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*Immunopathology and Infectious Disease*

# A Role for Natural Regulatory T Cells in the Pathogenesis of Experimental Cerebral Malaria

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**Cerebral malaria (CM) is a serious complication of *Plasmodium falciparum* infection that is responsible for a significant number of deaths in children and nonimmune adults. A failure to control blood parasitemia and subsequent sequestration of parasites to brain microvasculature are thought to be key events in many CM cases. Here, we show for the first time, to our knowledge, that $CD4^+CD25^+Foxp3^+$ natural regulatory T (Treg) cells contribute to pathogenesis by modulating immune responses in *P. berghei* ANKA (PbA)-infected mice. Depletion of Treg cells with anti-CD25 monoclonal antibody protected mice from experimental CM. The accumulation of parasites in the vasculature and brain was reduced in these animals, resulting in significantly lower parasite burdens compared with control animals. Mice lacking Treg cells had increased numbers of activated $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes, but $CD8^+$ T-cell recruitment to the brain was selectively reduced in these mice. Importantly, a non-Treg-cell source of interleukin-10 was critical in preventing experimental CM. Finally, we show that therapeutic administration of anti-CD25 monoclonal antibody, even when blood parasitemia is established, can prevent disease, confirming a critical and paradoxical role for Treg cells in experimental CM pathogenesis.** *(Am J Pathol 2007, 171:548–559; DOI: 10.2353/ajpath.2007.061033)*

Cerebral malaria (CM) is a major cause of death in people infected with *Plasmodium falciparum*, with most deaths occurring in young children in sub-Saharan Africa. An estimated 10 to 20% of children who develop CM die, and a significant proportion of survivors have permanent neurological damage.[1–3] CM can be associated with sequestration of parasitized red blood cells (pRBCs) in the brain microvasculature[4] and secretion of toxic molecules by parasites,[5] as well as inflammatory components of the host immune response, including secretion of cytokines[6] and recruitment of activated leukocytes to the brain.[7–9] An experimental model of CM (ECM) caused by infection of C57BL/6 and CBA mice with *P. berghei* ANKA (PbA) displays many features of human CM and has allowed the identification of several important factors in CM pathogenesis. Both $CD4^+$ and $CD8^+$ T cells contribute to the development of ECM,[10–13] and the spleen seems to be a key site for priming of PbA-specific T-cell responses.[14] In addition, the proinflammatory cytokines interferon (IFN)-$\gamma$,[15,16] tumor necrosis factor,[17] and LT$\alpha$,[18] as well as perforin,[13] all seem to play a role in ECM pathogenesis.

Although the risk factors that predispose individuals to develop CM remain largely unknown, high blood parasitemia is significantly correlated with increased risk of CM.[19] Effective immune responses to *Plasmodium* blood stages only emerge in people living in malaria-endemic regions after several years of repeated malaria infections.[20] Antibodies against the surface of the merozoite lifecycle stage of *P. falciparum* and cell-mediated immunity are both thought to be required for protective immunity, but they may also contribute to pathology.[21] Recently, $CD4^+CD25^+$ regulatory T (Treg) cells were shown to be rapidly induced *in vivo* in humans following *P. falciparum* infection, and this was associated with a burst of transforming growth factor-$\beta$ production, decreased parasite-specific immune responses, and higher rates of

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parasite growth.[22] Treg cells have also been shown to enhance *P. yoelii* infection in BALB/c mice.[23] Together, these reports support a detrimental role for Treg cells in controlling parasites during malaria infections, although their effect on CM pathogenesis is unknown.

Naturally occurring $CD25^+CD4^+$ Treg cells, constituting 5 to 10% of peripheral $CD4^+$ T cells in mice and humans, express the forkhead/winged helix transcription factor Foxp3.[24] They are produced in the thymus as a distinct and functionally mature population, but there is also evidence that they are induced in the periphery.[25] Treg cells play a critical role in the maintenance of immunological self-tolerance, as well as the control of immune responses to pathogens,[26] commensal microbes, and environmental antigens.[24] Treg cells mediate their effects by direct cell contact[27] or the secretion of anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor-$\beta$.[28] Here, we show that Treg cells play an important role in modulating the host immune response to PbA during the pathogenesis of ECM. This is one of the first examples of Treg cells contributing to a pathogenic process during an infectious disease.

## Materials and Methods

### Mice

Female C57BL/6 and CBA/CaH mice 5 to 6 weeks of age were purchased from the Australian Resource Centre (Canning Vale, Perth, Western Australia) and maintained under conventional conditions. Female C57BL/6 mice deficient in IL-10 (originally obtained from Jackson Laboratories, Bar Harbor, ME) were bred and maintained in house. All animal procedures were approved and monitored by the Queensland Institute of Medical Research Animal Ethics Committee.

### Parasites and Infections

*P. berghei* ANKA (PbA) was used in all experiments after one *in vivo* passage in mice. A transgenic PbA (231c1l) line expressing luciferase and green fluorescent protein under the control of the ef1-$\alpha$ promoter was used for experiments involving *in vivo* imaging.[29] All mice were infected by injecting $10^5$ pRBCs intravenously (i.v.) via the lateral tail vein. Blood parasitemia was monitored by examination of Diff-Quick (Lab Aids, Narrabeen, NSW, Australia)-stained thin blood smears obtained from tail bleeds. Anemia was estimated by measuring hemoglobin levels using a HemoCue Hb 201 analyzer according to the manufacturer's instructions (HemoCue AB, Angelholm, Sweden). For serum cytokine analysis, 100 $\mu$l of blood was collected via the lateral tail vein before infection and 5 days after PbA infection. Blood was allowed to clot, and serum was collected and stored at $-70$°C until required.

### Disease Assessment

Mice were monitored twice daily after day 5 postinfection (p.i.), and clinical ECM evaluated. Clinical ECM scores were defined by the presentation of the following signs: ruffled fur, hunching, wobbly gait, limb paralysis, convulsions, and coma. Each sign was given a score of 1. Animals with severe ECM (accumulative scores $\geq$4) were sacrificed by $CO_2$ asphyxiation according to ethics guidelines, and the day of death was deemed to be the following day.

### Antibodies

Allophycocyanin-conjugated anti-TCR$\beta$ chain, phycoerythrin (PE)-Cy5- or PE-conjugated anti-CD4, PE-conjugated anti-CD69, anti-CD25-biotin (7D4), PE-Cy5-conjugated anti-CD8, PE-conjugated anti-Ly6G, fluorescein isothiocyanate-conjugated anti-Ly6C, allophycocyanin-conjugated anti-B220, fluorescein isothiocyanate-conjugated anti-CD19, allophycocyanin-conjugated anti-CD11c, PE-Cy5-conjugated anti-CD11b, fluorescein isothiocyanate-conjugated anti-CD45.2, biotin-conjugated anti-NK1.1, anti-intercellular adhesion molecule (ICAM)-1, anti-vascular cell adhesion molecule (VCAM)-1 monoclonal antibodies (mAbs), and Alexa Fluor 488-conjugated streptavidin were purchased from Biolegend (San Diego, CA) or BD Biosciences (Franklin Lakes, NJ). PE-Cy5-conjugated $\alpha$-galactosylceramide ($\alpha$GalCer) mouse CD1d tetramers were a generous gift from Dale Godfrey and Daniel Pellicci (University of Melbourne, Melbourne, VIC, Australia). PE-labeled anti-mouse Foxp3 mAb was purchased from eBioscience (San Diego, CA). Anti-CD25 (PC61; rat IgG1) and isotype control mAb (MAC49; rat IgG1) were purified from culture supernatants by protein G column purification (Amersham, Uppsala, Sweden) followed by endotoxin removal (Mustang Membranes; Pall Life Sciences, East Hills, NY). Purified control rat IgG were also used in some experiments and purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

### Preparation of Tissue Mononuclear Cells

Spleen cells were isolated by digesting tissue in collagenase type 4 (1 mg/ml; Worthington Biochemical Corp., Lakewood, NJ) and deoxyribonuclease I (0.5 mg/ml; Worthington Biochemical) at room temperature for 40 minutes. Splenocytes and lymph node cells (from superficial cervical, axillary, brachial, mesenteric, and inguinal lymph nodes) were isolated by passing tissue through a 100-$\mu$m sieve and washing twice with phosphate-buffered saline supplemented with 2% (v/v) fetal calf serum (wash buffer). Red blood cells were then lysed using red cell lysis buffer (Sigma-Aldrich), according to the manufacturer's instructions, underlaid with fetal calf serum, and centrifuged at 443 $\times$ *g* for 5 minutes. Cell pellets were washed once more with wash buffer and cells counted. Brain mononuclear cells were isolated by digesting tissue as described above before passing through a 100-$\mu$m sieve and washing twice with wash

buffer. The cell pellet was resuspended in 33% (v/v) Percoll and centrifuged at 693 × *g* for 12 minutes at room temperature. Supernatant containing debris was removed, and the leukocyte pellet was washed once in wash buffer, depleted of red blood cells as described above, underlaid with fetal calf serum, and centrifuged at 443 × *g* for 5 minutes. Cell pellets were washed once more with wash buffer and cells counted. Peripheral blood leukocytes were prepared from heparinized blood that was depleted of red blood cells by three or four treatments with red cell lysis buffer (Sigma-Aldrich) according to the manufacturer's instructions.

### *Flow Cytometric Analysis*

For the staining of cell surface antigens, cells were incubated with fluorochrome-conjugated or biotinylated mAbs on ice for 30 minutes followed by Alexa Fluor 488-streptavidin incubation for an additional 30 minutes. Intracellular staining for Foxp3 was performed on fixed/permeabilized cells using PE-labeled anti-mouse Foxp3 kit (eBioscience), according to the manufacturer's instructions. Data were acquired on a FACSCalibur flow cytometer and analyzed using Cell Quest Pro software (BD Biosciences). Cell populations in the spleen and brain were defined as follows: $CD4^+$ T cells ($CD4^+TCR^+$), $CD8^+$ T cells ($CD8^+TCR^+$), B cells ($B220^+CD19^+$), neutrophils ($CD11b^+Ly6G^+$), macrophages/monocytes ($CD11b^+Ly6C^+$), dendritic cells (DC; $CD11c^{hi}$), NK cells ($NK1.1^+TCR^-$), NK T cells (CD1d $\alpha$GalCer tetramer$^+$ $NK1.1^+$), and microglia ($CD45^{intermediate\ (int)}CD11c^+$ or $CD11b^+$). Cytokines in serum samples collected 5 days p.i. were quantified using the cytometric bead array (CBA) inflammatory kit (BD Biosciences) on a FACScan cytometer equipped with Cell Quest Pro and CBA software (BD Biosciences).

### *$CD25^+$ T-Cell Depletion*

$CD25^+$ T-cell depletion was performed by intraperitoneal (i.p.) injection of 0.5 mg of anti-CD25 mAb (PC61) 1 day or 14 days before PbA infection. The efficacy of CD25 depletion was confirmed by fluorescence-activated cell sorting (FACS) analysis using anti-CD4, anti-CD25, and anti-*Foxp3* antibodies.

### *IFN$\gamma$ ELISPOT*

$CD4^+$ and $CD8^+$ T cells were positively selected from RBC-depleted splenocytes using magnetic activated cell sorting according to protocols recommended by the manufacturer of the metallo-conjugated anti-CD4 and anti-CD8 antibodies and positive selection columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells isolated by this procedure were greater than 98% pure as assessed by FACS. The IFN$\gamma$ ELISPOT was performed as previously described.[18]

### In Vivo *Bioluminescence Imaging*

Luciferase-expressing PbA pRBCs were visualized by imaging whole bodies or dissected organs with an I-CCD photon-counting video camera and *in vivo* imaging system (IVIS 100; Xenogen, Alameda, CA). On day 5 p.i., when ECM symptoms were observed in infected control animals, mice were anesthetized with fluorothane and injected subcutaneously with 0.1 ml of 5 mg/ml D-luciferin firefly potassium salt (Xenogen). Images were then captured on the IVIS 100 according to the manufacturer's instructions. Parasites were visualized in the brain after removal from mice that had been perfused with 20 ml of saline via the heart. Bioluminescence generated by luciferase transgenic PbA in mice or brain tissue was measured according to the manufacturer's instructions using the same regions of measurement for all samples being compared. The unit of measurement was photons/second/$cm^2$/steer radiant (p/sec/$cm^2$/sr).

### *Immunohistochemistry*

ICAM-1 and VCAM-1 staining was conducted on 6-$\mu$m acetone-fixed brain sections, and primary antibodies were detected with appropriate secondary detection reagents and horseradish peroxidase according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). Sections were dehydrated and mounted before microscopic examination. These sections were then used to count ICAM-1- and VCAM-1-positive vessels in 25 consecutive microscopic fields of view at a final magnification of 400×.

### *Real-Time Reverse Transcriptase-Polymerase Chain Reaction*

Total RNA was extracted from the spleen using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and an RNeasy Mini Kit with on-column DNase digestion (Qiagen, Valencia, CA). RNA samples were reverse-transcribed into cDNA using the cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The number of IFN$\gamma$ and IL-10 cDNA molecules in each sample were calculated by using TaqMan gene expression assays (Applied Biosystems), and the number of HPRT (forward: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; reverse: 5′-GATTCAACCTTGCGCTCATCTTAGGC-3′) (housekeeping gene) cDNA molecules in each sample were calculated by real-time reverse transcription-polymerase chain reaction using Platinum SYBR Green Master Mix (Invitrogen Life Technologies). All reverse transcription-polymerase chain reactions were performed on a Corbett Research RG-3000 Rotor Gene (Corbett Life Sciences, Sydney, NSW, Australia). Standard curves were generated with known amounts of cDNA for each gene, and the number of cytokine molecules per 1000 HPRT molecules in each sample was calculated.

### *Statistical Analysis*

Differences in survival of treatment groups were analyzed using the Kaplan-Meier log-rank test. Differences in parasitemia, cytokine levels, and bioluminescence were analyzed using either the Mann-Whitney *U*-test or the Stu-

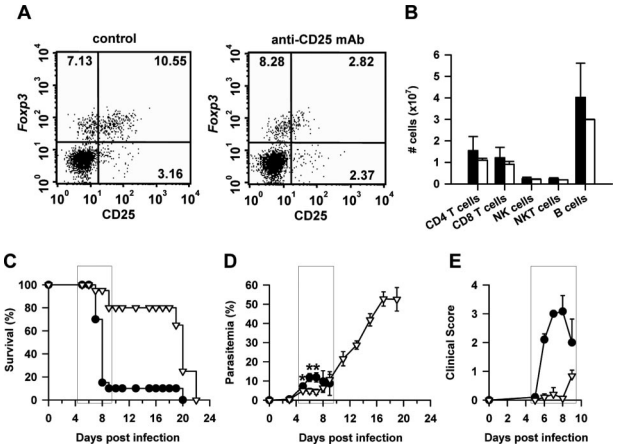


**Figure 1.** ECM is prevented in C57BL/6 mice lacking Treg cells. Mice were injected i.p. with 0.5 mg of anti-CD25 mAb or 0.5 mg of control mAb 1 day before infection with PbA. FACS profiles of CD25 and Foxp3 expression on splenic leukocytes gated on $CD4^+$ T cells from control mice or Treg-depleted mice, as indicated, are shown 1 day after antibody administration (**A**). Analysis of splenic $CD4^+$ T cell, $CD8^+$ T cell, NK cell, NK T cell, and B cell numbers from control mice (closed bars) or Treg-depleted mice (open bars) at the same time are also shown (**B**). Data are single representative samples (**A**) or mean ± SEM (**B**) of individual samples from five mice per treatment group. Results are from one representative experiment of three performed. Survival (**C**), parasitemia (mean ± SEM; $n = 5$ mice per group) (**D**), and clinical disease (mean score ± SEM; $n = 5$ mice per group) (**E**) were monitored in mice that received anti-CD25 mAb (open triangles) or control mAb (closed circles). Results in **C** represent data pooled from four separate experiments yielding similar results ($n = 20$ mice per treatment group), and the **open box** indicates the time when mice displayed ECM symptoms. Results in **D** and **E** are one representative experiment of four performed. Statistical differences of $^*P < 0.05$ are indicated.

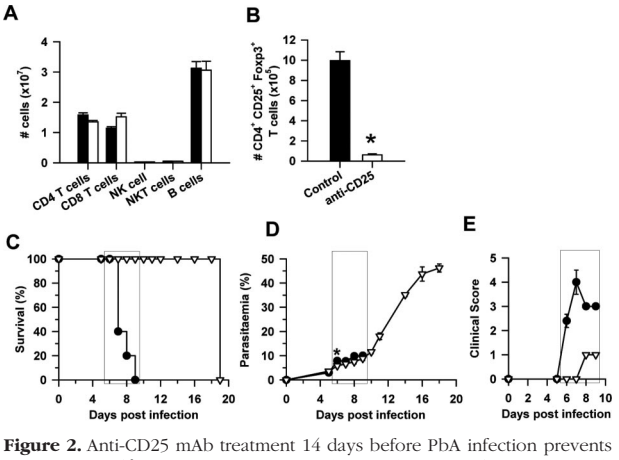


**Figure 2.** Anti-CD25 mAb treatment 14 days before PbA infection prevents ECM in C57BL/6 mice. Mice were injected i.p. with 0.5 mg of anti-CD25 mAb (open bars or open triangles) or 0.5 mg of control mAb (closed bars or closed circles) 14 days before infection with PbA. FACS analysis of splenic $CD4^+$, $CD8^+$, NK, NK T, and B cell numbers (**A**) as well as $CD4^+CD25^+Foxp3^+$ T cells (**B**) on the day of infection are shown. Data represent the mean ± SEM of individual samples from three mice per treatment group. Survival (**C**), parasitemia (mean ± SEM) (**D**), and clinical disease (mean score ± SEM) (**E**) were monitored. The **open box** in **C–E** indicates the time when mice displayed ECM symptoms. Data are from one representative experiment of four performed. Statistical differences of $^*P < 0.05$ are indicated.

dent's *t*-test where indicated. For all statistical tests, $P < 0.05$ was considered significant.

## *Results*

### *Mice Depleted of $CD4^+CD25^+Foxp3^+$ Treg Cells Do Not Develop ECM*

To establish whether natural Treg cells contribute to the development of ECM, we used an anti-CD25 mAb (PC61) to deplete $CD4^+CD25^+Foxp3^+$ Treg cells in C57BL/6 mice. Specific depletion of $CD4^+CD25^+Foxp3^+$ Treg cells and no other major lymphocyte population in the spleen was confirmed by flow cytometry 24 hours after antibody administration (Figure 1, A and B). Furthermore, we confirmed that $CD4^+CD25^+Foxp3^+$ Treg cells were depleted and had not simply down-regulated CD25 expression, as previously reported,[30] because there was no accumulation of $CD4^+CD25^-Foxp3^+$ Treg cells in mice receiving anti-CD25 mAb (data not shown). We also demonstrated that $CD4^+CD25^+Foxp3^+$ Treg cells were depleted by anti-CD25 mAb treatment in the lymph nodes, blood, and brain (see Supplemental Figure 1 at *http://ajp.amjpathol.org*). These cells remained at similar numbers in the spleen and lymph nodes up until the time that control animals died with ECM but increased over the course of infection in the blood and brain (see Supplemental Figure 1 at *http://ajp.amjpathol.org*).

Ninety percent of mice treated with an isotype control antibody the day before PbA infection developed severe neurological signs of ECM between days 6 and 9 p.i. and were subsequently sacrificed (Figure 1C). In contrast, mice depleted of $CD4^+CD25^+Foxp3^+$ Treg cells the day before infection showed a significant ($P < 0.001$) increase in survival (80% survival on day 18 p.i.) (Figure 1C) and a small but significant reduction ($P < 0.05$) in blood parasitemia at days 5, 6, and 7 p.i. compared with control-treated mice (Figure 1D). Clinical scores were also reduced in mice depleted of Treg cells (Figure 1E), and these animals developed no neurological signs of ECM and survived until 3 weeks after infection, when they developed hyperparasitemia (Figure 1D) and severe anemia (hemoglobin levels <20 g/L). Histological examination of the brain showed no cerebral hemorrhages in ECM-resistant mice that received anti-CD25 mAb, unlike in control PbA-infected animals (data not shown).

To exclude the possibility that anti-CD25 mAb might directly affect emerging antiparasitic immune responses, including the generation of inducible Treg cells, we next depleted Treg cells 14 days before PbA infection, a time period that allows continued Treg-cell depletion[31] as well as clearance of antibody from the blood[32] (see Supplemental Figure 2 at *http://ajp.amjpathol.org*). Specific depletion of $CD4^+CD25^+Foxp3^+$ Treg cells and no other major leukocyte population was confirmed by flow cytometry 13 days after anti-CD25 mAb administration and the day before infection (Figure 2, A and B), and we again demonstrated that $CD4^+CD25^+Foxp3^+$ Treg cells were depleted in the lymph nodes, blood, and brain using this anti-CD25 mAb treatment regime (see Supplemental Figure 1 at *http://ajp.amjpathol.org*).

Mice depleted of Treg cells 14 days before infection failed to develop ECM (Figure 2C), had significantly ($P < 0.05$) lower blood parasitemia on day 6 p.i. (Figure 2D), and had lower clinical scores (Figure 2E) compared with control mice. Again, these mice survived until the third

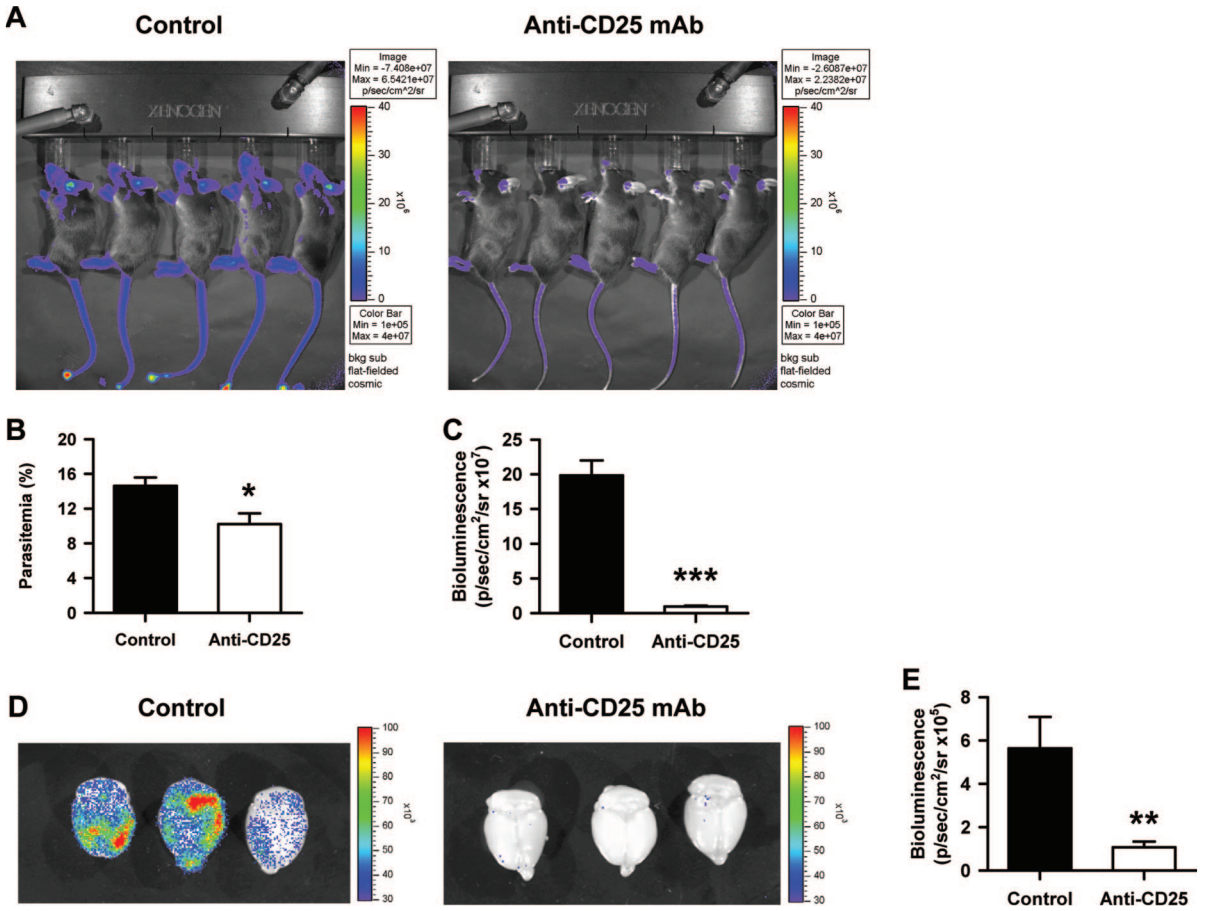


**Figure 3.** Reduced PbA accumulation in the vasculature and brain of mice lacking Treg cells. Mice were injected i.p. with 0.5 mg of anti-CD25 mAb or 0.5 mg of control mAb 14 days before infection with transgenic PbA expressing luciferase as indicated. Mice were injected with luciferin on day 5 p.i., and whole-body images recorded for 1 minute, 5 minutes after luciferin injection (**A**). Parasite burden determined by microscopic examination of blood smears (**B**; $n = 5$ mice per group) and bioluminescence (**C**; $n = 5$ mice per group) are shown. After whole-body imaging, mice were sacrificed and perfused, and brains were removed for recording images for 5 minutes, 1 hour after luciferin injection (**D**). Bioluminescence of brain tissue was also recorded (**E**; $n = 3$ mice per group). Data are from one representative experiment of two performed. Statistical differences of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are indicated.

week after infection with no signs of ECM and ultimately developed hyperparasitemia (Figure 2D) and severe anemia. Therefore, our data show that Treg-cell depletion 14 days before PbA infection results in the same outcome as Treg-cell depletion the day before infection and indicate a critical role for natural Treg cells, but not inducible Treg cells, in ECM pathogenesis.

## *Prevention of Cerebral PbA Accumulation in the Absence of Treg Cells*

Although small reductions in blood parasitemia were observed in the absence of Treg cells (Figures 1D and 2D), this may not reflect overall differences in parasite burden because of the ability of PbA to accumulate in vasculature and tissue. Therefore, to visualize the effect of Treg-cell depletion on parasite burden, we infected mice 14 days after anti-CD25 mAb treatment with a transgenic PbA line that constitutively expressed luciferase. Following injection of luciferin into mice at day 5 p.i., when ECM symptoms were present, parasites were observed as bioluminescence in extremities such as the tail, ears, nose, and foot pads, where blood vessels were close to the surface of the skin, as well as tissues such as the lungs and brain (Figure 3A). In this experiment, a small, but significant ($P < 0.05$), reduction in blood parasitemia was observed in mice depleted of Treg cells 14 days before infection, as determined by microscopic examination of blood smears (Figure 3B). However, when bioluminescence generated from parasites was measured, a much larger and highly significant reduction ($P < 0.001$) was observed in mice treated with anti-CD25 mAb compared with controls (Figure 3C). This indicated that parasite accumulation in tissue and vasculature was greatly reduced in mice depleted of Treg cells compared with controls. Furthermore, these data showed that parasite burdens in control animals were greatly underestimated, and differences in parasite burden between mice lacking Treg cells and controls were much greater than indicated by parasitemia values determined by microscopic examination of blood smears.

The presence of parasites in the brain is believed to contribute to CM,[4] although this is not always the case.[8] Therefore, to test if the accumulation of parasites in the brain was affected by the depletion of Treg cells, mice injected with luciferin were sacrificed and perfused with saline to remove all blood, leaving only pRBCs that had adhered to microvasculature or penetrated tissue via

hemorrhages. A striking and significant ($P < 0.01$) difference was observed in the bioluminescence emerging from parasites in the brains of control mice and those treated with anti-CD25 mAb following removal and imaging (Figure 3, D and E). Few parasites were visualized in the brains of mice lacking Treg cells. In contrast, random patterns of intense parasite accumulation were observed in brain tissue from control animals. The same results were obtained when mice were treated with anti-CD25 mAb 1 day before PbA infection (data not shown). Together, these data show that parasite accumulation in the brain was associated with the onset of ECM in C57BL/6 mice and that this process was prevented in the absence of Treg cells. In addition, blood parasitemia values in mice with ECM symptoms underestimated total parasite burden because of the accumulation of parasites in the brain, vasculature, and other tissue sites.

## *Alterations in Cellular Recruitment to the Brain in PbA-Infected Mice Depleted of Treg Cells*

The up-regulation of vascular cell adhesion molecules and the recruitment of leukocytes to the brain is a feature of ECM.[21,33] Therefore, we next assessed whether anti-CD25 mAb treatment 14 days before infection affected these markers of inflammation in the brain. The number of cerebral vessels expressing either VCAM-1 or ICAM-1, as determined by immunohistochemistry, was not altered between Treg-depleted mice and controls when the latter group was sacrificed with ECM (Figure 4, A and B), indicating that inflammation of the brain per se was not absent in mice treated with anti-CD25 mAb. The total number of leukocytes in the brains of both Treg-depleted mice and controls, when control mice were sacrificed with ECM, was also similarly increased (Figure 4C). Although numbers of $CD4^+$ T cells, B cells, NK cells, NK T cells, neutrophils, and DCs all increase in the brains of anti-CD25 mAb-treated mice and controls, they were not significantly different (data not shown). However, $CD8^+$ T-cell recruitment to the brain was significantly ($P < 0.01$) reduced in mice lacking Treg cells at this time (Figure 4D). In contrast, mice lacking Treg cells had significantly ($P < 0.05$) increased numbers of brain macrophages/monocytes (as determined by CD11b and Ly6C expression) compared with control animals (Figure 4E). Because microglia express CD11b and CD11c, we next tested whether the increase in macrophage/monocytes reflected an expansion of these cells in response to anti-CD25 mAb treatment. There was no increase in the numbers of $CD11b^+$ and $CD11c^+$ cells that expressed intermediate levels of CD45 (a characteristic of microglia[34,35]) in the absence of Treg cells, relative to control mice (data not shown), indicating that microglia were not affected by anti-CD25 mAb treatment and did not account for the increased number of $CD11b^+Ly6C^+$ cells in the brains of mice lacking Treg cells. The same results were also obtained when mice received the anti-CD25 mAb 1 day before PbA infection (see Supplemental Figure 3 at *http://ajp.amjpathol.org*). Together, these data indicate that cerebral inflammation was not prevented by the depletion of Treg cells but that cellular recruitment to the brain was selectively altered.

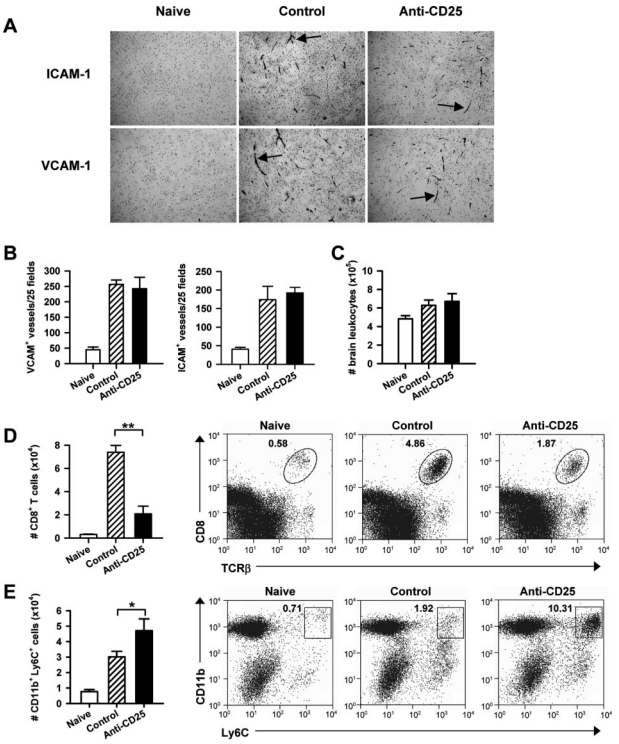


**Figure 4.** Changes in leukocyte recruitment to the brain of mice lacking Treg cells. **A:** VCAM-1 and ICAM-1 staining was performed on brain sections at ECM in control mice as indicated (antibodies administered 14 day before PbA infection). **Arrows** indicate cerebral vessels expressing adhesion molecules. **B:** The number of VCAM-1- and ICAM-1-positive vessels in the brains of mice injected i.p. with 0.5 mg of anti-CD25 mAb (closed bars) or 0.5 mg of control mAb (hashed bars) 14 days before PbA infection, as indicated, were determined when the latter group developed ECM and compared with expression in the brains of naïve mice (open bars). **C:** The total number of leukocytes in brain tissue was enumerated microscopically at the time of ECM in control-treated mice. FACS analysis of brain $CD8^+$ T cell (**D**) and macrophage/monocytes (**E**) at the same time are shown. Numbers above FACS gates indicate the percentage of gated cells. Data represent the mean ± SEM of individual samples in each group ($n$ = 3–5 mice/group) from one representative experiment of three performed. Statistical differences of $^*P < 0.01$ and $^{**}P < 0.001$ are indicated.

## *Enhanced T-Cell Activation in the Spleen and Lymph Nodes of PbA-Infected Mice Depleted of Treg Cells*

We next investigated whether Treg cells influenced the generation of immune responses during PbA infection. We first assessed the activation status of $CD4^+$ and $CD8^+$ T cells in mice treated with anti-CD25 mAb, relative to control animals, at the time of infection (day 0), when ECM symptoms were first detected (day 5 p.i.) and when control animals died with ECM (day 7 p.i.). Following Treg cell-depletion either 14 days (Figure 5) or 24 hours (see Supplemental Figure 4 at *http://ajp.amjpathol.org*) before infection, we found increased numbers of activated $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes at day 7 p.i., based on expression of CD69 (early activation marker identifying recently activated T cells). In contrast, there was either no difference or a decrease in the number of activated $CD4^+$ T

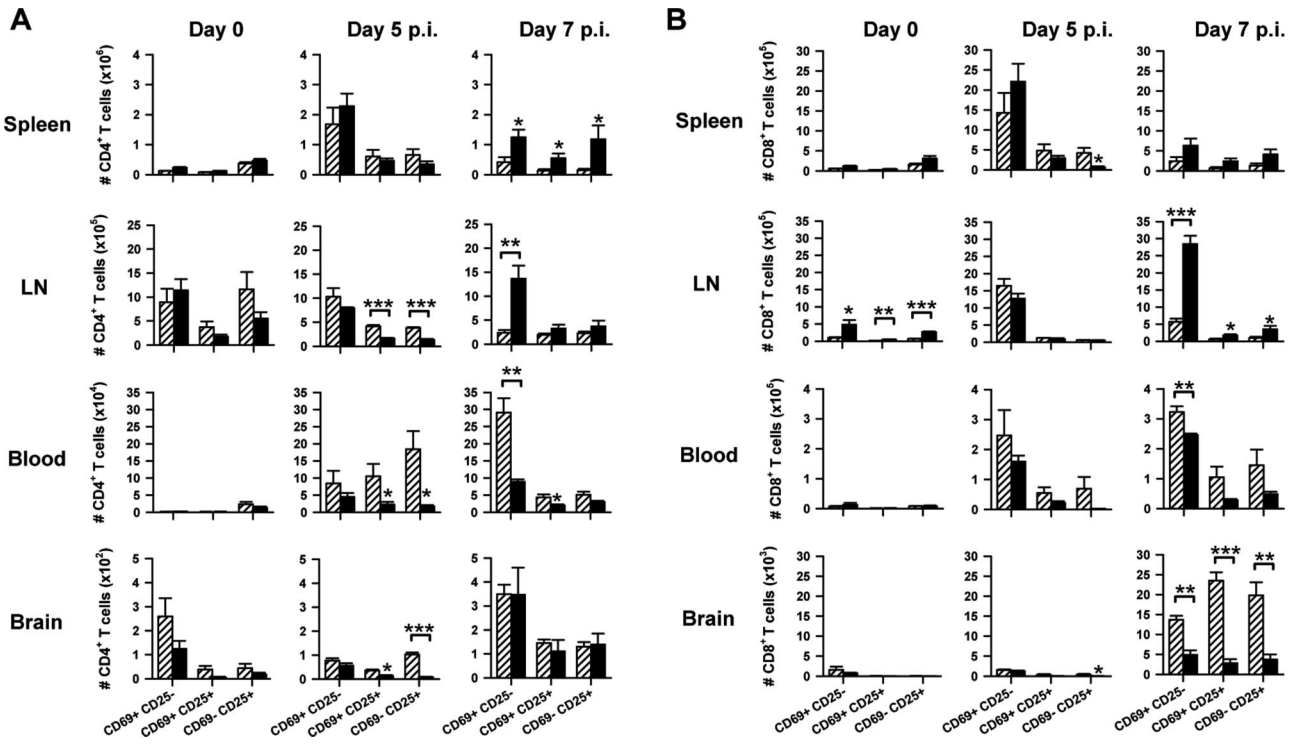


**Figure 5.** Increased T-cell activation in the absence of Treg cells during PbA infection. Mice were injected i.p. with 0.5 mg of anti-CD25 mAb (closed bars) or 0.5 mg of control mAb (hashed bars) 14 days before infection with PbA. The number (mean ± SEM) of $CD4^+$ T (**A**) and $CD8^+$ T (**B**) cells expressing CD25 and CD69 as indicated in the spleen, lymph nodes (LN), blood and brain were measured by FACS on the day of PbA infection (day 0) and days 5 and 7 p.i. These data are from three individual mice per group and are one representative experiment of two performed. Statistical differences of $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are indicated.

cells and $CD8^+$ T cells in the blood and brain at this time point (Figure 5). When CD25 expression was examined, similar patterns of expression emerged (Figure 5), indicating that the protective effect of anti-CD25 mAb administration did not result from the depletion of activated $CD25^+CD4^+$ or $CD25^+CD8^+$ T cells. These data also demonstrate that the reduction in $CD8^+$ T cells in the brains of mice depleted of Treg cells (Figure 4) was not simply caused by specific depletion of $CD25^+CD8^+$ T cells, because the number of $CD8^+CD25^-$ T cells in the brains of these mice was also reduced compared with control mice at day 7 p.i. (Figure 5).

## *Enhanced $CD4^+$ T-Cell IFNγ Production following PbA Infection in the Absence of Treg Cells*

IFNγ plays a role in ECM pathogenesis[15,16] but, paradoxically, is also required for efficient cell-mediated immunity during malaria.[36,37] Therefore, we next measured the number of splenic IFNγ-producing $CD4^+$ and $CD8^+$ T cells in mice lacking Treg cells (anti-CD25 mAb administered 14 days before infection) when control animals developed ECM symptoms (day 5 p.i.). The number of IFNγ-producing splenic $CD4^+$ T cells was significantly increased ($P < 0.01$) in Treg-depleted mice compared with control-treated mice (Figure 6A). There was no difference in the number of IFNγ-producing splenic $CD8^+$ T cells between treatment groups (Figure 6A). This did not reflect a general increase in cytokine production, because even though anti-CD25 mAb treatment did increase serum levels of MCP-1 significantly ($P < 0.01$), there was no significant increase in tumor necrosis factor or IL-6 levels, and serum IL-10 levels were significantly decreased ($P < 0.05$) compared with control mice at 5 days p.i. (Figure 6B). In addition, the depletion of Treg cells did not alter IFNγ or IL-10 mRNA accumulation in the spleen at this time (Figure 6C). Again, similar results were observed when mice received anti-CD25 mAb the day before PbA infection (see Supplemental Figure 5 at *http://ajp.amjpathol.org*). Collectively, these results demonstrate that the protection afforded by anti-CD25 mAb treatment was not due to inadvertent depletion of activated $CD4^+$ T cells. On the contrary, $CD4^+$ T-cell and $CD8^+$ T-cell activation was increased, and $CD4^+$ T-cell IFNγ production was selectively enhanced in mice lacking Treg cells.

## *Resistance to ECM in the Absence of Treg Cells Requires IL-10*

IL-10 blockade has previously been shown to increase the incidence of ECM in resistant BALB/c mice, and administration of IL-10 to CBA mice was found to reduce ECM symptoms,[38] suggesting a role for IL-10 in protection against ECM. We also observe a small, but consistent, increase in time to death in C57BL/6 mice lacking IL-10 compared with C57BL/6 control mice (mean time to

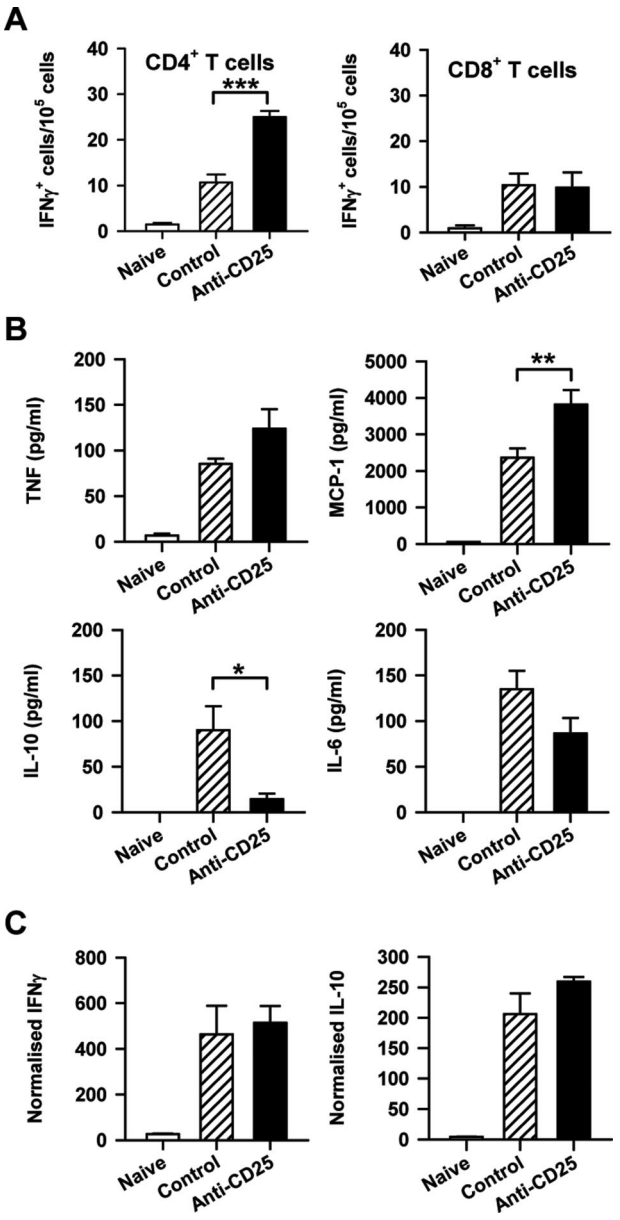


**Figure 6.** $CD4^+$ T-cell activation is enhanced in the absence of Treg cells resulting in improved IFNγ-dependent control of PbA. Mice were injected i.p. with 0.5 mg of anti-CD25 mAb (closed bars) or 0.5 mg of control mAb (hashed bars) 14 days before infection with PbA. The number (mean ± SEM) (**A**) of IFNγ-producing splenic $CD4^+$ and $CD8^+$ T cells were determined by ELISPOT 5 days after PbA infection and compared with naïve mice (open bars). These data are from three individual mice per group and are one representative experiment of two performed. Serum cytokine levels were measured 5 days p.i. (mean ± SEM of individual serum samples from five mice per treatment group) in naive mice, control mice and Treg cell-depleted mice, as indicated (**B**). Data are from one representative experiment of two performed. **C:** IFNγ and IL-10 mRNA levels were determined in spleen tissue taken from naive mice, control mice, and Treg cell-depleted mice at 5 days p.i. as indicated. IFNγ and IL-10 mRNA levels are presented relative to 1000 HPRT mRNA molecules (normalized) (mean ± SEM of individual serum samples from five mice per group). Statistical differences of $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ are indicated.

death 7 ± 0 days versus 7.8 ± 0.2 days, respectively). To determine whether IL-10 was necessary to prevent the development of ECM in the absence of Treg cells, IL-10-deficient mice were administered anti-CD25 mAb and then infected with PbA. Although ECM was significantly

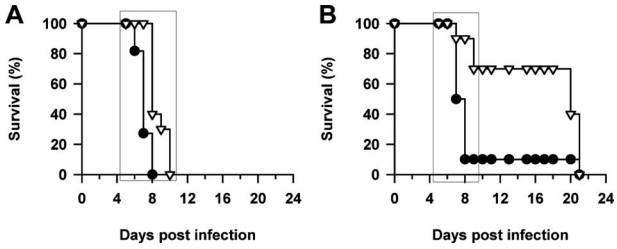


**Figure 7.** IL-10 is required for protection from ECM in the absence of Treg cells. B6.IL-$10^{-/-}$ mice (**A**) and C57BL/6 mice (**B**) were injected i.p. with 0.5 mg of control mAb (closed circle) or 0.5 mg of anti-CD25 mAb (open triangle) 1 day before infection with PbA and survival was monitored. Results represent data pooled from two separate experiments yielding similar results ($n$ = 10 mice per treatment group), and the **open box** indicates the time when mice displayed ECM symptoms.

($P < 0.01$) delayed by 24 to 48 hours in IL-10-deficient mice depleted of Treg cells compared with control IL-10-deficient mice (mean time to death 7.1 ± 0.4 days versus 8.7 ± 0.4 days, respectively), these animals still developed ECM (Figure 7A). In contrast, ECM was prevented ($P < 0.01$) in wild-type mice depleted of Treg cells as previously shown (Figure 7B). These results indicate a critical role for IL-10 in protection from ECM in the absence of Treg cells. The source of this IL-10 is unknown at present, but it is clearly not derived from a $CD4^+CD25^+Foxp3^+$ Treg cell. Furthermore, given the reduced levels of serum IL-10 in mice lacking Treg cells (Figure 6B) and no change in IL-10 mRNA accumulation in the spleens of these animals compared with control mice (Figure 6C), it is likely that the IL-10 required to ensure protection from ECM acts locally in specific tissue sites rather than systemically.

## *Depletion of Regulatory T Cells after PbA Infection Can Prevent CM*

Finally, to determine whether anti-CD25 mAb treatment had any therapeutic potential, PbA-infected C57BL/6 mice were administered this antibody at day 4 p.i., when blood parasitemia was readily detected. This resulted in a significant ($P < 0.05$) increase in survival, with 80% of mice surviving at day 20 p.i. (Figure 8). However, the therapeutic potential of anti-CD25 mAb treatment was

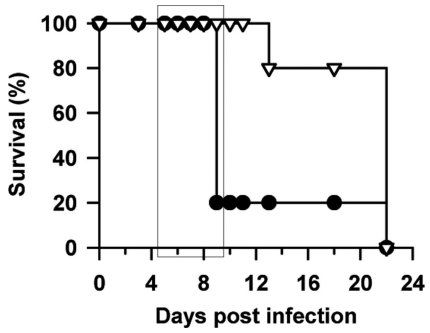


**Figure 8.** Depletion of Treg cells before onset of neurological symptoms prevents ECM in C57BL/6 mice. Mice were injected i.p. with 0.5 mg of anti-CD25 mAb (open triangle) or 0.5 mg of control mAb (closed circle) 4 days after infection with PbA ($n$ = 5 mice per group). A survival curve is shown, and the **open box** indicates the time when mice exhibited ECM symptoms. Data are from one representative experiment of two performed.

lost when initiated after the onset of neurological symptoms (data not shown).

## Discussion

In this study, we found that administration of anti-CD25 mAb before PbA infection resulted in the depletion of Treg cells, associated with reduced parasite load and protection from ECM. Imaging of PbA *in situ* (Figure 3, A and D) demonstrated that the overall parasite burden in control PbA-infected mice was much greater than indicated by blood smears due to accumulation of parasites in peripheral tissue sites and in the vasculature. Therefore, the effect of Treg depletion on parasite burdens was substantially greater than revealed from counting blood smears (Figures 1D and 2D). The difficulty in estimating the number of parasites sequestered in tissues has long hampered clinical investigations of malaria, because it is these parasites that are thought to be critical for disease pathogenesis.[39,40] Our data show that Treg depletion results in a major reduction in tissue parasites that may explain why these animals fail to develop ECM.

Recently, it was reported that treatment with anti-CD25 mAb protected mice from ECM when administered the day before and the day after PbA infection, but not when given 30 days before infection.[41] The protective effect of anti-CD25 mAb was attributed to the depletion of activated T cells, but this was not investigated. Our data indicate that the numbers of activated $CD4^+$ and $CD8^+$ T cells in PbA-infected mice increase significantly in several different tissue sites following anti-CD25 mAb treatment either 1 or 14 days before infection and that this enhanced T-cell activation allows improved control of parasitemia at a critical time in ECM pathogenesis, as well as reduced tissue accumulation and vascularization of pRBCs. One possible explanation that anti-CD25 mAb treatment 30 days before PbA infection failed to protect mice[41] is the rapid emergence of Treg cells after infection with this treatment regime. Recently, it was reported that Treg cells expand rapidly following *P. yoelli* infection,[42] and this is also found in PbA infection[41] (see Supplemental Figure 1 at *http://ajp.amjpathol.org*). Hence, the longer anti-CD25 mAb pretreatment period of 30 days in the previous study may have enabled the rapid emergence of Treg cells that prevented the enhanced T-cell activation and parasite control observed in our study. This remains to be tested experimentally.

Although the anti-CD25 mAb treatment reduced parasite burden significantly, it did not allow ultimate control of parasite growth. Instead, these animals go on to die with severe anemia and hyperparasitemia, as has been reported for all other mouse strains that are resistant to ECM induced by PbA, such as those deficient in IFN$\gamma$ receptor[16] and LT$\alpha$.[18] Several parasite-mediated changes to the host immune response may contribute to the failure of anti-CD25 mAb-treated mice to ultimately control PbA growth. First, parasite-specific $CD4^+$ T cells are depleted via an IFN$\gamma$-dependent mechanism after PbA infection but before elimination of parasites.[43] Second, $CD8^+$ DCs become nonresponsive after PbA infection, resulting in a failure to cross-prime $CD8^+$ T cells and possibly present parasite antigen to $CD4^+$ T cells.[44] Finally, in murine *P. chabaudi* infection, $CD8^-$ DCs become the major antigen presenting cell population later in infection.[45] If this change takes place during PbA infection and there are differences in the effectiveness of immune responses generated by these DCs, they may alter the ability of the host to control parasite growth. Together, these changes are likely to contribute to PbA escaping cell-mediated immune responses after the phase of cerebral malaria induction has passed in resistant mice. Although there has been little work on the role of antibody in this model, it is also possible that a failure to generate effective antiparasitic antibodies may also contribute to the failure to control PbA later in infection.

The depletion of Treg cells prevented ECM and the accumulation of parasites in the cerebral microvasculature, an event concomitant with many, but not all, human CM cases.[8] Because control-treated PbA-infected mice develop brain hemorrhages,[18] whereas mice depleted of Treg cells do not (data not shown), it is unclear whether parasite accumulation in the brains of control animals resulted from the trapping of parasites in hemorrhages, vascularization, or sequestration. In another study using PbA that expressed luciferase under the schizont-specific AMA-1 promoter, very few schizonts were sequestered in the brain during ECM.[29] The PbA line used in the current study expressed luciferase under the control of the ef1-$\alpha$ promoter that allows luciferase expression in all blood stages.[46] Therefore, it is likely that the intense bioluminescence observed in the brains taken from mice with ECM in our study arises from the accumulation of ring forms, trophozoites, and young schizonts in the hemorrhages associated with the cerebral vasculature of these animals. Nevertheless, the presence of detectable numbers of PbA pRBCs in the brain was always associated with the onset of ECM in our studies, and removal of Treg cells prevented this accumulation, possibly by preventing the formation of cerebral hemorrhages. It is increasingly recognized that CM is not a homogeneous condition but is a disease syndrome that comprises a number of pathological correlates and pathogenic processes.[47] The C57BL/6 ECM model seems to reflect some aspects of the human disease, including damage to the cerebral vasculature leading to perivascular hemorrhages. The prevention of microvascular endothelial cell damage in brain in the absence of Treg cells indicates a strong immunological involvement in this pathogenic process.

Both $CD4^+$ and $CD8^+$ T cells are involved in the pathogenesis of ECM[10–13] but are also required for the effective control of malaria parasites,[48] emphasizing the delicate balance that exists between host-mediated control of infection and disease development. $CD8^+$ T cells have been shown to sequester to the brain following PbA infection at the onset of cerebral symptoms[12,13] and are postulated to cause damage to the brain endothelium via the production of perforin.[13] We found reduced $CD8^+$ T-cell recruitment to the brains of PbA-infected mice depleted of Treg cells. This effect was not caused by a failure in $CD8^+$ T-cell activation in mice treated with anti-

CD25 mAb, because CD69 expression was increased and there was no reduction in IFNγ production compared with $CD8^+$ T cells from control-treated animals after PbA infection. In addition, the increase in $CD25^+CD8^+$ T cells in the spleen and lymph nodes at day 7 p.i. in mice lacking Treg cells demonstrates that the anti-CD25 mAb was not simply depleting these cells. This conclusion is also supported by the reduction in $CD25^-CD8^+$ T cells in the brains of Treg cell-depleted mice compared with controls, further confirming that there was no selective effect on $CD25^+CD8^+$ T cells. Therefore, Treg cells seem to play an important role in establishing the immune conditions necessary for the recruitment of $CD8^+$ T cells to the brain during ECM. This recruitment mechanism is currently under investigation.

The influence of Treg cells on $CD8^+$ T-cell recruitment to the brain during ECM was selective as the recruitment of no other major leukocyte population was reduced in the absence of Treg cells. The reduced accumulation of $CD8^+$ T cells in the brain of PbA-infected mice lacking Treg cells could either result from changes in the initial priming of parasite-specific $CD8^+$ T cells after infection or subsequent recruitment to the brain. The degree of $CD8^+$ T-cell trafficking following *Listeria monocytogenes* infection has been shown to depend on the length of antigen stimulus during the early stages of infection,[49] suggesting that early activation of $CD8^+$ T cells following PbA infection in the absence of Treg cells could alter the tissue homing properties of these cells. In addition, parasites or parasite products might induce local tissue changes in the brain to induce $CD8^+$ T-cell accumulation. Studies to investigate these possibilities are underway.

Importantly, the decrease in $CD8^+$ T-cell recruitment did not reflect a general reduction in brain inflammation in mice depleted of Treg cells, because total brain leukocyte accumulation and increased expression of ICAM-1 and VCAM-1 on cerebral vascular endothelium was not altered relative to control mice. Furthermore, the number of macrophage/monocytes ($CD11b^+Ly6C^+$) in the brain of mice that received anti-CD25 mAb was increased compared with control mice at the onset of ECM in the latter group. The increase in this cell population was unlikely to be caused by a relative increase due to reduced numbers of $CD8^+$ T cells because the numbers of no other leukocyte population in the brain increased in a similar way. Macrophages have potential anti-inflammatory activity and the ability to reduce pathology caused by infection.[50] However, whether the accumulation of these cells in the brain of mice lacking Treg cells contributes to survival is unknown at this time. Nevertheless, these data do suggest selective changes to the leukocytes recruited to the brain in PbA-infected mice depleted of Treg cells.

IFNγ has been shown to play a key role in ECM pathogenesis.[15,16] Human $CD4^+$ T cells are a significant source of IFNγ after exposure to *P. falciparum* antigens,[51] and IFNγ production by peripheral blood mononuclear cells in response to liver-stage or blood-stage antigens is associated with resistance to *P. falciparum* infection and disease.[36,37] Recently, elevated plasma IFNγ levels and the presence of IFNγ gene polymorphisms involved in increased gene transcription were also found to be associated with protection from CM in African children.[52] These data again reinforce the delicate balance that determines whether host immune factors such as IFNγ promote antiparasitic immunity or mediate disease pathology during malaria. The data in our study indicate that IFNγ can either promote or inhibit ECM, depending on the specific cellular interaction occurring in the host after PbA infection and the timing of this interaction. In particular, the presence of Treg cells following PbA infection skews the role of IFNγ from antiparasitic to pathogenic.

The resistance to ECM in mice depleted of Treg cells was also dependent on the presence of the regulatory cytokine IL-10. However, mice lacking Treg cells had reduced levels of serum IL-10 (Figure 6B) and no change in IL-10 mRNA accumulation in the spleen (Figure 6C) after PbA infection. Therefore, it is likely that IL-10 is produced and acts in very specific tissue locations to prevent ECM. The source of this IL-10, the relevant tissue where it is produced, and the target of its activity are currently under investigation. A recent study has shown that transforming growth factor-β production and the generation of inducible Treg cells following *P. falciparum* infection suppresses antiparasitic immunity.[22] Natural Treg cells but not inducible Treg cells were involved in ECM pathogenesis, as indicated by our studies that depleted Treg cells 14 days before infection. However, the data do not exclude the possibility that inducible Tregs could contribute to protection from ECM, potentially via production of regulatory cytokines such as IL-10. Alternatively, conventional parasite-specific $CD4^+$ T cells may be a critical source of this cytokine, as recently reported in experimental models of cutaneous leishmaniasis[53] and toxoplasmosis.[54] The ability of proinflammatory cytokines such as IFNγ and regulatory cytokines like IL-10 to either promote or protect from severe malaria are likely to depend on many factors, including host and parasite genetics and the immune status of an infected individual. Identifying the source of these cytokines during malaria infection will be an important first step in defining the cellular targets that either suppress antiparasitic immunity or protect from developing pathology. The specific tissue microenvironments where cytokine production and responses occur during malaria will also have a major impact on disease outcome.

The IL-2 receptor comprises the CD25 molecule and has been found on the myelin sheath of the mouse central nervous system.[55] Up-regulation of CD25 has also been reported in the brain tissue of mice after lipopolysaccharide stimulation.[56] Furthermore, IL-2 can modulate the function and behavior of neurons, glia, and oligodendrocytes *in vitro*.[57] In fact, there has been a report that anti-CD25 mAb could ameliorate the signs and symptoms of multiple sclerosis, although the mechanism responsible for this effect was not reported.[58] Therefore, one possibility for the lack of brain pathology in the current study was that the administration of anti-CD25 mAb had direct effects on the brain following PbA infection. However, we believe this explanation is unlikely because mice depleted of Treg cells 14 days before infection did

not develop ECM. In these naïve mice, antibody would be unable to cross the blood-brain barrier,[59] and it is unlikely that anti-CD25 mAb would be available when the mice were infected[32] (see Supplemental Figure 2 at *http://ajp.amjpathol.org*). In addition, given that mice depleted of Treg cells displayed no breakdown in the blood-brain barrier as discussed above, no residual antibody would be able to cross this barrier following infection. Furthermore, we observed no difference in microglia numbers in the brain of control and anti-CD25 mAb-treated mice. Hence, the most likely cause of mice failing to develop ECM in the absence of Treg cells was an effect on the evolving host immune response to the parasite.

Finally, we demonstrated that the depletion of Treg cells has therapeutic potential for preventing CM when blood parasitemia is detected in blood smears if conducted before the onset of CM symptoms. Reagents for such a therapy in humans are already in clinical use. However, the use of such a therapy in humans with severe malaria would need to consider the potential for Treg depletion to modulate pre-existing host immunity[26] and break immunological tolerance to self-antigens.[24] Interestingly, mice that were depleted of Treg cells before the onset of ECM symptoms but after PbA infection had been established, had no reduction in blood parasitemia compared with control animals (data not shown). This suggests that anti-CD25 mAb therapy may directly modulate host mediators of pathology when administered after the establishment of infection.

In conclusion, we found that Treg-cell depletion in mice with anti-CD25 mAb resulted in protection from ECM. This protection was associated with improved $CD4^+$ and $CD8^+$ T-cell activation, reduced accumulation of PbA in the vasculature and brain, and selective changes to cerebral leukocyte recruitment. The results in this study highlight the delicate balance that exists between immunity and pathology during malaria, and have important implications for understanding the pathogenesis of CM and the development of vaccines and therapies to prevent severe malaria. Significantly, this is the first report showing that Treg cells can contribute to pathogenesis during infectious disease by suppressing antiparasitic immunity.

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