Vascular endothelial growth factor (VEGF) and lovastatin suppress the inflammatory response to *Plasmodium berghei* infection and protect against experimental cerebral malaria

Miriam Canavese¹, Andrea Crisanti^{1,2}

¹Department of Experimental Medicine Centre of Genomics, University of Perugia, Italy, ²Department of Life Sciences, Imperial College London, London, United Kingdom

Cerebral malaria (CM) is a severe complication of *Plasmodium falciparum* infection, which is associated with high mortality and long-term cognitive impairment even when effective anti-parasitic treatment is administered.^{1,2} Supportive therapy is needed to improve both morbidity and mortality associated with this condition. In an accompanying paper, we have demonstrated that in the *Plasmodium berghei* ANKA (PbA) rodent model, CM can be effectively prevented by a treatment combining sub-lethal doses of lipopolysaccharide S (LPS) and vascular endothelial growth factor (VEGF). Since LPS is not suitable for human therapy, we investigated whether lovastatin would represent a suitable substitute. This compound, widely used to lower cholesterol levels in plasma, shares with LPS the ability to elicit an anti-inflammatory response by activating the Nrf-2 gene, and when given to *P. berghei*-infected mice prevents to some extent the onset of CM. We show here that lovastatin- and VEGF-treated mice did not develop CM and showed few signs, if any, of endothelial damage and systemic inflammation. The combination treatment was much more effective than lovastatin and VEGF alone. Immunohistochemistry and gene expression analysis indicated that VEGF and LPS together overturned the two pathogenic mechanisms responsible for the development of CM: endothelial damage and dysregulated activation of the inflammatory response. These findings provide the rationale for investigating the therapeutic potential of these compounds in human CM as well as in other inflammatory pathologies that respond poorly to steroid and non-steroid anti-inflammatory therapy.

Keywords: Vascular endothelial growth factor, Cerebral malaria, Cytokines, Statins

Introduction

The utilisation of the rodent malaria model *Plasmodium berghei* Anka (PbA) has provided a better understanding of both pathological features and molecular mechanisms leading to the development of cerebral malaria (CM).³ These involve a dysregulation of the inflammatory response to the parasite infection associated to extensive endothelial damage, each progressively potentiating the other.⁴ Compelling evidence implicates the cytokines, IFN-gamma and TNF-alpha, in driving the inflammatory response leading to experimental cerebral malaria (ECM).⁵⁻⁷ IFN-gamma is required for upregulating the expression of endothelial adhesion molecules,

which binds to infected erythrocytes in the brain vessels, and for inducing the synthesis of macrophage-derived TNF-alpha that in turn enhances the inflammatory response.⁸ Comparison of ECM susceptible C57BL/6 and ECM resistant Balb/c mice revealed that the latter ones significantly upregulated the gene haeme oxygenase-1 (HO-1) in response to PbA infection.⁹ Pharmacological induction of HO-1 prevented the development of ECM thus supporting the notion that this enzyme ought to have a protective role.^{9,10} This has been attributed to the ability of its end product, carbon monoxide (CO), to neutralise the oxidising properties of the free haeme generated as a consequence of parasite digestion of haemoglobin and erythrocyte rupture.¹¹ Accordingly, CO inhalation in PbA-infected C57BL6 mice inhibited blood-brain barrier

Correspondence to: Professor Andrea Crisanti, Department of Life Sciences, Imperial College, London, UK. Email: acrs@imperial.ac.uk

disruption, preserved vascular integrity and prevented monocyte infiltration.⁹ Likewise nitric oxide (NO), a potent inducer of HO-1, protected mice from developing ECM.⁹ The effect of NO on the expression of HO-1 appeared to be mediated by the nuclear factor erythroid (NF-E2)-related factor 2 (Nrf-2), a molecule that plays a critical function in down regulating the inflammatory response by inducing anti-oxidant genes.^{9,12} While the role of CO and NO in neutralising the oxidising activity of free haeme and in down regulating the inflammatory response, respectively, are well established, their functional relationships with the vascular endothelial growth factor (VEGF) have been underestimated. Nitric oxide, carbon monoxide and HO-1 are functionally correlated with VEGF. Vascular endothelial growth factor promotes NO production and also induces endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression in vascular endothelial cells *in vitro*.^{13–15} Vascular endothelial growth factor in turn is capable of up-regulating the expression of HO-1 in endothelial cells. Accordingly, endothelial cells from HO-1 knockout mice do not respond optimally to VEGF stimulation.^{13,16} The role of VEGF in modulating tissue pathology in response to *P. berghei* infection is supported by the observation that Axitinib, a potent inhibitor of VEGF signalling pathway, aggravated dramatically the course of *P. berghei* infection in both ECM resistant and ECM susceptible mice. Conversely, VEGF showed a protective activity against ECM in PbA-infected mice. These findings add to the understanding of the protective mechanism of NO and CO against ECM. Accordingly, they would exert a dual activity: downregulate the activation of the inflammatory response and protect the endothelial cells from damage thereby neutralising both major mechanisms implicated in the development of ECM.

Both NO and CO have a very limited therapeutic interval that makes them unsuitable for routine and unsupervised use in humans. Alternative therapy suitable for CM ought to prevent the activation of the inflammation process while protecting the endothelium. We showed that a combination regimen including VEGF and Lipopolysaccharide S (LPS), a molecule that at sub-lethal doses abolished the activation of the inflammatory response by up regulating both HO-1 and Nrf-2, met these requirements. Vascular endothelial growth factor-LPS treatment completely protected PbA-infected mice against ECM and prolonged their life span. Treated animals did not show signs of tissue pathology, endothelial damage and systemic inflammation. This observation prompted us to investigate whether lovastatin, a compound widely used in therapy to lower

cholesterol, could replace LPS given its ability to induce the transcription of the anti-inflammatory gene Nrf-2 and to exert some protective activity against ECM. Our results show that the addition of lovastatin to VEGF abolished the activation of both local and systemic inflammatory response in the brain, the spleen and liver of treated animals. Furthermore, the treatment completely prevented the induction of the inflammatory cytokine INF-gamma and TNF-alpha while up regulating the genes HO-1 and Nrf-2. Accordingly, lovastatin and VEGF treatment protected against ECM, prolonged life span of infected animals and prevented the development of splenomegaly. These findings provide the rationale for developing a supportive therapy for CM with compounds potentially suitable for human therapy.

Results

Lovastatin potentiates the protective activity of VEGF against ECM

We compared several treatment regimens to investigate whether lovastatin in combination with VEGF exerted a synergistic activity similar to that observed with LPS in protecting C57BL/6 mice against *P. berghei* ECM. Typically, PbA-infected C57BL/6 mice start showing signs of convulsion, ataxia, coma at around day 6–7 post infection and die within the following 24 hours (Fig. 1A). A treatment with lovastatin 20 mg/kg alone significantly ameliorated the signs of neuro-inflammation and prolonged the lifespan of the mice by 2 days in line with previous published reports.^{2,17} The administration of simvastatin 20 mg/kg alone, a statin that, like lovastatin, inhibits endogenous cholesterol production by competitive inhibition of HMG-CoA reductase, failed to protect against ECM (Fig. 1A), confirming previous studies.¹⁸ Difference in hydrophobicity and bioavailability may account for the observed differences. A combination treatment including lovastatin 20 mg/kg and VEGF 200 ng/mouse daily starting from the first day post infection completely blocked the development of ECM and prolonged the life span of the mice on average by 7 days without affecting the parasite load (Fig. 1A). This time course is identical to that observed in *P. berghei*-infected ECM-resistant BALB/c mice. Notably, no differences were observed when comparing the course of infection between VEGF-lovastatin and VEGF-LPS-treated animals (Fig. 1A). Morphological analysis of internal organs revealed that the lovastatin-VEGF combination treatment had a significant effect in preventing the development of splenomegaly in infected animals as previously observed when treating infected mice with VEGF-LPS (Fig. 1B).

Figure 1 Effect of Statins and vascular endothelial growth factor (VEGF) combination treatments on PbA-infected mice. (A) Survival rate and parasitaemia (inset) of infected C57BL/6 mice (dark blue filled triangle) treated with lovastatin alone (purple filled diamonds), simvastatin alone (pink filled cross) or with a combination of VEGF and lipopolysaccharide S (LPS) (red filled stars) or VEGF and lovastatin (violet open squares). The survival of non-infected and untreated mice (light blue filled squares) is also shown. (B) Photographs of spleens collected from non-infected, and PbA-infected C57BL/6 mice either not treated or treated with lovastatin, simvastatin, LPS + VEGF and lovastatin-VEGF collected at the end point. The images were taken using a Canon Power Shot A520, Scale bar, 50 μ m.

VEGF–lovastatin treatment suppresses the inflammatory response and protects the blood vessels' integrity

The internal organs (brain, liver, spleen and lungs) of all experimental groups were collected at day +3 post infection and on the day of death (end point) to carry out histology and immunohistochemistry analysis. The examination of the brains from non-treated infected animals showed a mononuclear cell infiltrate alongside with signs of oedema and blood vessel damage typically observed in ECM susceptible mice (Fig. 2A). Tissue sections taken from the spleens of

these animals also showed oedema and increased cellular content. Similarly, a significant cellular infiltrate was also observed in the lungs. Lovastatin and simvastatin when given alone did not improve the histological features of tissue pathology in all tissues and organs examined. Intriguingly, simvastatin apparently caused a worsening of the oedema and an increase of the mononuclear cellular infiltrate in the brain, the spleen and the liver, while lovastatin did not show the expected improvement anticipated on the basis of its protective effect on the development of ECM (Fig. 2A). On the contrary, the VEGF–lovastatin combination treatment

Figure 2 Immuno-histological analysis of tissue sections from infected *P. berghei* ANKA infected mice. (A) Brain, liver, spleen and lung sections from C57BL/6 mice non-infected, infected and infected plus treatments (lovastatin, simvastatin, lipopolysaccharide S (LPS) + vascular endothelial growth factor (VEGF) and lovastatin + VEGF). The specimens were collected at day +3 post infection and at end point, stained with H&E (A) or processed for immunohistochemistry with anti-CD3 and cleaved caspase 3 antibodies (B). Blue arrows show cleaved caspase 3 reactive endothelial cells. Scale bars are indicated.

completely prevented the formation of the cellular infiltrate in the brain and the development of oedema. Accordingly, no signs of inflammation could be detected in other tissues including the spleen. This morphological profile is almost identical to that observed in VEGF-LPS-treated mice in agreement with the progression of *P. berghei* infection in these animals. The immunohistochemistry analysis supported the conclusions initially inferred on the basis of the histology. At the time of death, infected animals either non-treated or statin-treated shared a similar immunohistochemistry reactivity pattern (Fig. 2B). The tissues from these animals showed a strong reactivity with an antibody directed against the lymphocyte antigen CD3 thus revealing the T cell nature of the mononuclear cell infiltrate. This analysis also demonstrated that capillaries and small veins were stained with an antibody directed against the activated form of caspase 3, a hallmark of apoptosis thus confirming the involvement of extensive endothelial cell damage (Fig. 2B). In agreement with the morphological observations, the monoclonal antibodies directed against the lymphocyte CD3 antigen and the activated form of caspase 3 did not show any reactivity in tissue sections from VEGF-lovastatin treated. This reactivity profile is identical to that observed in non-infected controls.

Analysis of cytokine and inflammatory markers in statin-treated animals

To study the activity of statins on *P. berghei* induced inflammatory response, we examined the expression of three groups of genes encoding pro-inflammatory cytokines (TNF-alpha and INF-gamma); inflammation regulatory elements (HO-1, Nrf-2 and IDO) and VEGF modulated signalling molecules (VEGF-R2 and S1Pr1). In line with previous observations, PbA infection induced in the brains of C57BL/6 mice 5- and 10-fold up-regulation of the TNF-alpha and INF-gamma genes, respectively, (Table 1). This was accompanied by a parallel increase of IDO. The treatment with lovastatin had a contrasting effect on the pro-inflammatory cytokines; it inhibited the induction of the INF-gamma while upregulating the transcription of TNF-alpha. Lovastatin also elicited a significant activation of the anti-inflammatory genes Nrf-2 and HO-1 (Table 1). On the contrary, the administration of simvastatin rather than activating the anti-inflammatory genes Nrf-2 and HO-1 upregulated the pro-inflammatory cytokines, TNF-alpha and INF-gamma, as well as IDO to levels higher than those observed in infected non-treated animals. The gene expression profile induced by simvastatin could explain its lack of protective activity against ECM and the worsening of the pathology in the tissues and organs examined. The VEGF-lovastatin treatment induced a completely different profile. Both Nrf-2

and HO-1 genes were highly induced already from day +3 post infection mirrored by a parallel increase in the transcription levels of S1Pr1, a gene involved in endothelial remodelling and repair (Table 1). The expression profile was very similar to that previously observed when treating infected mice with VEGF-LPS. As shown in an accompanying paper in this issue, *P. berghei* infection unleashed in the spleen a strong activation of pro-inflammatory cytokines reaching 200- and 260-fold induction over non-infected controls for TNF-alpha and INF-gamma, respectively. The genes IDO and VEGF-R2 were also strongly upregulated. The activation of IDO most likely represents a response to the high level of INF-gamma while the activation of VEGF-R2 could represent a compensatory response to parasite-mediated endothelial damage. Contrary to what has been observed in the brain, in other organs, both lovastatin and simvastatin were able to downregulate the induction of TNF-alpha and INF-gamma though the activation of VEGF-R2 was only marginally affected. The addition of VEGF to lovastatin enhanced the inhibitory activity on the inflammatory cytokines genes, reverted the activation of VEGF-R2 to nearly basal levels while up regulating the anti-inflammatory gene Nrf-2 (Table 1). The liver of untreated mice showed at day +3 post infection an expression profile that with the exception of VEGF-R2 was similar to that observed in the spleen; however, unlike the spleen a few days later, most pro-inflammatory genes were back to baseline levels (Table 1). Here statins treatment activated the anti-inflammatory gene Nrf-2 and downregulated the induction of both TNF-alpha and INF-gamma already by day +3 post infection; however, only the addition of VEGF completely reversed the pro-inflammatory cytokines to baseline levels. In the lung of infected animals, we did not observe significant changes in the expression levels of the genes analysed. The administration of lovastatin upregulated the transcription of both Nrf-2 and HO-1 while simvastatin failed to exert a similar activity (Table 1).

Discussion

Our findings confirmed the protective activity of lovastatin against ECM in PbA-infected mice. This is in agreement with the ability of statins to reduce iNOS expression, thereby protecting neuronal cells, while increasing endothelial NOS expression and increasing microcirculatory blood flow via local vasodilatation.^{2,19} However, not all statins share the same protective properties. Simvastatin and lovastatin are both known to inhibit HMG-CoA reductase but simvastatin does not exert the same protective activity against ECM. On the contrary, simvastatin-treated mice developed ECM and showed a marked worsening

Table 1 Fold change expression of inflammation markers in the brain and internal organs

	Mouse strain		Mouse strain		rf-2		HO-1		TNF-alpha IFN-gamma		IDO		VEGF-R2 S1Pr1		IDO		TNF-alpha IFN-gamma		HO-1		Nrf-2	
	Mouse strain	C57BL6	Mouse strain	C57BL6	rf-2	rf-2	HO-1	HO-1	TNF-alpha IFN-gamma	TNF-alpha IFN-gamma	IDO	IDO	VEGF-R2 S1Pr1	VEGF-R2 S1Pr1	IDO	IDO	TNF-alpha IFN-gamma	TNF-alpha IFN-gamma	HO-1	HO-1	Nrf-2	Nrf-2
Brain	Infected		Infected		Liver		1.51	1.91	4.04	1.18	3.72	1.87	5.95	1.87	3.72	1.87	5.95	1.87	1.51	1.91	0.22	0.68
	LPS-infected-VEGF		LPS-infected-VEGF				1.00	0.78	10.13	5.68	14.98	1.01	10.12	1.01	14.98	1.01	10.12	1.01	1.00	0.78	0.65	1.43
	Infected + - lovastatin		Infected + - lovastatin				3.30**	23.01**	0.95	0.80	1.0*	12.60**	1.74**	12.60**	1.0*	1.0*	0.95	0.80	3.30**	23.01**	1.07*	33.72**
	Infected + - lovastatin		Infected + - lovastatin				9.10**	25.21**	0.77**	1.40*	2.97*	5.63**	5.14**	5.63**	2.97*	2.97*	0.77**	1.40*	9.10**	25.21**	2.62**	4.98**
	Infected + - lovastatin		Infected + - lovastatin				3.41**	18.24**	6.48**	7.11**	2.97*	5.63**	5.14**	5.63**	2.97*	2.97*	6.48**	7.11**	3.41**	18.24**	1.23**	43.57**
	Infected + - lovastatin		Infected + - lovastatin				6.03**	23.78**	2.62**	11.28**	2.97*	5.63**	5.14**	5.63**	2.97*	2.97*	2.62**	11.28**	6.03**	23.78**	1.87**	19.66**
	Infected + - lovastatin		Infected + - lovastatin				6.11**	10.58**	1.25**	3.48**	2.22**	15.91**	1.78**	15.91**	2.22**	2.22**	1.25**	3.48**	6.11**	10.58**	1.21*	48.68**
	Infected + - lovastatin		Infected + - lovastatin				12.57**	13.60**	0.56**	3.85**	1.50**	14.70**	5.40**	14.70**	1.50**	1.50**	0.56**	3.85**	12.57**	13.60**	4.20**	3.54*
	Infected + - lovastatin		Infected + - lovastatin				2.45**	3.25**	14.55**	3.85**	15.54**	0.86**	2.10**	0.86**	15.54**	15.54**	14.55**	3.85**	2.45**	3.25**	16.01**	71.79**
	Infected + - lovastatin		Infected + - lovastatin				1.15*	2.04**	18.86**	6.55*	20.11**	0.72*	5.96**	0.72*	20.11**	20.11**	18.86**	6.55*	1.15*	2.04**	4.16**	10.9**
Spleen	Infected		Infected		Lung		0.15	0.70	15.79	23.31	18.68	1.95	597.06	1.95	18.68	1.95	597.06	1.95	0.15	0.70	0.75	0.62
	LPS-infected-VEGF		LPS-infected-VEGF				1.58	0.63	262.24	202.13	180.01	0.87	452.05	0.87	180.01	0.87	452.05	0.87	1.58	0.63	0.43	0.48
	Infected + - lovastatin		Infected + - lovastatin				0.84	61.02**	3.22**	8.41**	30.67**	11.66**	23.68**	11.66**	30.67**	30.67**	3.22**	8.41**	0.84	61.02**	1.92**	2.87**
	Infected + - lovastatin		Infected + - lovastatin				1.13*	2.78**	0.82**	1.13**	2.74**	5.87**	214.66**	5.87**	2.74**	2.74**	0.82**	1.13**	1.13*	2.78**	12.16**	6.47**
	Infected + - lovastatin		Infected + - lovastatin				1.38*	14.17**	4.12**	26.00**	27.81**	5.87**	214.66**	5.87**	27.81**	27.81**	4.12**	26.00**	1.38*	14.17**	1.91**	2.10**
	Infected + - lovastatin		Infected + - lovastatin				3.44**	7.44**	19.33**	3.36**	16.33**	7.45**	34.00**	7.45**	16.33**	16.33**	3.36**	3.36**	3.44**	7.44**	5.21**	4.39**
	Infected + - lovastatin		Infected + - lovastatin				0.76*	16.30**	5.36**	9.08**	44.00**	6.04**	82.66**	6.04**	44.00**	44.00**	5.36**	9.08**	0.76*	16.30**	2.61**	1.43**
	Infected + - lovastatin		Infected + - lovastatin				1.44	2.42**	1.18**	1.75**	4.50**	17.33**	13.0**	17.33**	4.50**	4.50**	1.18**	1.75**	1.44	2.42**	10.92**	7.20**
	Infected + - lovastatin		Infected + - lovastatin				2.70*	5.05**	9.18**	9.69**	11.77**	1.82	144.54*	1.82	11.77**	11.77**	9.18**	9.69**	2.70*	5.05**	1.37**	1.49**
	Infected + - lovastatin		Infected + - lovastatin				1.19	2.63**	31.04**	7.54**	17.33**	1.20*	81.54**	1.20*	17.33**	17.33**	7.54**	7.54**	1.19	2.63**	0.51	0.35*

Expression of inflammation markers in brain, liver, spleen and lung tissue of C57BL/6-infected vs -treated mice (lovastatin, simvastatin, lipopolysaccharide S (LPS) + vascular endothelial growth factor (VEGF) and lovastatin + VEGF), + 3days post infection (blue) and at the end point (red). Each value represents the mean of the fold changes obtained from three biological replicates. Induction relative to the non-infected control (fold change) was calculated using the comparative Ct method ($\Delta\Delta Ct$). Values obtained from non-treated vs treated mice were compared using a one-sample *t*-test to access statistical significance * ($0.01 \leq P < 0.05$) and ** ($P < 0.01$).

of tissue pathology as revealed by morphological and immunohistochemistry examination. Differences in the hydrophobicity of the two statins may affect their respective bioavailability; however, simvastatin and lovastatin could as well differ in their ability to interact with yet unidentified targets regulating the activation of the inflammatory response. Our findings confirmed that lovastatin had the ability to upregulate the expression of the anti-inflammatory genes, Nrf-2 and HO-1, in the brain. However, tissues and organs from treated mice still showed important signs of inflammation pathology including mononuclear cell infiltrate, endothelial damage and oedema. Furthermore, lovastatin treated mice died 7–10 days earlier than ECM-resistant Balb/c mice. Similar results were obtained when treating ECM susceptible mice with LPS alone thus suggesting that the induction of Nrf-2 and HO-1 is not sufficient to elicit full protection against cerebral and systemic inflammation.

The addition of VEGF to lovastatin enhanced its protective and anti-inflammatory activity. The treated animals did not develop CM and showed a much-prolonged post-infection life span (up to seven days). The morphological and immunohistochemistry analysis provided important clues to explain this improvement. Tissue sections did not reveal signs of inflammation pathology in all organs examined including brain, spleen liver and lungs. These tissues also failed to react with antibodies against the CD3 antigen and activated caspase 3 thus confirming the lack of both cellular infiltrate and endothelial damage. The analysis of gene expression showed an enhanced activation of the anti-inflammatory genes, Nrf-2 and HO-1, mirrored by a complete silencing of the pro-inflammatory cytokines TNF- α and IFN- γ . Interestingly, unlike statins, the VEGF-lovastatin treatment induced a strong activation of S1P, a gene encoding a sphingolipid signalling molecule implicated in regulating vascular integrity and endothelial remodelling to injury. This gene was upregulated in the brain, spleen and lung of mice possibly indicating an improved disease outcome, associated with preserved endothelium integrity, reduced host inflammation and T cell influx into these organs.

We have demonstrated that the synergy achieved by combining VEGF and LPS reflect their respective protective activities on the two main pathogenic mechanisms leading to CM: an unregulated activation of the inflammation response and endothelial cell damage. Here, we show that lovastatin could possibly replace LPS alongside VEGF, without an obvious loss of activity in blocking the activation of systemic inflammation. While this observation has important consequence for accelerating the development of effective supportive therapy against human CM, it may provide clues to develop new treatments for conditions where systemic inflammation plays a major contribution in disease pathology.

Materials and Methods

Animals

C57BL/6 females (age 4–6 weeks) were purchased from Harlan Laboratories, UK, Ltd. Oxon, England. All animal procedures and care conformed strictly to the United Kingdom Home Office Guidelines under the Animals (Scientific Procedures) Act 1986 and the protocols were approved by the Home Office of Great Britain (Licence number: 70/7237).

Parasite and infection procedure

The parasite PbA clone 2.34 was originally obtained from The University of Perugia, Dipartimento di Medicina Sperimentale, Italy. Serial passages of *P. berghei* were carried out by i.p. Inoculation of naïve C57BL/6 mice with 10^5 parasitised red blood cells to obtain parasitised erythrocytes stocks. In this study C57BL/6 mice were infected by i.p. injection of 10^5 infected erythrocytes at day 0. Parasitaemia was first measured 3 days post-infection followed by a daily measurement until the day of death. A drop of blood was collected by venesection of the tail of the mouse and transferred onto the edge of a microscope slide. The blood was drawn evenly across a second slide to make a thin blood film and allowed to dry at room temperature before staining with Giemsa stain. Five fields of approximately 200 cells each were counted and the parasitaemia was calculated as the percentage of the total red blood cells containing parasites.

Treatment regimen

Starting from day 1 post infection, the mice were treated daily with lovastatin 20 mg/kg (InSolution™ lovastatin Sodium Salt, Merck-Millipore, Nottingham, UK, # 438187) or Simvastatin 20 mg/kg (InSolution™ Simvastatin Sodium Salt, Merck-Millipore, # 567022) or Mouse VEGF-164 (mVEGF₁₆₄) 200 ng/mouse (Cell Signalling, Herts, UK, # 5211) or a combination treatment of lovastatin 20 mg/kg and subsequently Mouse VEGF-164 (mVEGF₁₆₄) 200 ng/mouse over a period of 5 days. Lipopolysaccharide S from *Escherichia coli* 055:B5 (LPS) 20 μ g/mouse (Sigma-Aldrich, Gillingham, Dorset, UK, # L6529) was injected i.p. route from *E. coli* 055:B5 5 days, prior infection.

Parameters used to assess the development of Cerebral Malaria

The onset of cerebral complications was determined by monitoring a number of clinical signs, such as ruffled fur, hunching, wobbly gait, limb paralysis, convulsion and death. Moreover, a drop in body temperature below 34°C was also used to confirm the onset of cerebral complications. Mice were monitored daily from the day of the infection. At the end of the experiment, mice underwent deep anaesthesia for blood sampling by cardiac

puncture. Afterwards, they were euthanised and organ collection (brain, spleen, lung, liver) took place.

Histopathology and immunohistochemistry

Formalin-fixed samples of brain, liver, spleen collected from infected, treated and WT littermates were embedded in paraffin blocks. Thin tissue sections (5 µm-thick) were obtained and routinely processed for histopathological examination (H&E stain) and for immunohistochemistry using antibodies to detect CD3 (anti-CD3 Antibody, Abcam, Cambridge, UK, ab5690/Antigen Retrieval: Citrate buffer 10 minutes – MW, dilution: 1:100 – 4°C overnight) and Cleaved Caspase 3 positive cells (Cleaved Caspase 3 Antibody, Cell Signalling #9661, Antigen Retrieval: Citrate buffer 10 minutes – MW, dilution: 1:100 – 4°C overnight). All the antibodies were visualised using the Vectastain Elite ABC kit (Vector Laboratories, #PK-6101) and DAB Peroxidase Substrate kit (Vector Laboratories, Peterborough, UK, # SK-4100). Additional serial tissue sections were also processed for indirect immunoperoxidase staining following the procedures described elsewhere.²⁰ Microphotographs of organs were taken using a Canon Power Shot A520 with a close-up lens 250D (5.7 zoom).

mRNA transcript quantification

Total RNA was extracted using Trizol (Life Technologies) and reverse transcribed using the SuperScript III First Strand Synthesis System kit (Life Technologies, Paisley, UK). Transcript abundance was measured using an Applied Biosystem Thermocycler and Fast SYBER Green Master Mix (Life Technologies).

GAPDH was used as housekeeping reference gene in a qPCR reaction. Induction relative to the non-infected control (fold change) was calculated using the comparative Ct method ($\Delta\Delta C_t$).

The specific mouse primer sequences (5'–3') used in this work to profile the cytokines and other immune-related factors were as follows.

GAPDH²¹ (forward) ATGACATCAAGAAGGTGGTG;

(reverse) CATAACAGGAAATGAGCTTG;

VEGF-R2²² (forward)GGCGGTGGTGACAGT-ATCTT;

(reverse)TCTCCGGCAAGCTCAAT;

S1Pr1²³ (forward)AACTTTGCGAGTGAGC-TGGT;

(reverse)CTAGAGGGCGAGGTTGAGTG;

TNF-alpha²⁴ (forward)CCCAGGGACCTCTCT-CTAATC;

(reverse)ATGGGCTACAGGCTTGTCAGT;

IFN-gamma²⁵ (forward)CTAATTATTCGGTAA-CTGACTTGA;

(reverse)ACAGTTCAGCCATCACTTGGA;

IDO²⁶ (forward)GGCTTCTTCCTCGTCTCTCT-ATTG;

(reverse)TGACGCTCTACTGCACTGGATAC;
HO-1^{27,28} (forward)GGTGATGGCTTCCTG-TACC;

(reverse)AGTGAGGCCCATAACCAGAAG;

Nrf-2^{29,30} (forward)TCTCCTCGCTGGAAAA-AGAA;

(reverse) AATGTGCTGGCTGTGCTTTA.

Statistical Analysis

All statistical analyses were performed using Graph-Pad Prism 6.0 (GraphPad Software Inc).

Fold changes obtained from real-time PCR data for each of the genes selected from individuals in different conditions were compared. More specifically, the pairings for comparison were done as follows (condition A vs condition B):

- Not treated (infected) versus
 - infected + lovastatin
 - infected + simvastatin,
 - infected + lovastatin + VEGF
 - infected + LPS + VEGF littermates (C57BL/6)
- infected + lovastatin versus infected + LPS + VEGF
- infected + simvastatin versus infected + LPS + VEGF
- infected + lovastatin + VEGF versus infected + LPS + VEGF

Each condition A versus condition B pairing was explored with biological triplicates. In order to detect the genes for whom the difference of expression between conditions was significant, the following procedure was adopted, individually onto each selected gene (by means of an Excel spreadsheet):

- the difference between condition-related expressions was computed as: $\text{diff} = \log_2(\text{expression}_{\text{condA}} / \text{expression}_{\text{condB}})$ so that no difference between expressions (i.e. $\text{expression}_{\text{condA}} / \text{expression}_{\text{condB}} = 1$) resulted in $\text{diff} = 0$;
- to test for statistical significance of expression difference, a one-sample *t*-test was constructed, the null hypothesis being: $H_0: \mu_{\text{diff}} = 0$, with a sample formed of the three diff values obtained from the three biological replicates. Rejection of the null hypothesis (P -value < 0.05) was used to identify significance.
- genes exhibiting significant differences were labelled with * ($0.01 \leq P < 0.05$) and ** ($P < 0.01$)

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Disclaimer statements

Contributors

M.C. conceived the study, performed all the experiments, analyzed and interpreted the data. A.C.

conceived, directed the experiments, interpreted the data and wrote the manuscript. M.C and A.C. discussed the results and commented on the manuscript.

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Conflict of interest

The authors declare no competing financial interests.

Ethics approval

All animal procedures and care conformed strictly to the United Kingdom Home Office Guidelines under the Animals (Scientific Procedures) Act 1986 and the protocols were approved by the Home Office of Great Britain (License number: 70/7237).

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