Brief Definitive Report

Locally Up-regulated Lymphotoxin α, Not Systemic Tumor Necrosis Factor α, Is the Principle Mediator of Murine Cerebral Malaria

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Abstract

Cerebral malaria (CM) causes death in children and nonimmune adults. TNF-α has been thought to play a key role in the development of CM. In contrast, the role of the related cytokine lymphotoxin α (LTα) in CM has been overlooked. Here we show that LTα, not TNFα, is the principal mediator of murine CM. Mice deficient in TNFα (B6.TNFα−/−) 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.TNFα−/− 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 LTα (B6.LTα−/−) 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.TNFα−/−, and B6.LTα−/− 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 LTα mRNA after irradiation and engraftment of LTα-deficient BM, indicating that LTα 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 TNFα 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 TNFα production in the brain during murine CM caused by Plasmodium berghei ANKA (4,5). In this experimental model of CM, anti-TNFα 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 TNFα-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). TNFα is thought to mediate pathology by either direct cytolytic effects or induction of an inflammatory cascade involving mediators such as IFN-γ and nitric oxide (NO; reference 9). However, the critical role of TNFα in human CM has recently been questioned by the failure of TNFα-neutralizing reagents to decrease the incidence of clinical CM (10,11).

Recently, lymphotoxin α (LTα), a related member of the TNF family, has been reported to have important, yet
distinct, roles in various infectious and autoimmune disease models (12, 13). LTα forms a soluble homotrimer that along with TNFα is capable of binding both the TNFR1 and TNFR2. In addition, LTα can form a cell-bound heterotrimer with two LTβ molecules, which binds the LTβ receptor (14). Given the shared receptor usage of TNFα and LTα (14), and the potential cross-reactivity of anti-TNFα antibodies with LTα (15), it is possible that LTα, and not TNFα, is the principal mediator of CM. To test this hypothesis, C57BL/6 mice deficient in TNFα or LTα were infected with *P. berghei* ANKA pRBCs and their susceptibility to CM was compared. Our results clearly show that LTα, and not TNFα, 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 TNFα (B6.TNFα+/−) and both TNFα and LTα (B6.TNFα/LTα+/−; reference 16) were obtained from Bantin & Kingman, while those deficient in LTα (B6.LTα−/−; reference 17) were obtained from The Jackson Laboratory. In some experiments, B6.LTα−/− mice generated from C57BL/6 mice embryonic stem cells (12) were purchased from Bantin & Kingman and used to confirm the resistance of LTα-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 107 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 107 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 Specialties). RBCs were counted using a hemocytometer by diluting 2 μl 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 levels increased in mice with CM. Therefore, we sought to investigate further if this cytokine played a role in murine CM.

**Statistical Analysis.** Statistical analysis was performed for IFN-γ and nitrate levels using Student’s *t* test and for survival curves using Kaplan-Meier plots and Log Rank tests.

**Results and Discussion**

**LTα-deficient Mice Do Not Die from CM.** To determine if LTα is involved in CM, we first tested for LTα production using LTα-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). LTα 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 TNFα and LTα in CM, we infected mice deficient in these cytokines (B6.TNFα+/− and B6.LTα−/− mice, respectively) with *P. berghei* (ANKA) and monitored animals for signs of CM. Strikingly, B6.TNFα−/− mice began to show neurological symptoms

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 LTα were AGGGGCCCAAGGACTCTCT (sense) and ACGATCGTGCGTTGCTCTC (antisense), and for amplification of the house keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) were GTTGAGTACAGCCGACATTCTGT (sense) and GATTCACCTTGGCGTCTCATCTTCAGGC (antisense). The number of LTα 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 manufacturer’s instructions. Standard curves were constructed with known amounts of LTα and HPRT cDNA, and the number of LTα molecules per 1,000 HPRT molecules in each sample was calculated.

**Measurement of Serum IFN-γ and Nitrate Levels.** Serum samples were diluted 1:2 and IFN-γ 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 μm 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 Axiosplan 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 ×40 objective (final magnification ×400).

**Statistics.** Statistical analysis was performed for IFN-γ and nitrate levels using Student’s *t* test and for survival curves using Kaplan-Meier plots and Log Rank tests.
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.TNFα−/− mice had to be killed (Fig. 1 b). In stark contrast, B6.LTα−/− mice and those deficient in both TNFα and LTα (B6.TNF/LTα−/− mice) failed to show any neurological symptoms associated with CM. However, hemoglobin levels in B6.LTα−/− 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 ~10^{10}/ml to 1–3 × 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.TNFα−/− mice died, parasitemia ranged from 12–24% pRBCs and was similar for all mouse strains (Fig. 1 c). B6.TNF/LTα−/− mice have previously been shown to be resistant to CM, but this resistance was attributed to the absence of TNFα (24). These results clearly show that LTα, and not TNFα, is the primary mediator of murine CM, and that the development of CM is independent of blood parasitemia.

Both nitric oxide (NO) and IFN-γ have been reported to play important roles in the development of murine CM (9, 24). Serum nitrate (an indirect measure of NO) and IFN-γ levels were reduced in mice deficient in either TNFα or LTα, 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-γ and nitrate levels in the serum of B6.LTα−/− and B6.TNF/LTα−/− 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-γ levels in P. berghei-infected B6.TNF/LTα−/− mice (24). These results suggest that both TNFα and LTα play a role in the production of NO and IFN-γ after infection with P. berghei. However, the development of CM in B6.TNFα−/− mice demonstrates that elevated serum NO and IFN-γ levels do not correlate with the development of CM.

Changes to the Cerebral Vasculature of TNF-α− and LTα-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.TNFα−/− 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 LTα 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.TNFα−/− mice. These data show that CM is independent of blood parasitemia.
Increased ICAM-1 expression is a feature of murine CM. Sections (3 μM) were prepared from formol saline-fixed brain tissue and stained with hematoxylin and eosin. Magnifications of x100 (a and b) and x400 (c and d) are shown for C57BL/6 and B6.TNFα−/− 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 LTα-deficient mice (e and f; original magnification: x100). 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.LTα−/− and B6.TNF/LTα−/− 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.TNFα−/− mice than in C57BL/6 mice (Fig. 3 b; 438 ± 22 ICAM-1+ vessels per 25 fields of view versus 894 ± 16 ICAM-1+ vessels per 25 fields of view, respectively), but increased when compared with expression in naive controls (Fig. 3 b; 245 ± 9 ICAM-1+ vessels per 25 fields of view). Expression of ICAM-1 was also reduced in B6.LTα−/− and B6.TNFα/LTα−/− mice at day 7 after infection (Fig. 3 b; 438 ± 23 ICAM-1+ vessels per 25 fields of view and 476 ± 23 ICAM-1+ vessels per 25 fields of view, respectively). Associated with the decreased ICAM-1 expression, in TNFα and LTα-deficient mice, there was also a decrease in the number of CR3-positive inflammatory cells found in the brain (Fig. 3 c; 100 ± 3, 427 ± 23, 160 ± 6, 165 ± 5, and 151 ± 7 CR3+ cells per 25 fields of view for naive C57BL/6 mice, and C57BL/6, B6.TNFα−/−, B6.LTα−/−, and B6.TNFα/LTα−/− mice at day 7 after infection, respectively). Together, these results indicate that TNFα and LTα 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.

**LTA Is Produced by a Radiation-resistant Cell Population.**

To assess whether leukocytes accumulating in the brain were the source of LTα 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 TNFα, LTα, 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 mice and B6.TNFα−/− mice died at the same time as C57BL/6 mice (day 7 after infection). Brain cryosections (6 μM) were stained with (a) anti-ICAM-1 antibodies, (b) anti-CR3 (5C6) mAbs with fluorescent secondary reagents. Original magnification is x400. The samples are representative of material from four experiments.

**Figure 2.** Perivascular hemorrhages are present in C57BL/6 and B6.TNFα−/− mice that develop CM. Sections (3 μM) were prepared from formol saline-fixed brain tissue and stained with hematoxylin and eosin. Magnifications of x100 (a and b) and x400 (c and d) are shown for C57BL/6 and B6.TNFα−/− 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 LTα-deficient mice (e and f; original magnification: x100). 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).

**Figure 3.** Comparative immunohistochemical analysis of brain sections taken from *P. berghei*-infected mice when C57BL/6 and B6.TNFα−/− mice developed CM (day 7 after infection). Brain cryosections (6 μM) 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 x400. The samples are representative of material from four experiments.
LTα 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.TNFα−/− (overlapping C57BL/6 mice and indicated by arrow), B6.LTα−/− (dashed line), and B6.TNF/LTα−/− (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.TNFα−/− mice and mice deficient in LTα were statistically different (χ² = 7.01, P < 0.01), as was the difference between mice engrafted with BM from B6.LTα−/− and B6.TNF/LTα−/− mice (χ² = 23.83, P < 0.0001). (b) LTα mRNA is detected by real-time RT-PCR in brain tissue from B6.LTα−/− mice (data not shown). Furthermore, when chimeric mice were prepared by irradiating B6.TNF/LTα−/− mice and engrafting with BM from C57BL/6, B6.TNFα−/−, or B6.LTα−/− 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/LTα−/− mice engrafted with BM from B6.TNFα−/− mice indicates a role for TNFα in the death of P. berghei–infected mice suffering from anemia and hyperparasitemia. Although the LTα-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 LTα in the brain. Similarly, we cannot yet rule out any involvement of immune defects associated with TNFα- and LTα-deficient mice in their different susceptibility to CM. Nevertheless, these data show that LTα produced by a radiation-resistant cell is the key factor for the development of murine CM.

In summary, we have shown that LTα 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 LTα, as all are radiation-resistant (27, 28) and capable of producing inflammatory mediators (27, 29). Given the constitutive expression of LTα mRNA in the brain, it is likely that LTα 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 LTα-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 LTα in human CM is unknown, but results from this work indicate that it should be investigated.

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Lymphotoxin-α Mediates Murine Cerebral Malaria

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