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Pathology of *Tnf*-deficient mice infected with *Plasmodium chabaudi adami* 408XZ

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Abstract

Tumor necrosis factor alpha (Tnf) plays a pleiotropic role in murine malaria. Some investigations have correlated Tnf with hypothermia, hyperlactatemia, hypoglycemia, and a suppression of the erythropoietic response, although others have not. In this study, we have evaluated parasitemia, survival rate and several pathological features in C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice after infection with *Plasmodium chabaudi adami* 408XZ. Compared to the C57BL/6J*Tnf*$^{+/+}$ mice, C57BL/6J*Tnf*$^{-/-}$ mice showed increased parasitemia and decreased survival rate, whereas blood glucose, blood lactate and body weight were not significantly different. However, C57BL/6J*Tnf*$^{-/-}$ mice suffered significantly more from severe anemia and hypothermia than C57BL/6J*Tnf*$^{+/+}$ mice. These results suggest that Tnf is an important mediator of parasite control, but not of anemia development. We hypothesize that the high mortality observed in the *Tnf* knock-out mice is due to increased anemia and pathology as a direct result of increased levels of parasitemia.

Index Descriptors and Abbreviations: Murine malaria; Tnf; C57BL/6J; *Plasmodium chabaudi adami* 408XZ; Parasitemia; Survival rate; Anemia; Hypothermia; RBC, red blood cells; pi, postinfection; Hb, hemoglobin; ANOVA, analysis of variance; SEM, standard error of the mean

1. Introduction

The immunological component of human resistance to infection with *Plasmodium falciparum* is complex and needs to be better understood, if successful strategies for control of malaria are to be realised. In the last years, many studies have been performed to investigate the role of TNF in pathology and resistance during the malarial infection. Several polymorphisms at the human *TNF* locus have been reported to be associated with cerebral malaria, severe malarial anemia, and mild malaria (McGuire et al., 1994 and McGuire et al., 1999; Knight et al., 1999; Flori et al., 2003 and Flori et al., 2005). High production of TNF is associated with human cerebral malaria and a fatal outcome (Kwiatkowski et al., 1990), although some studies have not supported such an association and have described it as a possible indicator for cure and a good prognosis (Shaffer et al., 1991; Mordmuller et al., 1997). High in vivo concentrations of TNF are correlated with hyperparasitemia, hyperlactatemia, hypoglycemia, fever, anemia, renal

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impairment, and pulmonary oedema in human malaria caused by *P. falciparum* (Shaffer et al., 1991; Krishna et al., 1994; Odeh, 2001).

Membrane-bound Tnf, Tnf receptor superfamily member 1B (Tnfr2) and lymphotoxin alpha (Lta) are involved in the development of murine cerebral malaria (Lucas et al., 1997; Engwerda et al., 2002). Treatment of susceptible A/J mice infected with *Plasmodium chabaudi chabaudi* AS with human recombinant TNF resulted in increased survival rates and alleviated the course of infection. A similar treatment of resistant C57BL/6J mice did not result in a significant change in the parasitemia levels, although the percentage of mice that succumbed to the infection increased notably (Stevenson et al., 1990). Treatment of C57BL/6J mice infected with *P. chabaudi chabaudi* AS with anti-Tnf antibodies resulted in significantly increased mortality but did not alter the peak parasitemia level (Jacobs et al., 1996). Several studies have correlated Tnf levels with hypoglycemia, hyperlactatemia, and hypothermia in uninfected mice and with hypothermia and suppression of the erythropoietic responses in infected mice with *P. chabaudi chabaudi* AS and *Plasmodium berghei* (Bauss et al., 1987; Cross and Langhorne, 1998; Miller et al., 1989). In contrast, other studies showed that Tnf was not associated with hypoglycemia and did not inhibit erythropoiesis in vitro during infections with *Plasmodium yoelii* and *P. chabaudi chabaudi* AS (Taylor et al., 1992; Yap and Stevenson, 1994).

Recently, we have suggested *Tnf* as a candidate gene within *char3* locus to control parasitemia levels during a *P. chabaudi chabaudi* 54X infection (Hernandez-Valladares et al., 2004a). In a later study, we have compared parasitemia levels, survival rates and pathology between a number of *P. chabaudi* sub-strains in susceptible A/J and resistant C57BL/6J mice, and found that *P. chabaudi adami* 408XZ induced the most severe pathology (Hernandez-Valladares et al., 2004b). To identify the role played by Tnf in parasitemia control and mortality, we have investigated the course of the infection with the virulent *P. chabaudi adami* 408XZ between wild-type and *Tnf*-deficient C57BL/6J mice. We have compared additional parameters of pathology such as anemia, lactate and glucose levels, hypothermia and loss of body weight between the two mouse strains to find out which factors might contribute to the increased parasitemia and mortality observed in infected C57BL/6J$Tnf^{-/-}$ mice.

2. Materials and methods

2.1. Experimental animals and murine parasite

Tnf-deficient mice were generated by gene targeting (Taniguchi et al., 1997). The *Tnf* gene was disrupted by homologous recombination in F_1 CBA × C57BL/6 embryonic stem cells, with allele disruption occurring on the C57BL/6 haplotype (originally derived from Charles River Laboratory stock, Wilmington, MA, USA). *Tnf* gene knock-out embryonic stem cells were transferred into C57BL/6J blastocysts, and the resulting chimeric animals were mated to C57BL/6J mice. The mutant animals were backcrossed with C57BL/6J mice for five more generations (Iraqi et al., 2001). C57BL/6J$Tnf^{-/-}$ and C57BL/6J$Tnf^{+/+}$ mice were obtained from crosses of C57BL/6J$Tnf^{+/-}$ mice. After confirmation of genotype by PCR amplification (Iraqi et al., 2001), C57BL/6J$Tnf^{-/-}$ and C57BL/6J$Tnf^{+/+}$ mice were selected for experimentation and housed in single-sex cages of five animals of the same strain until parasitic infection at 12–16 weeks of age. *P. chabaudi adami* 408XZ was originally obtained from Dr. S.J. Foote, The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia. The parasites were propagated from frozen stocks in male A/J mice, which were obtained from Harlan, UK, Ltd. (Shaws Farm, Blackthorn, Bicester, Oxon, OX6 OTP, UK). Fifteen males and fifteen females of each genotype were infected intraperitoneally with 10^5 *P. chabaudi adami* 408XZ parasitized RBC. Three mice from each sex and genotype served as uninfected control animals. A few mice failed to develop parasitemia and were removed from the experiment.

All experiments were performed in compliance with the laws and institutional guidelines.

2.2. Parasitemia

The percentage of parasitemia (parasitized RBC per 100 RBC) was determined by Giemsa-stained thick-film examination of a blood sample from the tail tip taken two or three times weekly.

2.3. Parameters of malaria-associated pathology

Total Hb concentrations were measured spectroscopically at 540 nm (Horecker, 1943). Samples of 2 μl of blood were collected from the tail and diluted in 150 μl of distilled water in a Costar 3799 plate (Corning Incorporation, Corning NY, USA). After 30 min at room temperature, the plate was centrifuged at 600*g* for 10 min. One hundred microliters of supernatant was transferred to a new plate and the optical density was measured at 540 nm in a Multiscan MCC/340 ELISA plate reader (Titertek Instruments, Huntsville, AL, USA). Measurements were carried out in triplicate. Total Hb concentrations were obtained from a regression equation whose gradient and intercept were calculated from known total Hb concentrations of a wild-type C57BL/6J mouse. Blood glucose was measured by spreading 10 μl of tail blood onto SureStep test strips (Lifescan, Johnson & Johnson, Milpitas, California, USA). The strips were analyzed using a One Touch glucometer according to the manufacturer's instructions (Lifescan, Johnson & Johnson, Milpitas, California, USA). Blood lactate was measured by spreading 15 μl of tail blood onto BM-Lactate test strips (Roche Diagnostics GmbH, Mannheim, Germany). The strips were analyzed using an Accutrend Lactate meter according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Body weight was measured in grams and it was presented as a percentage of

the initial body weight before the infection (day 0). Body temperature was measured using a rectal probe with a TES 1319 digital thermometer (TES Electrical Electronic Corp., Taipei, Taiwan).

All mice were scored daily for fitness, according to the Institute's animal care and use manual, on a scale of 1–4, with 1 appearing normal, 2 having abnormal posture and hairs standing on end, 3 showing change of respiration, and 4 being too weak to move after prodding. To prevent unnecessary suffering, animals with the highest score were sacrificed by CO_2 asphyxiation.

Each parameter was measured two or three times weekly in infected and control animals. The data were presented as the arithmetic mean of each mouse strain ± SEM.

2.4. Statistical analysis

Parasitemia counts and the parameters of pathology were analyzed by ANOVA for each time point using GENSTAT 6.1 (Payne et al., 2002). The analysis included fixed effects for strain and sex. The strain-sex interaction was not significant. Therefore, the results below represent significant differences between the pathological parameters from the two mouse strains. Age was included as a linear covariate. *P* values less than or equal to 0.05 were considered significant. Survival curves were obtained by Kaplan–Meier estimates of the survivor functions for the mouse strains. The survival rates of the different mouse strains were then compared using the Cox proportional hazards model.

3. Results

3.1. Course of infection

A total of 27 C57BL/6J*Tnf*$^{-/-}$ and 21 C57BL/6J*Tnf*$^{+/+}$ infected animals were analyzed. Severe clinical disease commenced on day 10 pi and finished on day 15 pi for most animals. The highest percentage of mice with a weak fitness (scored 3 or 4) was observed on day 12 pi in both groups of animals, at decreasing parasitic levels (Fig. 1a). The percentage of mice with weak fitness between day 10 pi and day 15 pi was higher in the *Tnf*-deficient group than in the wild-type group (data not shown). After day 16 pi, few C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice scored 3 or 4 and all surviving animals looked healthy after day 21 pi and until the end of the experiment. Non-surviving animals succumbed to the infection between days 10 and 21 pi. C57BL/6J*Tnf*$^{-/-}$ mice had higher parasitemia than C57BL/6J*Tnf*$^{+/+}$ mice at most of the time points (Fig. 1a). The percentage of parasitemia at the first peak of infection for C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice was 34 and 22%, respectively. The parasitemia levels between the two mouse strains were significantly different at day 7 ($P < 0.001$), at day 10 ($P < 0.05$) and at day 22 ($P < 0.05$) pi. The survival curves for C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice were significantly different ($P < 0.01$, Fig. 1b). Thirty percent of C57BL/6J*Tnf*$^{-/-}$ mice and seventy-one percent of C57BL/6J*Tnf*$^{+/+}$ mice survived the infection. The hazard ratio was 0.31, meaning that the risk of death for C57BL/6J*Tnf*$^{+/+}$ mice was lower than the risk for C57BL/6J*Tnf*$^{-/-}$ mice by 69%.

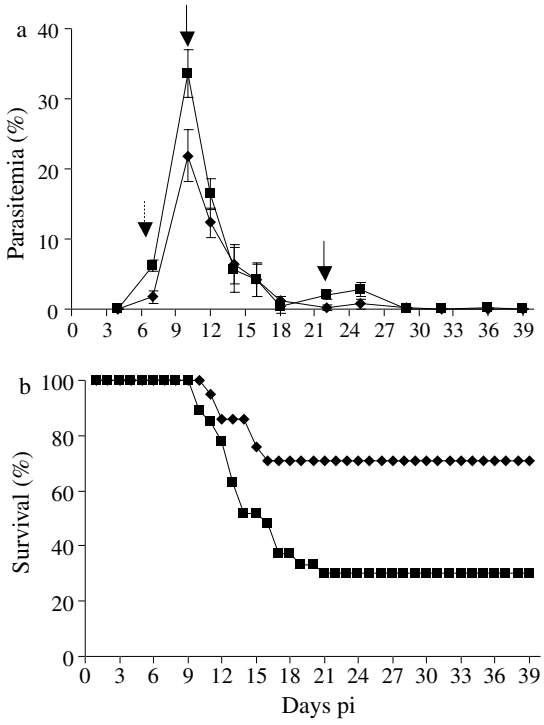

Fig. 1. Parasitemia (a) and survival curves (b) in C57BL/6J*Tnf*$^{-/-}$ (■) and C57BL/6J*Tnf*$^{+/+}$ (◆) mice over the course of a *P. chabaudi adami* 408XZ infection. The percentage of parasitemia is presented as the arithmetic mean of each mouse strain ± SEM. Differences in parasitemia levels between C57BL/6JT*nf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice with a *P* value below 0.05 are indicated with a solid arrow and with a value below 0.001 with a dashed arrow. The survival curves for the two mouse strains were significantly different ($P < 0.01$).

3.2. Hb profiles

Before the infection, Hb concentrations in C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice were 14.5 and 15.2 g/dL, respectively. Both values were similar to the Hb concentrations recently reported for wild-type C57BL/6J mice, 14.8 g/dL for male and 15.4 g/dL for female animals (Kile et al., 2003). Uninfected control C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice had Hb concentrations of 11.8 ± 0.4 and 12.3 ± 0.4 g/dL, respectively, throughout the experiment. Infected C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ animals became anemic after two weeks and they fully recovered their original Hb levels after 32 days pi (Fig. 2). C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice reached minimum Hb concentrations on day 12 pi (4.1 g/dL) and on day 14 pi (5.0 g/dL), respectively, the time points after the first peak of the infection. C57BL/6J*Tnf*$^{-/-}$ mice showed a more exacerbated anemia than C57BL/6J*Tnf*$^{+/+}$ mice. Hb concentrations were lower in C57BL/6J*Tnf*$^{-/-}$ mice than in C57BL/6J*Tnf*$^{+/+}$ mice at most of the time points. However, Hb levels between the two mouse strains were only significantly

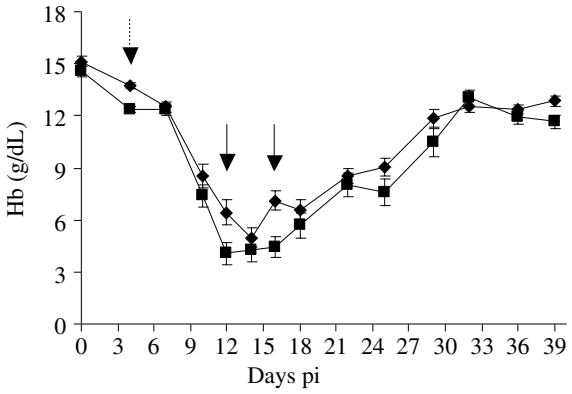

Fig. 2. Hb changes during *P. chabaudi adami* 408XZ infection in C57BL/6J*Tnf*$^{-/-}$ (■) and C57BL/6J*Tnf*$^{+/+}$ (◆) mice. Hb levels are presented as the arithmetic mean of each mouse strain ± SEM. Hb differences between the two mouse strains are indicated with a solid arrow when *P* is below 0.05 and with a dashed arrow when *P* is below 0.001.

different on day 4 ($P < 0.001$), day 12 ($P < 0.05$), and day 16 pi ($P < 0.05$).

3.3. Blood glucose and lactate profiles

The blood sugar concentrations of C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice were 5.7 and 5.9 mM (or 102.6 and 106.2 mg/dL), respectively, before the infection. Blood sugar levels in uninfected control C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ animals were 6.8 ± 0.3 and 6.6 ± 0.2 mM (or 122.4 ± 5.4 and 118.8 ± 3.6 mg/dL), respectively, throughout the experiment. These data were more similar to the blood glucose levels observed in resistant male B10.A mice, 120 mg/dL (Cross and Langhorne, 1998), and in male wild-type C57BL/6J mice, 126 mg/dL (Hernandez-Valladares et al., 2004b), than those reported in the Mouse Phenome Database for C57BL/6J mice, 234 and 224 mg/dL for male and female animals, respectively, after a 4 h fasting period (Naggert J.K., Paigen B., Svenson K.L. and Peters L.L. Diet effects on body composition and plasma glucose, leptin, and insulin levels; MPD: 143. The Jackson Laboratory, Bar Harbor, Maine, http://www.jax.org/phenome). After infection, C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice had similar blood glucose curves throughout the experiment. The blood glucose levels increased on days 12 and 14 pi (Fig. 3a). However, these hyperglycemic levels observed after the highest parasitemias were not significantly different between mice from both strains. C57BL/6J*Tnf*$^{-/-}$ mice had significantly lower blood glucose levels than C57BL/6J*Tnf*$^{+/+}$ mice on day 7 pi ($P < 0.01$).

Blood lactate concentrations from C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice were 5.5 and 5.2 mM, respectively, before the infection. Uninfected control C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice had blood lactate concentrations of 5.5 ± 0.2 and 5.2 ± 0.3 mM, respectively, throughout the experiment. These values were substantially higher than the serum lactate concentrations measured in female C57BL/6J mice (0.8–1.6 mM) in a previous experiment (Chang et al., 2001). After infection, blood lactate concentrations were similar in C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice throughout the experiment (Fig. 3b). Both mouse strains showed an increase of blood lactate levels between day 10 and 16 pi, the period between the first peak of parasitemia and the late stages of parasite clearance. Blood lactate concentrations remained in the pre-infection range after day 18 pi until the end of the experiment. Lactate concentrations from C57BL/6J*Tnf*$^{-/-}$ mice were significantly lower than those from C57BL/6J*Tnf*$^{+/+}$ mice on day 7 pi ($P < 0.001$).

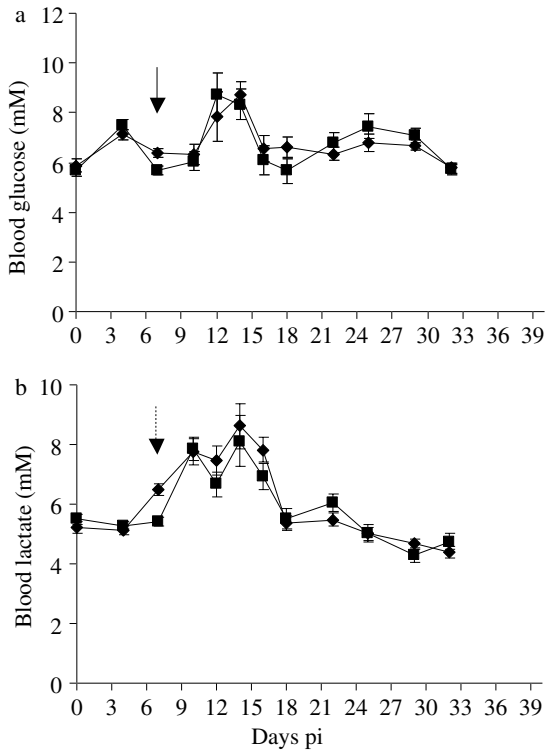

Fig. 3. Blood glucose (a) and lactate (b) concentrations in C57BL/6J*Tnf*$^{-/-}$ (■) and C57BL/6J*Tnf*$^{+/+}$ (◆) mice during *P. chabaudi adami* 408XZ infection. Glucose and lactate concentrations are presented as the arithmetic mean of each mouse strain ± SEM. Differences with a *P* value below 0.01 are indicated with a solid arrow and with a value below 0.001 with a dashed arrow.

3.4. Body temperature and weight changes

Before the infection, the body temperature of C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice was 34.7 and 35.1 °C, respectively. Body temperature of uninfected control C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ animals was 34.3 ± 0.3 and 34.6 ± 0.2 °C, respectively, throughout the experiment. Body temperatures of C57BL/6J*Tnf*$^{+/+}$ mice were similar to the temperatures measured previously in resistant male B10.A and B10.D2 mice, 36.5 °C (Cross and Langhorne, 1998) and in male wild-type C57BL/6J mice, 35.6 °C (Hernandez-Valladares et al., 2004b). Body temperatures of infected C57BL/6J*Tnf*$^{+/+}$ mice reached a minimum value of 31 °C on day 14 pi (Fig. 4a), when the parasitemia has decreased to a value of 5%. Temperatures recovered in C57BL/6J*Tnf*$^{+/+}$ mice from

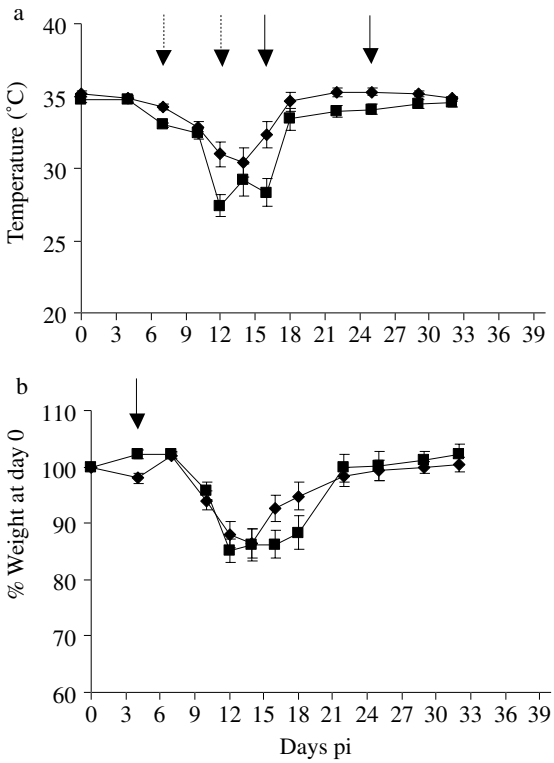

Fig. 4. Changes in body temperature (a) and weight (b) in C57BL/6J*Tnf*$^{-/-}$ (■) and C57BL/6J*Tnf*$^{+/+}$ (◆) mice during a *P. chabaudi adami* 408XZ infection. Weight data for each mouse strain are expressed as percentage of the corresponding body weights before infection. Data are presented as the arithmetic mean of each mouse strain ± SEM. Differences with a *P* value below 0.01 are indicated with a solid arrow and with a value below 0.001 with a dashed arrow.

day 16 pi onward. C57BL/6J*Tnf*$^{-/-}$ animals showed lower body temperatures than C57BL/6J*Tnf*$^{+/+}$ mice at most of the time points, having minimum body temperatures of 27 °C and 28 °C on day 12 and day 16 pi, respectively. Body temperatures between the two mouse strains were significantly different on day 7 ($P < 0.001$), day 12 ($P < 0.001$), day 16 ($P < 0.01$), and day 25 pi ($P < 0.01$).

Body weight of C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice was 23.8 and 25.4 g, respectively, before the infection. That of uninfected control C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice ranged between 100–103% and 98–101% of the body weight at day 0, respectively, throughout the experiment. Both infected mouse strains showed similar body weight profiles after infection, although C57BL/6J*Tnf*$^{-/-}$ mice showed a slower recovery of the initial body weights when compared to C57BL/6J*Tnf*$^{+/+}$ mice (Fig. 4b). Body weight of C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice decreased to 85 and 87% of their pre-infection values on day 12 and day 14 pi, respectively, as previously observed in male wild-type C57BL/6J infected with *P. chabaudi adami* 408XZ (Hernandez-Valladares et al., 2004b). Body weights from C57BL/6J*Tnf*$^{-/-}$ mice were significantly higher than those from C57BL/6J*Tnf*$^{+/+}$ mice on day 4 pi ($P < 0.01$). The mice from the two strains recovered their initial body weights by day 22 pi.

4. Discussion

Our observations demonstrate that endogenous Tnf has a protective role during *P. chabaudi adami* 408XZ malaria and enhances survival. This is in agreement with a previous study, which found correlation between Tnf levels in the spleen of C57BL/6J mice and resistance to *P. chabaudi chabaudi* AS malaria (Jacobs et al., 1996). The same study confirmed the causal relationship between Tnf and survival by treatment of resistant C57BL/6J mice with anti-Tnf antibodies that resulted in significantly increased mortality but did not alter the peak parasitaemia levels. Our data showed a protective role for Tnf on both parasitemia and survival. This discrepancy on the effect of Tnf on parasitemia control between the two experiments might be due to the use of different *P. chabaudi* substrains in both infections. Tnf-dependent control mechanisms might be less effective during the *P. chabaudi chabaudi* AS infection than during the *P. chabaudi adami* 408XZ infection. Because the *P. chabaudi adami* 408XZ substrain is more virulent and lethal that the *P. chabaudi chabaudi* AS substrain (Hernandez-Valladares et al., 2004b), it is likely that additional immune mechanisms will be needed to control growth of *P. chabaudi adami* 408XZ. Other responses, mediated by other cytokines such as interferon gamma, may be sufficient to curb milder forms of malaria, such as *P. chabaudi chabaudi* AS (Meding et al., 1990).

The precise mechanism by which Tnf mediates parasite control is not yet known. Tnf mediates different inflammatory responses such as the acute phase response, generation of reactive oxygen species, and even immune responses (Bouharoun-Tayoun et al., 1995). However, antibody responses in *Tnf*-deficient mice were not compromised during trypanosome infections (Naessens et al., 2004), suggesting that acquired responses in these mice are intact and are not the cause of genetic resistance. The fact that Tnf also plays an important role in resistance to the effects of *Trypanosoma congolense* infection in mice (Iraqi et al., 2001) raises the possibility the same Tnf-mediated responses may be effective against different parasites.

C57BL/6J*Tnf*$^{+/+}$ mice developed severe anemia after infection with *P. chabaudi adami* 408XZ, with minimum Hb levels after two weeks pi. A similar profile has been observed in our previous study comparing susceptible A/J with C57BL/6J mice in which anemia was measured in terms of RBC density (Hernandez-Valladares et al., 2004b). C57BL/6J*Tnf*$^{-/-}$ mice suffered from a more severe anemia having significantly lower Hb levels than C57BL/6J*Tnf*$^{+/+}$ mice on day 12 and day 16 pi. Mice from both strains that did not survive the infection were anemic before death. In our model, *Tnf*-deficiency does not prevent C57BL/6J mice from developing anemia, but rather enhances it at the most critical stages of the infection. This suggests that Tnf does not mediate the anemia associated with malaria caused by *P. chabaudi*, although other cytokines may still be involved. In agreement with our results, a previous study showed that Tnf was not involved in the inhibition of in vitro

erythropoiesis during *P. chabaudi chabaudi* AS malaria (Yap and Stevenson, 1994). In contrast, other studies with *Plasmodium vinckei* and *P. berghei* demonstrated that Tnf mediated malarial anemia in mice by inhibition of erythropoiesis and promotion of erythrophagocytosis (Miller et al., 1989; Clark and Chaudhri, 1988). Moreover, neutralization of Tnf in C57BL/6J*Il-10*$^{-/-}$ mice ameliorated anemia after infection with *P. chabaudi chabaudi* AS (Li et al., 2003). Further support for a role of Tnf in malarial anemia comes from human malaria studies, which showed that low IL-10 responses combined with high Tnf concentrations were associated with severe anemia (Kurtzhals et al., 1998; Othoro et al., 1999). All the above studies show that the involvement of Tnf in the development of anemia depends on the particular host–parasite model studied. Different malaria parasites will reach different densities during infection, resulting in a different antigenic load and additional responses, or they may have different deficiencies in activating macrophages and interacting with the immune system. On the host side, genetic differences will result in more or less efficient responses to the same parasite. From the results in this paper, it is clear that Tnf does not mediate anemia development in our model. The lack of Tnf in the knock-out mice rather resulted in enhanced anemia. This suggests that in our model, anemia is correlated with the level of parasitemia; Tnf only has a protective role and does not contribute to pathology. In studies of anemia associated with African trypanosomes in murine models, similar Tnf-dependent and independent mechanisms were observed in infections with different parasite strains (Naessens et al., 2005).

Blood glucose and lactate profiles during the *P. chabaudi adami* 408XZ infection were very similar for both C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice. Blood glucose concentrations were increased on day 12 and day 14 pi whereas blood lactate levels were increased from day 10 to day 16 pi, the time points between the maximum parasitemia and the late stages of parasitic clearance from the bloodstream. Blood glucose and lactate concentrations between the two mouse strains were only significantly different on day 7 pi and showed only minor differences. The blood glucose profile of C57BL/6J*Tnf*$^{+/+}$ mice did not show any hypoglycemic trend during the critical stages of the infection as that one observed in B10.A mice (Cross and Langhorne, 1998) but rather displayed a hyperglycemic trend, which was observed even more clearly in this experiment than in a previous one (Hernandez-Valladares et al., 2004b). Blood lactate concentrations of both mouse strains were increased at the time points when clinical symptoms were severe. A similar trend was observed in C57BL/6J mice infected with *P. berghei* ANKA showing neurological complications typical of murine cerebral malaria after 6 days pi (Chang et al., 2001).

There was no correlation between glucose level and imminence of death, and that in both mouse strains. As previously observed in wild-type C57BL/6J mice (Hernandez-Valladares et al., 2004b), blood glucose level is not an indicator of malaria fatal outcome in C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice. However, most, but not all, C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice showed increased levels of lactate before death when compared to the pre-infection values. Elevated serum lactate concentrations have been suggested as a predictor of mortality and morbidity (Husain et al., 2003). But since some C57BL/6J*Tnf*$^{-/-}$ and C57BL/6J*Tnf*$^{+/+}$ mice died with levels of blood lactate similar or lower than the levels measured before the infection, hyperlactatemia is not an indicator of death in the *P. chabaudi adami* 408XZ infection. In our model, Tnf does not mediate blood glucose and lactate levels during the plasmodial infection. A study of C57BL/6J*Il-10*$^{-/-}$ mice infected with *P. chabaudi chabaudi* AS and treated with anti-Tnf antibodies showed that the neutralization of Tnf did not affect glucose levels (Li et al., 2003) and confirmed our results. The past and current mouse data clearly indicate the need to address the differences in blood glucose profiles observed in mouse models infected with different *Plasmodium* substrains and to study the role of Tnf in controlling the glucose levels in these models. Studies of glucose levels in human malaria are also controversial: hypoglycemia is typically found in severe cases (Marsh et al., 1995), although other studies have conversely found that hyperglycemia is associated with cerebral malaria (Van Thien et al., 2001). TNF is associated with hyperlactatemia during *P. falciparum* infections (Krishna et al., 1994). Plasma lactate levels in uninfected mice were increased after treatment with recombinant human TNF (Bauss et al., 1987) and after a *P. berghei* infection (Chang et al., 2001). In contrast, hyperlactatemia was not controlled by Tnf in our mouse model, an additional result to support the strong dependence of the malaria pathologies on the particular host–parasite interactions.

C57BL/6J*Tnf*$^{+/+}$ mice suffered from hypothermia and loss of body weight after infection. The lowest body temperature and biggest loss of body weight occurred at day 14 pi, as observed in a previous *P. chabaudi adami* 408XZ infection in wild-type C57BL/6J mice (Hernandez-Valladares et al., 2004b). C57BL/6J*Tnf*$^{-/-}$ mice showed a more severe hypothermic profile than C57BL/6J*Tnf*$^{+/+}$ mice, having significantly lower body temperatures than C57BL/6J*Tnf*$^{+/+}$ mice on day 12 and day 16 pi. The body weight profile of C57BL/6J*Tnf*$^{-/-}$ did not significantly differ from the one of C57BL/6J*Tnf*$^{+/+}$ mice. Non-survivor mice from both mouse strains had low body temperature and body weight before death. These results indicated that Tnf does not play a crucial role in the development of malarial hypothermia and loss of body weight, but rather suggested that a lack of Tnf enhanced the two pathological features. So, these results did not support previous studies that suggested a role for Tnf in hypothermia and weight loss (Bauss et al., 1987; Li et al., 2003).

Pro-inflammatory cytokines such as Tnf, switch on an inflammatory process that helps to control parasite infections, but at the same time mediates pathology that can be harmful to the host. In our murine model, *Tnf*-deficient

mice had a higher parasitemia than wild-type mice, suggesting a protective role for the cytokine. There was no indication that Tnf was critical to any of the tested pathological features as they were present in the knock-out mice to the same extent or more than in the wild-type mice. However, the *Tnf*-deficient mice had higher mortalities, were more anemic and more hypothermic at the critical stages of the infection than the wild-type mice. This suggested that the increased mortality, anemia and hypothermia in the *Tnf*-deficient mice could be a consequence of the higher parasitemia. Furthermore, since anemia and hypothermia were present in all mice that succumbed to the disease, these two features may have directly contributed to mortality. The lack of a role for Tnf in pathology is not because the pathogen was of low virulence, as a previous comparative study has shown (Hernandez-Valladares et al., 2004b). Comparison of different host–parasite models should help in understanding the particular contributions of different responses and mediators to disease resistance.

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