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A ROLE FOR CD4+ AND CD8+ CELLS AND NOT FOR CD25+ CELLS IN THE CONTROL OF *PLASMODIUM BERGHEI* ANKA BLOOD STAGE PARASITES IN RATS

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Summary:

In previous studies of the infection of rats by *P. berghei* Anka, we have shown that primary blood stage infection induced the expansion of CD4+ T cells and CD8+ T cells in adult resistant rats while the number of CD4+CD25+ cells was found to be higher in young susceptible rats. In this work, the respective contribution of each cell population was determined in young and adult rats treated with monoclonal antibodies. Down-regulation of surface CD25 molecules, including those expressed by CD4+ cells did not significantly enhance the capacity of young rats to control the development of erythrocytic stages or modify the course of infection in adult infected rats. However, we observed a significant loss of protection when adult rats were treated with anti-CD4 mAb (W3/25) with higher blood parasitemia levels and ~ 50 % of rats succumbed to infection. More importantly and in contrast to earlier studies performed in mice, we found a significant increase in blood parasite levels and a significant delay in parasite clearance in adult rats treated with anti-CD8 mAb OX8, known to deplete CD8+ cells. These results suggest that CD8+ cells play a critical role in the development of immune responses in rats to control the replication of blood stage parasites.

KEY WORDS : CD4+ cells, CD8+ cells, CD25+ cells, *Plasmodium berghei*, rat.

Résumé : LE CONTRÔLE DES FORMES SANGUINES DE *PLASMODIUM BERGHEI* ANKA CHEZ LE RAT EST DÉPENDANT DES CELLULES CD4+ ET CD8+ MAIS INDÉPENDANT DES CELLULES CD25+

Nous avons précédemment montré que l'infection primaire de rats jeunes/adultes par Plasmodium berghei *Anka induisait une expansion des cellules T CD4+ et T CD8+ chez les rats adultes résistants alors que des taux de cellules CD4+CD25+ significativement plus élevés étaient retrouvés chez les rats jeunes susceptibles infectés. Dans ce travail, par des traitements avec des anticorps monoclonaux, nous avons déterminé la contribution de chacune de ces populations cellulaires chez les rats jeunes et adultes au cours de l'infection. Nous montrons ainsi que la diminution de l'expression du marqueur CD25, y compris à la surface des cellules CD4+, ne modifie en rien le déroulement et l'issue de l'infection, que ce soit chez les rats jeunes susceptibles ou chez les rats adultes résistants. À l'inverse, le traitement de rats adultes avec un anticorps monoclonal anti-CD4 (W3/25) induit une augmentation de la parasitémie et ~ 50 % des rats traités succombent à l'infection. De même, la déplétion des cellules CD8+ chez des rats adultes infectés induit une augmentation de la parasitémie. Ces résultats suggèrent que chez le rat, contrairement à ce qui a été montré chez la souris, les cellules CD8+ jouent un rôle critique dans le développement des réponses immunes impliquées dans le contrôle des stades érythrocytaires du* Plasmodium.

MOTS CLÉS : *cellules CD4+, cellules CD8+, cellules CD25+,* Plasmodium berghei, *rat.*

INTRODUCTION

In human malaria, although several studies have been focused on the relationship between immunity/pathology and immune responses, there is still a paucity of information concerning their functional role during infection. From experimental models, it appears that different combinations of inbred mouse and rodent *Plasmodium* species are necessary to investigate both the induced immune responses and related pathology. Findings from several rodent models have provided convergent evidence that T cells are central players in coordinating host immune responses in this infection. The mouse models in which the protective immune responses to blood-stage parasites have been mainly studied are infections by *Plasmodium chabaudi chabaudi* and *P. yoelii*. From these studies, it has been shown that CD4+ T cells were the main cells involved in the control of replication of blood-stage parasites followed by the involvement of B cells and humoral responses to clear blood parasites rapidly and totally (Langhorne *et al*., 1989; Langhorne *et al*., 1998).

Regarding the role of CD8+ T cells, most reports have demonstrated their essential role in the expression of protection against liver stages (Schofield *et al*., 1987; Weiss *et al*., 1988). Subsequently it has been shown that CD4+ T cells are also involved in protective immunity against liver stages of *Plasmodium* in helping B cells to induce anti-malarial antibodies, in assisting the induction of CD8+ T cell responses and in directly inhibiting the development of liver stage parasites (for review Tsuji & Zavala, 2003).

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In experimental cerebral malaria (ECM), the use of *P. berghei* Anka (PbA) clearly demonstrated the role of both CD8+ and CD4+ cells in the immunopathological reaction observed in mice (for review Renia *et al*., 2006). These observations indicate that the nature of action of CD4+ and CD8+ T cells could exhibit a dual activity, very likely depending not only on different *Plasmodium* and mouse strains combinations used but also on their kinetics of expression and their distribution (liver, spleen and brain).

Interestingly, in the rat model PbA infection does not induce cerebral malaria, however earliest experiments showed that age profoundly influenced the peak parasitemia and mortality (Singer *et al*., 1955; Zuckerman & Yoeli, 1954). A more detailed study taking into account the size of inoculum and the age of the rats revealed that young infected rats (95-100 %) succumbed to infection ~ 16 days p.i. from hyperparasitemia (> 60 %) while adult rats controlled the growth of parasites (< 20 %), cleared blood parasites at ~ 3 weeks p.i. and 90-100 % resisted infection (Pierrot *et al*., 2003 and unpublished observations). Thus our data indicate that a primary infection of young *versus* adult rats could be considered as relevant to human chronic infection with *Plasmodium falciparum* in endemic areas where children < 5 are more susceptible than adults. In addition, this rat experimental model seems to be a suitable model to study the immune responses related to the age during malaria infection. The analysis of T cell distribution in this model indicated a significant expansion of CD4+ and CD8+ T cells in spleens of adult infected rats when compared to those of young infected rats (Adam *et al*., 2003). Comparative studies of CD4+CD25+ cells in this model revealed a higher number of these cells in the PbA-infected young susceptible rats when compared to those observed in adult resistant rats. CD4+CD25+ T cells or Treg cells have been shown to produce high levels of IL-10, and are able to participate in the down-regulation of immune responses to infectious pathogens (Belkaid & Rouse, 2005). In our model, despite the fact that young susceptible rats produced significantly more circulating IL-10 than adult resistant rats, the *in vivo* neutralisation of this cytokine did not influence either the parasitemia levels or the issue of infection (Adam *et al*., 2003). This indicates that high production of IL-10 *per se* did not participate in the expression of susceptibility of young rats. With respect to the contribution of T cells, earlier report by Kamiyama *et al.* indicated that congenital athymic rats (rnu/rnu) exhibited high levels of parasitemia and died, while heterozygous littermates (rnu+) controlled the replication of blood parasites and survived (Kamiyama *et al*., 1987). These results indicated that T cells were essential in developing protective immunity in *P. berghei*-infected rats. However, the contribution of CD4+ and CD8+ cells in parasite clearance and host protection of adult rats, or the role for CD25+ cells in triggering death of young rats following PbA infection has not been evaluated so far. In this paper, we show that the apparent absence of CD25+ cells is not likely a major contributor to the severity of infection and associated mortality in young rats. On the contrary, the down-regulation of CD4 markers combined with high parasitemia and partial mortality of adult treated rats are in favour of a protective role of CD4+ cells. Regarding CD8+ cells, to our knowledge, this is the first report which clearly shows evidence of the role for these cells in the control of blood parasite replication.

MATERIALS AND METHODS

Parasites and animals

Plasmodium berghei ANKA (PbA) uncloned strain used in this study was described previously (Adam *et al*., 2003). A total of 187 F344 rats including 60 young rats and 127 adult rats were purchased from Harlan and raised in the specific pathogen-free animal facility of the Institut Pasteur de Lille. Young and adult rats (4- and 8 week-old at the beginning of the experiment) were infected with PbA as described previously (Adam *et al*., 2003). All experiments were conducted following the guidelines of laboratory animal care published by the French Ethical Committee and approved by the local Comité d'Ethique en Experimentation Animale Nord-Pas-de-Calais (CEEA AF 05/2009).

Treatments with anti-CD25, anti-CD4 and anti-CD8 monoclonal antibodies

Rat monoclonal antibodies (mAb) specific for CD8, CD4 and CD25 (OX8, W3/25 and OX39 respectively, IgG1 isotype) were a gift of Dr I. Anegon (Inserm UMR643, Nantes, France). OX8 mAb have been shown to be capable of depleting CD8+ cells *in vivo* (Davis, 2001). With respect to W3/25 and OX39 treatments, they led to a down regulation of surface CD4 and CD25 molecules respectively (Caballero *et al*., 1998 and personal observations). In order to determine the quantities of mAb required to observe and to maintain the down-regulation of surface CD4 or CD25 molecules, we performed preliminary experiments with young and adult uninfected/infected rats. These experiments led us to conceive long term treatments. Hence, for the depletion of CD8+ cells, groups of adult rats were i.p. injected with 2 mg/rat of purified OX8 five and two days before infection followed by further injections at days 1, 4, 7 and 13 post infection. This treatment allowed the depletion of CD8+ cells for about three

weeks post infection. For the down-regulation of surface CD4 molecules, groups of adult rats were i.p. injected with 4.5 mg/rat of purified W3/25 two days before the infection followed by further injections at days 1, 4, 7, 10 and 13 post infection. As for OX8-treated rats, W3/25-treated rats received a total of six injections. For the down-regulation of surface CD25 molecules, groups of young and adult rats were i.p. injected with 1 mg/rat or 2 mg/rat respectively of purified OX39 one day before infection followed by further injections at days 2, 6 and 9 post infection. For each mAb treatment, two separate experiments were performed using two different batches of purified antibodies and two different batches of parasitized red blood cells.

Flow cytometry

To check the efficiency of treatment with mAbs, blood and spleen cells were analyzed by one- or two-color fluorescence-activated cell sorting immunophenotyping, performed using FITC-, PE-, or biotin-conjugated mAbs: anti-CD25 (OX39), anti-CD4 (W3/25), anti-CD8 (OX8) as previously described (Pierrot, 2007).

Statistical analysis

The Mann-Whitney *U* test for nonparametric data was used for statistical comparisons between PbA blood parasitemia of treated rats and vehicle-treated controls. Statistical differences in survival were assessed by Fisher's Exact test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Treatment with anti-CD25 mAb does not affect either the susceptibility of young rats or the resistance of adult rats

In our previous work, we observed that PbA infection induced higher expansion of CD4+CD25+ cells in young rats when compared to adult infected rats (Adam, 2003). It should be noted that in rats CD25 is a cell surface marker for regulatory CD4+ T cells (Stephens *et al*., 2004). To examine whether CD4+CD25+ cells, may contribute to the suppression of the protective response, we treated young Fischer rats with anti-CD25 mAb and infected them with PbA. The efficacy of this treatment was checked by FACS (Fig. 1A, 1B). Infection with PbA of untreated and treated rats caused severe parasitemia and all rats succumbed to the infection (Fig. 1C). In parallel, we conducted the same experiments in adult rats, as CD25+ cells are also induced when compared to adult uninfected rat controls, and followed the course of infection. Again, the down-regulation of surface CD25 markers did not improve the capacity of adult rats to clear blood parasites (Fig. 1D). Taken together, these results indicate that a high expansion of CD4+CD25+ cells in young infected rats could be considered as a potential indicator of an unfavourable outcome of infection but does not appear to affect the replication of blood stage parasites.

In malaria, the role of CD4+ CD25+ T cells is still an open question and controversial since different observations have been made and substantial heterogeneity noted even when the same animal models were tested. Indeed, it was initially shown that CD4+CD25+ T cells, using different depletion strategies, were involved in the expression of ECM pathology related to PbA and could contribute to the fatal outcome of infected mice with *P. yoelii* due to hyperparasitemia (Amante *et al*., 2007; Hisaeda *et al*., 2004). In this context, a study revealed that the levels of CD4+CD25+ T cells and IL-10 were found to be higher in Balb/C susceptible mice when compared to DBA/2 mice infected with a non lethal clone of *P. yoelii* (Wu *et al*., 2007).

These findings contradict other studies indicating that the depletion of regulatory CD4+CD25+ T cells in mice failed to affect the outcome of malaria infection related either to PbA (Vigario *et al*., 2007) or *P. yoelii* (Couper *et al*., 2008). However, during *P. yoelii* infection, Couper *et al*. did show the implication of CD4+CD25-FoxP3- cells in the down regulation of pro-inflammatory responses and in modulating parasite clearance (Couper *et al*., 2008). In the case of rats infected with PbA, the treatment with anti-CD25 mAb failed to reveal any role for CD25+ cells during this infection. Most importantly, these results, along with those indicating that the neutralisation of IL-10 did not protect young infected rats likely suggest that the conversion of young susceptible rats to an immune phenotype cannot be achieved by modulating regulatory mechanisms.

Treatment with anti-CD4 mAb exacerbates PbA parasitemia and leads to the death of adult rats

Next, the role of CD4+ cells in the course of infection was evaluated by treating adult rats with anti-CD4 mAb (W3/25). Although the treatment down-regulated the CD4 surface marker in adult infected rats (Fig. 2A, 2B), it had no effect on parasitemia during the early phase of infection when compared with controls (parasitemia ~ 4 % in untreated and treated rats at day 9 p.i.) (Fig. 2C, 2D). However, after two weeks and thereafter we observed a significant increase in parasitemia in W3/25-treated rats. Indeed, the peak of mean parasitemia in the control group was ~ 15 % at day 13 p.i. and ~ 45 % at day 18 p.i. in W3/25-treated rats. Moreover, 54 % of treated rats succumbed to infection star-

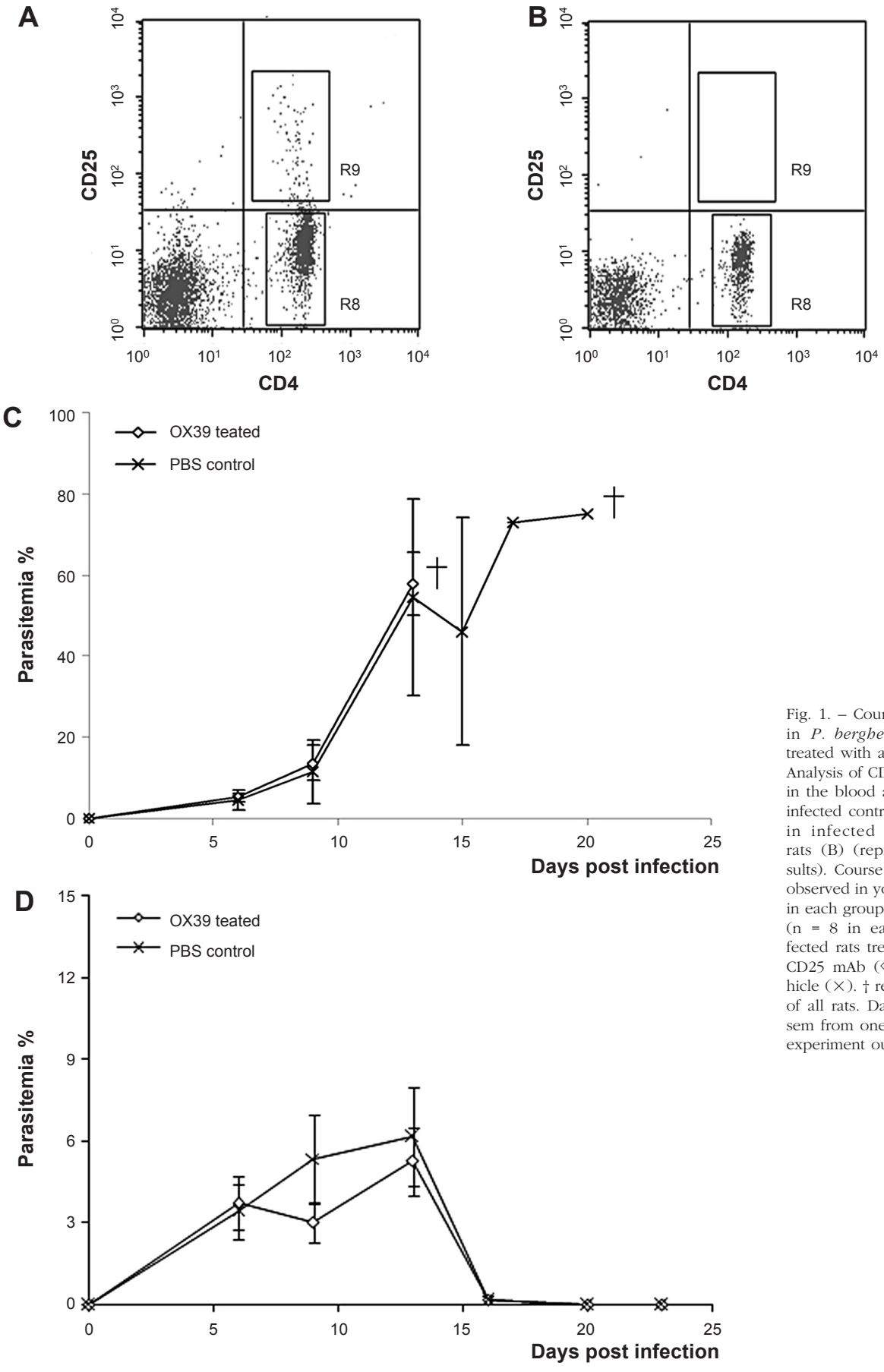

Fig. 1. – Course of infection in *P. berghei*-infected rats treated with anti-CD25 mAb. Analysis of CD4+CD25+ cells in the blood at day 6 p.i. in infected control rats (A) and in infected OX39-treated rats (B) (representative results). Course of parasitemia observed in young (C) (n = 6 in each group) and adult (D) (n = 8 in each group) infected rats treated with anti-CD25 mAb (◇) or with vehicle (×). † represents death of all rats. Data are mean ± sem from one representative experiment out of two.

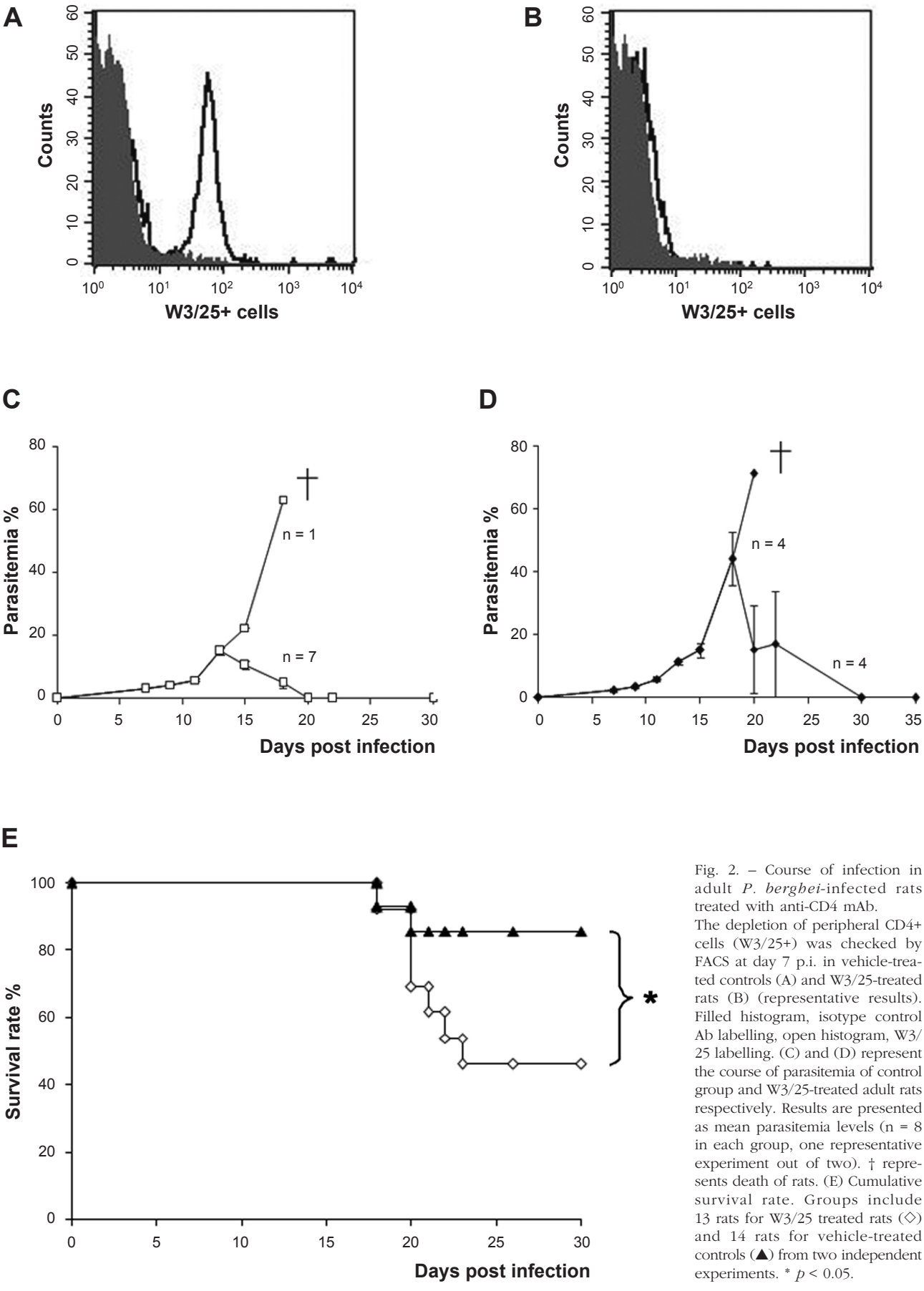

Fig. 2. – Course of infection in adult *P. berghei*-infected rats treated with anti-CD4 mAb.
The depletion of peripheral CD4+ cells (W3/25+) was checked by FACS at day 7 p.i. in vehicle-treated controls (A) and W3/25-treated rats (B) (representative results). Filled histogram, isotype control Ab labelling, open histogram, W3/25 labelling. (C) and (D) represent the course of parasitemia of control group and W3/25-treated adult rats respectively. Results are presented as mean parasitemia levels (n = 8 in each group, one representative experiment out of two). † represents death of rats. (E) Cumulative survival rate. Groups include 13 rats for W3/25 treated rats (◇) and 14 rats for vehicle-treated controls (▲) from two independent experiments. * $p < 0.05$.

ting from day 18 p.i. (Fig. 2E). The effect of anti-CD4 mAb treatment could not be related to a cross-linking of Fc receptors by IgG1 as the anti-CD25 mAb (IgG1 isotype) provides a useful control since the treatment with this mAb did not affect either the parasitemia or the issue of infection (Fig. 1). It is well known that CD4+ T cells play a critical role in protective immunity to blood malaria parasites by expressing direct effector functions as well as by helping B cells for antibody production (Stephens *et al*., 2005). The role of CD4+ cells is also supported by the fact that human CD4+ T cells are able to inhibit blood parasites *in vitro* (Fell *et al*., 1994).

Depletion of CD8+ cells exacerbates PbA parasitemia in adult rats

Preliminary experiments showed that the treatment with MRC OX-8 mAb resulted in a specific and dramatic reduction of CD8+ cells (up to 95 %, Fig. 3A, 3B) in both blood and spleens of adult rats. CD8+ depletion in adult infected rats strongly exacerbated PbA parasitemia compared with non-depleted controls (Fig. 3C). The significant increase in the parasite burden ($p < 0.01$) in treated adult rats was observed after day 15 p.i., indicating that CD8+ cells contribute to control the growth of blood-stage parasites. In addition, we observed a

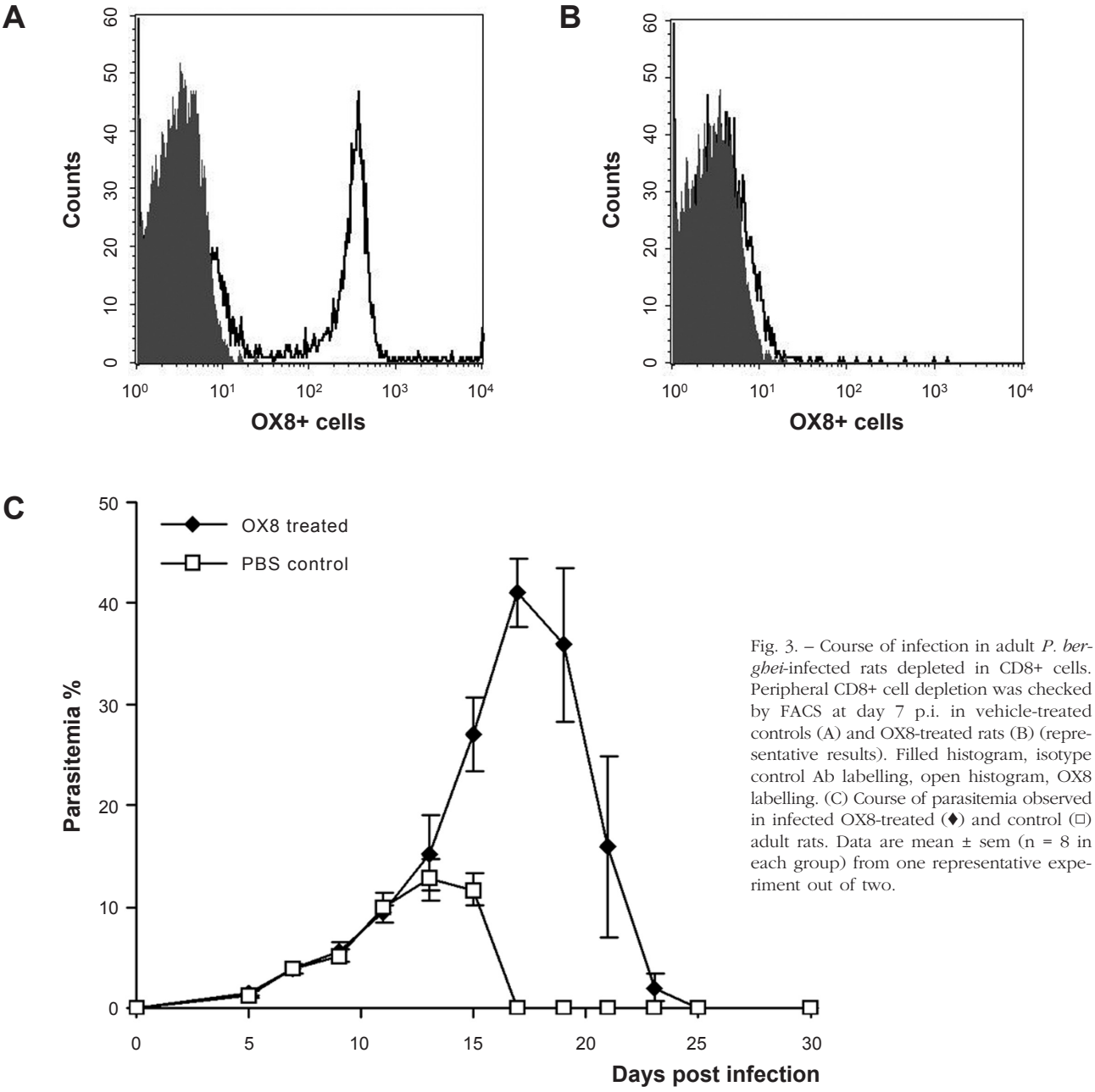

Fig. 3. – Course of infection in adult *P. bergbei*-infected rats depleted in CD8+ cells. Peripheral CD8+ cell depletion was checked by FACS at day 7 p.i. in vehicle-treated controls (A) and OX8-treated rats (B) (representative results). Filled histogram, isotype control Ab labelling, open histogram, OX8 labelling. (C) Course of parasitemia observed in infected OX8-treated (♦) and control (□) adult rats. Data are mean ± sem (n = 8 in each group) from one representative experiment out of two.

delay of ~ 1 week in parasite clearance in CD8+ depleted rats. Classically, CD8+ T cells have been shown to play a key role in protection against pre-erythrocytic stages of malaria infection (Tsuji & Zalava, 2003). With respect to the role of CD8+ T cells in the control of blood-stage parasites in infected mice, it seems that the transfer of immune CD8+ T cells fails to transfer protection and the depletion of CD8+ T cells in infected mice did not lead to a significant increase in the replication of blood parasites (Kumar & Miller, 1990; Suss *et al*., 1988; Vinetz *et al*., 1990). It is important to remember that in ECM, CD8+ T cells seem to be the principal actor in the development of this syndrome (for review Hafalla *et al*., 2006). Supporting this pathogenic role of CD8+ T cells in mice are recent studies using transgenic *P. berghei* expressing T and B cell epitopes (Lundie *et al*., 2008; Miyakoda *et al*., 2008). Indeed, both studies showed that malaria blood stage antigens can be cross-presented by APC to specific CD8+ T cells. These CD8+ T cells have been shown to be pathogen-specific cytotoxic lymphocytes and can contribute to cerebral malaria.

The use of rat experimental malaria involving PbA, a well known parasite strain provoking ECM and death in infected mice, revealed for the first time that the depletion of CD8+ cells of adult resistant rats both increased blood-stage parasite levels and delayed parasite clearance. These results suggest that the expansion of CD8+ T cells in infected rats is not pathogenic but rather associated with a better control of blood parasite replication. The characteristics of CD8+ T cell efficacy can be related to many functions that may include the release of cytokines such as IFNγ and/or IL-2, which exert pleiotropic effects to inhibit pathogen growth and have an important role in the amplification of cellular immune responses. The role of rat CD8+ cells is in line with data reporting the inhibitory role of human CD8+ T cells on the growth of *P. falciparum* blood parasites in the presence of adherent cells (Fell, 1994). Due to technical limitations, we were unable to recover a sufficient quantity of purified CD8+ cells in order to investigate their role in young susceptible rats. However, in a previous study we showed that total T cells from adult protected rats are capable of transferring immunity to young rats (Pierrot *et al*., 2007).

CONCLUSION

In conclusion, we found that both anti-CD4 treatment, which can affect not only T cells, but also macrophages and a dendritic cell subpopulation, and CD8+ cell depletion in rats (including T cells, NK cells, γδT cells and a dendritic cell subpopulation) resulted in a significant increase in blood parasitemia levels in adult rats. The role of T cell populations was further evidenced by the fact that nude adult rats (absence of thymic CD4+ and CD8+ cells) infected with the same line of PbA used throughout this work succumbed to infection (personal observations). In Fischer rats, CD4+ cells seem to be involved, at least in part, in the protection of adult animals from death. With respect to the role of CD25+ cells *in vivo*, although they were induced in infected rats, they do not seem to play a major role in the susceptibility of young rats to *P. berghei* Anka infection. More importantly, this work is the first report showing clearly that CD8+ cells can contribute to the control of blood parasite stages *in vivo*. Although we have not yet identified the pathway through which CD8+ cells inhibit blood parasite growth, these findings provide new insights regarding the role of CD8+ cells and may help to establish therapeutic strategies based on the induction of CD8+ cells against blood parasite stages. However, it should be kept in mind that these mechanisms might be distinct according to the animal species studied including humans and could change during infection. Thus, studying different infection models is important to ensure that we have as complete a picture as possible of the functions of induced immune responses.

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