELM $\alpha$  and RELM $\alpha$  expression in the intestinal mucosa following *C. rodentium* infection. We show that in addition to promoting Th17 cytokine responses, RELM $\alpha$  inhibits Th2 cytokine expression and Th2-cytokine effector macrophage responses in the *C. rodentium*-infected colons. Second, utilizing reporter *C. rodentium*, we examined RELM $\alpha$  expression and macrophage recruitment at the host pathogen interface. We observed infection-induced macrophage infiltration and RELM $\alpha$  expression by intestinal epithelial cells. The influence of infection-induced RELM $\alpha$  on macrophage recruitment in the intestine is discussed.

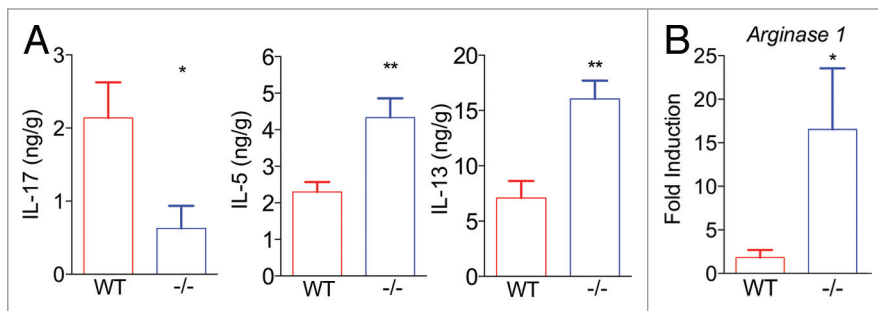
### Introduction

Mucosal surfaces, such as the intestine, are constantly exposed to the external environment, and development of a

balanced immune response is essential to prevent pathogen invasion while controlling excessive or unnecessary inflammation. Notably, macrophages, which constitute a significant proportion of the leukocytes within the gut, serve as initiators to polarize immune effector or regulatory responses following a variety of infectious or inflammatory stimuli.<sup>1</sup> In *Citrobacter rodentium* infection, a mouse model for enteropathogenic and enterhemorrhagic infections of humans,<sup>2</sup> we recently showed that Resistin-Like Molecule (RELM)  $\alpha$  promoted infection-induced intestinal inflammation via effects on macrophages.<sup>3</sup>

RELM $\alpha$  is a secreted protein that is most commonly associated with alternatively activated macrophages (AAMac), which are recruited in T helper type (Th) 2 cytokine-dominated environments, such as helminth infection and allergy.<sup>4-6</sup> In Th2 cytokine-biased immune responses, RELM $\alpha$  exhibited critical immunomodulatory functions.<sup>7-9</sup> In contrast, studies by Rothenberg and colleagues uncovered a pro-inflammatory function for RELM $\alpha$  in mouse models of inflammatory bowel disease.<sup>10,11</sup>

Our recent study focused on examining the role of RELM $\alpha$  in bacterial infection-induced inflammation.<sup>3</sup> Following *C. rodentium* infection, we observed that in comparison to wild-type (WT) mice, RELM $\alpha^{-/-}$  mice were protected from infection-induced intestinal inflammation. The ameliorated response observed in RELM $\alpha^{-/-}$  mice was associated with reduced Th17 cell-derived IL-17A. The Th17 cell-associated



**Figure 1.** RELM $\alpha$  promotes IL-17 expression while inhibiting Th2 effector responses in *Citrobacter rodentium*-infected colons. WT and RELM $\alpha^{-/-}$  mice were left naïve or infected for 10 d with *C. rodentium* followed by recovery of distal colon tissue for ELISA, plotted as ng cytokine per g colon tissue (A), or real-time RT-PCR, plotted as fold induction over naïve controls (B).

immune response, characterized by the production of cytokines IL-17 and IL-22, is critical for host immunity to several gastrointestinal pathogens including *C. rodentium*, *Helicobacter pylori*, and *Salmonella enterica*.<sup>12-14</sup> Infection-induced IL-17 promotes the recruitment of neutrophils and other effector cells, and IL-22 induces critical host defense mechanisms including antimicrobial peptide synthesis and mucus production.<sup>15-17</sup> Dysregulated Th17 cell responses are also associated with multiple inflammatory disorders, such as multiple sclerosis and inflammatory bowel disease (IBD).<sup>18</sup> Following *C. rodentium* infection, RELM $\alpha^{-/-}$  mice successfully cleared *C. rodentium* despite decreased accumulation of Th17 cells, suggesting that RELM $\alpha$ -induced Th17 cell responses promoted immunopathology. We identified that one mechanism of RELM $\alpha$ -mediated regulation was through macrophage production of the Th17 polarizing cytokine IL-23. In this addendum, we investigated the implications of RELM $\alpha$ -mediated regulation by examining the local cytokine environment in the *C. rodentium*-infected intestine, and by measuring macrophage infiltration and RELM $\alpha$  expression in the *C. rodentium*-colonized mucosa. Following *C. rodentium* infection, we show that RELM $\alpha^{-/-}$  mice exhibit significantly increased expression of Th2 cytokines and the Th2-effector AAMac gene *Arg1* that encodes for arginase. Given the counterbalance between Th2 and Th17 cell activation,<sup>19</sup> our data suggests that the RELM $\alpha$ -mediated stimulation of Th17 cell responses may be through

the inhibition of Th2 immune cytokines. Second, we characterized RELM $\alpha$  expression and macrophage responses in the infected intestinal mucosa by immunofluorescent staining and green fluorescent protein (GFP) reporter *C. rodentium*. In the *C. rodentium*-infected mucosa, we observed potent RELM $\alpha$  expression by intestinal epithelial cells. This was correlated with significant intestinal crypt elongation and increased macrophage accumulation at the interface with GFP-*C. rodentium*. In conclusion, these new studies reveal RELM $\alpha$  inhibition of the Th2 cytokine response at the site of *C. rodentium* infection and demonstrate increased macrophage infiltration and RELM $\alpha$  expression at the site of *C. rodentium* colonization.

## Results

### RELM $\alpha$ effects on the IL-23/Th17 Axis: Involvement of the Th2 cytokine response

Antigen presenting cells in the colon are critical initiators of the T helper cell response during infection.<sup>20</sup> Monocytes and macrophages can further shape the scope and magnitude of local innate and adaptive immune responses via cytokine production and polarization of innate lymphoid cells (ILCs) and CD4<sup>+</sup> T helper cells. A specific subset of macrophages, the CX<sub>3</sub>CR1<sup>+</sup> macrophages, have been ascribed a critical role in supporting host-protective ILC-derived IL-22 following *C. rodentium* infection.<sup>21</sup> Further, lineage-specific deletion of monocytes/macrophages resulted in impaired

accumulation of *C. rodentium* infection-induced IFN $\gamma$ <sup>+</sup> and IFN $\gamma$ <sup>+</sup>IL-17<sup>+</sup> CD4<sup>+</sup> T cells and host-protection.<sup>22</sup> Our recent data demonstrating that RELM $\alpha$  can promote expression of IL-12p40 and IL-23p19, cytokines that are critical for Th1/Th17 polarization, and the accumulation of Th17 CD4<sup>+</sup> T cells further highlights the importance of macrophages in shaping the local cytokine milieu.<sup>3</sup>

RELM $\alpha$  is most commonly associated with AAMacs and helminth infection, where it functions to negatively regulate expression of Th2 cytokines such as IL-13.<sup>7,8</sup> Given the existence of cross-regulation between Th2 and Th17 cytokine production,<sup>19</sup> we hypothesized that an additional mechanism by which RELM $\alpha$  promotes Th17 cytokine responses may involve indirect effects on the local cytokine environment. Consistent with this hypothesis, *C. rodentium*-infected RELM $\alpha^{-/-}$  mice exhibited significantly reduced colon IL-17 levels and increased colon IL-5 and IL-13 levels compared with WT controls (Fig. 1A). This switch to a Th2-associated cytokine response in RELM $\alpha^{-/-}$  mice correlated with increased expression of arginase1 (Fig. 1B), an enzyme that is expressed by Th2 cytokine-induced AAMacs. Previous helminth infection studies have shown that macrophage-derived arginase can inhibit Th2 cell cytokine production and proliferation and arginase-expressing macrophages can ameliorate intestinal inflammation.<sup>23,24</sup> Therefore, we hypothesize that *C. rodentium*-induced RELM $\alpha$  contributes to Th17 cell polarization both directly, through induction of IL-12p40 and IL-23p19, and indirectly by the inhibition of Th2 cytokines. In the absence of RELM $\alpha$ , the increased induction of Th2 cytokines and AAMac-derived arginase may act to limit effector CD4<sup>+</sup> T cell proliferation and contribute to intestinal tissue protection. Since studies originally identified RELM $\alpha$ , *arg1*, and *ym1* as coordinately expressed by AAMacs,<sup>5</sup> our data suggesting that RELM $\alpha$  may inhibit AAMac activation and *arg1* expression is unexpected. These data suggest that RELM $\alpha$  provides a cell-intrinsic negative feedback mechanism to inhibit

AAMac activation. Alternatively, during *C. rodentium* infection RELM $\alpha$  is expressed by cell types other than macrophages, including epithelial cells and eosinophils, and the cellular source of RELM $\alpha$  may influence functional outcomes. RELM $\alpha^{-/-}$  bone marrow chimeras may shed light on these differences and are currently being investigated. These studies have the potential to deepen our understanding of how RELM $\alpha$  regulates initiation and resolution of inflammation to various pathogenic insults at mucosal barrier surfaces, and may have implications for the understanding of the pathogenesis of human bacterial infection and intestinal inflammatory disorders.

RELM $\alpha$  belongs to the RELM family of secreted proteins that includes two human proteins (Resistin and RELM $\beta$ ), and investigating murine RELM proteins may give insight into understanding the function of human RELM proteins in health and disease.<sup>25</sup> Interestingly, a recent transcriptional profiling study by Loke and colleagues revealed a positive correlation between human resistin expression and Th17 cells in human ceca biopsies.<sup>26</sup> Taken together with our recent studies on RELM $\alpha$ , it is possible that murine RELM $\alpha$  is functionally comparable to resistin in humans. Although this study was performed on biopsies from healthy individuals, Th17 cell-associated responses have previously been associated with the pathogenesis of human IBD.<sup>27</sup> It is possible that resistin expression in the human intestine may contribute to this inflammatory environment and future studies on biopsies from IBD patients may give insight into a potential cytokine-stimulatory effect of resistin in human inflamed tissue.

#### RELM $\alpha$ expression and macrophage responses at the host-pathogen interface

Given our findings of RELM $\alpha$ -mediated effects on macrophages at the site of infection, we examined RELM $\alpha$  expression and macrophage recruitment at the *C. rodentium*-host interface. Using a GFP-expressing *C. rodentium*, we undertook imaging studies to investigate the cell types expressing *C. rodentium*-induced RELM $\alpha$ , localization of *C. rodentium* in the

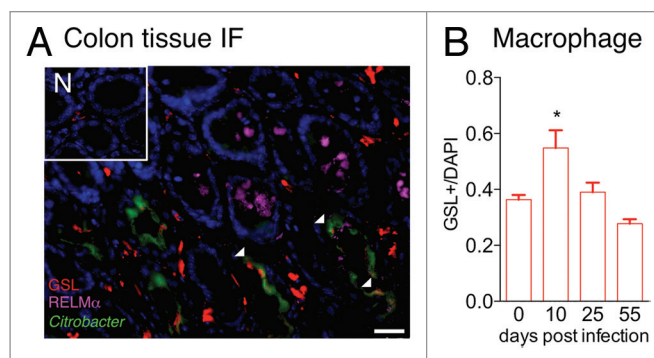
intestine, and the kinetics of colonic macrophage accumulation. Similar to the infection kinetics that we previously reported, infection of BL/6 mice with GFP-*C. rodentium* resulted in bacterial growth in the colon that peaked at day 10 post-infection, followed by clearance from the colon by day 25 post-infection (data not shown). Colon tissue cryosections from naïve or infected mice were stained with anti-RELM $\alpha$  (purple) and the macrophage binding lectin GSL (red) (Fig. 2A). We previously observed RELM $\alpha$  expression by macrophages, eosinophils, and intestinal epithelial cells by flow cytometry and immunohistochemistry. Surprisingly, these new localization studies demonstrate that in the *C. rodentium*-colonized crypts, RELM $\alpha$  was predominantly expressed by cells of goblet cell morphology rather than GSL<sup>+</sup> macrophages. The potential effect of goblet cell-derived RELM $\alpha$  on macrophage function at the host-pathogen interface is a new research avenue that we are investigating.

Previous reports have demonstrated *C. rodentium*-induced increase in CX<sub>3</sub>CR1<sup>+</sup> macrophages in the intestine.<sup>21</sup> We hypothesized that macrophages localized in the crypts are influenced by the proximity of the bacterial infection and by infection-induced factors such as epithelial-derived RELM $\alpha$ . At day 10 post-infection with *C. rodentium*, there was a marked increase in infiltrating GSL<sup>+</sup> macrophages in the colonized crypts. We also observed areas of GFP (green) and GSL (red) co-localization (Fig. 2A, white arrows), implicating macrophage

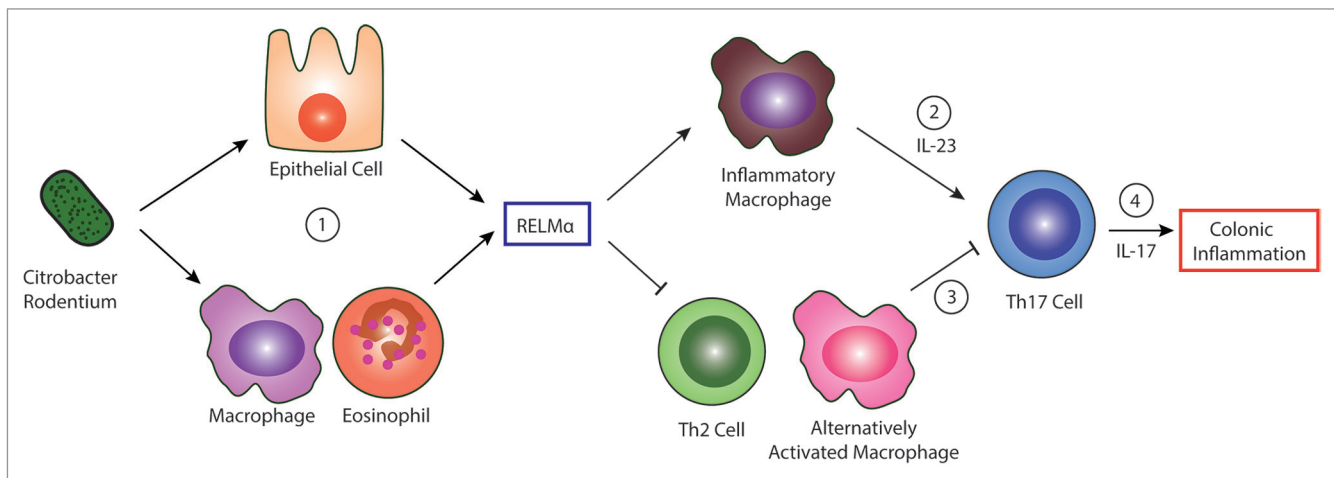
phagocytosis of *C. rodentium* as a host defense mechanism. Quantification of macrophage infiltration at the host-pathogen interface revealed that there was a significant increase in macrophage frequency at the peak of *C. rodentium* infection (day 10) that was resolved when *C. rodentium* had been cleared at day 25 and day 55 post-infection (Fig. 2B). The recruitment of macrophages to the host-pathogen interface has previously been shown to be critical for bacterial phagocytosis and clearance.<sup>21</sup> In addition, the infection-induced macrophages may also play a homeostatic role for the resolution of intestinal inflammation following bacterial clearance. For instance, Stappenbeck and colleagues reported the critical importance of macrophages in DSS-induced colitis.<sup>28</sup> Here, activated macrophages in the colonic crypts of DSS-treated mice produced regenerative factors that stimulated epithelial cell proliferation. Future studies in our lab will investigate the role of macrophages and macrophage-derived factors such as RELM $\alpha$  or arginase in the resolution of *C. rodentium*-induced inflammation, and the consequence of selective macrophage depletion at different time points post-infection.

#### Discussion

In our previous studies, we demonstrated a novel role for RELM $\alpha$  in contributing to a pathogenic Th17 response in the colon following infection



**Figure 2.** Macrophage recruitment and RELM $\alpha$  expression in *C. rodentium*-infected mice. (A) Immunofluorescent stained colon tissue sections from naïve (N) or day 10-infected WT mice reveal GSL<sup>+</sup> macrophages (red), RELM $\alpha$  (purple), *C. rodentium*-GFP (green), and DAPI (blue). Scale bar 25  $\mu$ m. (B) Quantification of GSL<sup>+</sup> macrophage frequency in naïve or infected mice was performed.



**Figure 3.** RELM $\alpha$  regulation of bacteria-induced intestinal inflammation. (1) *Citrobacter rodentium* infection induces RELM $\alpha$  expression by epithelial cells and leukocytes. (2) RELM $\alpha$  activates inflammatory macrophages to secrete IL-23 and activate Th17 cells. (3) RELM $\alpha$  inhibits Th2 cells and alternatively activated macrophages, which may counter-regulate the Th17 immune response and/or inhibit intestinal inflammation. (4) The elicited Th17 response causes colonic inflammation in the host.

with *C. rodentium* through effects on macrophages.<sup>3</sup> Here, we relate these findings to our other previous work demonstrating that RELM $\alpha$  limits Th2 inflammation.<sup>7</sup> These studies suggested that the RELM $\alpha$ -mediated effects observed might be a consequence of the counter-regulation of Th2/Th17 cytokine responses. Here, we show in new data that *C. rodentium*-infected RELM $\alpha$ <sup>-/-</sup> mice have increased Th2 cytokine responses in the intestine. Additionally, employing reporter GFP-*C. rodentium*, we observed infection-induced RELM $\alpha$  expression by goblet cells and significant increases in infection-induced macrophages in the *C. rodentium*-colonized mucosa. Real-time RT-PCR analysis of the infected intestine in WT or RELM $\alpha$ <sup>-/-</sup> mice revealed increased expression of arginase1, suggesting that RELM $\alpha$ <sup>-/-</sup> mice exhibited polarized alternative macrophage activation.

The importance of macrophage activation in bacterial infection has been the focus of several recent studies showing that classically activated macrophages (CAMacs) can limit bacterial burdens while AAMacs are detrimental to the host and sustain chronic bacterial infection.<sup>29-32</sup> CAMacs differentiate in response to toll-like receptor ligands such as LPS and Th1 cytokines, and promote bacterial killing through the production of proinflammatory cytokines

and microbicidal products. In contrast, AAMacs that differentiate in response to Th2 cytokines are considered anti-inflammatory and express regulatory molecules such as Transforming Growth Factor  $\beta$  and arginase1. Following infection with several different bacterial pathogens including *Salmonella typhimurium*, *Brucella abortus*, or *C. rodentium*, AAMacs impaired bacterial clearance by a variety of cell-intrinsic and cell-extrinsic mechanisms, resulting in a glucose-rich intracellular environment conducive for bacterial growth.<sup>30,32</sup> Additionally, AAMacs have deficient autophagy responses that are necessary for intracellular bacterial killing.<sup>31</sup> AAMacs may also impair bacterial clearance via cell-extrinsic mechanisms such as the inhibition of T cell activation or antibacterial CAMac responses.<sup>29</sup> However, whether AAMacs or their effector molecules mediate effects on infection-induced intestinal inflammation independently of effects on bacterial growth is less well defined. Our finding that the ameliorated intestinal inflammation in RELM $\alpha$ <sup>-/-</sup> mice was associated with increased arginase1 expression suggests that AAMac activation in the intestine may have a beneficial effect on *C. rodentium*-induced inflammation once the bacterial

burdens are cleared. Consistent with this hypothesis, previous studies have shown beneficial effects of SHIP<sup>-/-</sup> AAMacs in ameliorating intestinal inflammation induced by DSS.<sup>33</sup> Together with our results, these studies suggest that examination of AAMac responses during acute *C. rodentium* infection, as well as following clearance during the resolution of inflammation, is warranted.

In summary, studies in this addendum reveal that *C. rodentium* colonization of the mucosa promotes goblet cell expression of RELM $\alpha$  (Fig. 3 part 1). In addition to our previous report showing that RELM $\alpha$  stimulates inflammatory macrophage-derived IL-23 (Fig. 3 part 2), our new data in this addendum implicates RELM $\alpha$  inhibition of Th2 cytokines as an additional mechanism that promotes Th17 cell responses (Fig. 3 part 3). Finally, we show infection-induced macrophage responses in the intestine, and increased AAMac activation in the RELM $\alpha$ <sup>-/-</sup> mice that is associated with ameliorated intestinal inflammation (Fig. 3 part 4). The functional significance of macrophage activation and infiltration at the host-pathogen interface and the role that these macrophages may play in bacterial clearance or intestinal inflammation remain critical avenues of investigation that may help the treatment of bacterial-induced colitis.



## Materials and Methods

Mice and Infection: C57BL/6 mice were purchased from Jackson Laboratories and bred in-house. RELM $\alpha$ <sup>-/-</sup> mice were backcrossed onto Jackson C57BL/6 mice as previously described.<sup>7</sup> Mice were maintained in a specific pathogen-free facility at the University of California, Riverside, and all procedures were performed under the guidelines of the Institutional Animal Care and Use Committee. WT and GFP-*C. rodentium* (DBS-100) were provided by Bruce Vallance,<sup>15</sup> and infection was performed as previously described. In brief, mice were orally gavaged with 0.2 mL of  $\sim 5 \times 10^8$  colony forming units.

Histology: One-cm distal colon was removed, flushed with PBS, and was submerged in 4% formaldehyde for 4 h, followed by 30% sucrose overnight. Tissue was embedded in OCT (Biotek) and 8  $\mu$ m cryosections were cut using a cryostat (Leica). For immunofluorescent staining, sections were blocked with normal goat serum and then stained with rabbit anti-mouse RELM $\alpha$  antibody (Peprotech) at 4 °C overnight, followed by Cy3 conjugated anti-rabbit IgG (Abcam) for 30 min

at room temperature. After washing with PBS/0.1% Tween-20, the sections were blocked with Streptavidin Blocker, Biotin Blocker (Vector laboratories), and StartingBlock T20 block buffer (Thermo Scientific), 15 min each at room temperature, then stained with biotinylated GSL (Vector laboratories) for 1 h at room temperature and followed by Cy5 conjugated streptavidin (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. The sections were washed and covered with ProLong<sup>®</sup> Gold Antifade Reagent with DAPI and coverslip (Cell Signaling Technology). Microscopy was performed using a Leica immunofluorescent microscope with AF6000 software at 200 $\times$  and 400 $\times$  magnification. The gray-scale values of GSL+ macrophages was measured and corrected with that of DAPI (nuclei) from 3–5 random fields of each immunofluorescent section.

Real-time RT PCR and ELISA Analysis: 1 cm of distal colon tissue was recovered in RNeasy (Qiagen) followed by RNA extraction and real-time RT-PCR, as previously described. For cytokine ELISAs, 1 cm of distal colon tissue was weighed and homogenized in 0.5 mL PBS, followed by ELISA with anti

IL-17, anti IL-13, and anti IL-5 capture and detection antibodies (eBioscience).

Statistical Analysis: Graphs are analyzed with Graphpad Prism and are presented as mean  $\pm$  SEM. Statistical significance was confirmed when  $P < 0.05$ , using an unpaired two-tailed student T-test or when  $>2$  groups, a one way ANOVA test followed by the Dunnett's post-test.

### Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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