

**Brief Definitive Report****Locally Up-regulated Lymphotoxin  $\alpha$ , Not Systemic Tumor Necrosis Factor  $\alpha$ , Is the Principle Mediator of Murine Cerebral Malaria**Christian R. Engwerda,<sup>1</sup> Tracey L. Mynott,<sup>2</sup> Sanjeet Sawhney,<sup>1</sup> J. Brian De Souza,<sup>3</sup> Quentin D. Bickle,<sup>1</sup> and Paul M. Kaye<sup>1</sup><sup>1</sup>Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom<sup>2</sup>Centre for Molecular Microbiology and Infection, Imperial College of Science, Technology and Medicine, London SW7 2AZ, United Kingdom<sup>3</sup>Department of Immunology, Royal Free and University College London Medical School, Windeyer Institute of Medical Science, London W1P 6DB, United Kingdom**Abstract**

Cerebral malaria (CM) causes death in children and nonimmune adults. TNF- $\alpha$  has been thought to play a key role in the development of CM. In contrast, the role of the related cytokine lymphotoxin  $\alpha$  (LT $\alpha$ ) in CM has been overlooked. Here we show that LT $\alpha$ , not TNF $\alpha$ , is the principal mediator of murine CM. Mice deficient in TNF $\alpha$  (B6.TNF $\alpha^{-/-}$ ) were as susceptible to CM caused by *Plasmodium berghei* (ANKA) as C57BL/6 mice, and died 6 to 8 d after infection after developing neurological signs of CM, associated with perivascular brain hemorrhage. Significantly, the development of CM in B6.TNF $\alpha^{-/-}$  mice was not associated with increased intracellular adhesion molecule (ICAM)-1 expression on cerebral vasculature and the intraluminal accumulation of complement receptor 3 (CR3)-positive leukocytes was moderate. In contrast, mice deficient in LT $\alpha$  (B6.LT $\alpha^{-/-}$ ) were completely resistant to CM and died 11 to 14 d after infection with severe anemia and hyperparasitemia. No difference in blood parasite burden was found between C57BL/6, B6.TNF $\alpha^{-/-}$ , and B6.LT $\alpha^{-/-}$  mice at the onset of CM symptoms in the two susceptible strains. In addition, studies in bone marrow (BM) chimeric mice showed the persistence of cerebral LT $\alpha$  mRNA after irradiation and engraftment of LT $\alpha$ -deficient BM, indicating that LT $\alpha$  originated from a radiation-resistant cell population.

Key words: parasitic disease • protozoan infection • *Plasmodium berghei* • immunopathology • infection

**Introduction**

Cerebral malaria (CM) is a major cause of death in African children infected with *Plasmodium falciparum* (1). This serious neurological condition is characterized by the sequestration of parasitized erythrocytes in cerebral blood vessels (1). Because high levels of circulating TNF $\alpha$  are found in the serum of CM patients, this cytokine is thought to play an important role in CM (2, 3). This has been supported by reports of TNF $\alpha$  production in the brain during murine CM caused by *Plasmodium berghei* ANKA (4, 5). In this experimental model of CM, anti-TNF $\alpha$  antibodies (6) or soluble TNF receptor (TNFR; reference 7) have also been shown to attenuate the severity of symptoms and prevent

subsequent death. In addition, mice deficient in TNFR2 (p75) are resistant to CM (8).

In both human and murine CM, the blockage of cerebral vessels by parasitized RBCs (pRBCs) is thought to be caused by TNF $\alpha$ -mediated up-regulation of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, on the endothelium (1, 6). In mice, this is also associated with the accumulation of activated leukocytes in the lumen of brain micro-vessels (6). TNF $\alpha$  is thought to mediate pathology by either direct cytolytic effects or induction of an inflammatory cascade involving mediators such as IFN- $\gamma$  and nitric oxide (NO; reference 9). However, the critical role of TNF $\alpha$  in human CM has recently been questioned by the failure of TNF $\alpha$ -neutralizing reagents to decrease the incidence of clinical CM (10, 11).

Recently, lymphotoxin  $\alpha$  (LT $\alpha$ ), a related member of the TNF family, has been reported to have important, yet

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distinct, roles in various infectious and autoimmune disease models (12, 13). LT $\alpha$  forms a soluble homotrimer that along with TNF $\alpha$  is capable of binding both the TNFR1 and TNFR2. In addition, LT $\alpha$  can form a cell-bound heterotrimer with two LT $\beta$  molecules, which binds the LT $\beta$  receptor (14). Given the shared receptor usage of TNF $\alpha$  and LT $\alpha$  (14), and the potential cross-reactivity of anti-TNF $\alpha$  antibodies with LT $\alpha$  (15), it is possible that LT $\alpha$ , and not TNF $\alpha$ , is the principal mediator of CM. To test this hypothesis, C57BL/6 mice deficient in TNF $\alpha$  or LT $\alpha$  were infected with *P. berghei* ANKA pRBCs and their susceptibility to CM was compared. Our results clearly show that LT $\alpha$ , and not TNF $\alpha$ , is indeed the principal mediator of murine CM.

## Materials and Methods

**Mice.** C57BL/6 mice were purchased from Tuck and Co. and were housed under conventional conditions. Mice deficient in TNF $\alpha$  (B6.TNF $\alpha^{-/-}$ ) and both TNF $\alpha$  and LT $\alpha$  (B6.TNF $\alpha$ /LT $\alpha^{-/-}$ ; reference 16) were obtained from Bantin & Kingman, while those deficient in LT $\alpha$  (B6.LT $\alpha^{-/-}$ ; reference 17) were obtained from The Jackson Laboratory. In some experiments, B6.LT $\alpha^{-/-}$  mice generated from C57BL/6 mice embryonic stem cells (12) were purchased from Bantin & Kingman and used to confirm the resistance of LT $\alpha$ -deficient mice (17) to CM. B6.Ly5.1 mice were obtained from Charles River Laboratories (IFFA Credo). All mouse strains were bred at the London School of Hygiene and Tropical Medicine under barrier conditions. Mice used in all experiments were sex-matched and used at 6 wk of age. Chimeric mice were prepared by irradiating animals twice (48 h apart) with 5.5 Gy and then engrafting with  $10^7$  fresh bone marrow (BM) cells intravenously via the lateral tail vein within 2 h of the second radiation exposure. Mice were maintained on antibiotics for 4 wk after engraftment and infected with parasites 8 wk after receiving BM.

**Parasites, Infection, and Disease Assessment.** *P. berghei* (ANKA) was obtained from Dr. N. Wedderburn (Royal College of Surgeons, London, UK) and used in all experiments after one in vivo passage in C57BL/6 mice. Mice were infected by injecting  $10^4$  pRBCs intravenously via the lateral tail vein, as described previously (18). Animals were monitored for neurological signs of CM, including convulsions, ataxia and paralysis. Parasite burden was determined from Giemsa stained blood smears, as described previously (18), and expressed as the percentage of pRBCs. Blood hemoglobin levels were determined when collecting blood using a Hemoglobin meter, according to the manufacturer's instructions (Buffalo Medical Specialities). RBCs were counted using a hemocytometer by diluting 2  $\mu$ l of tail blood in 1 ml RPMI. Animals were monitored and killed by cervical dislocation when death was deemed inevitable, according to UK Home Office guidelines. Blood was harvested by cardiac puncture for serum isolation. Brain tissue was carefully removed and half was snap frozen on cork blocks in OCT embedding media solution (Raymond A. Lamb, Eastbourne, UK) and stored at  $-80^{\circ}\text{C}$  until required for immunohistochemistry. The other half was stored in RNAlater (Ambion) for 24 h at  $4^{\circ}\text{C}$ , before freezing at  $-80^{\circ}\text{C}$  until required for RNA extraction. Some tissue was fixed in formol saline (4% vol/vol; formaldehyde) for wax embedding and preparing tissue sections for hematoxylin and eosin staining.

**Real-time Reverse Transcription PCR.** RNA was isolated from brain tissue using Tri Reagent (Sigma-Aldrich), and an RNeasy Mini Kit with on-column DNase digestion (QIAGEN), according to the manufacturers' instructions. RNA was reverse transcribed into cDNA as described previously (19, 20). Oligonucleotides (5'-3') used for the specific amplification of LT $\alpha$  were AGGGGCCAGGGACTCTCT (sense) and ACGATCCGTGCTTGCTCTC (antisense), and for amplification of the house keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) were GTTGGATACAGGCCAGACTTTGTTG (sense) and GATTCAACCTTGCCTCATCTTAGGC (antisense). The number of LT $\alpha$  and HPRT cDNA molecules in each sample was calculated by real-time reverse transcription (RT)-PCR using QuantiTect SYBR green master mix (QIAGEN) and a LightCycler (Roche), according to the manufacturers' instructions. Standard curves were constructed with known amounts of LT $\alpha$  and HPRT cDNA, and the number of LT $\alpha$  molecules per 1,000 HPRT molecules in each sample was calculated.

**Measurement of Serum IFN- $\gamma$  and Nitrate Levels.** Serum samples were diluted 1:2 and IFN- $\gamma$  levels measured by ELISA, as described previously (21). Serum nitrate levels were measured by reducing nitrate to nitrite and using Griess reagent, as described previously (22).

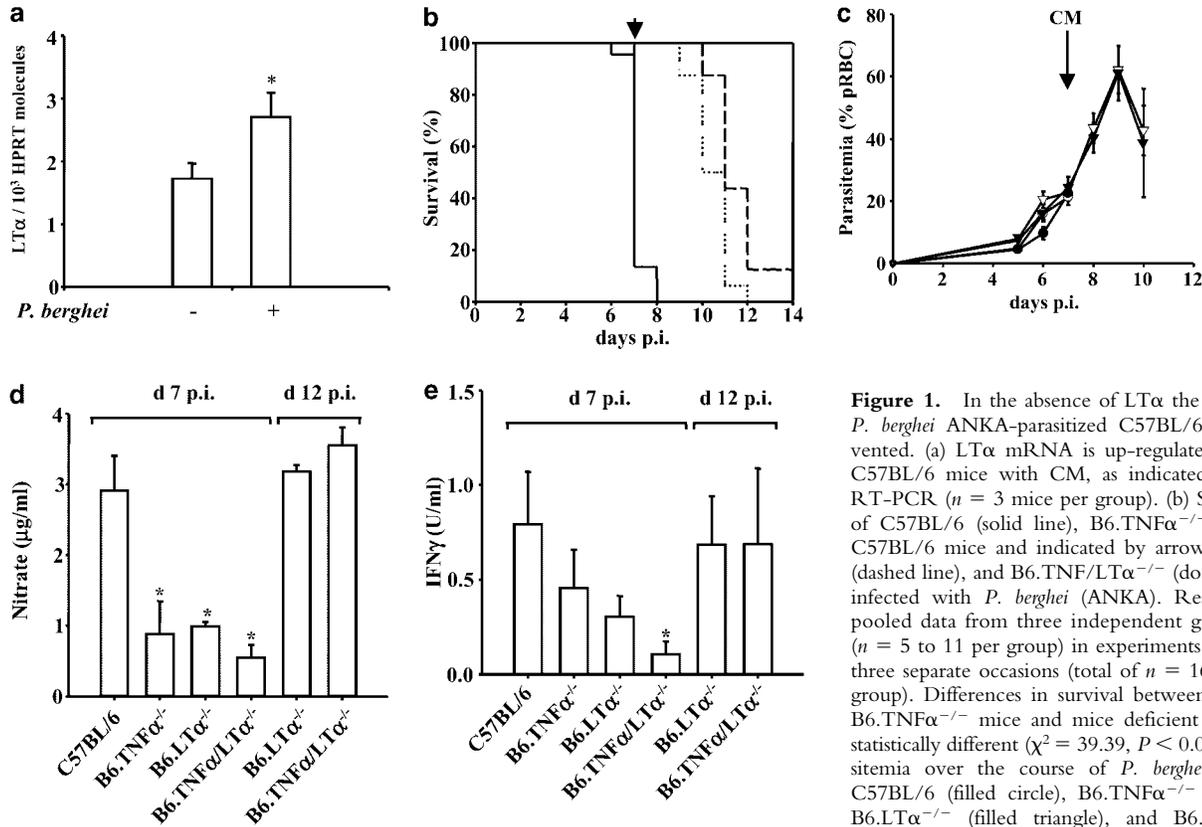
**Immunohistochemistry on Cryostat Sections of the Brain** Antibodies used for histology included mouse anti-*P. berghei* polyclonal antibody (18), anti-complement receptor 3 (CR3) (5C6; reference 23), and anti-CD54 (ICAM-1; KAT-1; Serotec). Brain tissue responses were analyzed on 6  $\mu$ m acetone-fixed cryostat tissue sections stained with the above primary antibodies and appropriate fluorescein-isothiocyanate (FITC)-conjugated secondary detection reagents according to the manufacturer's instructions (Vector Laboratories). Sections were mounted in Vectashield mounting media for fluorescence (Vector Laboratories), before analysis on an Axioplan fluorescent microscope (ZEISS). Images were recorded digitally using a MagnaFire Sp digital camera (Optronics). In some experiments, peroxidase-conjugated secondary reagents (Vector Laboratories) were used to detect ICAM-1 and CR3 expression in brain tissue. These sections were then used to count ICAM-1-positive vessels and CR3-positive cells in 25 consecutive microscope fields of view using a  $\times 40$  objective (final magnification  $\times 400$ ).

**Statistics.** Statistical analysis was performed for IFN- $\gamma$  and nitrate levels using Student's *t* test and for survival curves using Kaplan-Meier plots and Log Rank tests.

## Results and Discussion

**LT $\alpha$ -deficient Mice Do Not Die from CM.** To determine if LT $\alpha$  is involved in CM, we first tested for LT $\alpha$  production using LT $\alpha$ -specific primers in a real-time RT-PCR with mRNA isolated from the brains of C57BL/6 mice infected with *P. berghei* and showing signs of CM (Fig. 1 a). LT $\alpha$  mRNA was constitutively expressed in the mouse brain, and levels increased in mice with CM. Therefore, we sought to investigate further if this cytokine played a role in murine CM.

To distinguish the roles of TNF $\alpha$  and LT $\alpha$  in CM, we infected mice deficient in these cytokines (B6.TNF $\alpha^{-/-}$  and B6.LT $\alpha^{-/-}$  mice, respectively) with *P. berghei* (ANKA) and monitored animals for signs of CM. Strikingly, B6.TNF $\alpha^{-/-}$  mice began to show neurological symptoms



**Figure 1.** In the absence of LT $\alpha$  the early death of *P. berghei* ANKA-parasitized C57BL/6 mice is prevented. (a) LT $\alpha$  mRNA is up-regulated in brains of C57BL/6 mice with CM, as indicated by real-time RT-PCR ( $n = 3$  mice per group). (b) Survival curves of C57BL/6 (solid line), B6.TNF $\alpha^{-/-}$  (overlapping C57BL/6 mice and indicated by arrow), B6.LT $\alpha^{-/-}$  (dashed line), and B6.TNF/LT $\alpha^{-/-}$  (dotted line) mice infected with *P. berghei* (ANKA). Results represent pooled data from three independent groups of mice ( $n = 5$  to 11 per group) in experiments conducted on three separate occasions (total of  $n = 16$ –22 mice per group). Differences in survival between C57BL/6 or B6.TNF $\alpha^{-/-}$  mice and mice deficient in LT $\alpha$  were statistically different ( $\chi^2 = 39.39$ ,  $P < 0.0001$ ). (c) Parasitemia over the course of *P. berghei* infection in C57BL/6 (filled circle), B6.TNF $\alpha^{-/-}$  (open circle), B6.LT $\alpha^{-/-}$  (filled triangle), and B6.TNF/LT $\alpha^{-/-}$  (open triangle) mice. Arrow indicates the time point

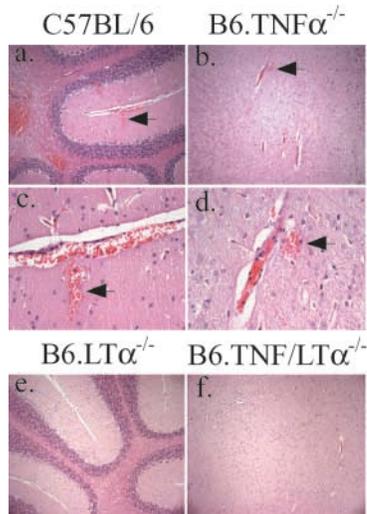
(day 7) that CM developed in C57BL/6 and B6.TNF $\alpha^{-/-}$  mice. Data shown are from one experiment that is representative of the four performed. (d) Serum nitrate and (e) serum IFN- $\gamma$  levels at day 7 and 12 postinfection (p.i.), as indicated. Statistically significant differences of  $P < 0.05$  are indicated (\*). Data shown are from one experiment that is representative of the three performed.

of CM, including ataxia, convulsions, and palsy, at the same time as infected C57BL/6 mice (days 6–8 after infection). Within 12–24 h of the onset of neurological symptoms, all C57BL/6 and B6.TNF $\alpha^{-/-}$  mice had to be killed (Fig. 1 b). In stark contrast, B6.LT $\alpha^{-/-}$  mice and those deficient in both TNF $\alpha$  and LT $\alpha$  (B6.TNF/LT $\alpha^{-/-}$  mice) failed to show any neurological symptoms associated with CM. However, hemoglobin levels in B6.LT $\alpha^{-/-}$  mice dropped from 14.5–16 g/dL (at day 0 p.i.) to less than 4–6 g/dL (day 12 after infection), and RBC numbers decreased from  $\sim 10^{10}$ /ml to  $1$ – $3 \times 10^9$ /ml. These mice died between days 11–15 after infection. Such decreases in hemoglobin and RBC numbers have previously been associated with death by complications arising from anemia (9). Importantly, when C57BL/6 and B6.TNF $\alpha^{-/-}$  mice died, parasitemia ranged from 12–24% pRBCs and was similar for all mouse strains (Fig. 1 c). B6.TNF/LT $\alpha^{-/-}$  mice have previously been shown to be resistant to CM, but this resistance was attributed to the absence of TNF $\alpha$  (24). These results clearly show that LT $\alpha$ , and not TNF $\alpha$ , is the primary mediator of murine CM, and that the development of CM is independent of blood parasitemia.

Both nitric oxide (NO) and IFN- $\gamma$  have been reported to play important roles in the development of murine CM (9, 24). Serum nitrate (an indirect measure of NO) and IFN- $\gamma$  levels were reduced in mice deficient in either

TNF $\alpha$  or LT $\alpha$ , compared with C57BL/6 mice at the onset of CM symptoms in susceptible strains (Fig. 1, d and e, day 7 after infection). Subsequently, both IFN- $\gamma$  and nitrate levels in the serum of B6.LT $\alpha^{-/-}$  and B6.TNF/LT $\alpha^{-/-}$  mice rose such that by day 12 after infection they were similar to the levels found in C57BL/6 mice. These data are in agreement with previous studies that show reduced NO and IFN- $\gamma$  levels in *P. berghei*-infected B6.TNF/LT $\alpha^{-/-}$  mice (24). These results suggest that both TNF $\alpha$  and LT $\alpha$  play a role in the production of NO and IFN- $\gamma$  after infection with *P. berghei*. However, the development of CM in B6.TNF $\alpha^{-/-}$  mice demonstrates that elevated serum NO and IFN- $\gamma$  levels do not correlate with the development of CM.

*Changes to the Cerebral Vasculature of TNF- $\alpha$ - and LT $\alpha$ -deficient Mice after *P. berghei* Infection.* Histological examination of brain sections revealed the presence of RBCs laden with malaria pigment (hemozoin) in C57BL/6 and B6.TNF $\alpha^{-/-}$  mice at the time of death (Fig. 2, a–d). Small vascular hemorrhages were also observed in these animals (Fig. 2, a–d), but not in animals deficient in LT $\alpha$  at the same time (Fig. 2, e and f). To determine if these anatomical features were associated with the presence of pRBCs in the brain, sections were stained with anti-*P. berghei* antibody (Fig. 3 a). Significant numbers of pRBCs were found in the cerebral blood vessels of C57BL/6 and B6.TNF $\alpha^{-/-}$



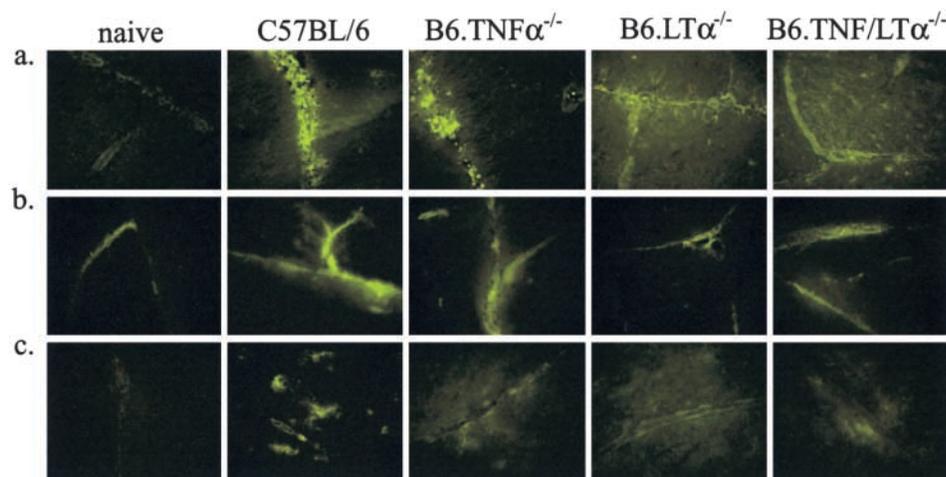
**Figure 2.** Perivascular hemorrhages are present in C57BL/6 and B6.TNF $\alpha^{-/-}$  mice that develop CM. Sections (3  $\mu$ M) were prepared from formal saline-fixed brain tissue and stained with hematoxylin and eosin. Magnifications of  $\times 100$  (a and b) and  $\times 400$  (c and d) are shown for C57BL/6 and B6.TNF $\alpha^{-/-}$  mice that died with CM. Areas within the cerebellum (a, c, and e) and cerebrum (b, d, and f) are shown. Arrows highlight sites of perivascular hemorrhage. These hemorrhages were not observed in LT $\alpha$ -deficient mice (e and f; original magnification:  $\times 100$ ). These data are representative of material obtained in four experiments (at least 25 microscope fields from 12 or more samples from each mouse strain were examined).

mice at the time of death (Fig. 3 a), but not in B6.LT $\alpha^{-/-}$  and B6.TNF/LT $\alpha^{-/-}$  mice at the same time (day 7 after infection; Fig. 3 a), despite blood parasitemias being the same in all mouse strains at this time (Fig. 1 b). These data suggest that pRBC were being retained in cerebral blood vessels of mice that succumbed to CM.

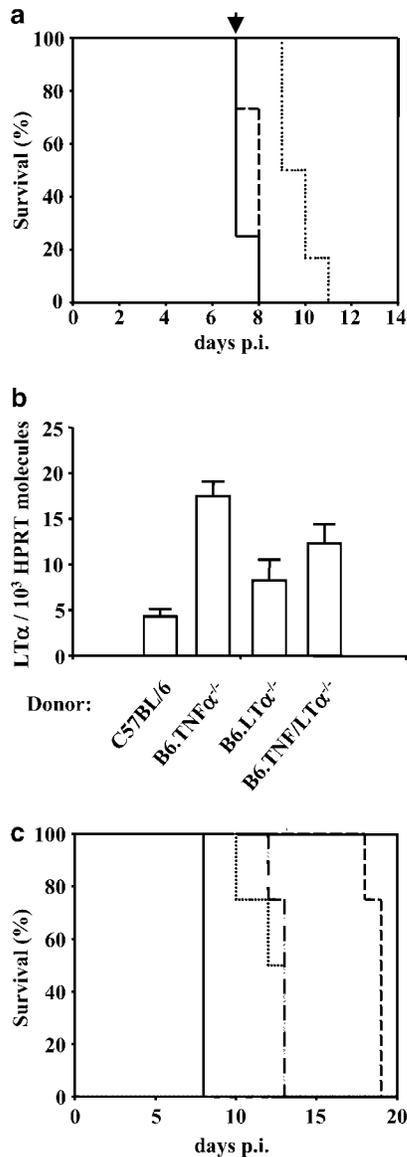
Increased ICAM-1 expression is a feature of murine CM and is thought to mediate binding of leukocytes and possibly pRBCs to micro-vessels in the brain (25, 26). ICAM-1 expression on vascular endothelial cells in the brain was less in B6.TNF $\alpha^{-/-}$  mice than in C57BL/6 mice (Fig. 3 b;  $438 \pm 22$  ICAM-1 $^{+}$  vessels per 25 fields of

view versus  $894 \pm 16$  ICAM-1 $^{+}$  vessels per 25 fields of view, respectively), but increased when compared with expression in naive controls (Fig. 3 b;  $245 \pm 9$  ICAM-1 $^{+}$  vessels per 25 fields of view). Expression of ICAM-1 was also reduced in B6.LT $\alpha^{-/-}$  and B6.TNF $\alpha$ /LT $\alpha^{-/-}$  mice at day 7 after (Fig. 3 b;  $438 \pm 23$  ICAM-1 $^{+}$  vessels per 25 fields of view and  $476 \pm 23$  ICAM-1 $^{+}$  vessels per 25 fields of view, respectively). Associated with the decreased ICAM-1 expression, in TNF $\alpha$  and LT $\alpha$ -deficient mice, there was also a decrease in the number of CR3-positive inflammatory cells found in the brain (Fig. 3 c;  $100 \pm 3$ ,  $427 \pm 23$ ,  $160 \pm 6$ ,  $165 \pm 5$ , and  $151 \pm 7$  CR3 $^{+}$  cells per 25 fields of view for naive C57BL/6 mice, and C57BL/6, B6.TNF $\alpha^{-/-}$ , B6.LT $\alpha^{-/-}$ , and B6.TNF $\alpha$ /LT $\alpha^{-/-}$  mice at day 7 after infection, respectively). Together, these results indicate that TNF $\alpha$  and LT $\alpha$  are both required for optimal ICAM-1 up-regulation and leukocyte accumulation in the brain during murine CM. However, these data also show that these events do not necessarily predict the development of CM.

*LT $\alpha$  Is Produced by a Radiation-resistant Cell Population.* To assess whether leukocytes accumulating in the brain were the source of LT $\alpha$  that mediated CM, BM chimeric mice were constructed. Congenic B6.Ly5.1 mice were irradiated and engrafted with BM from C57BL/6 mice or animals lacking TNF $\alpha$ , LT $\alpha$ , or both (all express the Ly5.2 CD45 allele). After engraftment, chimeric mice contained fewer than 2% Ly5.1-positive leukocytes in the spleen and peripheral blood (data not shown). After infection with *P. berghei*, chimeric mice engrafted with BM from C57BL/6 and B6.TNF $\alpha^{-/-}$  mice died at the same time as C57BL/6 and B6.TNF $\alpha^{-/-}$  mice (Fig. 4 a), and showed similar signs of CM. The leukocytes that accumulated in the brain in chimeric mice that developed CM were identified as being of donor origin by staining brain tissue with anti-Ly5.2 antibodies (data not shown). Surprisingly, chimeric mice engrafted with B6.LT $\alpha^{-/-}$  BM died in the ensuing 24 h of CM (Fig. 4, a and c), in contrast to the prolonged survival and lack of CM in B6.LT $\alpha^{-/-}$  mice. This observation indicates that a radiation-resistant cell population in the brain



**Figure 3.** Comparative immunohistochemical analysis of brain sections taken from *P. berghei*-infected mice when C57BL/6 and B6.TNF $\alpha^{-/-}$  mice developed CM (day 7 after infection). Brain cryosections (6  $\mu$ M) were stained with (a) anti-*P. berghei* antibodies, (b) anti-ICAM-1, and (c), anti CR3 (5C6) mAbs with fluorescent secondary reagents. Original magnification is  $\times 400$ . The samples are representative of material from four experiments.



**Figure 4.** LT $\alpha$  is produced by a radiation-resistant cell population in the brain. (a) Survival curves of chimeric B6.Ly5.1 mice engrafted with BM from C57BL/6 (solid line), B6.TNF $\alpha^{-/-}$  (overlapping C57BL/6 mice and indicated by arrow), B6.LT $\alpha^{-/-}$  (dashed line), and B6.TNF/LT $\alpha^{-/-}$  (dotted line) mice infected with *P. berghei*. Results represent pooled data from three independent groups of mice ( $n = 4-6$  per group) in experiments conducted on three separate occasions (total of  $n = 12-16$  mice per group). Differences in survival between B6.Ly5.1 mice engrafted with BM from C57BL/6 or B6.TNF $\alpha^{-/-}$  mice and mice deficient in LT $\alpha$  were statistically different ( $\chi^2 = 7.01$ ,  $P < 0.01$ ), as was the difference between mice engrafted with BM from B6.LT $\alpha^{-/-}$  and B6.TNF/LT $\alpha^{-/-}$  mice ( $\chi^2 = 23.83$ ,  $P < 0.0001$ ). (b) LT $\alpha$  mRNA is detected by real-time RT-PCR in the brain of all chimeric B6.Ly5.1 mice following *P. berghei* infection (brain tissue collected at time of death). The source of engrafted BM is indicated. (c) Survival curve of chimeric B6.Ly5.1 mice engrafted with BM from C57BL/6, B6.TNF $\alpha^{-/-}$  or B6.LT $\alpha^{-/-}$  mice (all groups overlap; solid line), and chimeric B6.TNF/LT $\alpha^{-/-}$  mice engrafted with BM from C57BL/6 (dotted line), B6.TNF $\alpha^{-/-}$  (dashed line), or B6.LT $\alpha^{-/-}$  (dashed and dotted line) mice ( $n = 4$  per group). Differences in survival between B6.Ly5.1 mice engrafted with BM from C57BL/6 and B6.TNF/LT $\alpha^{-/-}$  mice engrafted with BM from C57BL/6, B6.TNF $\alpha^{-/-}$ , or B6.LT $\alpha^{-/-}$  mice were statistically different ( $\chi^2 = 15$ ,  $P < 0.0001$ ), as was the increased survival time of B6.TNF/LT $\alpha^{-/-}$  mice engrafted with BM from B6.TNF $\alpha^{-/-}$  mice over all other groups ( $\chi^2 = 6.628$ ,  $P < 0.01$ ).

is the major source of LT $\alpha$  during murine CM. However, the fact that chimeric mice engrafted with BM from B6.TNF/LT $\alpha^{-/-}$  mice survived a further 2-3 d (Fig. 4 a) and died showing few signs of CM indicates that either TNF $\alpha$  or LT $\alpha$  produced by leukocytes may contribute to the pathology induced by a LT $\alpha$ -producing, radiation-resistant cell population. LT $\alpha$  mRNA was found in the brains of all chimeric mice, including those engrafted with BM from LT $\alpha$ -deficient mice (Fig. 4 b), confirming that a radiation-resistant cell population was the source of LT $\alpha$ . No LT $\alpha$  mRNA molecules were ever detected by real-time RT-PCR in brain tissue from B6.LT $\alpha^{-/-}$  mice (data not shown). Furthermore, when chimeric mice were prepared by irradiating B6.TNF/LT $\alpha^{-/-}$  mice and engrafting with BM from C57BL/6, B6.TNF $\alpha^{-/-}$ , or B6.LT $\alpha^{-/-}$  mice, all mice failed to develop CM (Fig. 4 c), and died with anemia and hyperparasitemia (data not shown). The extended survival of irradiated B6.TNF/LT $\alpha^{-/-}$  mice engrafted with BM from B6.TNF $\alpha^{-/-}$  mice indicates a role for TNF $\alpha$  in the death of *P. berghei*-infected mice suffering from anemia and hyperparasitemia. Although the LT $\alpha$ -producing cell population is likely to be of nonhematopoietic origin, we cannot rule out the possibility that a long-lived, radiation-resistant, BM-derived cell population is a source of LT $\alpha$  in the brain. Similarly, we cannot yet rule out any involvement of immune defects associated with TNF $\alpha$ - and LT $\alpha$ -deficient mice in their different susceptibility to CM. Nevertheless, these data show that LT $\alpha$  produced by a radiation-resistant cell is the key factor for the development of murine CM.

In summary, we have shown that LT $\alpha$  produced by a radiation-resistant cell population is the principal mediator of murine CM. Both endothelial cells that line the brain vasculature, microglial cells or astrocytes in the brain parenchyma are potential sources of LT $\alpha$ , as all are radiation-resistant (27, 28) and capable of producing inflammatory mediators (27, 29). Given the constitutive expression of LT $\alpha$  mRNA in the brain, it is likely that LT $\alpha$  acts in synergy with a parasite product or a parasite-induced host mediator. In fact, mice that die with CM accumulate pRBC in cerebral vessels (Fig. 3 a), despite having similar blood parasitemia to LT $\alpha$ -deficient mice (Fig. 1 c), potentially providing increased levels of these products in the brain. The identity of these factors is unknown, but a candidate parasite product for this role is glycosylphosphatidylinositol (GPI), the membrane anchor for various parasite surface antigens that has been shown to increase expression of inflammatory mediators by macrophages and vascular endothelial cells (30). The involvement of LT $\alpha$  in human CM is unknown, but results from this work indicate that it should be investigated.

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## References

- Miller, L.H., M.F. Good, and G. Milon. 1994. Malaria pathogenesis. *Science*. 264:1878–1883.
- Grau, G.E., T.E. Taylor, M.E. Molyneux, J.J. Wirima, P. Vassalli, M. Hommel, and P.H. Lambert. 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. *N. Engl. J. Med.* 320:1586–1591.
- Kwiatkowski, D., A.V. Hill, I. Sambou, P. Twumasi, J. Cast-racane, K.R. Manogue, A. Cerami, D.R. Brewster, and B.M. Greenwood. 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet*. 336:1201–1204.
- de Kossodo, S., and G.E. Grau. 1993. Role of cytokines and adhesion molecules in malaria immunopathology. *Stem Cells*. 11:41–48.
- Medana, I.M., N.H. Hunt, and G. Chaudhri. 1997. Tumor necrosis factor- $\alpha$  expression in the brain during fatal murine cerebral malaria: evidence for production by microglia and astrocytes. *Am. J. Pathol.* 150:1473–1486.
- Grau, G.E., L.F. Fajardo, P.F. Piguet, B. Allet, P.H. Lambert, and P. Vassalli. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science*. 237:1210–1212.
- Garcia, I., Y. Miyazaki, K. Araki, M. Araki, R. Lucas, G.E. Grau, G. Milon, Y. Belkaid, C. Montixi, W. Lesslauer, et al. 1995. Transgenic mice expressing high levels of soluble TNF-R1 fusion protein are protected from lethal septic shock and cerebral malaria, and are highly sensitive to *Listeria monocytogenes* and *Leishmania major* infections. *Eur. J. Immunol.* 25:2401–2407.
- Lucas, R., J.N. Lou, P. Juillard, M. Moore, H. Bluethmann, and G.E. Grau. 1997. Respective role of TNF receptors in the development of experimental cerebral malaria. *J. Neuroimmunol.* 72:143–148.
- Amani, V., A.M. Vigario, E. Belnoue, M. Marussig, L. Fonseca, D. Mazier, and L. Renia. 2000. Involvement of IFN- $\gamma$  receptor-mediated signaling in pathology and anti-malarial immunity induced by *Plasmodium berghei* infection. *Eur. J. Immunol.* 30:1646–1655.
- Kwiatkowski, D., M.E. Molyneux, S. Stephens, N. Curtis, N. Klein, P. Pointaire, M. Smit, R. Allan, D.R. Brewster, G.E. Grau, et al. 1993. Anti-TNF therapy inhibits fever in cerebral malaria. *Q. J. Med.* 86:91–98.
- van Hensbroek, M.B., A. Palmer, E. Onyiorah, G. Schneider, S. Jaffar, G. Dolan, H. Memming, J. Frenkel, G. Enwere, S. Bennett, et al. 1996. The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. *J. Infect. Dis.* 174:1091–1097.
- Riminton, S.D., H. Korner, D.H. Strickland, F.A. Lemckert, J.D. Pollard, and J.D. Sedgwick. 1998. Challenging cytokine redundancy: inflammatory cell movement and clinical course of experimental autoimmune encephalomyelitis are normal in lymphotoxin-deficient, but not tumor necrosis factor-deficient, mice. *J. Exp. Med.* 187:1517–1528.
- Roach, D.R., H. Briscoe, B. Saunders, M.P. France, S. Riminton, and W.J. Britton. 2001. Secreted lymphotoxin- $\alpha$  is essential for the control of an intracellular bacterial infection. *J. Exp. Med.* 193:239–246.
- Bazzoni, F., and B. Beutler. 1996. The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med.* 334:1717–1725.
- Sheehan, K.C., N.H. Ruddle, and R.D. Schreiber. 1989. Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. *J. Immunol.* 142:3884–3893.
- Korner, H., M. Cook, D.S. Riminton, F.A. Lemckert, R.M. Hoek, B. Ledermann, F. Kontgen, B. Fazekas de St Groth, and J.D. Sedgwick. 1997. Distinct roles for lymphotoxin- $\alpha$  and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue. *Eur. J. Immunol.* 27:2600–2609.
- De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariathasan, S.C. Smith, R. Carlson, L.P. Shornick, J. Strauss-Schoenberger, et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science*. 264:703–707.
- Hearn, J., N. Rayment, D.N. Landon, D.R. Katz, and J.B. de Souza. 2000. Immunopathology of cerebral malaria: morphological evidence of parasite sequestration in murine brain microvasculature. *Infect. Immun.* 68:5364–5376.
- Engwerda, C.R., S.C. Smelt, and P.M. Kaye. 1996. An in vivo analysis of cytokine production during *Leishmania donovani* infection in scid mice. *Exp. Parasitol.* 84:195–202.
- Banks, T.A., B.T. Rouse, M.K. Kerley, P.J. Blair, V.L. Godfrey, N.A. Kuklin, D.M. Bouley, J. Thomas, S. Kanangat, and M.L. Mucenski. 1995. Lymphotoxin- $\alpha$ -deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685–1693.
- Kaye, P.M., and G.J. Bancroft. 1992. *Leishmania donovani* infection in scid mice: lack of tissue response and in vivo macrophage activation correlates with failure to trigger natural killer cell-derived gamma interferon production in vitro. *Infect. Immun.* 60:4335–4342.
- Amante, F.H., and M.F. Good. 1997. Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. *Parasite Immunol.* 19:111–126.
- Rosen, H. 1990. Role of CR3 in induced myelomonocytic recruitment: insights from in vivo monoclonal antibody studies in the mouse. *J. Leukoc. Biol.* 48:465–469.
- Rudin, W., H.P. Eugster, G. Bordmann, J. Bonato, M. Muller, M. Yamage, and B. Ryffel. 1997. Resistance to cerebral malaria in tumor necrosis factor- $\alpha$ /beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response. *Am. J. Pathol.* 150:257–266.
- Grau, G.E., P. Pointaire, P.F. Piguet, C. Vesin, H. Rosen, I. Stamenkovic, F. Takei, and P. Vassalli. 1991. Late administration of monoclonal antibody to leukocyte function-antigen 1 abrogates incipient murine cerebral malaria. *Eur. J. Immunol.* 21:2265–2267.
- Lucas, R., P. Juillard, E. Decoster, M. Redard, D. Burger, Y. Donati, C. Giroud, C. Monso-Hinard, T. De Kesel, W.A. Buurman, et al. 1997. Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. *Eur. J. Immunol.* 27:1719–1725.

27. Sedgwick, J.D., D.S. Riminton, J.G. Cyster, and H. Korner. 2000. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol. Today*. 21:110–113.
28. Gao, Z., V.C. McAlister, and G.M. Williams. 2001. Repopulation of liver endothelium by bone-marrow-derived cells. *Lancet*. 357:932–933.
29. Pober, J.S., and R.S. Cotran. 1990. Cytokines and endothelial cell biology. *Physiol. Rev.* 70:427–451.
30. Schofield, L., S. Novakovic, P. Gerold, R.T. Schwarz, M.J. McConville, and S.D. Tachado. 1996. Glycosylphosphatidylinositol toxin of *Plasmodium* up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. *J. Immunol.* 156:1886–1896.