Lipoxin A\textsubscript{4} and 15-Epi-Lipoxin A\textsubscript{4} Protect against Experimental Cerebral Malaria by Inhibiting IL-12/IFN-\gamma in the Brain

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Abstract
Cerebral malaria is caused by infection with \textit{Plasmodium falciparum} and can lead to severe neurological manifestations and predominantly affects sub-Saharan African children. The pathogenesis of this disease involves unbalanced over-production of pro-inflammatory cytokines. It is clear that signaling though IL-12 receptor is a critical step for development of cerebral malaria, IL-12 genetic deficiency failed to show the same effect, suggesting that there is redundancy among the soluble mediators which leads to immunopathology and death. Consequently, counter-regulatory mediators might protect the host during cerebral malaria. We have previously showed that endogenously produced lipoxins, which are anti-inflammatory mediators generated by 5-lipoxygenase (5-LO)-dependent metabolism of arachidonic acid, limit host damage in a model of mouse toxoplasmosis. We postulated here that lipoxins might also play a counter-regulatory role during cerebral malaria. To test this hypothesis, we infected 5-LO-deficient hosts with \textit{P. berghei} ANKA strain, which induces a mouse model of cerebral malaria (ECM). Our results show accelerated mortality concomitant with exuberant IL-12 and IFN-\gamma production in the absence of 5-lipoxygenase. Moreover, in vivo administration of lipoxin to 5-LO-deficient hosts prevented early mortality and reduced the accumulation of CD8+IFN-\gamma\textsuperscript{+} cells in the brain. Surprisingly, WT animals treated with lipoxin either at the time of infection or 3 days post-inoculum also showed prolonged survival and diminished brain inflammation, indicating that although protective, endogenous lipoxin production is not sufficient to optimally protect the host from brain damage in cerebral malaria. These observations establish 5-LO/LXA\textsubscript{4} as a host protective pathway and suggest a new therapeutic approach against human cerebral malaria (HCM). (255 words).

Introduction
Cerebral malaria is a severe neurological complication of infection with \textit{Plasmodium falciparum}. This disease predominantly occurs in children in sub-Saharan Africa, where approximately 500,000 people are affected annually, with fatality rates ranging from 17.5 to 19.2\%. Moreover, approximately 25\% of cerebral malaria survivors develop long-term neurological sequelae even after the appropriate antimalarial treatment [1–5].

The pathogenesis of cerebral malaria involves the sequestration of parasitized red blood cells in the brain microvasculature, the accumulation of mononuclear cells in brain tissue, and the increased expression of pro-inflammatory cytokines, including IFN-\gamma [6–22]. The large proportion of deaths that occurs in hospitals before anti-parasitic treatment can take effect highlights the importance of understanding the pathogenesis of this disease and of implementing new, rapidly acting interventions in combination with anti-plasmodium treatment [23–31].

Experimental cerebral malaria (ECM) caused by infection of C57BL/6 mice with \textit{Plasmodium berghei} ANKA has been useful in identifying host factors involved in the pathogenesis of cerebral malaria and displays many features of the human disease[32–35]. Development of the mouse model requires an immune response against the parasites. Dendritic cells, CD4+ and CD8+ T cells, NK T cells, NK cells, and platelets have all been involved in disease induction and regulation. Additionally, while on one hand, IL-12 receptor is a critical component for the development of cerebral malaria, the use of single knockout mutants for several pro-inflammatory cytokines, including IL-12 have failed to show an obvious influence on cerebral malaria pathogenesis [6,7,9,11,14,16,18,20,22], suggesting that redundancy among those mediators might take place in vivo.

A balance between host pro-inflammatory and anti-inflammatory immune responses is a key determinant for the pathogenesis of cerebral malaria. Weaker pro-inflammatory responses could allow parasite persistence and proliferation, whilst exuberant pro-inflammatory responses could trigger lethal immunopathology,
including cerebral malaria. Consequently, the identification of potent counter-regulatory pathways and mediators that control and/or inhibit the pathogenesis of cerebral malaria without promoting parasite proliferation and survival is important for the development of novel therapeutic interventions against this disease.

Lipoxins are a class of anti-inflammatory/pro-resolution lipid mediators derived from lipoxygenase-mediated metabolism of arachidonic acid. In recent years, a growing list of counter-regulatory actions has been attributed to lipoxins, including inhibition of chemotaxis, pro-inflammatory cytokine and chemokine production and NK cell activation, among others[36,37].

5-lipoxygenase (5-LO), one of the enzymes required to generate lipoxin A₄ (LXA₄), is also needed to synthesize other mediators, such as leukotriene B₄ (LTB₄). Previously, we have used 5-lipoxygenase-deficient (Alox5⁻/⁻) mice to study the potential role of endogenously produced lipoxins in regulating the intensity and extent of pro-inflammatory response to infectious diseases, including toxoplasmosis and tuberculosis [38–40]. In hosts infected with these pathogens, we unveiled a lipoxin-triggered common regulatory mechanism—modulation of dendritic cell-IL-12 production. However, the outcome on immunopathology and survival after those infections depended on the nature of the pathogen. While the increased inflammatory response led to mortality in T. gondii infected mice, it triggered enhanced resistance to M. tuberculosis. Consistent with this, polymorphisms within the 5-lipoxygenase gene in humans are associated with resistance to tuberculosis in endemic areas in Africa, making it the first identified TB susceptibility gene in humans[41]. Lipoxins and its epimers (including 15-epi-LXA₄) have also been shown to be produced in a 5-LO-independent manner in vivo after aspirin (via acetylated COX2) or statins (via S-nitrosylation of COX2)[42,43]. 15-epi-LXA₄ presented longer half-life in vivo when compared to LXA₄ [44], nevertheless both molecules have shown overlapping biological actions.

Taking into account the intensity of inflammation during cerebral malaria and the results we observed during both T. gondii and M. tuberculosis infections, we hypothesized that the anti-inflammatory actions of lipoxins play a host-protective role during the pathogenesis of ECM. To test this hypothesis, we infected Alox5⁻/⁻ mice with P. berghei ANKA strain. The results shown here indicate that endogenously generated LXA₄ protects mice against ECM by inhibiting IL-12 production and accumulation of IFN-γ-producing cells in the brains of infected mice. In addition, we found that administration of 15-epi-LXA₄ (a more stable endogenous epimer of LXA₄) prolongs survival and damps pro-inflammatory responses in P. berghei-infected WT mice. These observations provide a proof-of-concept for a potential new therapy for cerebral malaria in humans (HCM).

**Results**

5-LO-deficient mice present accelerated mortality after P. berghei ANKA infection

Cerebral malaria induced by P. berghei ANKA infection is typically characterized by intense CNS cellular infiltration with vascular and tissue damage, despite relatively low levels of parasitemia. Given the intensity of the inflammatory response, we hypothesized that 5-lipoxygenase-dependent arachidonic acid metabolism might either contribute to the severity of the disease, via synthesis of leukotrienes, or mediate host protective responses, via production of lipoxins. To distinguish between these possibilities, we infected both WT and Alox5⁻/⁻ mice with P. berghei ANKA-parasitized red cells. Mean survival time (MST) was 8 days for WT mice, but only 3 days for Alox5⁻/⁻ mice (Figure 1A). In contrast, parasitemia levels were similar in infected WT and Alox5⁻/⁻ mice (Figure 1B). On the other hand, we found a trend for reduction in the levels of parasite 18S rRNA in the CNS at 5 days after infection (Figure 1C), therefore excluding the possibility that the more severe pathology is associated with...
increased parasite sequestration. Thus, 5-lipoxygenase may contribute to host survival by limiting inflammation rather than by limiting parasite proliferation or survival.

5-LO-dependent control of IL-12p70 and IFN-γ during *P. berghei* ANKA infection

Type 1 cytokines, including IL-12 and IFN-γ, are associated with murine cerebral malaria pathogenesis. Despite its protective role during the liver stage of infection, IFN-γ can damage the host during blood stage of severe forms of malaria, including cerebral malaria. We hypothesized that counter-regulatory pathways might be required to limit host damage caused by an overly exuberant type 1 cytokine response. Consequently, we tested whether the accelerated mortality of *Alox5*−/− mice might be due to higher levels of IL-12 and IFN-γ after *P. berghei* ANKA infection. *Alox5*−/− mice had significantly increased serum levels of both cytokines 3 days after infection, as compared to WT mice (Figure 2). Consistent with this, *il12a*, *il12b*, *ifng*, *il6*, *il17a* mRNA expression were considerably increased in the brains of *P. berghei* ANKA-infected *Alox5*-KO vs. WT mice at 5 days after infection (Fig. 2C–E and figure S1).

5-LO-mediated synthesis of LXA₄ during ECM

The results shown so far indicate that the absence of 5-lipoxygenase led to aberrant IL-12 and IFN-γ production during ECM, suggesting a potential defective counter-regulatory pathway. Because 5-lipoxygenase mediates the synthesis of several arachidonic acid-derived lipid mediators, including leukotrienes and lipoxins, we investigated whether genetic deficiency of 5-lipoxygenase would alter the profile of arachidonic acid-derived mediators. Serum LTB₄ and LXA₄ levels were significantly reduced, while PGE₂ and 15-HETE levels were not significantly altered by 5-lipoxygenase deficiency during *P. berghei* ANKA infection (Figure 3A–E). Interestingly, serum TXB₂ concentration was increased 3, but not 5 days after infection. Taking together, the data indicate that 5-lipoxygenase mediates synthesis of LXA₄ and LTB₄ during *P. berghei* ANKA infection without significantly affecting the levels of other lipid mediators.

Increased CD4+ and CD8+ T cell infiltration, IL-12+ and IFN-γ+ cells in infected 5-LO-deficient hosts

The increased mRNA and protein levels of both IFN-γ and IL-12 in *P. berghei* ANKA-infected *Alox5*−/− mice suggested that the lack of an endogenous 5-LO-dependent anti-inflammatory pathway led to either higher cytokine production by pathogen-specific cells or increased accumulation/proliferation of cytokine producing cells along the perivascular areas of the brains of infected mice. To evaluate this possibility, we enumerated CD4+ and CD8+ T cells, as well as IFN-γ+ cells in the brains of *P. berghei* ANKA-infected WT and *Alox5*−/− mice 5 days after infection (Figure 4A–B). Increased frequency of densely stained areas surrounded by
foamy weakly stained tissue was noted in brain sections from *P. berghei* ANKA-infected *Alox5<sup>−/−</sup>* mice, suggesting tissue damage. Although increased frequency of CD4<sup>+</sup>IFN-γ<sup>+</sup> cells in brains of both WT and *Alox5<sup>−/−</sup>* infected mice did not differ significantly (Fig. 4C, D and G), there was increased detection of CD8<sup>+</sup>IFN-γ<sup>+</sup> cells in the brains of infected *Alox5<sup>−/−</sup>* versus WT mice (*p* = 0.0183) [Fig. 4E, F and H]. Our additional observation that CD8<sup>+</sup> T cell IFN-γ<sup>+</sup> response in *P. berghei* ANKA-infected C57Bl/6 mice is consistent with previous reports of the presence of CD8<sup>+</sup> T cell IFN-γ<sup>+</sup> response in *P. berghei* ANKA-infected C57Bl/6 mice [8,10,12].

15-epi-Lipoxin A<sub>d</sub> treatment prevents the onset of experimental cerebral malaria

The results presented so far indicated that endogenous 5-LO provides some protection against *P. berghei* ANKA infection. Earlier studies have shown that lipoxins can promote resolution by inducing anti-microbial peptides and subsequent bacterial killing and clearance [45,46]. To determine whether the effect seen in the absence of Alox5 is resulting from production of anti-inflammatory lipoxins, such as LXA<sub>4</sub>, we investigated whether in vivo delivery of 15-epi-LXA<sub>4</sub> to mice could prolong survival while reducing type 1 cytokine production after *P. berghei* ANKA infection. Our results show that lipoxin treatment at the time of infection significantly prolonged survival of both infected *Alox5<sup>−/−</sup>* mice (MST 17.5 vs. 3.5 days) and WT mice (MST 20 vs. 5 days) for 15-epi-LXA<sub>4</sub>-treated and PBS-treated groups, respectively (Figure 5A). In agreement with our previous findings and with the changes in survival rates, 15-epi-LXA<sub>4</sub> treatment lowered brain *Il12a*, *Il12b* and *Ifng* mRNA expression in infected WT and *Alox5<sup>−/−</sup>* mice (Figures 5B–D), concomitant with increased expression of *Socs2* mRNA (Figure 5E). Thus, despite its production during *P. berghei* ANKA infection, increased lipoxin levels are beneficial to the host, diminishing the severity of the pro-inflammatory response and prolonging survival. In order to further support the therapeutic

**Figure 3.** *P. berghei* ANKA infection induces LO-dependent lipid mediator synthesis. C57Bl/6 WT and *Alox5<sup>−/−</sup>* (*n* = 4 mice/group) mice were infected i.p. with *P. berghei* ANKA strain. At 1 and 3 days after infection, animals were bled and serum levels of LXA<sub>4</sub> (A), LTB<sub>4</sub> (B), 15HETE (C), TXB<sub>2</sub> (D) and PGE<sub>2</sub> (E) were determined by ELISA. Data shown is representative of one out of three independent experiments performed. Statistical analysis of difference among groups was determined using Mann Whitney test.

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potential of lipoxin-based interventions during cerebral malaria, we compared whether delayed treatment (starting 3 days after inoculum) would affect survival and cytokine mRNA profile of PBS-treated infected WT mice. In fact, as can be seen in figure 5F, mice that received 15-epi-LXA4 from day 3 through 7 after infection, presented a significant delay of mortality rates (gray squares) when compared to PBS-treated WT controls (black circles). Notably, the mortality rates did not significantly differ whether treatment with 15-epi-LXA4 initiated at the day of infection or three days later. The levels of \textit{il12a} and \textit{ifng} mRNA expression in the brain were not significantly different among the experimental and control groups (Figures 5G and 5I). On the other hand, treatment with 15-epi-LXA4 either at the time of infection or three days post-inoculum caused a significant reduction in the expression of \textit{il12b} (Figure 5H), while significantly increased the expression levels of \textit{socs2} (Figure 5J). While CNS \textit{ifng} expression was dramatically reduced in Allox5\textsuperscript{−/−} mice after 15-epi-LXA4 (Figure 5D), both forms of 15-epi-LXA4 treatment failed to significantly change its expression levels in WT mice (Figure 5D and 5I). Taken together this set of results support that treatment 15-epi-LXA4 diminishes expression of some pro-inflammatory mediators and prevents mortality due to cerebral malaria even when mediator delivery is initiated three days after infection, thus providing support for the potential development of a novel supportive therapeutic venue that may prolong patient survival for sufficient time to allow anti-malarial drugs to take effect.
Figure 5. Exogenous delivery of 15-epi-LXA4 prolongs survival and reduces pro-inflammatory cytokine production of both WT and 5-LO-deficient mice after *P. berghei* ANKA infection. C57Bl/6 WT and *Alox5*<sup>-/-</sup> (*n* = 8 mice/group) mice were infected i.p. with *P. berghei* ANKA strain and treated with PBS alone or with 15-epi-LXA4 (1 µg/mouse) from day 1 to 7 after infection (shaded area in A and F) or from day 3 to 7 after infection (striped area in F). Survival was monitored (A and F) up to 20 days after inoculum. Five days after infection, mice were sacrificed and brains harvested. Tissues were homogenized, total RNA extracted and reverse transcribed for real-time RT-PCR determination of *il12a* (B and G), *il12b* (C and H), *ifng* (D and I) and *socs2* (E and J) expression. Data shown boxes/mean bar with min-max whiskers and are representative of three independent experiments performed. (* = *p* < 0.05, ** = *p* < 0.01 and *** = *p* < 0.0001) as determined by One-way ANOVA with Tukey multiple comparison test. doi:10.1371/journal.pone.0061882.g005
Discussion

Cerebral malaria is a lethal severe form of the disease whose pathogenesis is complex and incompletely understood. Observations from animal models (e.g., *P. berghei* ANKA infection in mice) and *P. falciparum*-infected humans support a hypothesis that sequestration of parasitized red cells by the brain microvasculature leads to vascular obstruction, edema, leukocyte activation and extravasation, hypoxia and necrosis. Although the precise phenotype of cerebral malaria can vary considerably, it is widely agreed that the pro-inflammatory cytokines, such as IFN-γ contribute to its pathogenesis and clinical features

[1,3,6,7,9,10,12,19–22,32–34].

Activation of brain endothelial cells, infected red cells and circulating leukocytes by both IFN-γ and TNF during cerebral malaria most likely triggers the cascade of events that swiftly transforms the brain microenvironment. Consequently, these pro-inflammatory cytokines may well determine the intensity of tissue damage and, subsequently, the severity of the disease. With this in mind, we postulated that parasite-triggered host IFN-γ production is modulated by anti-inflammatory/counter-regulatory pathways. Our results show that ECM induces production of LXA₄, a 5-lipoxygenase-derived arachidonic acid metabolite that dampens IL-12/IFN-γ production during infection with any of several pathogens [47] and up-regulates production of proteins that interfere with inflammatory cytokine signaling, such as SOCS2 [48] and promotes resolution [49].

The amount of LXA₄ produced during infection influences host survival. On one end of the spectrum, the absence of LXA₄ production during infection with the highly virulent parasite, *Toxoplasma gondii*, increases production of IL-12 and IFN-γ and exacerbates inflammation, resulting in host death from encephalitis [39]. On the other end of the spectrum, enhancement of IL-12 and IFN-γ production by LXA₄ deficiency protects hosts infected with *M. tuberculosis* by increasing clearance of the bacilli from the lungs [40]. Consistent with this, clinical studies in *M. tuberculosis* endemic areas associate ALOX5 promoter polymorphisms that decrease gene expression with a lower frequency of active disease [41]. Thus, by regulating inflammation that, in different conditions, can protect the host by suppressing the pathogen or kill the host by damaging essential organs, LXA₄ may determine infection outcome.

The results shown here establish that alox3-dependent LXA₄ production modulates disease severity in *P. berghei*-infected mice. Absence of LXA₄ production during *P. berghei* ANKA infection was associated with higher systemic levels of IL-12 and IFN-γ and increased il12a, il12b and ifng mRNA expression in the brain. Importantly, the accelerated mortality of alox5-deficient mice could be prevented by in vivo delivery of 15-epi-LXA₄. This is a critical observation, because Alox5 is involved in the synthetic pathways of several arachidonic acid-derived mediators, including LTB₄. Consistent with this, alox5-deficient mice failed to produce both LXA₄ and LTB₄ after infection, although serum levels of other arachidonic acid metabolites, including PGE₂, TXB₂ and 15HETE, were not affected. However, the residual production of LXA₄ detected here suggests that alternative pathways play a minor, but detectable role in the generation of LXA₄.

Furthermore, in support to a protective role of lipoxins, in vivo delivery of 15-epi-LXA₄ to infected WT mice significantly prolonged survival beyond the ECM mortality period, while reducing il12b mRNA expression levels in the brain. The effects of 15-epi-LXA₄ treatment did not significantly reduced ifng expression in WT mice, yet the treatment showed protection against ECM mortality. It is possible that lipoxins promoted protection via inhibition of cytokine-mediated host damage. This more targeted effect could explain the apparent discrepancy between the weak inhibition of ifng expression while significantly prolonging survival. The protective results with 15-epi-LXA₄ treatment in WT mice indicates that the endogenous production of LXA₄ is not sufficient to optimally protect the host from inflammatory brain damage during cerebral malaria and suggests that genetic variability in LXA₄ production may influence the risk of developing cerebral disease or other severe forms of malaria. If so, selective pressures that may favor increased LXA₄ production, such as during *T. gondii* and *P. falciparum* infection, and selective pressures that may favor decreased LXA₄ production, such as *M. tuberculosis* infection, could interplay in order to maintain considerable genetic diversity in regulation of 5-LO production in areas where all of these pathogens are endemic. Another closely related mediator that could potentially be involved here is LXβ₃ that has been shown to be equally active in vivo when compared to LXA₄ [50]. However, further studies are required to address whether this mediator is produced and biologically active during ECM.

Our observations also suggest the possibility of using LXA₄ stable analogs [51] as part of a therapeutic approach for severe malaria. Although such agents would not cure infection, they may prolong host survival sufficiently for traditional anti-malarial chemotherapeutics to effect a cure. A similar strategy has been attempted with corticosteroids [52–57] and aspirin [58,59], with little or no success. Some aspirin-dependent anti-inflammatory actions are mediated by aspirin-triggered lipoxins [60]. Although aspirin also initiates several lipoxin-independent pathways, including suppression of prostaglandin production and platelet function, our results presented here provide support that lipoxins can provide a more targeted effect in controlling inflammation during ECM and, potentially HCM, as compared to those treatments previously tested. Consequently the potential use of lipoxins and its analogs in a more targeted approach against HCM is likely to promote survival and is worthy of investigation.

Materials and Methods

Mice

C57Bl/6j and 5-lipoxygenase-deficient (Alox5⁻/⁻) mice were bred and maintained in a specific pathogen free animal facility at Cincinnati Children’s Hospital Medical Center. All procedures shown here were reviewed and approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee.

Parasites and infections

*P. berghei* ANKA strain clone c115cy1 was maintained by continuous passage in vivo. For experimental infections, mice were inoculated with 10,000 infected red blood cells by intraperitoneal injection. Parasitemia was determined by counting infected red blood cells in Wright-Giemsa-stained blood smears.

Cytokine and lipid mediators and ELISA kits

15-epi-LXA₄ was obtained from Cayman Chemicals. Due to its labile nature, long-term storage stock solution was kept at −80°C. For in vivo treatments stock aliquots were diluted in PBS immediately at the time of use. IL-12p70, IL-12p40 and IFN-γ levels were measured using commercial ELISA kits (BD biosciences). LTB₄, 15HETE, PGE₂ and TXB₂ ELISA kits were from Cayman Chemicals and an LXA₄ ELISA kit was from Oxford. Detection limits for these assays were: 15 pg/mL (IL-12p40), 39 pg/mL (IL-12p70), 31 pg/mL (IFN-γ), 13 pg/mL (LTB₄), 11 pg/mL (TXB₂), 170 pg/mL (15HETE), 36 pg/mL (PGE₂) and
20 pg/mL (LXA₄) for in vivo experiments, infected mice (n = 4–8) were bled for assessment of plasma cytokine and lipid mediator levels.

Real-time RT-PCR

Total RNA was isolated from tissues using the Trizol LS reagent according to the instructions of the manufacturer. cDNA was synthesized with TaqMan Reverse Transcriptase (Applied Biosystems, Foster City, CA) and mRNA expression of cytokines (IL-12p35, IL-12p40, IL-23p19, IFN-γ, IL-6, IL-17A and IL-23p19) and β-actin were analyzed by RT-PCR. P. berghei 18S expression levels were determined in perfused brains harvested 5 days after infection. Real-time RT-PCR was performed on an ABI-Prism 7000 PCR cycler (Applied Biosystems).

Microscopy

Brains were removed from mice up to 7 days after infection, and frozen sections were processed and stained with a biotinylated IL-12p35, CD4 and CD30 Ab, followed by a double incubation with Alexa Fluor 488- or 594-conjugated antibodies (Invitrogen). The slides were counterstained for nuclei with DAPI (Invitrogen). Images were acquired using a microscope (Axiocamt, Carl Zeiss MicroImaging, Inc.) with the AxioVision software (Carl Zeiss MicroImaging, Inc.) and analyzed using ImageJ software.

Statistical analysis

The statistical significance of differences in mean values between experimental versus control or vehicle treated samples was evaluated using the methods indicated in the figure legends. Differences were considered to be significant at p<0.05 unless otherwise indicated.

Supporting Information

Figure S1 Enhanced il6 and il17a mRNA expression in P. berghei ANKA infected Alox5-deficient mice. C57Bl/6 WT and Alox5⁻/⁻ (n = 4 mice/group) mice were infected i.p. with P. berghei ANKA strain. Five days after infection, mice were sacrificed and brains, livers and spleens harvested, homogenized, total RNA extracted and reverse transcribed. Real-time RT-PCR was performed for determination of il6 (A), il23a (B) and il17a (C) expression. Data shown are representative of one out of three independent experiments performed. Statistical differences were determined using Mann Whitney test. (TIF)

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Author Contributions

Conceived and designed the experiments: JA FTMC CM RMSG. Performed the experiments: NS CM RMSG SJ. Analyzed the data: JA. Wrote the paper: JA.

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