

# Hemostatic and protein C pathway dysfunction in the pathogenesis of experimental cerebral malaria

Accumulating data suggest that hemostatic dysfunction contributes to *Plasmodium falciparum* malaria pathogenesis.<sup>1</sup> In addition, specific mechanisms through which the protein C pathway modulates *P. falciparum* pathogenesis have been described.<sup>1</sup> We hypothesized that the anticoagulant and anti-inflammatory activities of recombinant activated protein C (APC) may possess therapeutic value in the setting of cerebral malaria (CM). In order to address this hypothesis, we assessed hemostatic parameters in an established murine model of experimental cerebral malaria (ECM), and using the same model, investigated the ability of recombinant APC to ameliorate ECM. In keeping with findings in patients with severe *P. falciparum* malaria, we observed that dysregulated thrombin generation and protein C pathway dysfunction were both late features of ECM. Furthermore, pretreatment with a monoclonal anti-EPCR antibody that blocks protein C/APC binding prior to *P. berghei* inoculation significantly reduced overall survival. Conversely, mice treated with recombinant APC exhibited a marked attenuation in clinical ECM progression and parasitemia, in parallel with a significant increase in overall survival. All together, these findings confirm that hemostatic and protein C pathway dysfunction are both consistent features in human and ECM, and demonstrate for the first time a role for recombinant APC in reducing clinical progression and mortality in ECM.

CM is a life-threatening complication of *P. falciparum* infection characterized by ataxia, seizures, altered consciousness and coma. Although CM is associated with significant mortality, the biological mechanisms underlying its pathogenesis remain poorly defined. Significant coagulation cascade activation including elevations in levels of fibrin degradation products (FDP) and thrombin-antithrombin (TAT) complexes are common in patients with *P. falciparum* infection.<sup>2,3</sup> Furthermore, Moxon *et al.* recently described overt DIC in 19% of children with retinopathy-positive CM.<sup>3</sup> Together, these findings suggest that hemostatic dysfunction may contribute to malaria pathogenesis. This hypothesis is supported by the observation that coagulation activation correlates with peripheral blood parasitemia levels, and is inversely-related to overall survival.<sup>4,5</sup> Moreover, microvascular fibrin deposition has also been reported in postmortem studies of patients with fatal CM.<sup>6</sup> A number of molecular mechanisms through which *P. falciparum* infection triggers coagulation activation have been described.<sup>1</sup> Recent studies have also highlighted specific mechanisms through which the protein C pathway influences *P. falciparum* pathogenesis.<sup>6,7</sup> Importantly, both

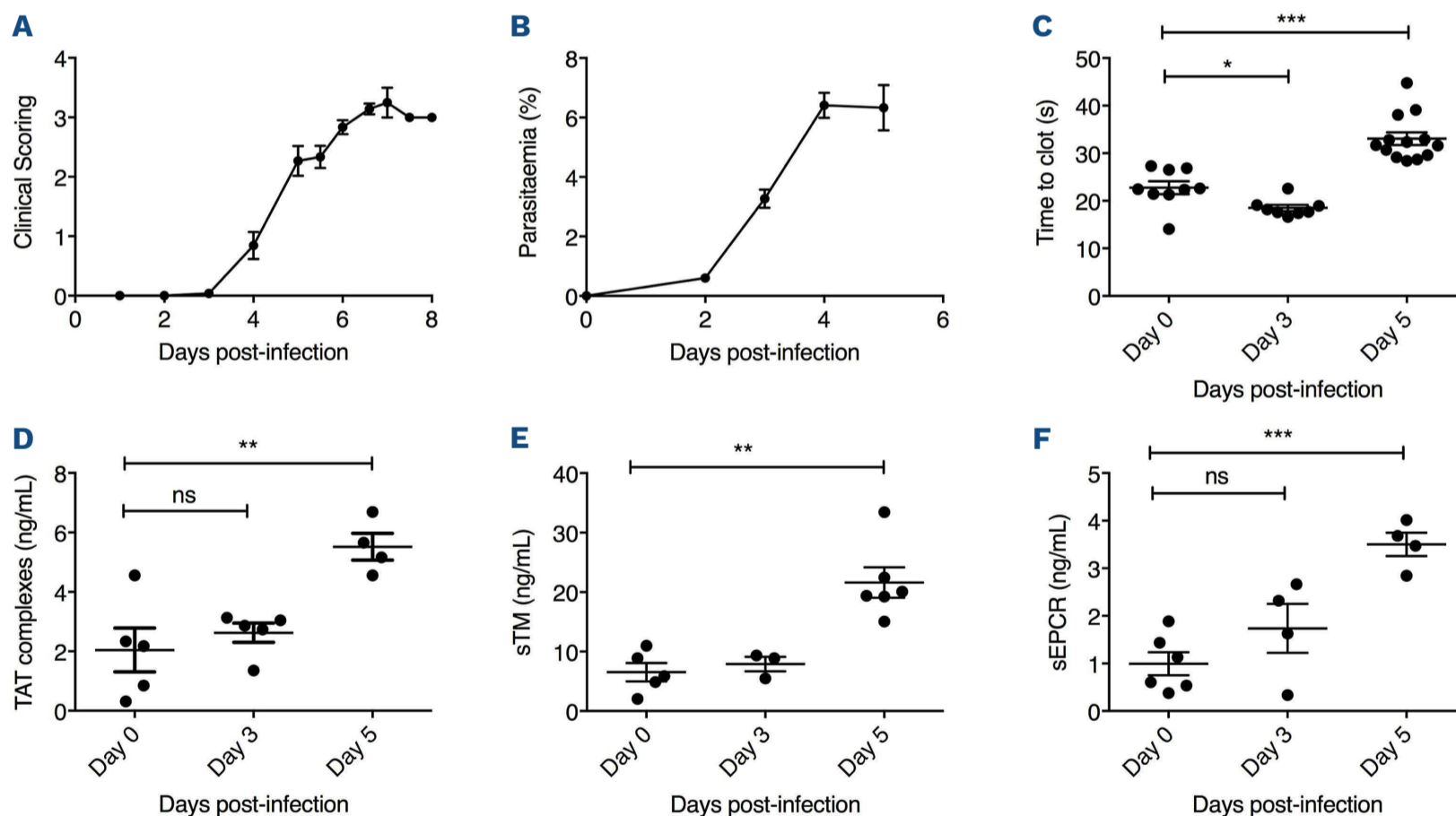
thrombomodulin (TM) and the endothelial protein C receptor (EPCR) can bind to PfEMP1 expressed on *P. falciparum* to infected erythrocyte (IE) surfaces,<sup>7,8</sup> thereby facilitating IE cytoadhesion to endothelial cells (EC). PfEMP1 binding to EPCR also limits generation of anticoagulant activated protein C (APC), and inhibits EPCR-dependent PAR1-mediated protection of EC barrier integrity.<sup>9,10</sup> The typical late clinical presentation of patients with CM makes it difficult to determine whether hemostatic dysfunction directly contributes to the pathogenesis of CM, or whether coagulation activation merely constitutes a secondary epiphenomenon. Therefore, in this study we sought to further investigate the role of coagulation activation and the protein C pathway in malaria pathogenesis using an established murine model of ECM, in which C57BL/6J mice were infected with *P. berghei* ANKA.<sup>11</sup>

We have previously described in detail the murine experimental cerebral malaria (ECM) model. All mouse experiments were performed in compliance with the Irish Medicines Board and approved by the Trinity College Dublin BioResource Ethics Committee. Mice aged 8 to 10 weeks were infected by intraperitoneal injection of  $2 \times 10^6$  *P. berghei* ANKA. Following inoculation, malaria progression was monitored using a previously validated ECM clinical scoring system.<sup>11</sup> *P. berghei* parasitemia levels were monitored by Giemsa-stained thin blood smears. Platelet counts were measured using a Sysmex haematology analyser (KX-21N). In order to prepare platelet-poor plasma, blood samples were centrifuged at 1,500 g for 15 minutes at 20°C, aliquoted and stored at -80°C until use. Murine plasma activated partial thromboplastin time (APTT) was determined using a commercial kit (C.K. Prest, Stago) and time for clot formation was determined using Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland). Similarly, PT was determined using Hemosil recombiplastin 2G according to the manufacturer's guidelines. Plasma levels of thrombin-antithrombin (TAT) complexes, soluble thrombomodulin (sTM) and soluble endothelial cell protein C receptor (EPCR) were quantified using specific commercial enzyme-linked immunosorbant assays (ELISA) (Abcam, Cambridge, UK and R&D Systems, UK) according to the manufacturer's instructions. In order to study the role of EPCR, ECM progression was assessed in mice pretreated with 50 µg of an EPCR blocking antibody RCR-16,<sup>12</sup> or isotype control antibody, immediately prior to *P. berghei* infection. Recombinant murine APC was generated, expressed, and purified as previously described.<sup>13</sup> Consistent with previous studies

assessing the anti-inflammatory properties of recombinant APC in murine endotoxemia models,<sup>14</sup> mice were treated with 10 µg mAPC administered intravenously immediately prior to *P. berghei* infection. Subsequently another 10 µg of mAPC was administered 4 hours post-infection. Alternatively, mice were treated with two 10 µg mAPC boluses administered 4 hours apart on day +3 following *P. berghei* inoculation.

Progressive clinical features similar to those observed in human CM were evident in mice from day +3 following infection (Figure 1A). Peripheral *P. berghei* parasitemia levels increased progressively (Figure 1B), and mice typically died within 7-10 days. In keeping with previous findings in patients with severe *P. falciparum* malaria, a significant increase in APTT was observed by day +5 following *P. berghei* inoculation (Figure 1C).<sup>2</sup> Again in keeping with human studies,<sup>3</sup> no significant changes in either PT or murine fibrinogen levels were seen through the course of *P. berghei* infection (data not shown). Nevertheless, by day +5 after inoculation, median plasma TAT levels were increased approximately 2.5 fold (2.0 ng/mL at day 0 vs. 5.5 ng/mL at day +5;  $P<0.01$ ) (Figure 1D), which was similar in magnitude to the increase in plasma TAT levels observed in patients

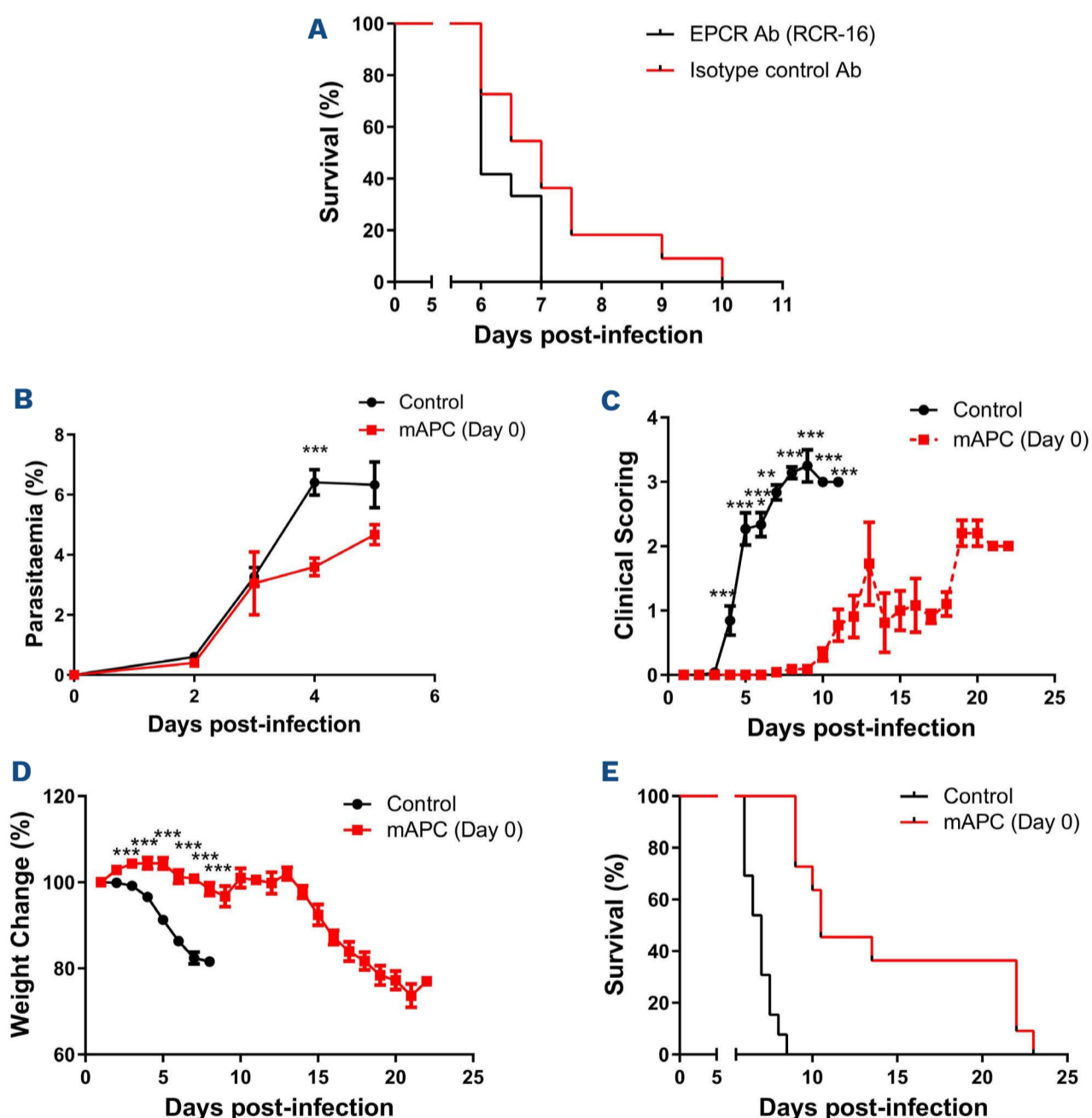
with CM.<sup>2,4,6</sup> In contrast to the early increase in plasma VWF levels that occurs within 24 hours following *P. berghei* infection,<sup>11</sup> plasma TAT levels remained normal for 72 hours post-infection. Murine plasma protein C and antithrombin levels were not significantly reduced during *P. berghei* infection (data not shown). However, again as reported in previous findings in children infected with *P. falciparum*,<sup>3,6</sup> plasma levels of both sTM and sEPCR were significantly elevated in C57BL/6J mice following *P. berghei* infection (sTM 6.5 ng/mL at day 0 vs. 21.6 ng/mL at day +5;  $P<0.001$  and sEPCR 0.99 ng/mL at day 0 vs. 3.50 ng/mL at day +5;  $P<0.001$ ) (Figure 1E and F). In keeping with the hypothesis that hemostatic dysfunction is a relatively late feature in ECM, the increases in plasma sTM and sEPCR were not observed until day +5 following inoculation. Collectively, these findings demonstrate that dysregulated thrombin generation represents a consistent feature of both human and experimental murine CM. Critically however, unlike the acute EC activation that represents an early hallmark in both murine and human malaria, hemostatic and protein C pathway dysfunction both develop at a much later stage. In order to investigate whether protein C pathway dysfunction contributes to ECM pathogenesis, mice were pre-



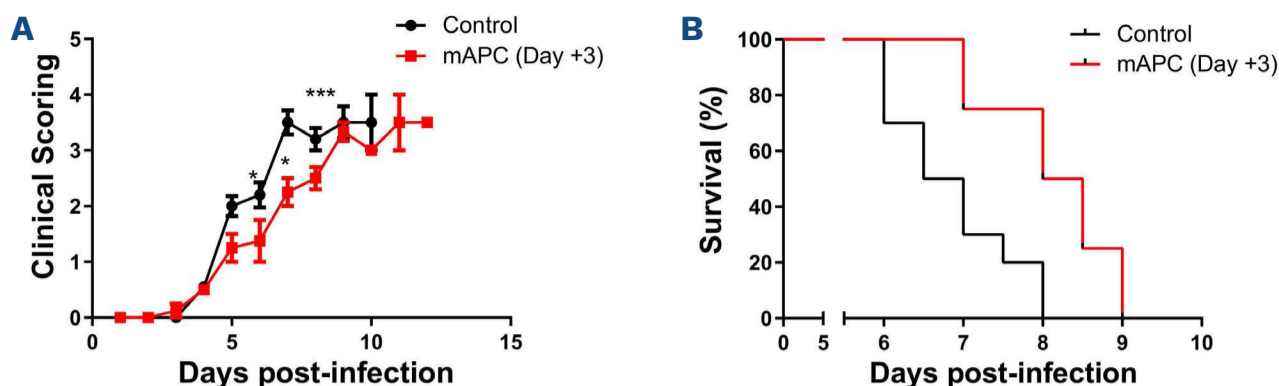
**Figure 1. Hemostatic and protein C pathway dysfunction constitute late features of experimental cerebral malaria.** (A) Following intraperitoneal inoculation with  $2 \times 10^6$  *Plasmodium berghei* ANKA parasites, experimental cerebral malaria (ECM) progression was followed in wild-type (WT) C57BL/6J mice ( $n=12$ ) using a validated clinical scoring algorithm. Results presented represent the mean values  $\pm$  standard error of the mean (SEM) unless otherwise stated. (B) Following *P. berghei* infection, peripheral blood parasitemia levels were determined each day using Giemsa-stained smears ( $n=12$ ). By day +5 following *P. berghei* infection, murine activated partial thromboplastin time (APTT) levels (C) and plasma thrombin-antithrombin (TAT) complex levels (D) were both significantly increased ( $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.0001$  respectively). Similarly, plasma levels of soluble endothelial cell protein C receptor (sEPCR) (E) and soluble thrombomodulin (sTM) (F) were also both significantly elevated by day +5. Data are expressed as mean values  $\pm$  SEM. In order to assess statistical differences, data were analyzed using Student's unpaired 2-tailed *t*-test. ECM clinical scoring data were assessed by two-way ANOVA analysis.

treated with a monoclonal anti-EPCR antibody (RCR-16) previously shown to block protein C/APC binding,<sup>12</sup> or an isotype control antibody, immediately prior to *P. berghei* inoculation. We observed significantly reduced overall survival ( $P<0.05$ ) in the cohort of mice treated with RCR-16 (Figure 2A), suggesting that EPCR-dependent APC generation and/or signaling is important for controlling ECM development. Case studies involving a small number of patients with severe *P. falciparum* malaria treated with APC have reported variable effects.<sup>15</sup> Consequently, we further investigated whether administration of recombinant mAPC influenced ECM pathogenesis. Mice were treated with 10  $\mu\text{g}$  mAPC immediately prior to *P. berghei* infection, and a subsequent second 10  $\mu\text{g}$  mAPC dose was administered 4

hour later. Mice treated with recombinant mAPC exhibited a mild but significant reduction in parasitemia at day +4 (Figure 2B;  $P<0.001$ ). In contrast however, the APC-treated mice demonstrated significantly attenuated clinical ECM progression (Figure 2C;  $P<0.001$ ) and weight loss (Figure 2D;  $P<0.001$ ). Furthermore, mAPC administration also caused a significant increase in overall survival (Figure 2E;  $P<0.001$ ). In order to investigate whether mAPC administered later in the course of ECM can still influence clinical progression, the effect of administering mAPC on day +3 was investigated. Once again, clinical progression (Figure 3A) and overall survival (Figure 3B) were both marginally improved in mice treated with mAPC compared to controls ( $P<0.05$ ). Interestingly, however, the magnitude of this ef-



**Figure 2. Recombinant anticoagulant activated protein C significantly attenuates clinical progression and markedly improves overall survival in experimental cerebral malaria.** (A) In order to determine if endothelial protein C receptor (EPCR) plays a role in experimental cerebral malaria (ECM) pathogenesis, mice were pretreated with the EPCR blocking antibody RCR-16 or an isotype control antibody prior to infection with  $2 \times 10^6$  *Plasmodium berghei* ANKA parasites. Twelve mice were treated in the RCR-16 and 11 in the isotype control groups. Overall survival was significantly reduced in mice treated with RCR-16. In order to investigate whether recombinant murine anticoagulant activated protein C (mAPC) administration influences ECM progression, mice were pretreated with 10  $\mu\text{g}$  mAPC immediately prior to *P. berghei* infection and a second 10  $\mu\text{g}$  mAPC dose was administered 4 hour later. Twelve mice were treated in the mAPC and control groups. Mice treated with recombinant mAPC exhibited (B) mildly reduced parasitemia at day +4, (C) attenuated clinical ECM progression (D) reduced weight loss and (E) significantly increased overall survival ( $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$ ).



**Figure 3. Recombinant anticoagulant activated protein C later in the course of experimental cerebral malaria has only mild effects on clinical progression and survival.** In order to investigate whether murine anticoagulant activated protein C (mAPC) later in the course of experimental cerebral malaria (ECM) can still influence clinical progression, 2 doses of mAPC were administered on day +3 following *Plasmodium berghei* inoculation. Once again, (A) clinical progression and (B) overall survival were both mildly improved in mice treated with mAPC compared to controls. Mouse survival data were compared using a log-rank (Mantel-Cox) Test.

fect was markedly less than that observed in mice treated with mAPC on day 0. This attenuated efficacy of APC administered later in the disease course is in keeping with the concept that CM is associated with progressive shedding of EPCR and TM from EC surfaces.<sup>10</sup>

In conclusion, our findings demonstrate that hemostatic and protein C pathway dysfunction are both consistent features in human and experimental murine CM. We also show for the first time that recombinant mAPC markedly reduces clinical progression and overall mortality in ECM. Further studies will be required to elucidate the molecular mechanisms through which APC modulates ECM pathogenesis, together with the optimal APC dosing and treatment regimen. Nevertheless, given the significant morbidity and mortality that are still associated with CM, novel adjunctive therapies to limit vascular dysfunction and slow disease progression are urgently required.

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### Contributions

NO'R, KG, EMG, JMOS, CD, ND, AC, and TMB performed experiments; NO'R, KG, EMG, JMOS, KJ, TMB, OPS, RJSP and JSO'D designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

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### Data-sharing statement

All original data and protocols can be made available to other investigators upon request.

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