Potential cerebral malaria therapy: intramuscular arteether and vitamin D co-administration

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SUMMARY

Cerebral malaria (CM) shows lethality rate of 15–25% despite effective antimalarial chemotherapy. The effective adjunct treatment to counteract the CM pathogenesis is urgently required. In murine CM model, most interventions studied till date are administered before the onset of CM symptoms, which belittle its translational value to human. We studied intramuscular arteether–vitamin D (ART–VD) combination treatment for CM outcome improvement after the onset of neurological symptoms. The intramuscular dose of 50 μg kg^{-1} VD for 3 days combined with a loading dose of 25 mg kg^{-1} α/β arteether followed by 12·5 mg kg^{-1} dose for two consecutive days led to significant improvement in survival (73% in combination group *vs* 29 and 0% in arteether and VD monotherapy, respectively) and clinical recovery. The treatment in all the groups partially restored the blood–brain barrier integrity and reduced the level of serum proinflammatory cytokines tumour necrosis factor-α and interferon-γ. The brain transcripts of inflammatory chemokines *viz*. CXCL10, CXCL9, CCL4 and CCL5 and T cell migration in the brain microvasculature were significantly diminished in all the treatment groups. ART–VD treatment significantly reduced intercellular cell adhesion molecule-1 expression. Taken together, our findings show that coordinated actions of ART–VD improve the outcome of experimental CM.

Key words: cerebral malaria, arteether, vitamin D, adjunct therapy, *Plasmodium berghei* ANKA.

INTRODUCTION

Cerebral malaria (CM) is a complex neurological manifestation of *Plasmodium falciparum* with high lethality rate amongst children below 5 years of age in Africa and afflict adults in Southeast Asia despite the treatment with highly effective antimalarials (Mishra and Newton, 2009; Idro *et al*. 2010). Thus, antimalarials alone can control the parasite propagation but not the disease progression. The CM pathogenesis is attributed to parasite sequestration in the microvasculature (Berendt *et al*. 1994; Ponsford *et al*. 2012) and dysregulated immune response (Clark and Rockett, 1994). The excessive production of proinflammatory cytokines and attenuation of Th2 response leads to upregulation of cell adhesion receptors such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, thereby, augmenting the cytoadhesion (Armah *et al*. 2005*a*). The endothelial activation causes loss of blood–brain barrier (BBB) function and increased permeability leads to focal haemorrhage in the brain tissue, microglial activation, neuronal damage, brain oedema, coma and eventually death of the patients in the absence of proper treatment (Renia *et al*. 2012). A number of other pathways such as rho-kinase pathway (Taoufiq *et al*. 2008) and kynurenine pathway (Clark *et al*. 2005) are altered in CM. The intervention strategies with the CM inducing factors may improve the outcome of the disease. A number of agents have been or are being evaluated against animal model and in human trials. Adjunctive therapies that have been tested in patients with CM or severe malaria include immunomodulatory agents such as dexamethasone, intravenous immunoglobulin, monoclonal antibodies to tumour necrosis factor alpha (TNF-α), pentoxifylline, curdlan sulphate, antioxidant *N*-acetylcystein, e plasma expander albumin, cytoadherence inhibitor levamisole and neuroprotective agent erythropoietin (John *et al*. 2010). Most of the trials were disappointing with a very little success rate. It would be prudent to target multitude pathways to improve CM outcome.

Vitamin D is a fat soluble vitamin with two dominant forms, vitamins D_2 (ergocalciferol) and D_3 (cholecalciferol). It is either supplied in diet or photosynthesized in the skin by the action of solar ultraviolet (UV) B radiation on 7-dehydrocholesterol. Besides the conventional role in calcium homeostasis and bone health, vitamin D insufficiency is associated with several other diseases, including multiple sclerosis (Sandberg *et al*. 2016), diabetes, cardiovascular disease (Papandreou and Hamid, 2015), rheumatoid arthritis, asthma, cancers, upper respiratory tract infections (Esposito and Lelii, 2015) and tuberculosis. Growing evidences indicate that the active

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form of VD, i.e. $1,25(OH)_2D_3$ shifts the balance of Th1 *vs* Th2-mediated functions towards the Th2 cells (Hayes *et al*. 2003), affects dendritic cell maturation and macrophage differentiation (Berer *et al*. 2000).Vitamin D stabilizes the quiescent endothelium, repairs the damaged endothelium *in vitro* and *in vivo* (Ni *et al*. 2014), protects the endothelium from oxidant injury through modulation between apoptosis and autophagy (Polidoro *et al*. 2013; Uberti *et al*. 2014), prevents endothelial activation (Equils *et al*. 2006; Martinesi *et al*. 2006), attenuates platelet activation and the expression of VCAM-1 in human endothelial cells (Stach *et al*. 2011). Several experimental models indicate the potential value of VD pharmacological analogues in neurodegenerative and neuroimmune diseases (Garcion *et al*. 2002). Vitamin D is less explored in malaria with varying results. VD administered orally provides significant survival benefits in the murine experimental CM (ECM) model by suppressing host inflammatory responses (He *et al*. 2014) whereas in another study vitamin D intraperitoneal injection did not improve survival in CM (Waisberg *et al*. 2012).

The *Plasmodium berghei* ANKA (PbA)-infected C57BL/6 mice are the widely accepted ECM model as it manifests many of the neurological features of human CM. However, the murine model should be carefully exploited when potential therapeutic applications are the focus of the study as treatment prior to onset of symptoms in mice may offer insight into disease progression, but may not yield viable therapy (Craig *et al*. 2012). In the present communication, we report the efficacy of α/β arteether in combination with VD against late-stage ECM.

MATERIAL AND METHODS

Infection and drug treatment

Female C57BL/6 mice (6–7 weeks old) weighing 17–18 g were used for all experiments. Animals were housed in polypropylene cages under standard conditions of temperature (24 ± 1 °C) and humidity (55–68%) and fed standard pellet diet and water *ad libitum*. *Plasmodium berghei* ANKA, (MRA-311) strain was obtained from Malaria Research and Reference Reagent Resource Center (MR4), ATCC Manassas, Virginia. All experimental mice were infected intraperitoneally (i.p.) with 3×10^6 PbA parasitized red blood cells (RBC), which were withdrawn from a previously infected mouse of the same strain. Parasitaemia was monitored daily by counting the number of pRBCs per 10 000 RBCs by microscopic examination of Giemsa-stained thin smears from mice tail blood. Mice were monitored daily for survival and neurologic signs of CM such as deviation of head, ruffled hair, hind limb paralysis, ataxia, convulsions and coma as reported (Engwerda *et al*. 2005). Eighty-ninety per cent of the PbA-infected animals showed typical CM symptoms between days 6 and 8 post-infection. After onset of symptoms on day 6, mice were randomized in four groups: PbA-infected (PbA; $n = 18$), PbA-infected VD treated (PbA + VD; $n = 9$), PbA-infected α/β arteether treated (PbA + ART; $n = 7$) and PbA-infected α/β arteether and VD supplemented group (PbA + ART-VD; $n = 15$) for survival record and parasitaemia trend. PbA group was the control group and received no treatment. In all other groups, treatment began day 6 onwards after ascertaining the cerebral symptoms. Mice in PbA +VD group received daily intramuscular injection of 50 μg kg^{-1} for 3 days. Mice in PbA + ART group received intramuscular injection of 25 mg kg^{-1} arteether on day 6 followed by 12·5 mg kg^{-1} arteether on day 7 and 8. Mice in PbA + ART-VD group received intramuscular injection of 25 mg kg^{-1} arteether on day 6 followed by 12·5 mg kg^{-1} on day 7 and 8 along with 50 μg kg^{-1} VD on days 6, 7 and 8. VD stock was prepared in DMSO (dimethyl sulfoxide) and diluted to appropriate dilution in groundnut oil. α/β Arteether was also prepared in ground nut oil. For the combination treatment, both the drugs were simultaneously dissolved in groundnut oil in appropriate quantity. Animal handling and experiments were conducted in accordance to the Institutional Animal Ethics Committee guidelines.

BBB permeability

To evaluate integrity of the BBB, 200 μL of 2% (w/v) solution of Evans blue in phosphate-buffered saline (PBS) was injected via intravenous route into each mouse. After 2 h, brains were excised and blue dye was extracted from the brain tissue in 100% formamide. The absorbance of the extracted dye was measured at 620 nm after 48 h (Baptista *et al*. 2010). The concentration of Evans blue dye was calculated using a standard curve. The data are expressed as μg of Evans blue dye per g of brain tissue.

Brain histology

Brains from uninfected, *P. berghei*-infected and treated mice were carefully removed, fixed in 4% buffered formaldehyde for at least 48 h, and paraffin-embedded. Five-micrometre-thick sections were cut and stained with haematoxylin and eosin (H&E). The cerebral microvasculature was analysed, occluded and non-occluded vessels were checked under light microscope under 400 × magnification and images were captured. Percentage of occluded vessels in each group was calculated by counting the total number of vessels.

Flow cytometry of brain sequestered leucocytes

Brains from mice of naïve, symptomatic PbA-infected mice (on day 6 post-infection) and

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treatment groups (on day 8, i.e. after the completion of treatment) were removed and passed through 70 μm sterile meshes to obtain a single-cell suspension. The suspension was overlaid on a 30% Percoll gradient and centrifuged at 1800 *g* for 10 min. The pellet was collected, washed twice and the supernatant was discarded. To examine T cell activation, 1×10^6 cells/sample were suspended in FACS staining buffer (1 × PBS with 2 mM EDTA and 2% FBS) and surface stained with FITC-labelled anti-CD4 (RM4-5, BD Biosciences) and PE-labelled anti-CD8 (53–6·7, BD Biosciences). Flow cytometric acquisition was performed using a FACSCalibur (BD Immunocytometry Systems) and analyses done using FlowJo software (TreeStar, Ashland).

Western blot analyses

Brains were removed from mice and homogenized in ice-cold lysate buffer (200 mM HEPES (pH 7·5), 250 mM sucrose, 1 mM dithiothreitol, 1·5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA) freshly supplemented with phosphatase and protease inhibitors (0·1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4 and 1× protease inhibitor cocktail, Sigma-Aldrich).The final protein concentration in each sample was determined using the Bradford reagent. Aliquots containing 50 μg of protein were re-suspended in SDS–PAGE loading buffer, resolved on SDS–10% PAGE and transferred onto PVDF (polyvinylidene difluoride) membranes. Membranes were blocked in non-fat dry milk (5%) in PBS–Tween 20 for 1 h followed by incubation with primary antibodies (1:1000) for overnight at 4 °C. Then, membranes were incubated with horseradish peroxidase-labelled secondary antibodies. Mouse monoclonal anti- ICAM-1, rabbit polyclonal anti-VCAM-1, anti-actin and horseradish peroxidase-labelled goat monoclonal anti-mouse antibodies were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-labelled goat polyclonal anti-rabbit antibody was obtained from Sigma. Signal was developed using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK) using Image Quant LAS 4000 (GE Healthcare). The probed membranes were stripped and re-probed with rabbit polyclonal β-actin antibody to detect total levels of protein. Band intensity was analysed by Total Lab Quant gel analysis software, version 5.01 (nonlinear dynamic, CA).

RNA extraction and real-time PCR

Total RNA was extracted from isolated brains by TRIzol reagent and 1 μg of RNA was reverse transcribed using oligo (dT) primer with the Revert aid H Minus first strand cDNA synthesis kit (Thermo-scientific). The prepared cDNA was used for qPCR. Real-time PCR was carried out using ABI StepOnePlusTM system. Housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. *CXCR3, CCR5, CXCL9, CXCL10, CCL4 and CCL5* transcripts were quantified in the brain using primers listed. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. Supplementary Table 1 (available from http://journals.cambridge.org/PAR) lists the sequence of primers used in the present study.

Cytokine ELISA

The concentrations of proinflammatory cytokines TNF-α, interferon-γ (IFN-γ) and anti-inflammatory cytokine interleukin (IL)-10 were determined in serum obtained from coagulated blood (15 min at 37 °C then 30 min at 4 °C, stored at −20 °C until analysis) by ELISA using commercially available kits according to the manufacturer's instructions. TNF-α and IFN-γ were detected using ELISA kits from BD Bioscience, while for IL-10, ELISA kit from Biolegend was used.

Statistical analysis

Data are presented as the mean ± S.E.M. (standard error of the mean). Survival analysis was performed using the Kaplan–Meier log rank test (Mantel–Cox) test. The statistical significance of the differences was analysed by the one-way analysis of variance (ANOVA) (Graphpad Prism5 software). A P value of <0·05 was considered significant. P value summary is mentioned as $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$; ns – not significant.

RESULTS

Effects of the ART–VD combination on mortality and parasitaemia in ECM

C57BL/6 mice infected with *P. berghei* ANKA through the i.p. route developed typical ECM symptoms between days 6 and 10 post-infection, characterized by ruffled fur, wobbly gait, paralysis and ataxia, finally ending up with coma and death. In the PbA group with no treatment, all mice died by D10 with specific signs of CM and with parasitaemia ~20% (12–22%) (Fig. 1a and b). The symptomatic mice died usually between 10 and 24 h after onset of symptoms. A significant difference in survival was observed in the entire treatment groups *vs* infected control group. In the PbA + VD and PbA + ART groups, more than half of the mice died even before completion of treatment. The majority of the deaths in all the treated groups were within 24 h after appearance of the symptoms suggesting that 12–24 h window is a critical time point to attain clinical recovery if the treatment is effective. In the PbA + ART–VD group, the significant survival of 73% was observed

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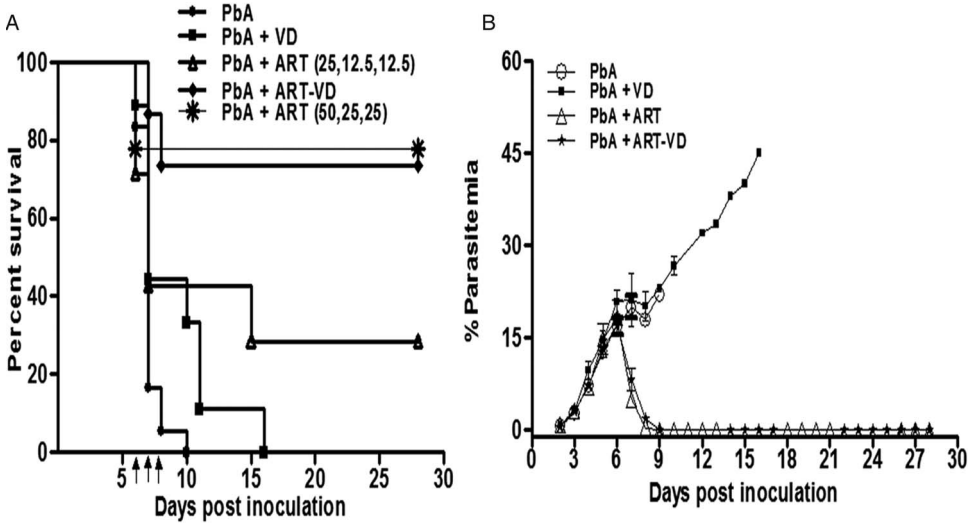

Fig. 1. Treatment of mice with ART–VD combination improves survival in mice and prevents ECM. (A) Mice were infected with *P. berghei* ANKA (*Pb*A) by intraperitoneal injection of 3×10^6 infected RBC and treated with VD, ART, ART–VD (doses mentioned in Material and Method section) from D6 to D8 post-infection (p.i.). ($P^{**} = 0{\cdot}004$ PbA + ART *vs* PbA + ART – VD, $P^{***} < 0{\cdot}0001$ (PbA + VD *vs* PbA + ART – VD), $P^{***} < 0{\cdot}0001$ (PbA *vs* PbA + ART – VD). (B) Parasitaemia trend in all the experimental groups.

as compared to other treated groups ($P^{**} = 0{\cdot}004$ PbA + ART–VD *vs* PbA + ART, $P^{***} < 0{\cdot}0001$ (PbA + ART–VD *vs* PbA + VD), $P^{***} < 0{\cdot}0001$ (PbA + ART–VD *vs* PbA). In the PbA + ART group, 43% mice recovered from ECM and remained negative throughout, but one mouse died on day 15 without any parasitaemia, thus reducing the survival from 43 to 29%. The mice that died in between was due to anaemia but no parasitaemia suggesting that even the effective antimalarial like arteether can control parasitaemia but the neurological deficit post-treatment leads to death of a certain per cent of survivors. A sharp decline in parasitaemia from $18{\cdot}34 \pm 2{\cdot}94$ and $17{\cdot}20 \pm 1{\cdot}50$ on day 6 to $0{\cdot}30 \pm 0{\cdot}10$ and $1{\cdot}90 \pm 0{\cdot}70$ on day 8 was evident in the surviving mice of PbA + ART and PbA + ART–VD group respectively. VD *per se* did not show any antimalarial activity as the mice survived of ECM displayed gradual rise in parasitaemia and died of anaemia (Fig. 1b). The mean survival time (MST) in PbA, PbA + VD and PbA + ART group were $6{\cdot}1 \pm 0{\cdot}9$ days, $8{\cdot}1 \pm 3{\cdot}2$ days and $13{\cdot}1 \pm 10{\cdot}6$ days, respectively. In the combination (ART–VD), MST was enhanced up to $22{\cdot}0 \pm 10{\cdot}3$ days. Furthermore, we assessed ECM outcome at high dose of arteether (PbA + ART 50,25,25) and results show that a higher loading dose of 50 mg kg^{-1} followed by 25 mg kg^{-1} dose for two consecutive days increased the survival upto 77% (MST: $22{\cdot}8 \pm 10{\cdot}1$ days) with arteether monotherapy.

Effect of ART–VD on BBB

BBB breakdown is a characteristic feature of ECM. Evans blue, a vascular marker has the ability to bind to albumin, so it can detect plasma protein leakage across the BBB into the cerebral parenchyma. Brains from PbA infected mice with symptomatic ECM appeared macroscopically blue. The per cent BBB restoration (with respect to PbA group) in treatment with VD, ART and ART–VD was 31·8, 54·02 and 69·12%, respectively, and it was evident from differential Evans blue staining of brains of untreated and treated *P. berghei*-infected mice (Fig. 2). The restoration in BBB dysfunction was equivalent in ART alone and combination group.

Effect of ART–VD on leucocyte accumulation

Leucocyte accumulation in capillaries and haemorrhage in the brain are the key features of murine CM. The results of brain histology (Fig. 3) showed profuse microvascular obstruction in PbA-infected untreated group (Fig. 3b). Upon treatment, the percentages of occluded vessels in the brain were diminished, accompanied by reduction in number of leucocytes per occluded vessel. Compared with 62·7% occluded brain microvessels in PbA control group mice, the capillary congestion was decreased up to 38·5, 29 and 24% in VD, ART and ART–VD treated groups.{$P^{***} < 0{\cdot}001$ (PbA + ART – VD) *vs* PbA, $P^{**} < 0{\cdot}01$ (PbA + ART *vs* PbA), $P^{*} < 0{\cdot}05$ (PbA + VD *vs* PbA)}.

Effect of ART–VD on T cell trafficking to the brain

ECM is associated with increased trafficking and accumulation of CD8 and CD4 T cells to the brain mediated through the IFN-γ inducible chemokines

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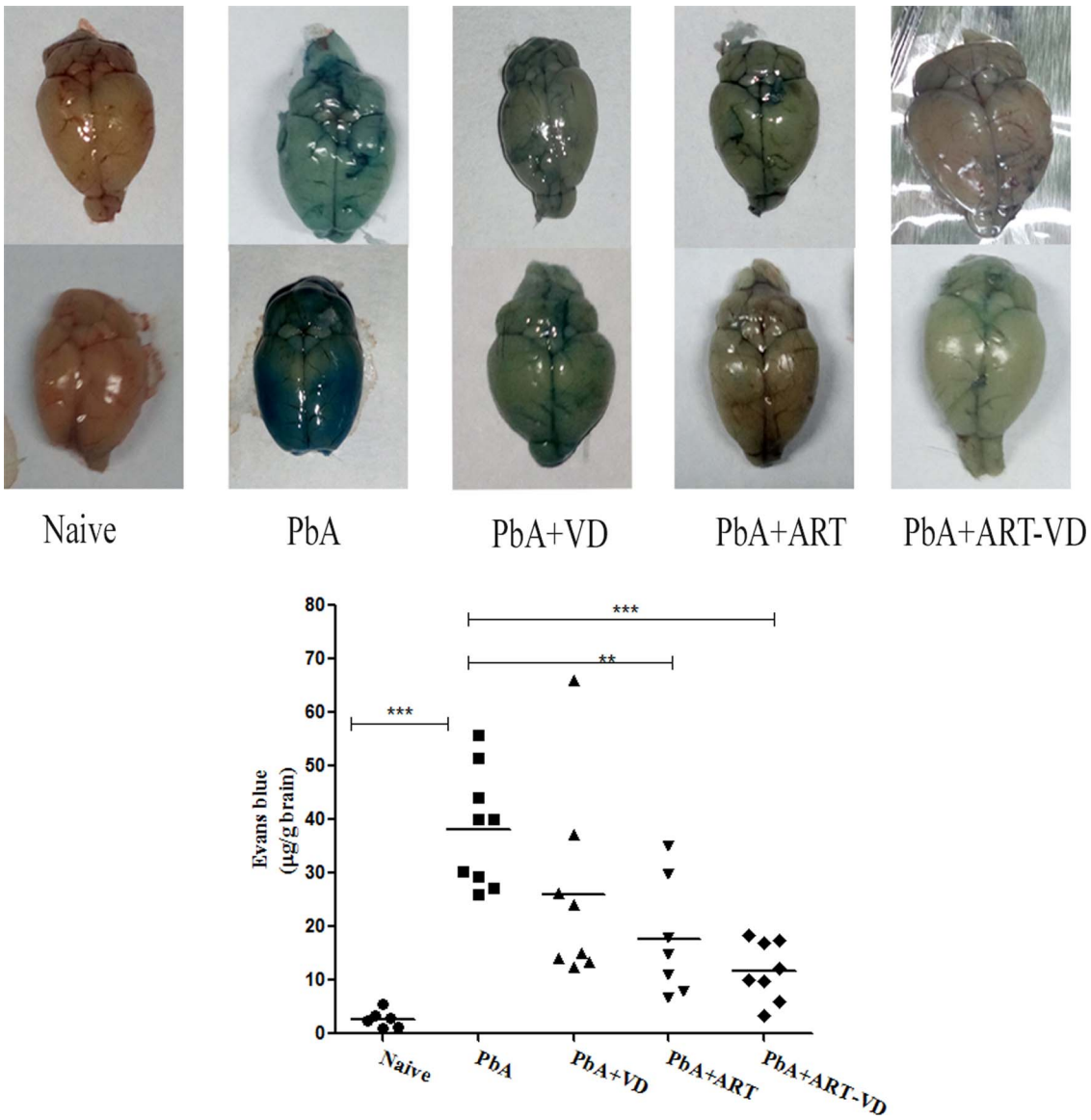

Fig. 2. ART–VD treatment prevents BBB dysfunction in the brains of *Pb*A-infected mice. Mice were infected with *Pb*A and left untreated or treated with ART, VD, ART–VD from D6 to D8. On day 6 p.i after the onset of symptoms, the mice from PbA group and uninfected mice were injected with Evans blue, and 2 h later, the mice were anesthetized and the brains removed, dye extracted in formamide and read at 620 nm after 48 h. Similar procedure was followed for mice from treatment groups after cessation of treatment. Images of a representative brain from each group and the corresponding bar graph showing quantification of Evan's blue leakage in all brains are shown. Statistically significant differences compared with the PbA are indicated by *; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

CXCL9 and CXCL10. To investigate the outcome of treatment on the migration and accumulation of T cells in the brains of mice, brain-sequestered leucocytes were stained for $CD8^+$ and $CD4^+$ cells. The PbA infection promoted the accumulation of both $CD8^+$ and $CD4^+$ T cells which was significantly reduced in treatment groups. The trafficking of $CD8^+$ T cells was more pronounced in ECM and a significant reduction of approximately 70 and 74% was observed in VD and ART-treated groups. Upon combination treatment, population of $CD8^+$ T cells was 80% less compared with PbA-infected mice. The $CD4^+$T cell were reduced upto 35, 56 and 62% upon VD, ART and ART–VD treatment, respectively (Fig. 4).

Effect of ART–VD on endothelial activation markers

The treatment with VD and ART inhibits endothelial activation markers during ECM. The expression of endothelial activation marker ICAM-1, VCAM-1 and E-selectin was quantified in brain tissue of *P. berghei*-infected mice, treated or not treated with drug using western blotting (Fig. 5). ICAM-1 expression was markedly increased in PbA-infected mice than in uninfected mice and reduced by 33·1,

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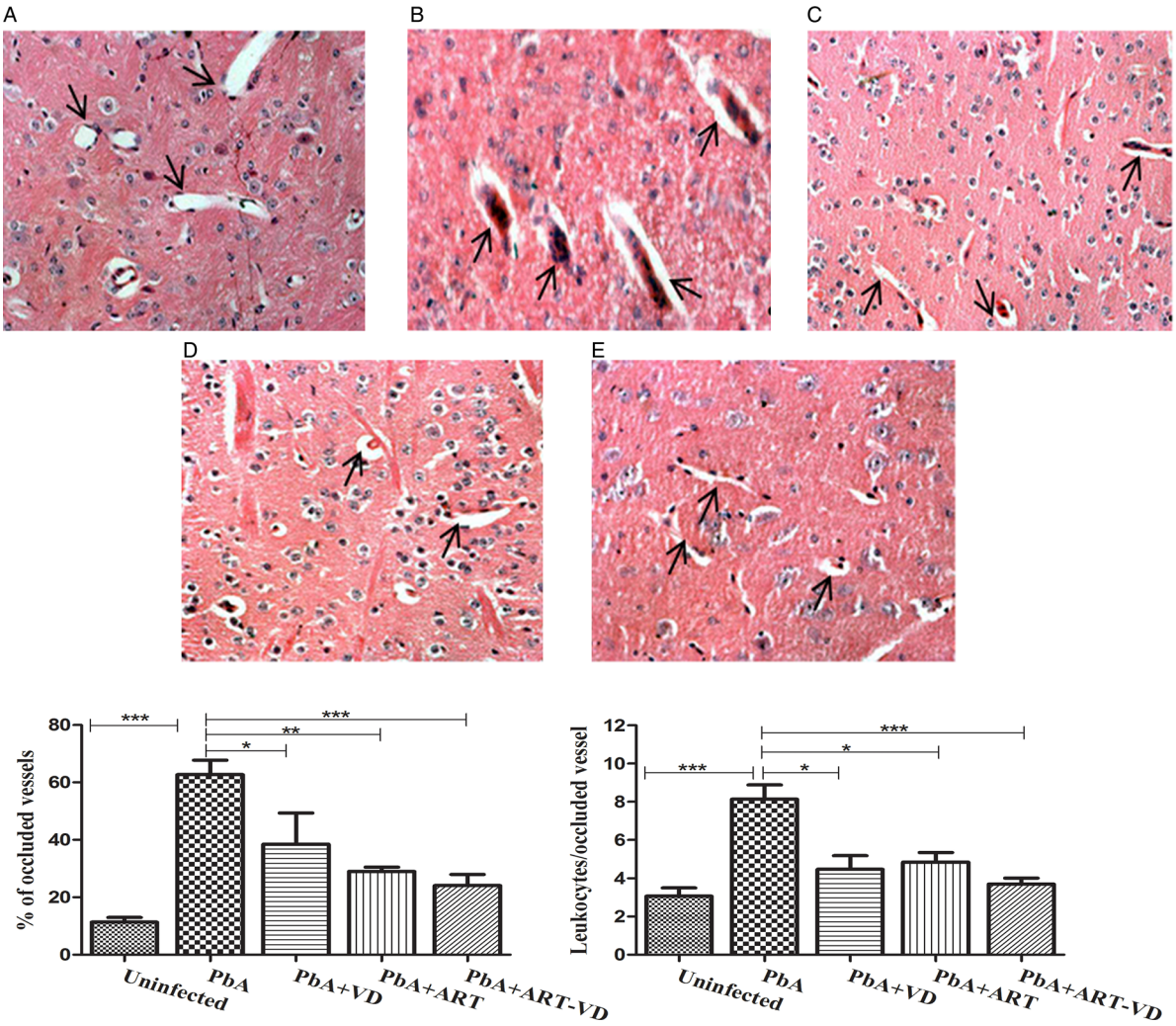

Fig. 3. H&E-stained brain sections. Images show representative brain section from (A) uninfected, (B) *P. berghei* ANKA-infected, (C) VD treated, (D) ART treated and (E) ART–VD-treated mice demonstrating occluded parenchymal vessels (black arrows). The PbA-infected mice shows heavy microvascular congestion (B). VD treatment reduced the obstruction as evident by fewer numbers of leucocytes in the occluded vessels (C). ART treatment shows relatively few obstructed vessels (D) and ART–VD treatment reduced the number of occluded vessels with few remnants of leucocyte (E). Bar graphs shows percentage of occluded vessels and number of leucocytes adhered in occluded vessels ($n = 3$).

34·8 and 51·2% upon VD, ART and ART–VD treatment. The VCAM-1 expression was however significantly reduced in all the treated groups. No significant alteration was observed in E-selectin expression though the expression was reduced in VD treated mice.

Effect of ART–VD on inflammation markers

The increased proinflammatory cytokine, certain chemokine and their receptors are the hall mark features of ECM. To ascertain the inflammatory parameters, we quantified the mRNA expression of chemokine receptors CXCR3, CCR5 and chemokine ligands CXCL10, CXCL9, CCL4 and CCL5 in the brain of mice from all the experimental groups (Fig. 6). The CXCR3–CXCL9–CXCL10 nexus plays an important role in the recruitment of T-cells in the brain microvasculature. The expression of CXCL10, a marker-associated with CM was induced to 42-fold in PbA-infected mice. A significant decline in expression of CXCL10 was observed upon treatment. The expression was 8-fold in ART–VD and 10-fold in ART alone treatment. Likewise, CXCL9 showed 30-fold increase in PbA-infected mice, 12-fold in ART-treated and 8-fold in ART–VD-treated mice. Similar results were obtained with CCL4 and CCL5 mRNA expression. Furthermore, the mRNA level of chemokine receptors CXCR3 and CCR5 experienced a significant decline in treated mice as compared with untreated infected mice. The serum level of pro-inflammatory cytokines IFN-γ and TNF-α was reduced in all the treatment groups, whereas anti-inflammatory cytokine IL-10 was increased (Fig. 7).

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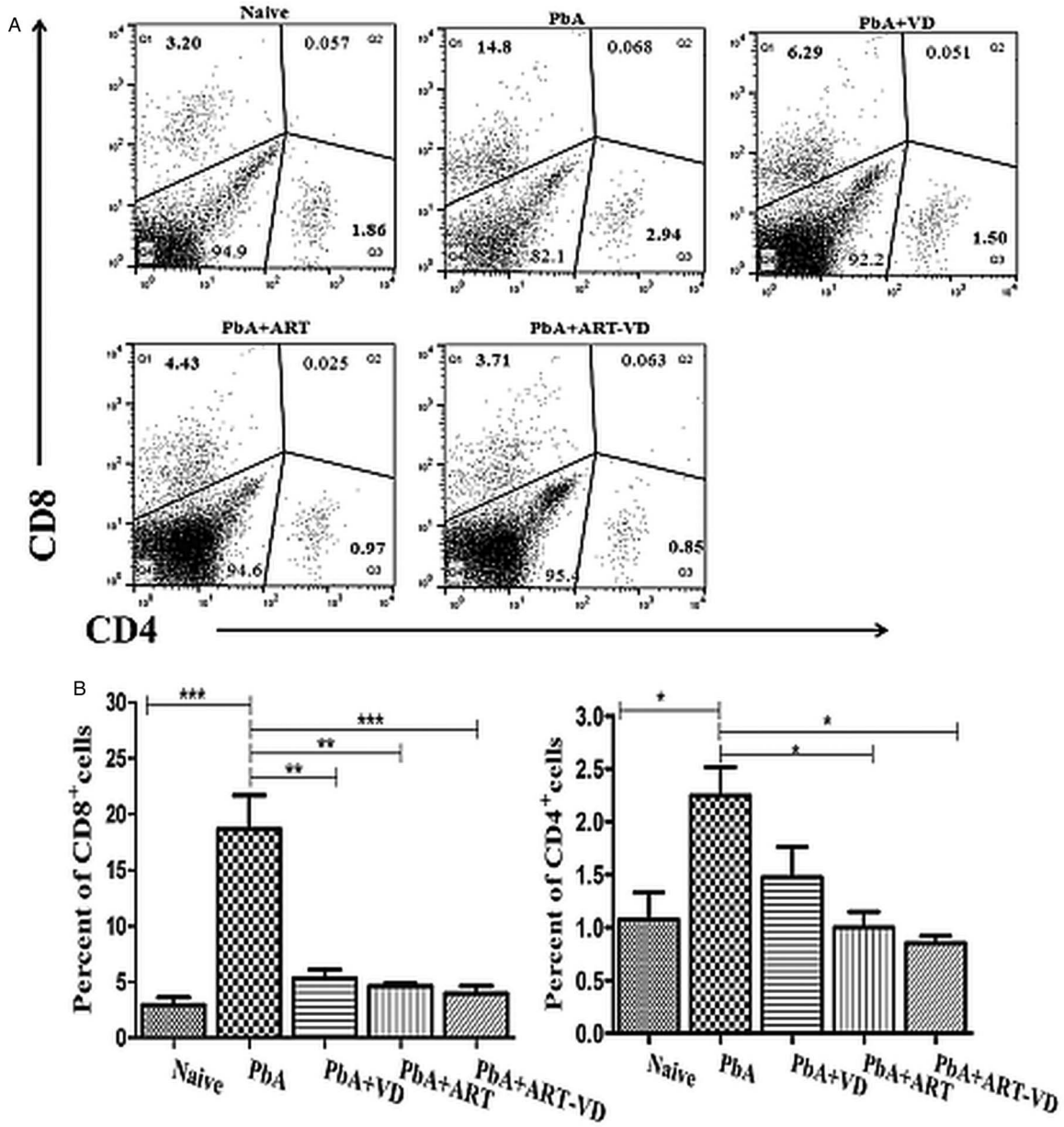

Fig. 4. Effect of treatment on pathogenic T-cell sequestration in the brain. FACS analysis of CD8 and CD4 T cells were carried out in brain samples from all the groups. Representative dot plots showing the proportions of both the T-cell populations in all the experimental groups (no. of mice in each group; $n = 3–5$). Bar graph shows per cent population of CD8 and CD4 T cells in the brain.

DISCUSSION

Human CM is a complex syndrome with varied pathology in African children and South-East Asian adults (Idro *et al*. 2005; Wassmer *et al*. 2015). The former shows neurological basis of disease characterized by rapid onset of coma, seizures and anaemia (Newton *et al*. 1998; Miller *et al*. 2013) while in the latter case central nervous system (CNS) dysfunction is accompanied by renal and respiratory distress (Sahu *et al*. 2015). The diverse outcome in host and the multifactorial pathogenesis of CM makes the goal of improving the survival of patients suffering from CM even more difficult. The highly effective antimalarials quinine and artemisinin derivatives are the mainstay of CM treatment, but due to rapid occurrence of terminal complications, the antimalarial action alone is insufficient. Adjunct treatments that act through alternate mechanisms and address one or more of the pathogenic processes are urgently needed for a full rate recovery. Till date, a number of agents such as exogenous nitric oxide (NO) donor-dipropylenetriamine-NONOate (Zanini *et al*. 2012), membrane stabilizer citicoline (El-Assaad *et al*. 2014), mTOR inhibitor rapamycin (Gordon *et al*. 2015) have been tested in ECM model as effective adjunct treatment. Atorvastatin, a lipid-lowering agent prevents cytoadhesion and endothelial damage under *in vitro*

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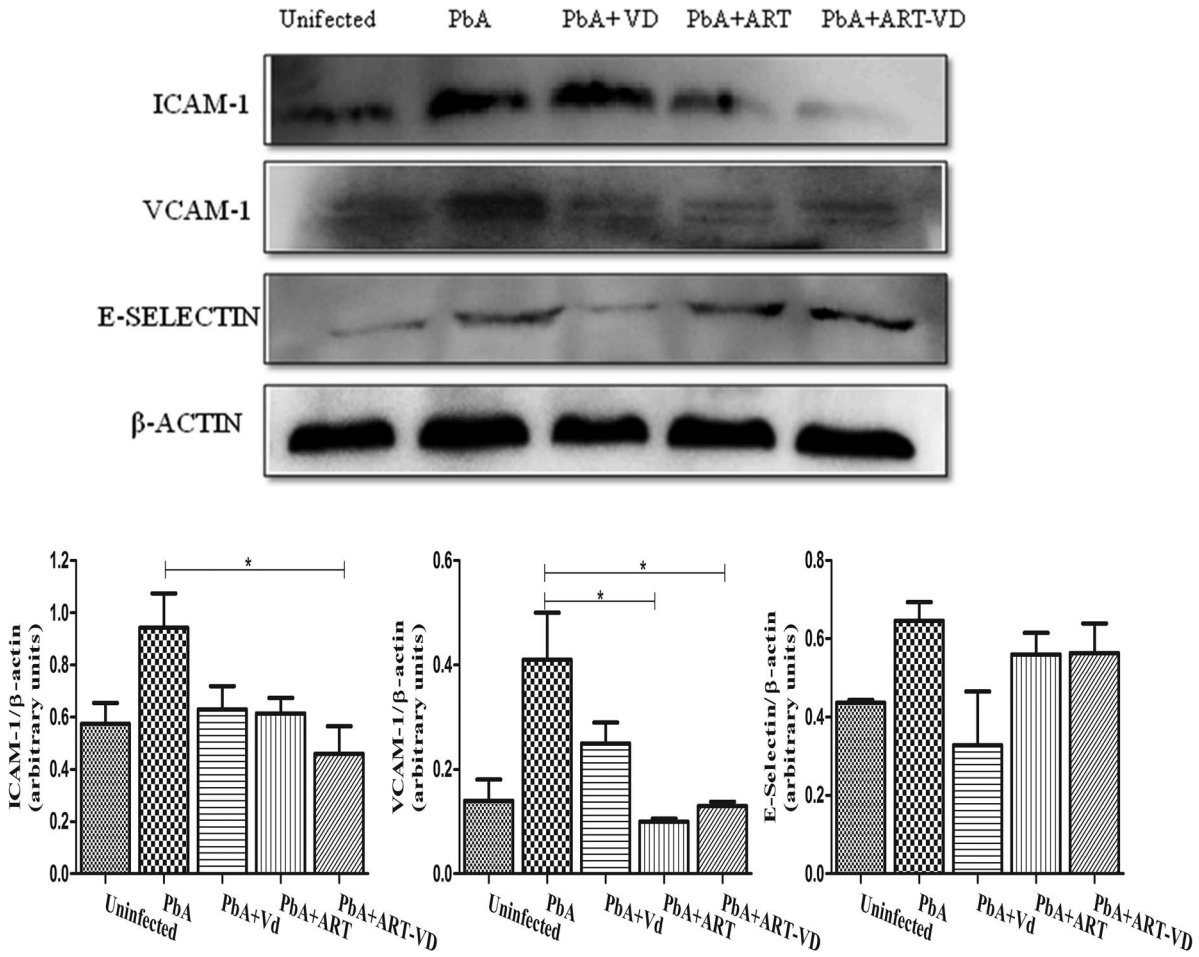

Fig. 5. Effect of treatment on endothelial activation marker. The expression of ICAM-1, VCAM-1 and E-selectin was assessed by Western blot analysis and data expressed as a ratio with respect to β-actin in the bar diagram.

condition (Taoufiq *et al*. 2011) and had shown protection in ECM model in combination with dihydroartemisinin (Dormoi *et al*. 2013) and mefloquine (Souraud *et al*. 2012). However, the prophylactic drug administration i.e. before the onset of symptoms undermines the utility of such treatments. In this context, a recent study reported the potential of curcumin to reduce ECM severity by suppressing NF-κB activation in brain and spleen, thereby preventing $CD8^{+}$T cell activation, parasite sequestration and breakdown of BBB. Curcumin in concert with arteether in late-stage ECM led to 100% survival compared with 50% in arteether monotherapy (Dende *et al*. 2015). Since arteether is developed by our institute so we became interested in this combination but unfortunately instead of 100% survival we could get only 73% survival with arteether and curcumin treatment at the same dose reported by Dende *et al*. (data not shown). Difference in survival could be due to different weight of animals used in both studies. Another study documented the efficacy of CXCL10 inhibitor atorvastatin in combination with artemether to be a potential adjunct treatment of ECM (Wilson *et al*. 2013). In the above-mentioned study, 100% survival in combination treatment was observed compared with 70% survival from ECM in artemether monotherapy but the period of observation of parasitaemia and survival was only 15 and 21 days, respectively.

In the past few years, vitamin D has gained immense importance in various diseases besides maintaining calcium and bone metabolism. VD is considered a neuroactive steroid linked to brain development and function (Kesby *et al*. 2011). Low vitamin D is associated with a number of psychiatric conditions (Eyles *et al*. 2013). Recently, a review mentioned that addition of cod liver oil or vitamin D and dicalcium phosphate to quinine reduced death rate in *P. berghei*-infected mice (Luong and Nguyen, 2015). VD when orally administered shows protective response in ECM through dampened Th1 response, Treg cell expansion and upregulation of IL-10 and all the treated mice progressed to delayed death (He *et al*. 2014). Considering the neuroprotective role and immunomodulatory role of VD, we assessed the α/β arteether combination with VD against ECM. Arteether is a rapidly acting blood schizonticide with higher safety margin than other artemisinin derivatives (Tripathi

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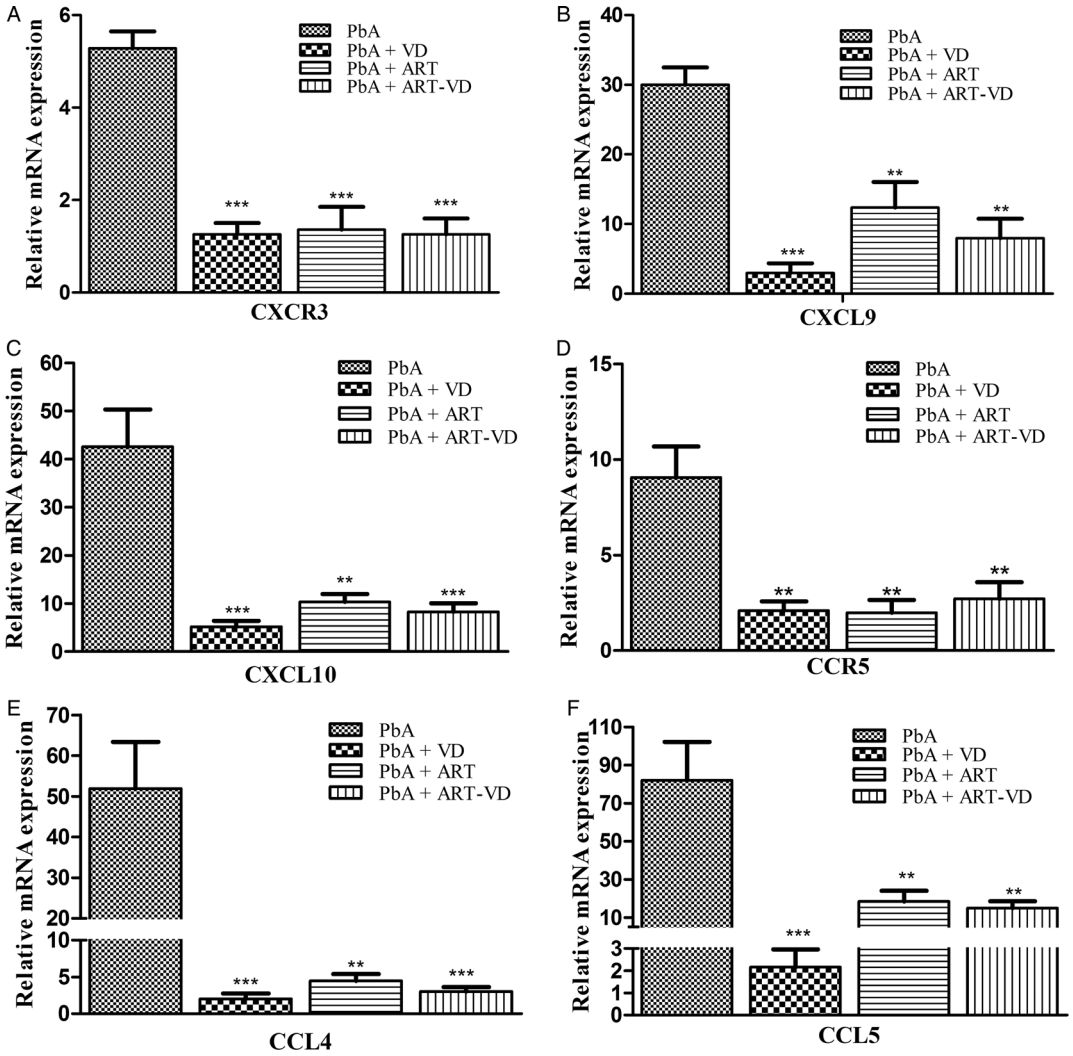

Fig. 6. (A–F) Treatment suppressed markers of inflammation in the brain. qPCR analysis was carried out from RNA isolated from brains of *P. berghei*-infected and treated mice. *CXCR3, CCR5, CXCL9, CXCL10, CCL4* and *CCL5* were significantly reduced in treatment groups. The data are normalized to *GAPDH* and given as fold changes with respect to RNA from the brain of uninfected animals. Statistically significant differences compared with the PbA are indicated by *, $*P < 0{\cdot}05$; $**P < 0{\cdot}001$; $***P < 0{\cdot}0001$.

et al. 2008). To rapidly achieve the parasiticidal level of antimalarial drug for treatment of severe malaria, a loading dose is preferred (Pasvol, 2005). Therefore, we administered a higher dose of arteether (25 mg kg^{-1}) on day 6 after onset of symptoms, followed by reduced doses (12·5 mg kg^{-1}) on two consecutive days. The combination of arteether with VD significantly improved survival and conferred protection from ECM compared with ART or VD monotherapy. The survival was 73% as observed in PbA + ART – VD group *vs* 29% in PbA + ART and no survival in PbA + VD group. An escalated loading dose of 50 mg kg^{-1} arteether alone on D6 followed by 25 mg kg^{-1} on D7 and D8 conferred 77% survival. During CM, an overwhelming pro-inflammatory response alters the blood brain barrier permeability. In our study, the BBB integrity was restored in all the treatment groups as evident by reduced Evans blue extravasation. The endothelial activation marker, ICAM-1, plays role in leucocyte trafficking to CNS (Dietrich, 2002) and pathogenesis of human as well as murine CM (Armah *et al*. 2005*b*; Chakravorty and Craig, 2005). The ICAM-1-deficient mice were protected from ECM due to decreased macrophage trapping in brain microvasculature (Favre *et al*. 1999).Our study corroborate these findings as decrease in ICAM-1 expression in the treated group is consistent with reduced occlusion in capillaries observed in H&E-stained brain sections (Fig. 3). The percentage of occluded vessels in brain were as low as 24·1 ± 3·8% in the ART–VD group compared with 29·1 ± 1·4%, 38·5 ± 10·8% and 62·6 ± 5% congested vessels in ART, VD and PbA groups. Amongst the treated mice, the numbers of leukocytes per occluded vessel were least in the combination group relative to PbA-infected untreated mice. The results further coincide with decreased trafficking of pathogenic $CD8^+$ T cells in the mice brains of

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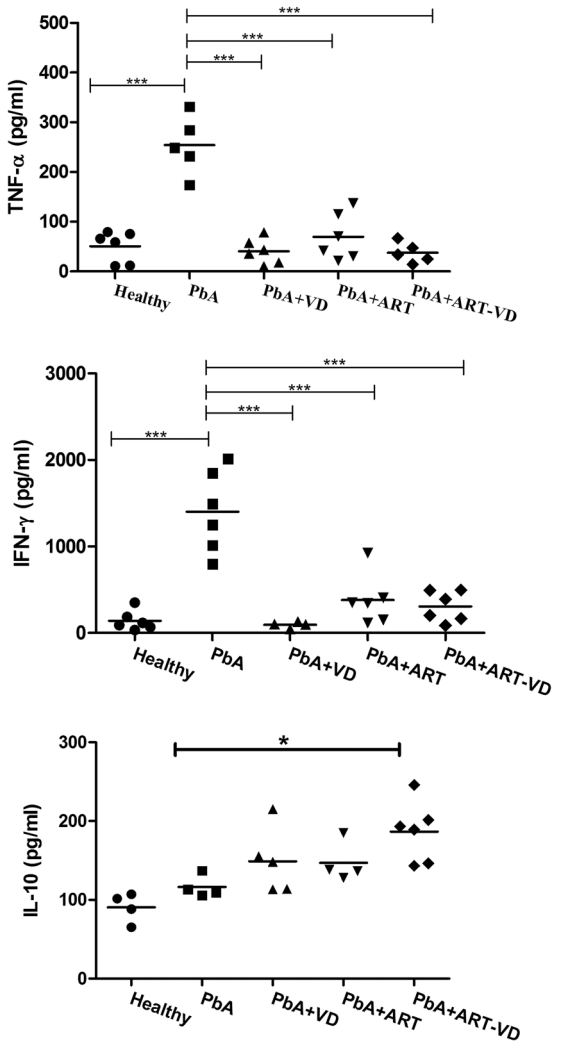

Fig. 7. Treatment suppressed circulating level of proinflammatory cytokines TNF-α and IFN-γ in the serum while anti-inflammatory IL=-10 level was increased in all the treated groups. Cytokine levels were assessed by ELISA using kits following manufacturer's instructions.

treatment groups. Compared with PbA-infected untreated mice, the reduction in $CD8^+$ T cell population was evident in all three treatment batches of mice, but no significant difference amongst the groups was seen. However, a significant but comparable reduction in $CD4^+$ T-cell population in the brain was observed in PbA + ART and PbA + ART – VD group, whereas moderate but insignificant decrease in CD4 cell recruitment in the VD group was evident. Chemo-attractant cytokines or chemokines are the key regulators of leucocyte trafficking (Ioannidis *et al*. 2014) and inflammatory chemokines produced under pathological condition attracts immune cells to the site of inflammation. The chemokine receptor CXCR3 and its ligands CXCL10 and CXCL9 are responsible for $CD8^+$ T cells trafficking in murine CM (Campanella *et al*. 2008; Nie *et al*. 2009). We found highly induced mRNA level of inflammatory chemokines CXCL10, CXCL9 and CXCR3 in the PbA group and was significantly reduced in all the treatment groups. CXCL10 is the predictor of fatal CM (Wilson *et al*. 2011). The CXCL10 reduction (88%) was more pronounced in VD alone group. The combination of ART with VD reduced the expression of CXCL10 by 81% compared to 76% reduction in ART alone treatment. Similarly, the systemic level of the proinflammatory cytokine TNF-α and IFN-γ were reduced in the treatment groups. The considerable upregulation of anti-inflammatory cytokine IL-10 in the combination treatment was observed correlating with the ECM protective attributes of ART–VD combination. Despite reduction in ECM related markers, VD alone could not rescue majority of mice due to its administration during the terminal stage of the disease and no anti-parasitic effect. Arteether checked parasitaemia and rescued 43% mice from ECM and cured them completely but finally only 29% could survive. Remaining one mouse died of anaemia with no parasitaemia. In the nutshell, improved cumulative immunological response in ART–VD treatment may be the possible reason for improvement in CM outcome. In conclusion, the present study clearly demonstrates that ART–VD combination confers protection from ECM.

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ETHICS STATEMENT

The study received ethical approval vide permit number: IAEC/2010/142 from CSIR-Central Drug Research Institute's 'Institutional Animal Care and Use Committee' recognized by 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)', Government of India. All animals were sacrificed using deep ether anaesthesia during or after the study and all efforts were made to minimize suffering.

CSIR–CDRI communication number: 9263

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182016001207.

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