Natural regulatory (CD4+CD25+FOXP+) T cells control the production of pro-inflammatory cytokines during *Plasmodium chabaudi adami* infection and do not contribute to immune evasion.

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Research paper.

Abstract.

Different functions have been attributed to natural regulatory $CD4^+CD25^+FOXP^+$ (Treg) cells during malaria infection. Herein, we assessed the role for T reg cells during infections with lethal (DS) and non-lethal (DK) *Plasmodium chabaudi adami* parasites, contrasting in the levels of parasitemia, inflammation and anaemia. Independent of the parasite virulence, the population of splenic Treg cells expanded during infection, and the absolute numbers of activated $CD69^+$Treg cells were higher in DS-infected mice. *In vivo* depletion of $CD25^+$T cells, which eliminated 80% of $CD4^+FOXP3^+CD25^+$ T cells and 60-70% of $CD4^+FOXP3^+$ T cells, significantly decreased the number of $CD69^+$ Treg cells in mice with lethal malaria. As a result, higher parasite burden and morbidity were measured in the latter, whereas the kinetics of infection with non-lethal parasites remained unaffected. In absence of Treg cells, parasite specific IFN-γ responses by $CD4^+$ T cells significantly increased both in mice with lethal and non lethal infection, whereas IL-2 production was only stimulated in mice with non-lethal malaria. Following the depletion, the production of IL-10 by $CD90^-$ cells was also enhanced in infected mice and interestingly, a potent induction of TNF-α and IFN-γ production by $CD4^+$ and $CD90^-$ lymphocytes was measured in DS-infected mice, which suffered earlier of severe anaemia. Taken together, our data suggest that the expansion and activation of natural Treg cells represents a counter-regulatory response to the overwhelming inflammation associated with lethal *P.c. adami* DS infection which involves TH1 lymphocytes as well as cells from the innate immune system.

Keywords: **rodent malaria,** *Plasmodium* **virulence, natural regulatory T cells, inflammation.**

1. Introduction

Natural Treg cells represent a population of $CD4^{+}CD45RB^{low}$ T cells constitutively expressing the α chain of the IL-2 receptor (CD25) (Schwartz, 2005) and which in mice comprise more than 85% of the cells expressing the Fork Head BOX P3 transcriptional factor (FOXP3) (Khattri et al., 2003; Fontenot et al., 2005). These cells play determinant roles in the preservation of self tolerance and in the control of graft and tumour rejection and inflammation, and their abrogation leads to autoimmunity and inflammatory diseases in several experimental models (Fontenot et al., 2005; Schwartz 2005).

In addition to their function in self-tolerance, Treg cells also participate in the control of overwhelming responses to infectious agents such as viruses, bacteria and protozoan parasites (Raghavan and Holmgrem, 2005; Belkaid et al., 2006; Demengeot et al., 2006; Suvas and Rouse, 2006). In malaria, T reg cells expand during *P. berghei* ANKA infections (Nie et al., 2007; Vigario et al., 2007), and have been shown to inhibit the development of pathogenic TH1 cells, responsible for cerebral disease in resistant BALB/c mice (Nie et al., 2007). These results contrast with the detrimental effects associated with T reg cells during *P. berghei* ANKA infection in susceptible C57BL/6 mice (Amante et al., 2007). In this infection model, depletion of T reg cells results in a significant increase in survival, a minor but significant reduction in blood parasitemia and an important reduction in parasite load in the brain and vasculature. A comparable delay in the onset of peak parasitemia has been reported during *P. berghei* NK65 infection in mice depleted of Treg cells (Long et al., 2003), and in the *P. yoelii* 17XL infection model, elimination of Treg cells allows BALB/c mice to control otherwise lethal infections (Hisaeda et al., 2004). Moreover, data provided by *P. falciparum* infections in humans correlates the expansion of natural Treg cells and the production of TGF-β with higher parasite multiplication rates (Walther et al., 2005). Altogether, these observations attribute contrasting functions to natural Treg cells during *Plasmodium* infections.

Herein, the role of natural Treg cells in the lethality associated with *P. c. adami* infection has been evaluated using two parasite strains with contrasting virulence, sustained by distinct parasite multiplication rates, and by the severity of inflammation and anaemia generated. Our results indicate that during *P.c. adami* infection, Treg cells contribute to the control of overwhelming inflammatory responses, and do not impair protective immune responses.

2. Materials and Methods.

2.1. **Parasites, mice and infections.**

The *P. c. adami* DS and DK strains derive from two different isolates (Congo-Brazaville, 1972). Female BALB/c mice, 4-6 weeks old (Charles River) were infected with 10^5 parasitized red blood cells (PRBC) by the intraperitoneal route. Parasitemia was measured daily in methanol fixed tail blood smears stained with a 10% Giemsa solution in PBS.

2.2. **Cell purification.**

Single-cell suspensions of splenocytes from naive and *P. c. adami* infected mice (peak parasitemia) were prepared. In experiments assessing the contribution of $CD90^-$ lymphocytes in the inflammatory response, T cells were removed using MACS CD90 microbeads (Miltenyi Biotec, USA), and levels of purity >95% were attained. $CD4^+$ T cells were enriched by negative selection using the EasySep® Mouse $CD4^+$ T cell enrichment kit (STemCell Technologies, Canada), and purity levels of 90-95% were obtained. Syngeneic adherent peritoneal macrophages were used as accessory cells, and were shown to be >85% $CD11b^+$ cells following staining with anti-mouse CD11b monoclonal antibody (Mab) (clone CL8941F, Cedarlane Laboratories).

2.3. **Assessment of $CD4^+$ T cell function.**

Purified $CD4^+$ T cells from naïve and infected mice (untreated or depleted of $CD25^+$ T cells *in vivo*) were co-cultured in 96 well plates ($2x10^5$ cells /well) with naive peritoneal

macrophages (2 x 10^4 cells/well), and were stimulated with low endotoxin azide-free anti-CD3 Mab (4μg/ml, clone C363.29B, Cedarlane Laboratories) or with 60,000 homologous PRBC. Culture supernatants from stimulated $CD4^+$ T cell were recovered 24h or 72h later for IL-2 and IFN-γ measurements by ELISA, respectively. All samples were assessed in triplicates.

2.4. **Phenotypic characterization of natural Treg cells by flow cytometry.**

Splenocytes from naive and infected mice (moderate and peak infection) were co-stained with FITC-conjugated anti-mouse CD4/L3T4 (clone GK1.5, Southern Biotech), allophycocyanin (APC)-conjugated anti-mouse CD25/IL-2 receptor alpha (IL-1Rα, clone 7D4, Southern Biotech) and PE/Cy-7conjugated anti-mouse CD69 (clone HI-2F3, Biolegend) Mabs following standard procedures. The cells were fixed and permeabilized in a fixation/permeabilization solution and permeabilization buffer (eBioscience, USA) and were incubated with anti-mouse FOXP3-PE Mab (clone FJK-16S, eBioscience, USA). The percentages and absolute numbers of $FOXP3^+$ T cells within the $CD4^+CD25^+$ population were determined using a FSC/SSC gate on lymphocytes and a gate on $CD4^+$ $CD25^+$T cells. CD69 expression in $FOXP3^+$ and $FOXP3^-$ $CD4^+$ T cells and in $CD4^-$ lymphocytes was determined by a multiparametric analysis using a FACScan (Becton Dickinson, USA).

2.5 *In vivo* depletion of $CD4^+CD25^+$ T cells.

Depletion of $CD25^+$ T cells was carried out by intravenous administration of anti-mouse CD25 Mab (clone PC61, rat IgG1). The antibody was purified from supernatants from confluent cell cultures of the TIB-222 hybridoma (kindly provided by Dr. C. Piccirillo, McGill University) on a Hi-Trap Protein G column according to the instructions provided by the manufacturer. Mice received 500μg of the PC61 Mab by intra-peritoneal injection a day prior to and a day after infection. Depletion efficiency, assessed by FACS analysis of

125 $CD4^+CD25^+$ cells, ranged from 85% to 95%, and the effect was shown to persist for more
126 than 10 days.

127 **2.6 Quantification of IL-2, IL-10, IFN-γ and TNF-α.**

128 Interleukin-2 (IL-2) was measured in 24h culture supernatants from $CD4^+$ T cells harvested
129 from naïve and infected mice (untreated or depleted of CD25+ T cells), following stimulation
130 with homologous PRBC (60,000) or with anti-CD3 Mab (4μg/ml). The capture (Clone JES6-
131 1A2) and detector (Clone JES6-5H4) anti-mouse IL-2 Mabs (Caltag laboratories, USA) were
132 used. Antigen-antibody complexes were detected with a streptavidin-horseradish peroxydase
133 conjugate (Amersham Biosciences, USA) and the reactions were developed by addition of a
134 TMB solution (3, 3', 5, 5'-tetramethylbenzidine, Sigma). Absorbance at 450nm was measured
135 in a BioRad Model 550 microplate reader. IL-2 concentrations in samples were calculated
136 against a standard curve generated with recombinant mouse IL-2 (Cedarlane Laboratories
137 Ltd, CA).

138 Interleukin-10, IFN-γ and TNF-α were measured in 72h cultures of non-stimulated
139 splenocytes ($4x10^6$ cells/ml), $CD4^+$ T cells ($4x10^6$ cells/ml) and $CD90^-$ lymphocytes ($4x10^6$
140 cells/ml) from naive and infected mice (untreated or depleted of CD25+ T cells) using the BD
141 OptEIA™ sets for mouse IL-10, IFN-γ and TNF-α (BD Biosciences, USA). IFN-γ was also
142 quantified in 72h cultures of $CD4^+$ T cells from uninfected and infected mice, deprived or not
143 of $CD25^+$ T cells, following stimulation with PRBC or with anti-CD3 Mab as described
144 previously.

145 **2.7. Determination of hemoglobin in blood.**

146 Hemoglobin concentrations were measured in control mice and in mice depleted of $CD25^+$ T
147 cells (n=7 per group) immediately before and every two days following infection. Briefly, 2
148 μl tail-vein blood was diluted in 500μl Drabkin's solution (Sigma) and hemoglobin was
149 assayed in 96-well microtiter plates (Costar, Cambrifge, MA) in a volume of 100 μl by

measuring the absorption at 540nm in a microplate reader. Values were converted to milligrams per millilitre using a standard curve of human hemoglobin (Sigma) prepared in Drabkin's solution. All samples were assessed in duplicates.

2.8. Statistical analysis.

Statistical analysis was performed using a Mann Whitney test for the comparison of parasite burden and blood hemoglobin content in control and anti-CD25 treated mice. A one way ANOVA and Bartlett's tests for equal variances was used when comparing three or more groups (≥6 individual mice per category) using the Prism Software.

3. Results

3.1. Pro-inflammatory cytokines responses are enhanced during lethal *P.c. adami* infection.

In BALB/c mice and in other inbred and outbred mouse strains, inoculation of *P. c. adami* DS PRBC results in elevated parasitemia (45-65%) and 100% mortality (Scorza et al., 2005 and unpublished results) (Fig. 1A). In contrast, similar infective doses with DK parasites result in moderate infection (13-20% parasitemia) that resolves 12 to 13 days later in BALB/c mice (Fig. 1 A).

We compared the levels of TNF-α, IFN-γ, and IL-10 in 72h culture supernatants of splenic cells from BALB/c mice with lethal and non-lethal infection, harvested at the moment of peak parasitemia without further stimulation. Significantly higher concentrations of IFN-γ (Fig. 1B), TNF-α (Fig. 1C) and IL-10 (Fig 1D) were measured in unstimulated splenic cell cultures from mice with lethal infection.

3.2. Parasite-specific TH1 effector cells are activated during lethal and non-lethal infection, but fail to produce IL-2 in response to anti-CD3 stimulation.

The functional state of purified $CD4^+$ T cells from infected mice was assessed at the moment of highest parasite burden, corresponding to peak parasitemia at day 9 and 10 following infection with non-lethal DK or lethal DS parasites, respectively. Specific IL-2 production by $CD4^+$ T cells in response to stimulation with homologous PRBC was measured during the two infections, and was shown to be significantly higher in mice with lethal malaria (Fig. 2A). Interestingly, in comparison with cells from uninfected mice, an important reduction in IL-2 production in response to stimulation with anti-CD3 Mab was measured in $CD4^+$ T cells from infected mice. The impaired IL-2 response was more pronounced during non-lethal malaria (86±8% compared to 64±7% inhibition during DS infection) (Fig. 2B). Independent of the virulence, comparable specific IFN-γ responses were detected when stimulating $CD4^+$ T cells from infected mice with PRBC (Fig. 2C), as well as following stimulation with anti-CD3 Mab (Fig 2D).

3.3. The absolute numbers of activated Treg cells significantly increase during lethal infection but their depletion does not abrogate the lethality associated with DS parasites.

Treg cells have been shown to control exacerbated inflammatory responses during *P.berghei* ANKA infections (Nie al at., 2007). As the production of pro-inflammatory TNF-α and IFN-γ was shown to be significantly up regulated during DS infection, we investigated whether Treg cells preferentially expanded in mice with lethal malaria. A lymphocyte gate was created to determine the percentages and absolute numbers of $FOXP3^+$ T cells within the splenic $CD4^+$ T cell population in naive and infected mice (Fig. 3 A, B). When compared to uninfected controls, higher absolute numbers of $CD4^+FOXP3^+$ T cells were measured at peak infection with DS and DK parasites, and were shown to be in particular enhanced during lethal infection at moderate parasitemia (17-21%) (Fig. 3 C). The total number of $CD4^+$T cells increased during infection and tend to be higher in DK-

infected mice (Fig. 3D), in correlation with the enhanced splenomegaly observed during non-lethal infection. A significant expansion of $CD25^+CD4^+FOXP3^+$ T cells was measured during lethal and non-lethal infection (Fig. 3E), and the total numbers of $CD69^+$ $CD4^+FOXP3^+$cells were significantly higher in mice infected with lethal DS parasites (Fig 3F).

We assessed the effect of the PC61 Mab on the kinetics of infection with the DS and DK strains. Two doses of antibody, administered a day prior to and a day after infection, eliminated more than 80% of $CD4^+FOXP3^+CD25^+$ cells in the infected mice and the effect was shown to persist until resolution of non-lethal infection or until the attainment of peak lethal infection (Fig. 4 A). Treatment with the depleting Mab led to a comparable and significant decrease in the absolute numbers of $CD4^+FOXP3^+$ cells in infected mice (Fig. 4B). At the moment of peak infection (8 days following treatment), more than 70% of $CD4^+FOXP3^+$ T cells were eliminated in infected mice, whereas a 30% reduction was measured in uninfected animals. As expected, treatment with the anti-CD25 Mab resulted in a significant drop in $CD4^+CD25^+$ T cell numbers in infected and naïve mice (Fig. 4C). A significant drop (>79%) in the total numbers of $CD69^+$ Treg cells was measured in mice with lethal infection, whereas a marginal inhibitory effect was observed in DK-infected mice (Fig 4D).

The depletion of $CD25^+$ T cells did not abrogate the virulence associated with *P. c. adami* DS infection. In a first experiment, higher parasite burden and enhanced distress (lethargy, ruffled fur and hypothermia) became apparent from day 7 post-infection in the treated group of mice. All the animals were sacrificed at day 8 post-infection due to severe morbidity, and peak parasitemia was higher in the group of depleted mice the day of death (Fig. 5A). In a second experiment, the parasite burden was higher in the group of depleted mice at day 7 post-infection when all these mice died, corresponding to a day earlier than the control

group (Fig. 5B). The differences in parasitemia at day 7 of infection with lethal DS parasites were shown to be statistically significant between the treated and untreated groups (Fig. 5C). In contrast, infections with non-lethal DK parasites were characterized by a marginal decrease in peak parasitemia in a first experiment (Fig. 5D) or remained unaffected by the treatment in a second experiment (Fig 5E).

3.4 Pro-inflammatory responses are further enhanced in absence of Treg cells.

The expression of CD69, an early activation marker of T cells, NK cells, B cells and other cells was compared in control and depleted infected mice. A significant increase in the absolute numbers of $CD4^+FOXP3^-CD69^+$ T cells was measured at peak infection with lethal and non lethal parasites, and in absence of $CD25^+$ T cells, this number further increased in DK-infected mice, whereas a marginal drop occurred in DS-infected animals (Fig. 6A). As expected, the treatment with the PC61 Mab led to a drop in the relatively low numbers of $CD4^+$ $FOXP3^-CD25^+$ T cells that increased during infection (Fig. 6B). The elimination of $CD25^+$T cells did not affect the absolute numbers of $CD4^-CD69^+$ lymphocytes, which remained high and comparable at peak lethal and non-lethal infections (Fig. 6C).

As the PC61 Mab may deplete activated effector $CD4^+$ T cells that transiently express the CD25 receptor, $CD4^+$ T cell effector responses (IL-2 and IFN-γ) to PRBC and anti-CD3 stimulation were compared in control and depleted mice at peak infection. In absence of $CD25^+$ T cells, parasite-specific IL-2 responses significantly increased in mice with non-lethal infection, whereas they remained unchanged in DS-infected mice (Fig 7A). Interleukin-2 production in response to stimulation with the anti-CD3 Mab marginally increased in mice depleted of $CD25^+$ cells, and this treatment did not restore the impaired IL-2 response in infected mice (Fig. 7B). Interestingly, elimination of $CD25^+$ cells resulted in a dramatic increase in IFN-γ production by $CD4^+$ T cells from mice with lethal infection (Fig 7C). In general, IFN-γ responses to TCR stimulation with the anti-CD3 Mab were significantly

enhanced in all the experimental groups in absence of $CD25^+$ cells (Fig. 7D). Theses results contrasted with those measured when eliminating $CD25^+$ cells within the population of purified $CD4^+$T cells by magnetic sorting, which resulted in a significant decrease of both IL-2 and IFN-γ responses (data not shown).

As pro-inflammatory cytokines are responsible for malaria disease (Clark et al, 2006), TNF-α, IFN-γ as well as anti-inflammatory IL-10 responses were compared in control and CD25-depleted mice. $CD90^-$ lymphocytes and $CD4^+$ T cells were purified from infected mice at peak infection and were cultured for 72h without further stimulation. In mice with virulent infection, the treatment with the PC61 antibody resulted in enhanced production of TNF-α and IFN-γ by $CD90^-$ cells from DS-infected mice (Fig. 8 A, B), and a comparable effect was observed for TNF-α in cells from naïve mice (Fig. 8A). Low levels of IL-10 were detected in $CD90^-$ cell cultures from infected mice, and were significantly enhanced in infected mice by the depletion (Fig 8C). IFN-γ production by $CD4^+$ T cells significantly increased in naïve and in infected mice (Fig. 8D), and surprisingly IL-10 responses by CD4+T cells were low (0-40 pg/ml) and remained comparable in treated and untreated mice (data not shown).

3.5. Severe anaemia is generated during lethal infection and is enhanced in absence of Treg cells.

A final objective in our study concerned the characterization of anaemia in mice depleted of Treg cells, as it is partially consequent to the inhibitory effects of inflammatory cytokines on erythropoiesis (Clark et al., 2006). Indeed severe anaemia was a hallmark of infection with lethal parasites and hemoglobin values in DS-infected mice drop below 80 mg/ml at days 7 and 8 post-infection (Fig. 9A), contrasting with the moderate anaemia during non-lethal DK infection (Fig. 9B). The depletion of $CD25^+$T cells accelerated the onset of severe anaemia during lethal malaria, which became apparent at day 6 post-infection (Fig 9A). This treatment

also significantly reduced blood hemoglobin at peak infection with non-lethal DK parasites (Fig. 9B), but the levels of anaemia remained moderate.

4.Discussion.

In the present study we show that the population of natural Treg cells significantly expands during lethal *P. c. adami* DS infections and in this malaria infection model, T reg cells contribute to the down-regulation of the severe inflammatory response, but fail to protect the mice from the development of lethal anaemia and death.

Regulatory T cells participate in the attenuation of overwhelming inflammatory responses during infections with Protozoa and Helminths (Belkaid et al., 2006). In BALB/c mice, resistant to *P. berghei* ANKA cerebral disease, the expansion of Treg cells has been shown to correlate with the control of pathogenic TH1 responses (Nie et al., 2007). In contrast, detrimental effects have been associated with Treg cells in susceptible C57BL/6 mice, for which the *in vivo* elimination of T reg cells prevents the development of cerebral disease and leads to an important reduction of parasite load in the vasculature and brain. Interestingly, although the inflammatory response (up regulation of vascular adhesion molecules, the recruitment of leukocytes to the brain and the production of TNF-α and IL-6) is not modified in C57BL/6 mice deprived of Treg cells prior to infection, a significant drop in the recruitment of pathogenic $CD8^+$ T cells to the brain is measured (Amante et al., 2007).

Our data indicates an enhanced inflammatory response during lethal *P. c. adami* DS infection which is accompanied by a significant increase in the number of activated Treg cells, and their elimination results in exacerbated inflammation, anaemia and blood parasite burden. Our results with e *P.c. adami* infections, as well as those described during *P. berghei* ANKA and *P. yoeli*i infections reveal contrasting roles for T reg cells in different malaria models. Indeed, the detrimental versus beneficial functions attributed to Treg cells during malaria may depend

299 on the pathology associated with the parasite species and strain (cerebral disease versus
300 anaemia), the MHC haplotype and genetic background of the host as well as on the timing
301 and magnitude of the counter-inflammatory responses induced. Malaria infections are
302 characterized by systemic inflammation which is responsible for disease in humans and in
303 experimental hosts (Clark et al., 2006; Schofield and Grau, 2005). As suggested by numerous
304 studies in mice, an early inflammatory response is essential for the control of parasite
305 multiplication (Stevenson et al., 1995; Mohan et al., 1997; Mitchell et al., 2005). However,
306 excessive inflammation is also a major contributor to the cerebral pathology and haemolytic
307 anaemia (Kurtzhals et al., 1998; Othoro et al., 1999), and thus, counter regulatory
308 mechanisms are required for its control. In this context, the ability to survive *P. berghei* K173
309 or *P. c. chabaudi* AS infection correlates with the timing and magnitude of TGF-β production
310 and its inhibitory effect on TNF-α release by immune cells (Omer et al., 1998; Omer et al.,
311 2003). Interleukin-10 also plays a protective role in experimental models of cerebral malaria
312 (Kossodo et al., 1997), and has been shown to be essential for the control of inflammation
313 during *P. chabaudi* infection (Linke et al., 1996).
314 In contrast to the results described for the virulent *P. yoelii* 17XL infection model (Hisaeda et
315 al., 2004) we failed to detect inhibitory activities associated to Treg cells which could account
316 for *P.c. adami* DS virulence. Secretion of IL-2 by $CD4^+$T cells in response to PRBC was in
317 particular enhanced during lethal DS infection and interestingly, independent of the infection,
318 IL-2 production in response to stimulation with anti-CD3 Mab was severely impaired in
319 $CD4^+$T cells. This scenario remained unchanged in the absence of Treg cells, albeit the fact
320 that specific IL-2 responses to PRBC were significantly restored in $CD4^+$ T cells from DK-
321 infected mice. In contrast, robust parasite-specific IFN-γ responses were measured in by
322 $CD4^+$ T cells DS and DK-infected animals, indicating comparable TH1 effector responses. It
323 is possible that the failure to produce IL-2 during infection may be consequent to a state of

exhaustion in T cells. A comparable phenomenon has been described in chronic HIV and SIV infections, in which $CD8^+$ T cells expressing the program cell death receptor PD-1 (a negative regulator of activated T cells), exhibit an "exhausted" phenotype, failing to produce IL-2 and to proliferate (Day et al., 2006, Petrova et al., 2007).

The depletion of Treg cells had a major positive impact on the production of pro-inflammatory cytokines during lethal DS infection. Significantly higher TNF-α and IFN-γ responses were induced in cells other than T cells, and IFN-γ production by $CD4^+$ T cells was further stimulated *in vivo* and *in vitro* in absence of $CD25^+$ T cells. Indeed, the expansion and activation of Treg cells could represent a counter-regulatory response to the exacerbated inflammation during lethal infection. Parasitemia becomes higher only at late DS infection whereas the total numbers of Treg cells have significantly expanded when the mice have moderate parasite burdens. Interleukin-10 production was significantly induced during lethal infection, as measured in splenic cell culture supernatants. The levels of this regulatory cytokine were relatively low in $CD90^-$ cell culture supernatants from infected mice, and were greatly enhanced in absence of Treg cells during infection. It is tempting to suggest that $CD8^+$ T cells and not $CD4^+$ T cells may represent an important source of IL-10 during *P.c. adami* DS infection, as moderate levels of this cytokine (300-500 pg/ml) were detected in purified T cell cultures (data not shown). In early studies, $CD8^+$ T cells have been involved in the down regulation of lymphoproliferative responses to native or recombinant malaria antigens (Riley et al., 1989; Mshana et al., 1990; Riley et al., 1993) and these cells are an important source of TGF-β during blood stage infection (Omer et al., 2003). The discrepancies between our results with total splenic cells, $CD90^-$ and $CD4^+$ cell cultures suggests that other cells besides $CD4^+$ T cells and $CD90^-$ lymphocytes are responsible for the high IL-10 response measured during lethal infection.

348 An obvious detrimental effect consequent to the depletion of Treg cells was the
349 exacerbation of severe anaemia, which may explain the enhanced morbidity observed in
350 DS-infected mice prior to attainment of peak parasitemia. Our results also illustrate the
351 striking incapacity of the enhanced inflammatory response to control parasite
352 multiplication, which was further enhanced during lethal infection. In addition, our data
353 strongly suggests that the IL-10 response induced during lethal DS infection is not sufficient
354 to control the inflammation and pathology generated.

355 An alternative hypothesis for the contrasting effect of Treg cell depletion in *P. yoelii*
356 17XL and *P. c. adami* DS infections may rely on the nature of the protective responses
357 required to control parasite burden. During *P. yoelii* 17XNL infections Treg cells could
358 exert their detrimental effects by suppressing B cell-dependent antibody production, which
359 has been shown to be essential for the control of *P. yoelii* parasites (Roberts and Weidanz
360 1979). In a study developed by Lim et al (2005) a direct suppressive effect of natural Treg
361 cells on B cells was suggested. Eddahri et al. (2006) proposed an indirect mechanism of
362 action mediated by the suppression of the T helper cell activity required to generate
363 antibodies and blocked by TGF-β neutralizing antibodies.

364 That additional factors may participate in *Plasmodium* virulence has been underlined by
365 several studies such as the one developed by Fakey and Spitalny (1987) in which the lethality
366 of *P. yoelii* YM parasites was associated with rapid parasite growth and the consequent
367 failure of the immune system to cope in time with the infection. In line with this assumption,
368 independent studies by Falanga and da Silva (1989) and Yap and Stevenson (1994) have
369 demonstrated the rescue of *P. chabaudi* infected mice by blood transfusion, which allowed
370 the development of protective immunity. Indeed, a rapid parasite multiplication rate, the
371 anaemia resulting from the destruction of parasitized red blood cells by the parasite as well as

372 from the inhibitory effects that inflammatory cytokines have on erythropoiesis, may all
373 contribute to malaria lethality.
374 We remain cautious in respect to the nature of the population of $FOXP3^+$ cells expanding
375 during *P.c. adami* infection, as in humans transient expression of $FOXP3^+$ has been described
376 in activated T cells, which also adopt suppressive phenotypes (Walker et al., 2003; Pillai et
377 al., 2007). Indeed, significantly marked T cell activation, measured as increased numbers of
378 $CD4^+FOXP3^-CD25^+$ T cells was observed during lethal infection, correlating with the
379 absolute numbers of $CD4^+FOXP3^+CD69^+$ T cells. Thus, the possibility that activated mouse
380 effector T cells express FOXP3 to transiently down-regulate exacerbated effector responses
381 cannot be excluded.
382 Future work will focus on the identification of the parasite factors responsible for the
383 uncontrolled inflammation during lethal DS infections. A delicate balance is required to cope
384 with rapid parasite multiplication rates, which is initially under the control of the pro-
385 inflammatory environment relying on phagocytes and NK cells (Urban et al., 2005). Indeed a
386 complex pattern of interactions participate in the pathology associated with *Plasmodium*
387 infections, and dissecting the factors and mechanisms involved remains an important and
388 challenging task for the scientific community addressing malaria.
389
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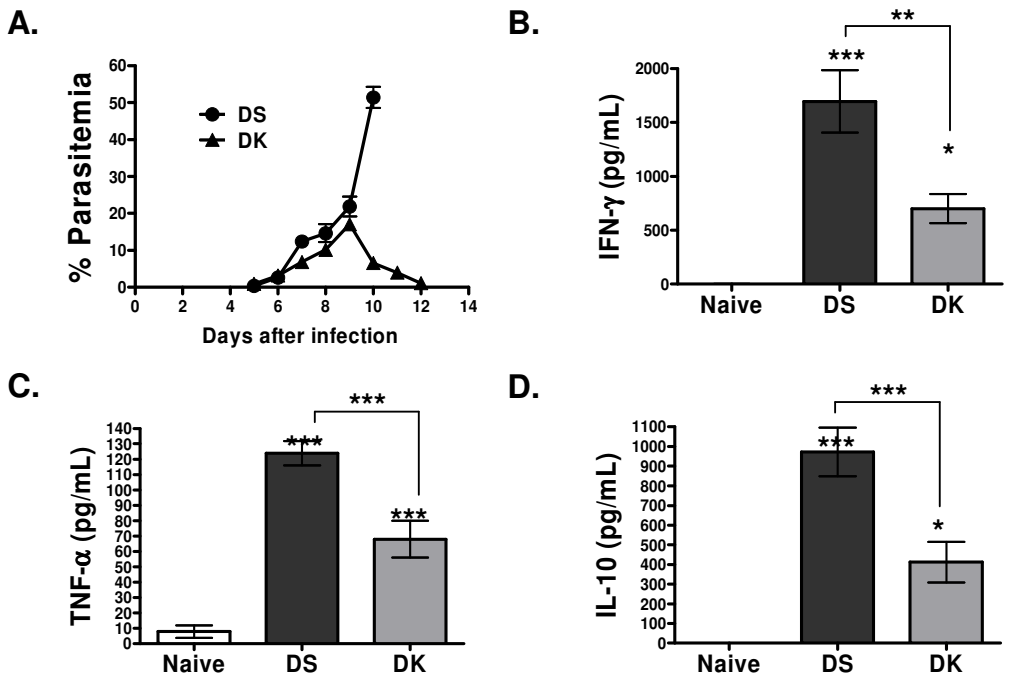
509 **Legends to Figures.**

Fig. 1



510

511 Fig. 1. The production of pro-inflammatory cytokines is enhanced during lethal *P. c. adami*
512 infection. A, Following intraperitoneal injection of 10^5 PRBC, *P. c. adami* DS infections
513 (●) develop steadily, attaining high parasitemia and 100% mortality when compared to
514 infection with non-lethal DK parasites (▲) (n=5 mice per group). Seventy two hours
515 unstimulated splenic cells culture supernatants (4 x 10^6 cells /ml) from naïve, *P.c. adami*
516 DS and DK-infected mice were assessed for IFN-γ (B), TNF-α (C) and IL-10 (D) content
517 (pg/ml), by ELISA. Bars represent the average ± SEM of 11 individual mice per group.
518 Statistical analysis was performed using a one way ANOVA and a Bartlett's test for equal
519 variances. ***P<0.001; **P<0.01, *P<0.05.

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Fig.

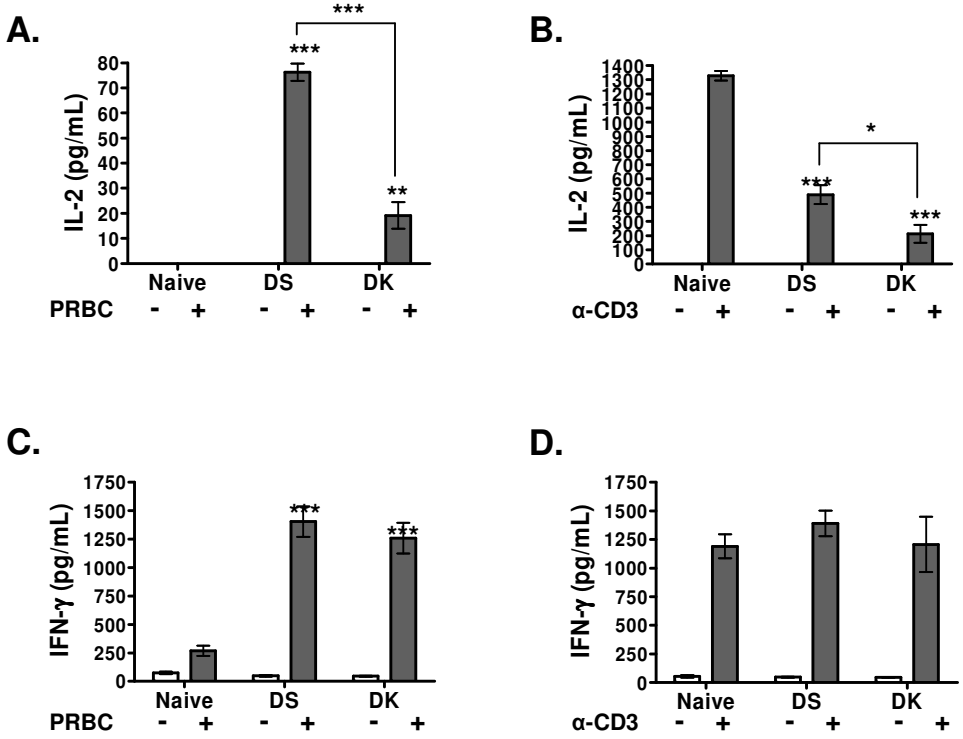


Fig. 2. Parasite-specific TH1 effector cells are activated during infection, but fail to produce IL-2 in response to anti-CD3 stimulation. $CD4^+$ T cells from naïve and infected mice (peak infection) were purified by negative selection, and were stimulated (5 $x10^5$ cells /well) for 24h (IL-2) or 72h (IFN-γ) with homologous PRBC (60,000) or with anti-CD3 Mab (4 µg/ml), in the presence of syngeneic naïve peritoneal macrophages (2x 10^4 cells /well). Interleukin-2 (A,B) and IFN-γ (C, D) levels were measured in 24 and 72h culture supernatants from unstimulated and stimulated $CD4^+$T cells. Bars represent the average ± SEM of 6 individual mice per group. Statistical analysis was performed using a one way ANOVA and a Bartlett's test for equal variances. ***P<0.001; **P<0.01, *P<0.05.

Fig.

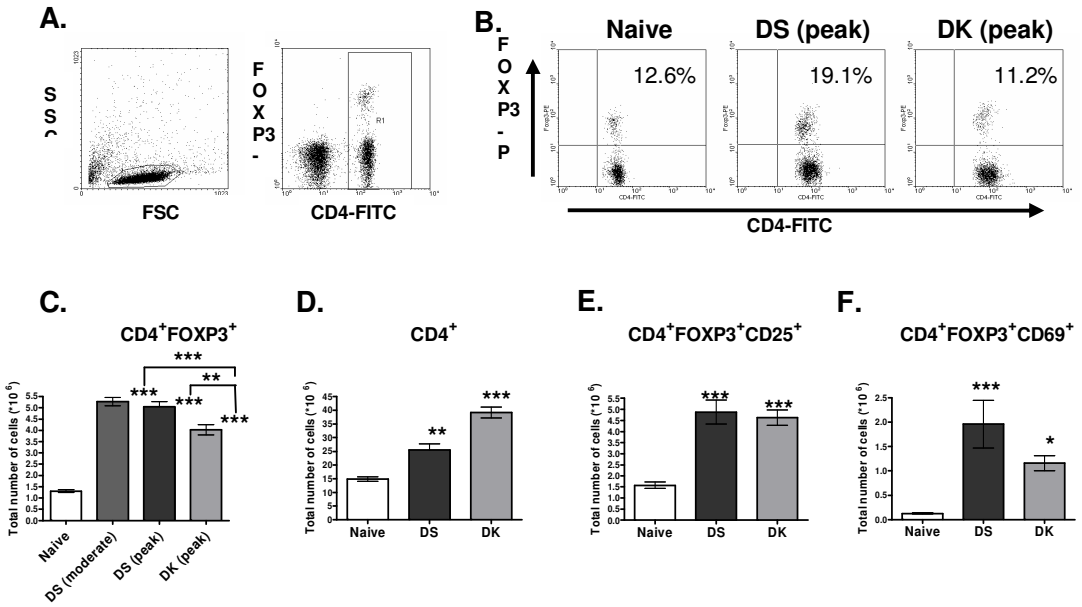


Fig. 3. The total numbers of activated Treg cells significantly increase during lethal infection. Using a lymphocyte gate (A,B), the absolute numbers of $CD4^+FOXP3^+$ (C), $CD4^+$ (D), $CD4^+FOXP3^+CD69^+$ (E) and $CD4^+CD25^+FOXP3^+$ (F) T cells were determined by flow cytometry in naive and *P. c. adami*-infected mice (peak parasitemia). Numbers in quadrants (B) show the percentages of $FOXP3^+$ cells within the $CD4^+CD25^+$ T cell population. The analysis in Fig. 3C includes *P.c. adami* DS infected mice with moderate (17-20%) and peak (45-60%) parasitemia, and DK infected mice with peak parasitemia (17-20%). Bars represent the average ± SEM of 7 individual mice per group. Statistical analysis was performed using a one way ANOVA and a Bartlett's test for equal variances. ***P<0.001, **P<0.01.

Fig.

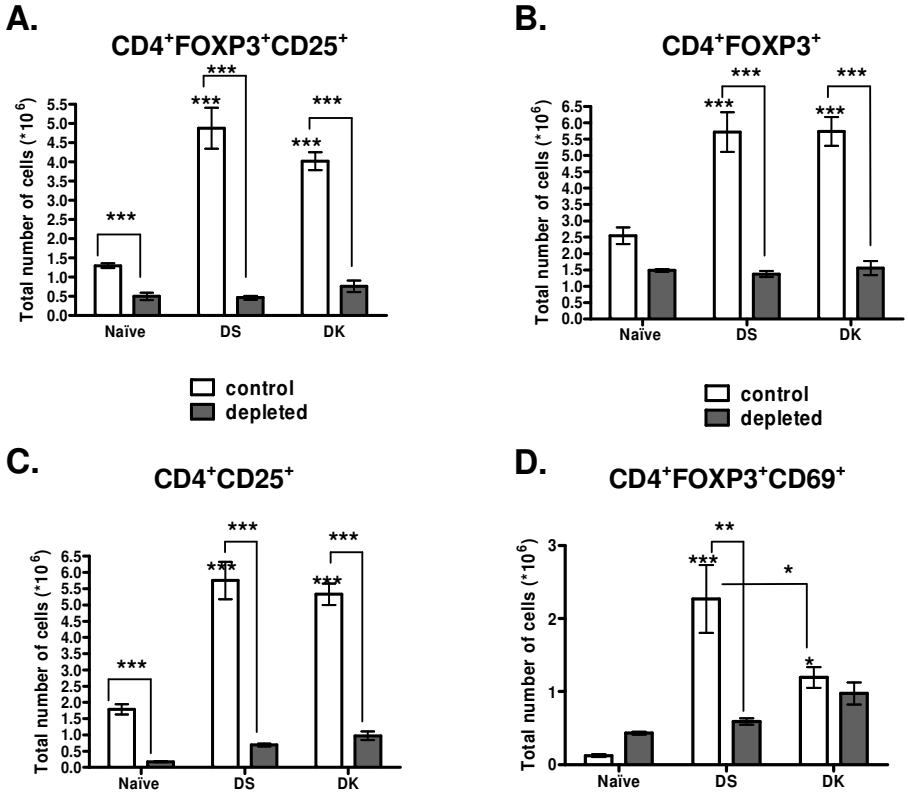
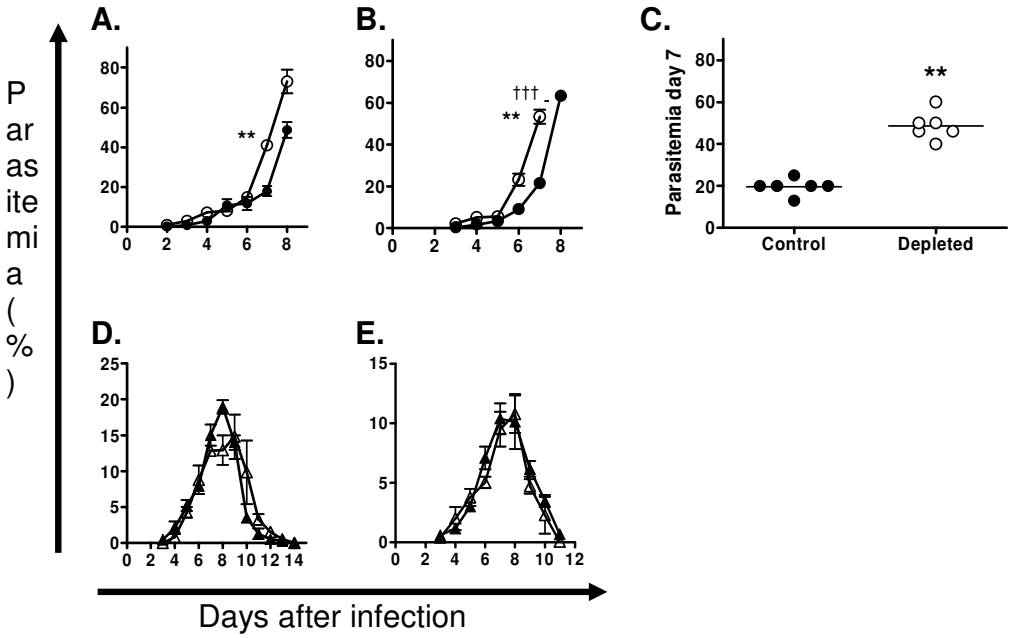


Fig. 4. Treatment with the PC61 Mab significantly eliminates the total numbers of activated Treg cells in naïve and infected mice. Groups of 7 mice received 500µg of anti-mouse CD25 monoclonal by intraperitoneal injection a day prior to and a day after infection. The absolute numbers of $CD4^+FOXP3^+CD25^+$ (A), $CD4^+FOXP3^+$ (B), $CD4^+CD25^+$ (C) and $CD4^+FOXP3^+CD69^+$ (D) cells were determined at peak infection by flow cytometry, using a gate on lymphocytes. Bars represent the average ± SEM of 7 individual mice per group. Statistical analysis was performed using a one way ANOVA and a Bartlett's test for equal variances. ***P<0.001, **P<0.01.

Fig.



559

560 Fig. 5. Depletion of Treg cells exacerbates the parasite burden in mice with *P. c. adami* DS
561 infection. Mice received 500µg of anti-mouse CD25 monoclonal by intraperitoneal
562 injection a day prior to and a day after infection. Parasitemia (% PRBC ±SEM) was
563 followed daily starting from day 3 post-infection until the moment of peak parasite burden
564 (DS), or until resolution of infection (DK). The progression of parasitemia in *P. c. adami*
565 DS (●; A, B) and DK (▲; D, E) infected control (closed symbols) and depleted (opened
566 symbols) mice was compared. C. Peak parasitemia values at day 7 post-infection in mice
567 from experiments A and B were grouped for analysis using a non-parametric Mann
568 Whitney test. Three mice were included per group in all 4 experiments. **P<0.01.

Fig.6

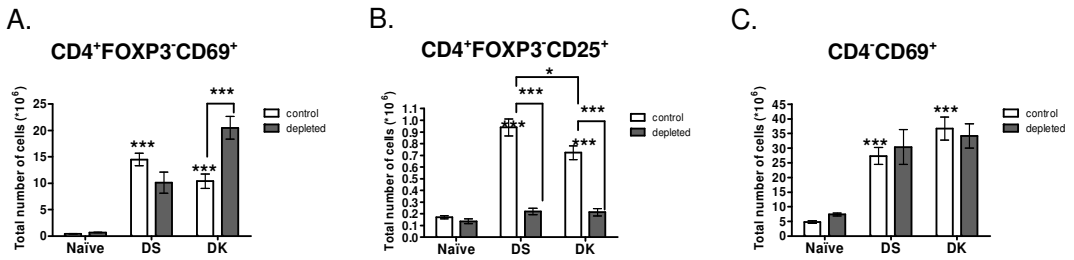
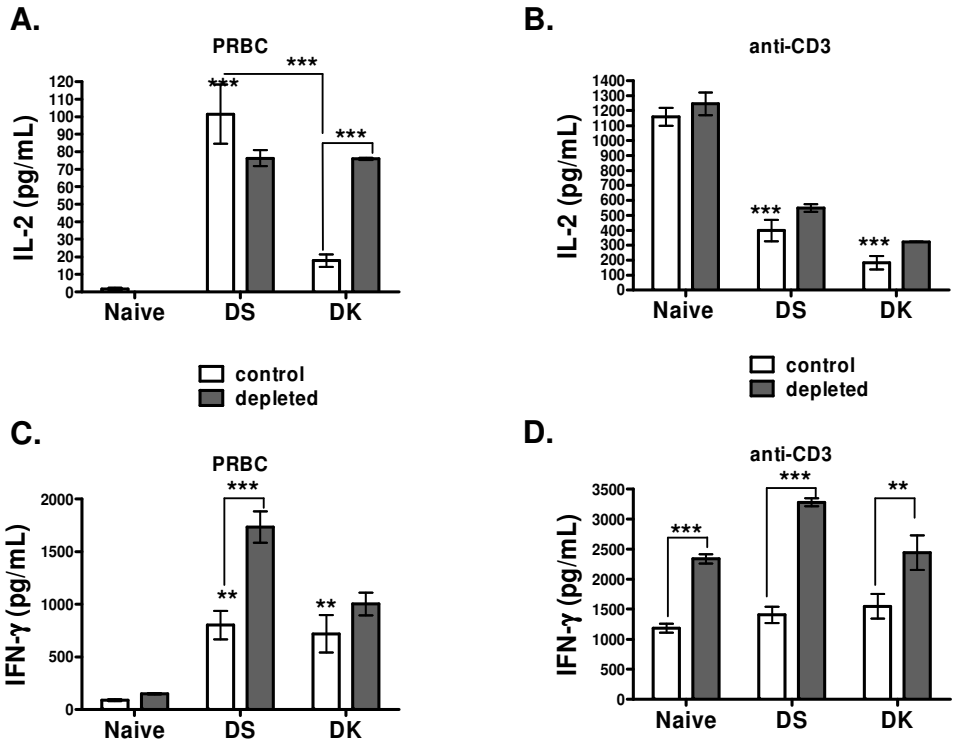


Fig. 6. *In vivo* administration of the PC61 Mab decreases the total numbers of activated $CD4^+FOXP3^-$ T cells but does not affect the numbers of activated $CD4^-$ lymphocytes during infection. Absolute numbers of $CD4^+FOXP3^-CD69^+$ (A), $CD4^+FOXP3^-CD25^+$ (B) and $CD4^-CD69^+$ (C) lymphocytes in control mice and in mice depleted of Treg cells prior to infection. Bars represent the average ± SEM of 10 individual mice per group. Statistical analysis was performed using a one way ANOVA and a Bartlett's test for equal variances. ***P<0.001, **P<0.01, *P<0.05.

Fig.



582

583 Fig. 7. Parasite specific IL-2 and IFN-γ responses by $CD4^+$ T cells are restored in absence
584 of Treg cells. Seventy-two hours culture supernatants of $CD4^+$ T cell from naïve, *P.c. adami*
585 DS and DK-infected mice (5 $x10^5$ cells/well) and naïve syngeneic macrophages ($2x10^4$
586 cells/well) were assessed for IL-2 (A, B) and IFN-γ (C, D) content (pg/ml) following
587 stimulation with 60,000 homologous PRBC (A,C) or anti-CD3 Mab (4ug/ml) (B,D) by
588 ELISA. Bars represent the average ± SEM of 6 individual mice per group. Statistical
589 analysis was performed using a one way ANOVA and a Bartlett's test for equal
590 variances. ***P<0.001, **P<0.01.

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Fig. 8

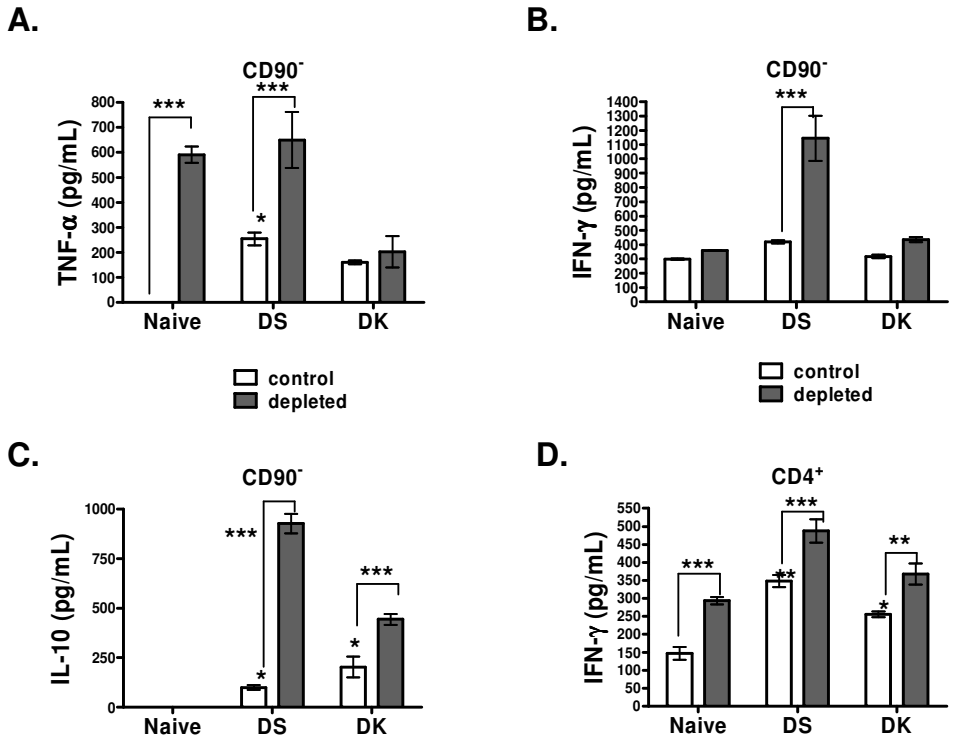
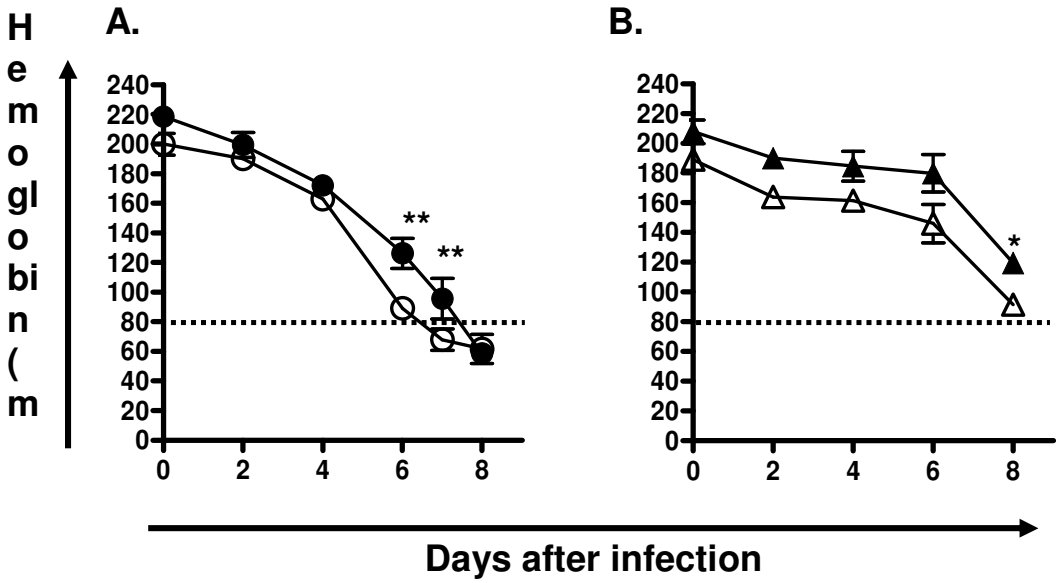


Fig. 8. The production of pro-inflammatory TNF-α and IFN-γ is enhanced in absence of Treg cells. Concentrations of TNF-α (A), IFN-γ (B,D) and IL-10 (C) in 72h cultures of $CD90^-$ lymphocytes (A-C) or $CD4^+$ T cells (D) from untreated (open bars) and Treg cell depleted (closed bars) naive and *P. c. adami* infected mice (peak infection). Similar concentrations of cells ($4x10^6$ cells/ml) were assessed for the $CD90^-$ and $CD4^+$ populations. Bars represent the average ± SEM of 7 individual mice per group. Statistical analysis was performed using a one way ANOVA and a Bartlett's test for equal variances.

*** P<0.001; **P<0.01; *P<0.05.

Fig.9



606

607 Fig.9. Anaemia is exacerbated in absence of Treg cells. The concentration of hemoglobin in
608 blood (mg/ml) was compared in control (closed symbols) and CD25-depleted (opened
609 symbols) mice during *P.c. adami* DS (●, A) and DK (▲, B) infection. Hemoglobin
610 measurements with the Drabkin's reagent were taken immediately before administration of
611 10^5 PRBC, and every 2 days until attainment of peak parasite burden. Seven mice were
612 included per group. Concentrations of hemoglobin inferior to 80 mg/ml (dotted lines) were
613 considered indicatives of severe anaemia. Statistical analysis was performed using a one
614 way ANOVA and a Bartlett's test for equal variances. **P<0.01; *P<0.05.

615