# Targeting the IL-33-NLRP3 axis improves therapy for experimental cerebral malaria

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**Cerebral malaria (CM) is a serious neurological complication caused by *Plasmodium falciparum* infection. Currently the only treatment for CM is the provision of anti-malarial drugs; however, such treatment by itself often fails to prevent death or development of neurological sequelae. To identify potential improved treatments for CM, we performed a non-biased whole brain transcriptomic time-course analysis of anti-malarial drug chemotherapy of murine experimental CM (ECM). Bioinformatics analyses revealed IL33 as a critical regulator of neuro-inflammation and cerebral pathology that was down regulated in the brain during fatal ECM and in the acute period following treatment of ECM. Consistent with this, administration of IL33 alongside anti-malarial drugs significantly improved the treatment success of established ECM. Mechanistically, IL33 treatment reduced inflammasome activation and IL1β production in microglia and intracerebral monocytes in the acute recovery period following treatment of ECM. Moreover, treatment with the NLRP3-inflammasome inhibitor MCC950 alongside anti-malarial drugs phenocopied the protective effect of IL33 therapy in improving the recovery from established ECM. We further showed that IL1β release from macrophages was stimulated by haemozoin and anti-malarial drugs and that this was inhibited by MCC950. Our results therefore demonstrate that manipulation of the IL33-NLRP3 axis may be an effective therapy to suppress neuroinflammation and improve the efficacy of anti-malarial drug treatment of CM.**

IL33 | NLRP3 | inflammasome | malaria | inflammation

## Introduction

Cerebral malaria (CM) is a severe manifestation of *Plasmodium falciparum* infection, which affects 2-3 million people each year, mainly young children in Africa (1). The only treatment for CM is anti-malarial drugs, typically in the form of parenteral artesunate or quinine compounds. Such treatment fails to prevent mortality in a quarter of CM patients, leading to the death of approximately 300,000 people each year (1-3). Moreover, up to 26% of individuals develop residual neurological deficits following anti-malarial drug treatment and recovery from CM (4-5). Thus, CM remains a leading cause of mortality and neuro-disability in tropical regions (1-5). Consequently, there is a critical clinical need for development of more effective therapies for CM that will enhance the protective effects of anti-malarial drugs.

The cerebral processes contributing to the pathophysiology of CM and those that undermine recovery from the syndrome after anti-malarial drug treatment are poorly understood (1, 6-8). However, there is a growing consensus that targeting the host pro-inflammatory immune response to infection may be an effective strategy to enhance anti-malarial drug treatment success of CM (7, 8). Indeed, serological and/or cerebral spinal fluid (CSF) concentrations of pro-inflammatory cytokines and chemokines, including TNFα, IL6, IL1β, IFN-γ and CXCL10, frequently correlate with the development of CM and, in some cases, severity of CM (7, 8). Pro-inflammatory processes may disrupt CM recovery by activating the brain endothelium, causing permeability of the blood brain barrier, activation of astrocytes and microglia, disruption of neuronal signalling, and recruitment of circulating leukocytes (1, 7-9). All of these events have been observed in brains of individuals with fatal CM (1, 6-9). In particular, it is believed that cerebrovascular dysfunction is a critical pathological process in CM development and fatal outcome (1, 7, 9). Therefore, intracerebral inflammatory responses at time of treatment may prevent re-establishment of brain homeostasis, leading to the failure of anti-malarial drug treatment.

In this study, to identify novel immune candidates for therapy of CM, we optimised a pre-clinical model of *P. berghei* (Pb) ANKA-induced murine experimental cerebral malaria (ECM) (10) where anti-malarial drug treatment of established ECM leads to sub-optimal recovery, associated with significant mortality and development of severe cerebral pathology. Using this infection-drug cure model of ECM, we have performed non-biased whole brain RNA-seq time-course analysis during anti-malarial drug chemotherapy. We subsequently identified IL33 as a key regulator of cerebral inflammatory pathways during fatal ECM, and in the acute period post-anti-malarial drug treatment. Injection of IL33 alongside anti-malarial drugs significantly improved the recovery of mice with established ECM, potentially through reduction of NLRP3-dependent inflammasome activation. Consistent with this, direct inhibition of the NLRP3 inflammasome using the specific inhibitor MCC950 phenocopied the protective capacity of IL33 in improving recovery from ECM. Overall, these data indicate that pharmacological strategies targeting the IL33-NLRP3 axis could potentially be beneficial for the treatment of CM.

### Significance

**Cerebral Malaria (CM) is a neurological complication of malaria infection that, despite anti-malarial drug treatment, results in fatality or neuro-disability in approximately 25% of cases. Thus, there is an urgent clinical need to develop therapies that can improve the efficacy of anti-malarial drugs to prevent or reverse cerebral pathology. Here, we show in an experimental mouse model of CM (ECM) that IL33 administration can improve survival and reduce pathology in the brain over anti-malarial drugs alone. Mechanistically, we demonstrate that IL33 enhances recovery from ECM by inhibiting NLRP3 inflammasome-induced inflammatory responses within the brain. These results suggest that IL33 and NLRP3 inflammasome inhibitors may be effective adjunctive therapies for CM.**

**Reserved for Publication Footnotes**

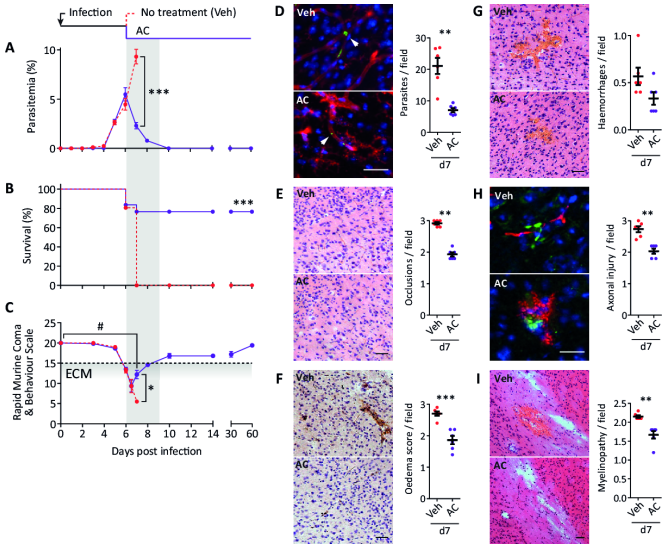


**Fig. 1.** . Anti-malarial drug treatment promotes sub-optimal recovery from ECM. Mice were infected with Pb ANKA GFP and treated with artesunate and chloroquine (AC) or vehicle (Veh) at the onset of ECM. (*A*) Peripheral parasitemia, (*B*) survival curves, and (*C*) RMCBS scores of mice after infection (d0) and drug treatment (grey box). (*D-I*) Brains were examined 16-24 h after treatment (d7) for: (*D*) GFP$^+$ parasites (green), co-stained with lectin (red) and DAPI (blue); (*E*) erythrocyte-congested vessels indicative of haemostasis (H&E); (*F*) extravascular IgG indicative of vasogenic odema (DAB counter-stained with haematoxylin); (*G*) haemorrhage (H&E); (*H*) β-APP accumulation (green) indicative of axonal injury, co-stained with erythrocytes (red) and DAPI (blue); (*I*) myelin damage (H&E). Data is presented as means ±SEM. (A-C) n=12-97 from 2-10 infections, (*D-I*) n=6 from 2 infections. Scale bar: 25μm. #p<0.05 d0 versus d7 in AC treated. (A, C) Specified comparisons for parasitemia and RMCBS were made by Mann-Whitney tests. (B) Comparison made by log rank test (D-I) Comparisons made by Mann-Whitney or t-tests as detailed in methods. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (all versus Veh), # *p<0.05 AC group day 7 versus day 0.

## Results

### Anti-malarial drugs promote sub-optimal recovery from established ECM

In order to study the recovery from established malaria-induced cerebral pathology, we adapted the conventional Pb ANKA ECM model (10) to recapitulate the clinical settings associated with the treatment of CM. C57BL/6 mice infected with Pb ANKA were treated daily with the anti-malarial drugs artesunate (the front line drug for treatment of severe malaria [2]) and chloroquine (as a representative quinine compound), both at 30 mg/kg, or vehicle alone. Treatment began at the onset of neurological dysfunction, as defined by a rapid murine coma and behaviour scale (RMCBS) score of ≤15 (11), on day 6 post infection (d6) (Fig. S1).

Peripheral parasitemia developed exponentially before rapidly reducing upon anti-malarial drug treatment (Fig. 1A). Despite their potent parasiticidal activity, administration of anti-malarial drugs (AC) failed to prevent mortality in approximately 25% of mice (Fig. 1B). Interestingly, in these cases where anti-malarial drug treatment was unsuccessful, drug treated mice succumbed more rapidly to ECM than vehicle treated controls (80% compared with 20% of deaths on day 6, respectively) (Fig. 1B). Anti-malarial drug treatment also failed to prevent significant deterioration in neurological function in the critical 6-12 h period post-treatment (d6.5), with drug-treated mice exhibiting comparable levels of neurological dysfunction as vehicle treated mice (Fig. 1C). Drug-treated mice still exhibited substantial neurological impairment at 24 h post-administration (d7), although this was ameliorated compared with the level of neurological dysfunction observed in untreated mice with fatal ECM (Fig. 1C). We then compared the neuropathology between mice that survived following treatment with anti-malarial drugs (d7: 16-24 hr post treatment), with mice which were not drug-treated and were therefore in the agonal stages of the disease (d7: 16-24 hr post vehicle treatment). Consistent with observations of residual neurological deficits in drug-treated mice (Fig. 1C), mice that survived following treatment with anti-malarial drugs (d7) exhibited a reduction in, but not complete abrogation, of various neuropathological features associated with CM (1, 6-10) including: cerebrovascular parasitized red blood cell (pRBC) accumulation (Fig. 1D), haemostasis (Fig. 1E), vascular leakage (Fig. 1F), haemorrhage (Fig. 1G) axonal injury (Fig. 1H), and myelin damage (Fig. 1I). None of the neuropathological features were observed in naïve mice (as we have previously shown in reference 10). Collectively, these data demonstrate that administration of anti-malarial drugs to mice with established ECM resulted in a similar mortality rate as anti-malarial drug treatment of CM (2, 3), and did not fully prevent or reverse associated neuropathology.

### Whole brain transcriptomics identifies IL33 as a potential therapy for ECM

As therapeutic strategies targeting only the parasite failed to prevent substantial mortality or morbidity, we utilised a non-biased systems approach to identify potential targets for additional therapy. We compared the cerebral (whole-brain) transcriptomes of mice by RNA-seq prior to infection (d0), at the onset of ECM (d6), in late-stage (agonal) ECM without drug treatment (d7), and at various time points after drug treatment (d7+AC, d10, d14, d30 and d60). Principal component analysis (PCA) demonstrated that anti-malarial drug administration led to a rapid change in the brain transcriptome (d7+AC and d10) compared with that in mice with early onset ECM (d6) and agonal ECM (d7), the latter two of which exhibited largely overlapping PCA transcriptome signatures (Fig. 2A). The brain transcriptome returned to homeostasis quickly post-resolution of ECM, with d14, d30 and d60 samples clustering with d0 (Fig. 2A). Anti-malarial drug administration did not reverse the majority of the gene changes (< or > 1.5 fold change and q value <0.05, compared with d0) that were established in the brain at onset of ECM, and which were also observed in fatal ECM (Fig. 2B). Drug treatment did, however, lead to segregated expression of many genes compared with agonal ECM (Fig. 2B). Very few genes were differentially expressed in brains at d14, d30 or d60 compared with d0 (Fig. 2C).

We then sought to understand in more detail the transcriptional responses that undermined the effectiveness of anti-malarial drug treatment of established ECM. 4825 differentially expressed genes (DEGs) were identified when all time points were compared, separately, to d0. DEGs were clustered by k-means into 8 clusters and ranked by hierarchical clustering (Fig. 2D, with gene expression pattern in each cluster visually represented in Fig. S2A). We then performed gene ontology analysis to assess the biological processes significantly enriched within each cluster (Fig. S2B). In general, anti-malarial drug treatment did not acutely modify the expression of the majority of the biological processes involved in inflammation and immunological activation (cluster I, VI and VII) established at the onset of ECM at d6 (Fig. 2D). Instead, anti-malarial drug treatment altered the expression of genes involved in nervous system development, metabolism and axogenesis (cluster VIII), transcription, apoptosis and cell adhesion (clusters II and IV) and DNA repair and regulation of lymphocyte activation (cluster V) (Fig. S2B). Together, these data show that anti-malarial drugs failed to rapidly alter the intracerebral expression of large numbers of genes defining the inflammatory signature of the brain during and post-ECM. Instead, in the surviving mice, anti-malarial drug administration

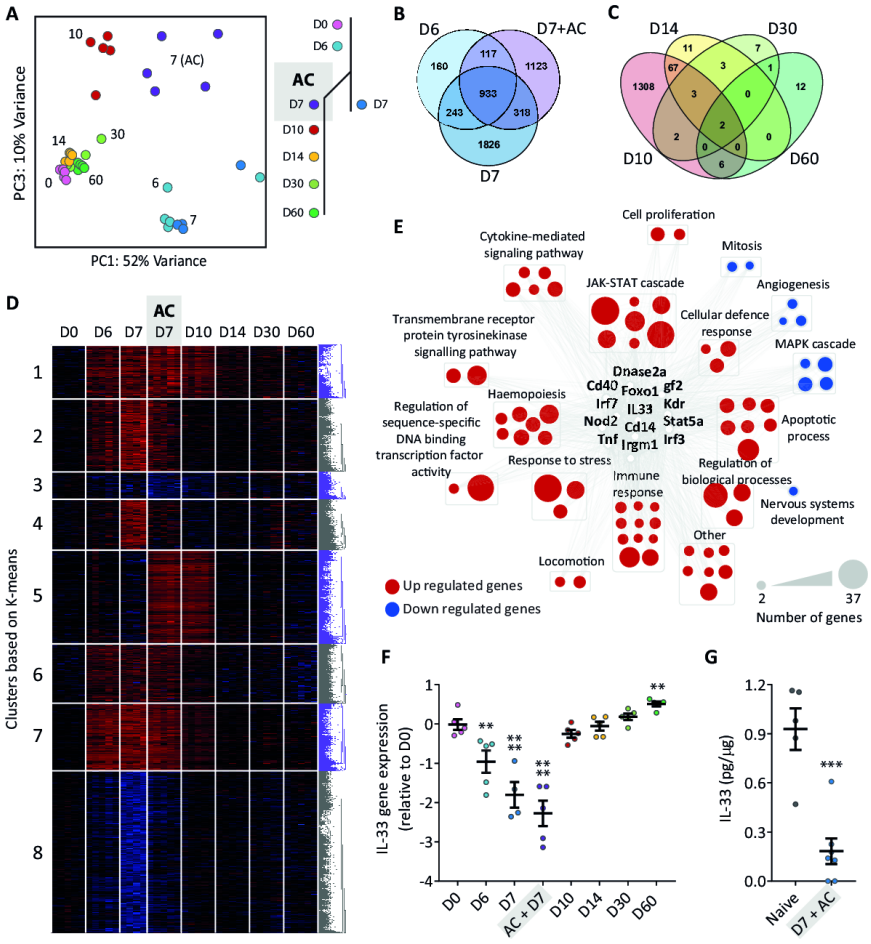


**Fig. 2.** . Expression profiling and pathway analyses indicate that IL33 is a potentially important gene negatively regulating pathogenesis in the late-stages of ECM. Whole-brain transcriptomic analyses were performed prior to infection (d0), at the onset of ECM (d6), in agonal ECM (d7), and after anti-malarial treatment (d7+AC, d10, d14, d30, d60). (*A*) Principal component analysis of whole brain transcriptomes. (*B, C*) Venn diagrams defining overlap of differentially expressed genes (DEGs) (<or> 1.5 fold change and q value <0.05) (*D*) K-means and hierarchical clustering of DEGs (normalised to d0) (*E*) Bipartite Cytoscape network defining enriched (GO slim) biological processes within the 13 filtered upstream regulators' combined protein-protein interaction networks (DEGs within protein-protein interactions network identified within d7 and / or d7+AC groups, compared with d0). (*F*) IL33 gene expression in the brain compared by one-way ANOVA (*G*) IL33 protein in brain homogenates measured by BioLegend LEGENDPlex, compared by t-test. Data presented as mean ±SEM. **p<0.01, ***p<0.001, ****p<0.0001 all versus d0 or naïve.

appeared to significantly modulate expression of genes involved in brain function.

To define the key genes controlling the cerebral transcriptional landscape during agonal ECM and following anti-malarial drug treatment, we identified the upstream regulators (URs) within each cluster (Fig. S2C). A transcription factor (TF) enrichment analysis revealed that most of the URs were controlled by a genetic regulatory network involving several TFs. Based on this information, we filtered this list to identify 13 URs whose expression were not regulated by TFs, as we hypothesised that these genes were strong candidates for independently and rapidly controlling the transcriptome of the brain during and following treatment of ECM. Importantly, these 13 genes were predicted to control multiple inflammation and immune-related processes in the brain during agonal ECM (d7) and immediately following anti-malarial drug treatment (d7+AC) (Fig. 2E).

Of the 13 identified independently-controlled master URs, IL33, which was present in cluster VIII of the heat map (Fig. 2D), was of particular interest due its protective role in other inflammatory neuropathologies, including Alzheimer's disease, stroke and spinal cord injury (12-15). IL33 gene expression was significantly downregulated in the brain during agonal ECM and in the acute phase post-anti-malarial drug treatment, before returning to levels observed in naïve mice from day 10 (Fig. 2F). IL33 protein levels were similarly reduced in the brain following anti-malarial drug treatment of ECM (d7+AC) compared with levels in naïve mice (Fig. 2G). These data identified IL33 as a potential immunotherapy to dampen inflammation, re-establish homeostasis in the brain, and improve the success of anti-malarial drug treatment of established ECM.

**IL33 enhances the effectiveness of anti-malarial drug treatment of ECM**

To investigate whether administration of IL33 could reduce the mortality and/or neuropathology associated with anti-malarial chemotherapy of established ECM, we administered anti-malarial drugs alone or together with IL33 to Pb ANKA infected mice at the onset of neurological dysfunction (d6). IL33 was administered as a single dose (0.02 mg/kg, human equivalent dose [HED] 0.0016mg/kg) alongside anti-malarial drugs (both at 30 mg/kg) on the first day of treatment. IL33 administration did not alter peripheral parasitaemia (Fig. 3A); however, IL33 treatment significantly improved survival over anti-malarial drugs alone (100% with IL33, versus 71% without) (Fig. 3B). Furthermore, IL33 significantly improved RMCBS scores of mice compared with mice treated with anti-malarial drugs alone, at both 6-12 (d6.5) and 16-24 (d7) h after treatment (Fig. 3C). We then assessed the effects of IL33 on the neuropathology we had previously observed in mice that survived following anti-malarial drug treatment (Fig. 1). We compared neuropathology between mice treated with anti-malarial drugs alone (d7), with those which were treated with combined IL33 and anti-malarial drugs (d7). IL33 administration significantly reduced a number of indices of cerebral pathology, including: cerebrovascular pRBC accumulation (Fig. 3D), haemostasis (Fig. 3E), vascular leakage (Fig. 3F), haemorrhage (Fig. 3G), and axonal injury (Fig. 3H). Myelin damage was unaltered (Fig. 3I). When IL33 treatment was administered without anti-malarial chemotherapy (on d6), all

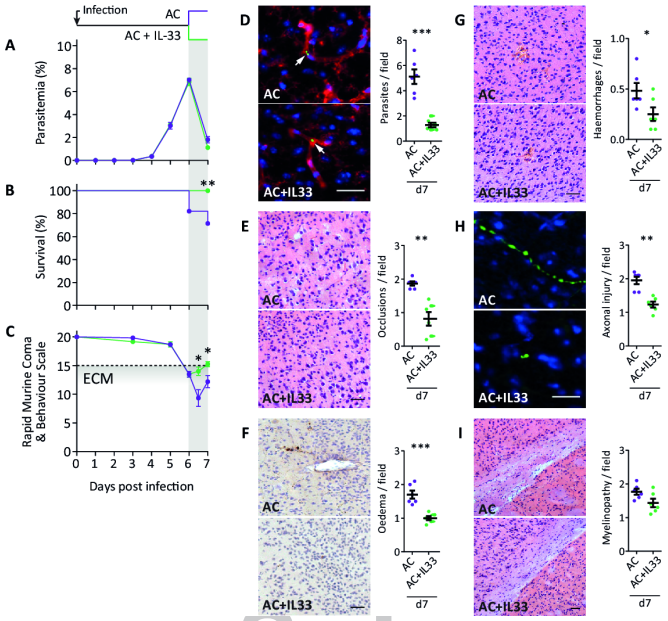

**Fig. 3.** . IL33 improves efficacy of anti-malarial drug treatment of ECM. Mice were infected with Pb ANKA (n=12-28 from 2-6 infections), and treated with anti-malarial drugs, either alone (AC) or together with IL33 (AC+IL33), at the onset of ECM. (*A*) Peripheral parasitemia, (*B*) survival curves, and (*C*) RMCBS scores of mice after infection (d0) and drug treatment (grey box). (*D-I*) Brains were examined at 16-24 h after treatment (d7) for: (*D*) GFP$^+$ parasites (green), co-stained with lectin (red) and DAPI (blue); (*E*) erythrocyte-congested vessels indicative of haemostasis (H&E); (*F*) extravascular IgG indicative of vasogenic odema (DAB counterstained with haematoxylin); (*G*) haemorrhage (H&E); (*H*) β-APP accumulation (green) indicative of axonal injury, co-stained with erythrocytes (red) and DAPI (blue); (*I*) myelin damage (H&E). Data is presented as means ±SEM. (*A-C*) n=12-28 from 2-6 infections. (*D-I*) n=6 from 2 infections. Scale bar: 25μm. (A, C) Separate comparisons were made between groups at d6.5 and d7 by Mann-Whitney test. (B) Comparison made by log rank test (D-I) Comparisons made by Mann-Whitney or t-test as detailed in methods. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus AC

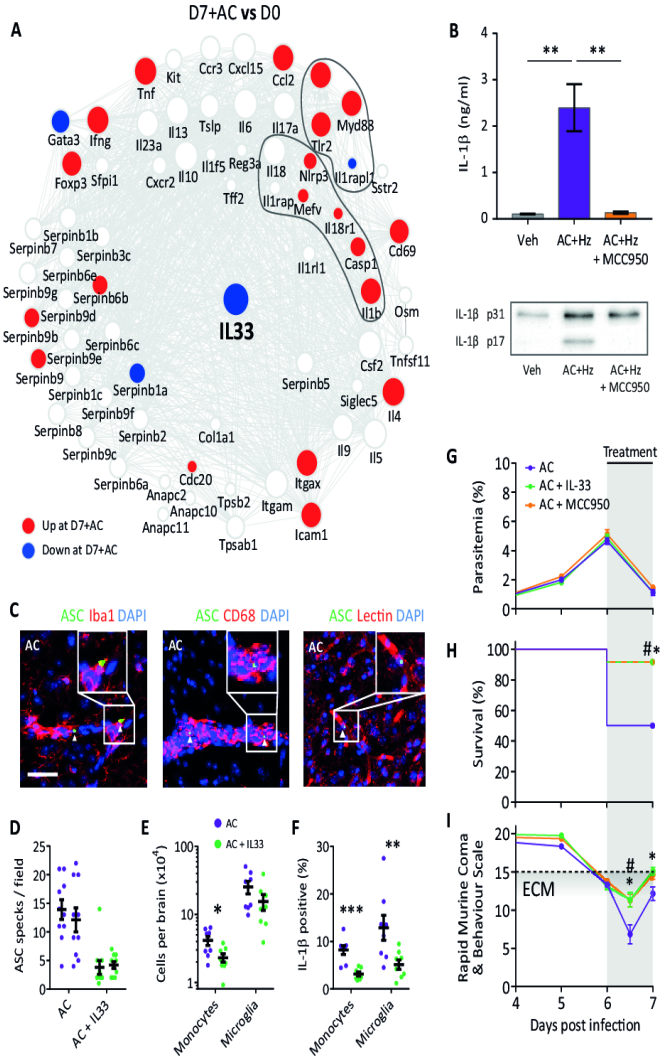

**Fig. 4.** . IL33 suppresses NLRP3 and IL-1β responses that undermine anti-malarial drug treatment of ECM. (*A*) Cytoscape network defining differentially expressed genes (DEGs) in IL33 protein-protein interaction network in brains 16-24 h post drug treatment (d7+AC) compared with d0. (*B*) BMDMs were treated with anti-malarial drugs and haemozoin (AC+Hz) with or without the NLRP3 inhibitor MCC950, with IL1 release measured by ELISA (n=4), and mature IL1β in the supernatant confirmed by Western blot. (*C, D*) Pb ANKA infected ASC-citrine reporter mice were treated at ECM onset with AC alone (AC), or together with IL33 (AC+IL33). (*C*) Cortical grey matter of AC-treated mice showing ASC specks associated with Iba1$^+$ microglia, intravascular CD68$^+$ monocytes, or lectin$^+$ endothelial cells. (*D*) ASC specks per field of view (20 fields total from n=2 for each group). (*E-F*) Pb ANKA-infected C57BL/6 mice were treated at ECM onset with AC alone (AC), or together with IL33 (AC+IL33), and brains examined by flow cytometry (n=8 from 2 infections). (*E*) Total numbers of microglial cells and intracerebral monocytes. (*F*) Production of IL1β by microglia and monocytes. (*G-I*) Pb ANKA-infected C57BL/6 mice (n=12 from 2 infections) were treated at ECM onset with AC alone (AC), together with IL33 (AC+IL33) or MCC950 (AC+MCC950). (*G*) Peripheral parasitaemia, (*H*) survival curves and (*I*) RMCBS scores. Data is presented as means ±SEM. (B) Comparisons made by ANOVA. (E, F) Comparisons made by Mann-Whitney tests. (G, I) Separate comparisons were made between groups at d6.5 and d7 by Kruskal-Wallis test, with Dunn's correction for multiple comparisons. (H) Comparisons made by log-rank test. (B) *p<0.05, **p<0.01, versus AC+Hz. (D-I) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, all versus AC. #p<0.05 MCC950 vs AC.

mice succumbed to ECM on d7, demonstrating that IL33 alone was not able to promote recovery from established ECM (Fig. S3). These results demonstrate that IL33 significantly improved the effectiveness of anti-malarial drug treatment of established ECM.

**IL33 suppresses NLRP3 inflammasome formation and inhibits IL-1β production in the brain**

We next examined the mechanism(s) through which IL33 improved the recovery from ECM. Analysing IL33's protein-protein interaction network revealed that a large number of IL33-regulated genes significantly upregulated in the brains of mice following anti-malarial drug treatment were directly or indirectly related to the NLRP3 inflammasome pathway (Fig. 4A and Fig. S4). It has previously been shown that the malarial parasite product haemozoin (Hz) can directly activate the NLRP3 inflammasome to promote IL1β production (16, 17). Consistent with this, we found that Hz induced release of mature IL1β from bone marrow-derived macrophages (BMDMs) (Fig. S5A). We also found that artesunate and chloroquine, as well as pyrimethamine, another commonly used anti-malarial drug (18), induced IL1β release from BMDMs (Fig. S5B & C). The release of IL1β from BMDMs induced by Hz and anti-malarial drug stimulation, individually and in combination, was completely inhibited by MCC950, a selective inhibitor of the NLRP3 inflammasome (19) (Fig. 4B and Fig. S5D and E). These data, therefore, implied that anti-malarial drugs and malaria-parasite products may directly induce damaging inflammasome-induced neuroinflammation, possibly undermining recovery from ECM. In agreement, ASC specks, indicative of inflammasome activation, were observed extensively within the brains of infected mice 16-24 h after anti-malarial drug

treatment of ECM (Fig. 4C). ASC specks were visualised adjacent to, and within, microglial cells, intravascular monocytes and endothelial cells (Fig. 4C). Critically, IL33 treatment significantly reduced the number of ASC specks in the brain, compared with mice treated only with anti-malarial drugs (Fig. 4D).

As our results indicated that IL33 administration reduced numbers of monocytes and microglia expressing inflammasomes, we examined whether IL33 treatment modified the polarisation or activation of the cells. Both microglia and recruited monocytes / macrophages expressed the IL33 receptor ST2 following drug-treatment of established ECM (Fig. S6A-C). IL33 administration reduced the numbers of monocytes, but not microglia, at 16-24 h post-treatment, and significantly reduced IL1β production in both cell types (Fig. 4E and F). This effect of IL33 was not mediated through alteration in M1 (based on TNFα and CD40) or M2 (based upon CD36, PDL1, and Relmα expression) polarisation in either monocytes or microglia (Fig. S6D). Collectively, these data indicate that IL33 therapy selectively inhibited the NLRP3 inflammasome-IL1β axis in microglia and monocytes during the acute recovery period following treatment of ECM.

$CD8^+$ T-cells have been shown to play an important role in the development of ECM (20). Although intracerebral $CD8^+$ T cells also expressed the ST2 receptor following anti-malarial drug treatment of ECM (Fig. S7A), IL33 administration did not significantly alter $CD8^+$ T-cells accumulation in the brain (Fig. S7B). IL33 also had no effect on intracerebral $CD8^+$ T cell effector functions, as defined by intracellular levels of Granzyme B and cell surface expression of the degranulation marker CD107a (Fig. S7C-D).

**NLRP3 inhibitor MCC950 improves anti-malarial drug treatment success of ECM**

We then assessed whether administration of a selective NLRP3 inhibitor alongside anti-malarial drugs could improve ECM recovery. MCC950 was administered as a single dose (50 mg/kg, HED 4.0541 mg/kg) alongside anti-malarial drugs (both at 30 mg/kg) on the first day of treatment (d6). MCC950 did not significantly alter peripheral parasitemia (Fig.4G). However, comparable to IL33, MCC950 co-treatment along with anti-malarial drugs significantly improved survival from established ECM (Fig. 4H). Furthermore, MCC950 administration also significantly improved the RMCBS scores of mice 6-12 h (d6.5) after treatment, compared with mice treated with anti-malarial drugs alone (Fig. 4I). Consistent with our findings regarding IL33 monotherapy, MCC950 administration alone (on d6) did not promote improved recovery from ECM (Fig. S8). Thus, NLRP3 inhibitor treatment also significantly improved the efficacy of anti-malarial drug treatment of ECM comparable to the effects of IL33 treatment.

## Discussion

In this study we have shown that anti-malarial drugs are unable to prevent mortality in a quarter of mice with established ECM, analogous to the failure rates for CM treatment (2, 3). Furthermore, even when anti-malarial drug treatment was successful and animals survived, they were left with significant levels of residual neuropathology. This is consistent with the long-lasting neurological sequelae commonly found in drug-cured CM patients (4, 5). Therefore, our experimental model effectively recapitulates both the primary and secondary clinical challenges associated with the anti-malarial drug treatment of CM. Using this model we assessed the effectiveness of adjunctive therapies in improving existing anti-malarial drug therapy. We have discovered that adjunctive IL33 or NLRP3 inhibitor therapy dramatically improved the survival and enhanced the recovery of mice that underwent anti-malarial drug treatment.

Our analysis of the brain transcriptome following anti-malaria drug treatment provided new insights into why anti-malarial drugs fail to promote optimal recovery from ECM. Specifically, our data highlights that the neuroinflammatory response associated with agonal ECM is not rapidly downregulated by anti-malarial drugs alone. Importantly, many of the inflammatory pathways that continue to be upregulated in the brains of mice following anti-malarial drug treatment of ECM (e.g. response to interferon gamma, cytotoxic T-cell and macrophage activation, and blood coagulation) likely converge to affect the activation of brain endothelial cells (1, 6-9). Concordantly, significant vasculopathy was still evident in mice 24 h after anti-malarial drug treatment of established ECM. Thus, our data are consistent with the notion that suboptimal recovery from CM is associated with excessive levels of neuroinflammation and continued disruption to the neurovascular unit (1, 6-9).

Analysis of the upstream regulators controlling the brain transcriptional response during ECM identified 13 genes that could potentially be targeted by additional therapies. We prioritised IL33 because exogenous administration of IL33 has been shown to resolve inflammation and promote repair in other neuropathologies, including Alzheimer's disease, stroke, and spinal cord injury (12, 13, 15). Moreover, we have previously shown that IL33 administration (without concurrent anti-malarial treatment) can attenuate ECM development when given at early stages of infection (21). We hypothesised that the observed reduction in cerebral IL33 during ECM allowed cerebral inflammation to become dysregulated, and undermined the success of anti-malarial drug treatment. Consistent with this, adjunctive administration of IL33 significantly improved survival and reduced neurological dysfunction in drug-treated mice, compared with anti-malarial drugs alone. Importantly, in addition to reducing parasite levels in the brain of surviving mice (examined 16-24 h post-treatment), IL33 therapy protected against ECM-induced cerebrovascular damage, as shown by reduced levels of vascular occlusion, oedema and haemorrhage.

Our gene expression analysis from anti-malarial drug treated animals suggested that there was an interaction between the decrease in IL33 gene expression and an increase in expression of genes in the NLRP3 inflammasome pathway (Fig. 4A). While the NLRP3 inflammasome is reportedly not a contributor to the development of ECM (22), its activation could account for the mortality observed after drug treatment of CM and ECM. Indeed, high levels of IL1β have been observed in the brains of individuals with fatal CM (23, 24). Moreover, the NLRP3-IL1β axis is a key driver of acute cerebrovascular dysfunction (25) and progressive neuroinflammation in a number of brain pathologies (26). We observed that administration of IL33 reduced ASC speck formation and IL1β production in the brain compared with mice given anti-malarial drugs alone. Furthermore, the selective NLRP3-inflammasome inhibitor MCC950 also significantly improved recovery of mice following anti-malarial drug therapy (as with IL33, MCC950 treatment by itself without anti-malarial drugs was not protective). Together, our results therefore suggest that IL33 improves anti-malarial drug treatment of ECM by altering the brain transcriptome, resulting in suppression of NLRP3-dependent inflammation. This model of protection is in agreement with reports suggesting that administration of IL33 supresses the expression of NLRP3-inflammasome components in an Alzheimer's disease model (12), and in a model of intracerebral haemorrhage (27). Our results are, however, in contrast to a recent report that oligodendrocyte-derived IL33 acts to promote production of IL1β from microglia, subsequently causing cognitive deficits and ECM development (28). Where we examined the NLRP3-supresssing effects of IL33 *in vivo*, Reverchon et al (28) defined the IL33-IL1β cycle in an in vitro mixed glial culture derived from naïve mice. IL33 treatment may exert fundamentally different direct and/or indirect activities *in vivo* within an established inflammatory brain

environment than in in vitro mixed glial cultures in the absence of any other inflammatory or pathogenic signal(s) (29, 30).

NLRP3 inflammasome activation in the acute recovery period following treatment with anti-malarial drugs could be caused by the drugs themselves, malaria parasite products, or damage-associated signalling molecules. Consistent with previous studies (16, 17), haemozoin, which we postulate phagocytic cells will be exposed to in significant amounts following anti-malarial drug treatment and death of high numbers of parasites, induced NLRP3-dependent release of mature IL1β from BMDMs. A variety of anti-malarial drugs (chloroquine, artesunate, and pyrimethamine) also induced predominantly NLRP3-dependent mature IL1β release from BMDMs. Thus, we speculate that anti-malarial drug treatment of CM may directly and indirectly provoke inflammasome activation in intracerebral mononuclear phagocytes, impairing the effectiveness of anti-parasitic chemotherapy to resolve malaria-induced cerebral pathology. In support of this, we consistently observed accelerated neurological dysfunction and mortality within the subset of mice that succumbed to ECM following anti-malarial drug treatment, compared with vehicle treated controls. Collectively, our data therefore suggests that fatality and neurological sequelae in anti-malarial drug treatment of CM may occur, at least partially, as a result of related iatrogenic effects, which can be prevented through IL33 or NLRP3 inhibitor administration.



## Methods

### Mice, infections and analyses

All animal work was approved following local ethical review by the University of Manchester Animal Procedures and Ethics Committees and was performed in accordance with the U. K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O. Project Licenses 70/7293 and P8829D3B4). Female and male C57BL/6 mice (8-10 week old) were purchased from Charles River. ASC-citrine reporter mice (31) mice were bred at the University of Manchester. All mice were maintained in individually ventilated cages. Cryopreserved Pb ANKA GFP parasites (32) or Pb ANKA parasites (33) were thawed and passaged once through C57BL/6 mice before being used to infect experimental animals. Animals were infected via intravenous injection of $1x10^4$ pRBCs. Peripheral parasite burdens of infected mice were followed from day 3 by microscopic examination of giemsa-stained thin blood smears. The development of, and subsequent recovery from, ECM was assessed using the RMCBS (12). Mice exhibiting early signs of ECM (score ≤15 on the RMCBS, invariably d6) received up to 6 daily intraperitoneal (i.p.) injections of 30 mg/kg artesunate (Sigma) and 30 mg/kg chloroquine (Sigma) in PBS or, alternatively, PBS alone. In some experiments mice received single doses of 0.02 mg/kg (HED 0.0016mg/kg, calculations based on ref. 34) recombinant IL33 (Biolegend), or 50 mg/kg (HED 4.0451mg/kg) MCC950 (Sigma) on day 6 via i.p. injection, concomitant with anti-malarial drug administration. Detailed information describing protocols for microscopy of brain pathology, RNA purification from whole brain and paired-end RNA-seq analysis, and flow cytometry of intracerebral leucocytes are provided in SI Appendix method.

### BMDM activation and assessment of IL1β secretion

BMDMs, generated as described in SI Appendix method, were seeded at 100,000 cells per well in 96-well plates then left to adhere overnight before priming with 1 μg/ml lipopolysaccharide (LPS, 0127:B8, Sigma) for 4 h. Following priming, media was replaced with fresh DMEM containing 10% FBS for haemozoin (Hz, Invivogen) or serum-free for anti-malarial drug treatments. MCC950 (CP-456773, Sigma) or vehicle control were pre-incubated for 15 min prior to inflammasome activation. For Hz assays, cells were treated with Hz or PBS for 24 h. Malaria drugs or appropriate vehicles were incubated for 5 h. In the case of co-incubation of Hz and drugs, cells were treated for 24 h. Supernatants were removed and analysed for IL1β content by ELISA (DuoSet, R&D systems). IL-1β cleavage within activated BMDMs was performed by Western blot as described in SI Appendix method.

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