

Macrophage-Mediated but Gamma Interferon-Independent Innate Immune Responses Control the Primary Wave of *Plasmodium yoelii* Parasitemia[∇]

Kevin N. Couper,¹ Daniel G. Blount,¹ Julius C. R. Hafalla,¹ Nico van Rooijen,²
J. Brian de Souza,^{1,3} and Eleanor M. Riley^{1*}

Immunology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom¹; Department of Molecular Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands²; and Department of Immunology and Molecular Pathology, University College London Medical School, 46 Cleveland Street, London W1T 4JF, United Kingdom³

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In most models of blood-stage malaria infection, proinflammatory immune responses are required for control of infection and elimination of parasites. We hypothesized therefore that the fulminant infections caused in mice by the lethal strain of *Plasmodium yoelii* (17XL) might be due to failure to activate a sufficient inflammatory response. Here we have compared the adaptive CD4⁺ T-cell and innate immune response to *P. yoelii* 17XL with that induced by the self-resolving, nonlethal strain of *P. yoelii*, 17X(NL). During the first 7 to 9 days of infection, splenic effector CD4⁺ T-cell responses were similar in mice with lethal and nonlethal infections with similar levels of activation in vivo and equivalent proliferation in vitro following mitogenic stimulation. Nonspecific T-cell hyporesponsiveness was observed at similar levels during both infections and was due, in part, to suppression mediated by CD11b⁺ cells. Importantly, however, RAG^{-/-} mice were able to control the initial growth phase of nonlethal *P. yoelii* infection as effectively as wild-type mice, indicating that T cells and/or B cells play little, if any, role in control of the primary peak of parasitemia. Somewhat unexpectedly, we could find no clear role for either NK cells or gamma interferon (IFN- γ) in controlling primary *P. yoelii* infection. In contrast, depletion of monocytes/macrophages exacerbated parasite growth and anemia during both lethal and nonlethal acute *P. yoelii* infections, indicating that there is an IFN- γ -, NK cell-, and T-cell-independent pathway for induction of effector macrophages during acute malaria infection.

Proinflammatory cytokines are essential for the control of intraerythrocytic *Plasmodium* infections in humans and in experimental animals (23, 25, 54). Antigen-presenting cells (APCs) produce interleukin 12 (IL-12), IL-15, IL-18, and tumor necrosis factor alpha (TNF- α) (17, 26, 31, 32, 37, 38, 40, 44, 45, 48), which can rapidly activate cells of the innate immune system to produce gamma interferon (IFN- γ) (31, 32, 44, 64). After several days (around 5 days in mice), IFN- γ from $\alpha\beta$ T cells begins to dominate the response. It has been reported that NK cell-derived IFN- γ contributes to the early control of *Plasmodium chabaudi*, *Plasmodium berghei*, and *Plasmodium yoelii* infections (6, 10, 21, 30, 31) and that NK cell depletion enhances the virulence of nonlethal *P. yoelii* and *P. chabaudi* infections (6, 20, 31), although the latter finding has recently been debated (42). Conversely, $\gamma\delta$ T cells play only a minor role in the control of the acute primary wave of malaria infection, as TCR $\gamma\delta$ ^{-/-} or $\gamma\delta$ T-cell-depleted mice develop comparable peak parasitemia with little or no delay in parasite clearance (24, 43). However, mice deficient in both B cells and $\gamma\delta$ T cells develop significantly higher levels of parasitemia, which takes longer to clear, than B-cell or $\gamma\delta$ T-cell single deficient animals (46, 55), indicative of some redundancy in the protection afforded by these two cell types.

CD4⁺ $\alpha\beta$ ⁺ T cells are critically required for effective clearance of parasitized red blood cells (pRBC), as evidenced by impaired parasite clearance leading to chronic infection and, ultimately, death among athymic and T-cell-deficient mice during infection with a number of malaria parasite species and strains (1, 5, 24, 27, 43, 61). Moreover, adoptive transfer of naive CD4⁺ T cells to nude mice can confer resistance to malaria infection (4), and adoptive transfer of malaria-specific effector CD4⁺ T cells can significantly reduce primary parasitemia (2, 3). In contrast, CD8⁺ T cells do not contribute significantly to anti-blood-stage immunity (43, 59).

The observation that primary peak parasitemia in T-cell-deficient mice tends to be comparable to or lower than that of wild-type (WT) mice (10, 27, 42) is consistent with exacerbation of NK cell function in the absence of T cells, perhaps reflecting compensation for lack of T- and B-cell-mediated effector mechanisms and lack of homeostatic control from regulatory T cells (13, 32). Exacerbation of innate immune responses in T-cell-deficient mice limits their utility for assessing the natural role of innate immune mechanisms; furthermore, studies in T-cell-deficient mice cannot address the possibility that induction of Th2 or regulatory T-cell responses, rather than lack of protective Th1 responses, may prevent control of virulent malaria infections.

By comparing the immune responses of C57BL/6 mice to two closely related strains of a rodent malaria parasite, we have previously shown that very rapid induction of anti-inflammatory cytokines (IL-10 and transforming growth factor β [TGF- β]) by the rapidly lethal 17XL strain of *P. yoelii* (PyL) is correlated with the inability to control and clear parasites and

* Corresponding author. Mailing address: Immunology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom. Phone: (44) 207 927 2706. Fax: (44) 207 927 2807. E-mail: eleanor.riley@lshtm.ac.uk.

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that this situation can be reversed, in part, by neutralization of these two cytokines (35, 36). In contrast, infection with the self-resolving, nonlethal *P. yoelii* 17X(NL) (PyNL) is characterized by an early IFN- γ and TNF- α response which is believed to contribute directly to parasite clearance (6, 10, 35, 36). Using a similar model, Hisaeda et al. (15) have reported that depletion of naturally occurring regulatory T cells by treatment of mice with anti-CD25 antibody prior to infection with PyL allows the infection to be resolved. Although this latter observation has not yet been confirmed by other investigators, it would be consistent with suppression of protective type 1 immune responses by IL-10- or TGF- β -producing regulatory T cells. Since both innate and adaptive immune responses are vulnerable to suppression by regulatory T cells and anti-inflammatory cytokines, we have carried out a detailed comparison of the cellular immune response to these two strains of *P. yoelii* in order to determine the effector mechanisms by which this parasite can be controlled.

Somewhat surprisingly, no significant differences in ex vivo CD4⁺ T-cell activation, polarization, proinflammatory cytokine production, or mitogen-induced T-cell proliferation were observed between mice with lethal and nonlethal infections. Furthermore, RAG^{-/-}, NK cell-depleted, and IFN- γ ^{-/-} mice controlled the primary wave of malaria parasitemia as well (or as badly) as WT mice did, suggesting that the major mechanism of parasite control during primary *P. yoelii* infections is IFN- γ independent and thus very different from that previously described for *P. chabaudi* or *P. berghei* infections. Instead, using a macrophage depletion model, we demonstrate a significant role for monocytes/macrophages in controlling the primary wave of parasitemia in both lethal and nonlethal *P. yoelii* infections.

MATERIALS AND METHODS

Mice and parasites. C57BL/6 WT mice, IFN- γ ^{-/-}, and RAG-1^{-/-} (fully backcrossed to C57BL/6 mice) were bred in-house or purchased from Harlan UK Ltd. (Oxon, United Kingdom). Mice were maintained under specific-pathogen-free conditions and used between 7 and 9 weeks of age. Male and female mice were used in different studies. Preliminary experiments showed that there were no significant sex-dependent differences in the outcome of infection.

Cryopreserved *Plasmodium yoelii* 17X(NL) (PyNL) (nonlethal) and *P. yoelii* 17XL (PyL) (lethal) parasites were passaged once through mice before being used in experimental animals. All infections were performed by intravenous (i.v.) injection of 10⁴ pRBC. Uninfected control mice received an equal number of uninfected RBC. Parasitemia was determined daily for the first 7 days of infection and then every other day. Body weight and erythrocyte numbers (Z2 Coulter particle counter; Beckman Coulter, Miami, FL) were assessed every other day.

In vitro restimulation assays. Single-cell suspensions of spleen cells were prepared by homogenization through a 70- μ m cell strainer (BD Biosciences), and live cells were enumerated by trypan blue exclusion. Cells were stimulated with concanavalin A (ConA) (1 μ g/ml), PyL or PyNL lysate (both at 1 \times 10⁶ pRBC equivalents/ml), or uninfected RBC lysate (1 \times 10⁶ RBC/ml) or given no stimulation. For flow cytometric analysis, 1 \times 10⁶ live cells were added to each well of a 48-well tissue culture plate in 1 ml culture medium (RPMI 1640 plus 1% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin), incubated at 37°C in 5% CO₂ for 18 h, and then aspirated and scraped from the plate, washed, and incubated with appropriate fluorochrome-labeled antibodies. For proliferation experiments, 5 \times 10⁵ live cells were added to each well of a 96-well flat-bottomed tissue culture plate in a final volume of 200 μ l and incubated at 37°C with 5% CO₂ for 60 h. One hundred microliters of culture supernatant was collected for cytokine analysis, and cells were pulsed with [*methyl*-³H]thymidine (TRK120; Amersham, United Kingdom) at 0.5 μ Ci/well and incubated for a further 12 h before thymidine incorporation was assessed by liquid scintillation counting.

Cell sorting. Splenocytes were incubated with Fc block and fetal calf serum. CD4⁺ T cells were then positively selected using anti-mouse CD4-conjugated midiMACS beads (Miltenyi Biotec, Germany; 1 μ l beads per 10⁶ CD4⁺ cells). CD11b⁺ cells were positively selected by incubation with phycoerythrin-labeled anti-CD11b antibodies (M1/70; rat IgG2b; eBioscience) and anti-phycoerythrin-conjugated midiMACS beads (Miltenyi Biotec; 2 μ l beads per 10⁶ CD11b⁺ cells). Cells were washed before being added to LS positive selection columns (Miltenyi Biotec). Column-bound cells were eluted, and cell purity was checked by flow cytometry.

Flow cytometry. The activation status and phenotype of cells were determined by flow cytometry using the following antibodies: anti-mouse CD4 (RM4-5; rat immunoglobulin G2a [IgG2a]; BD Biosciences), anti-mouse CD8 (53-6.7; rat IgG2a; BD Biosciences), anti-mouse CD25 (PC61; rat IgG1; eBioscience), anti-mouse CD44 (IM7; rat IgG2b; eBioscience), anti-mouse CD69 (H1.2F3; Armenian hamster IgG; eBioscience), anti-mouse Foxp3 (FJK-16S; rat IgG2a; eBioscience), anti-mouse major histocompatibility complex (MHC-II) (M5/114.15.2; rat IgG2b; eBioscience), and anti-mouse CD11c (N418; Armenian hamster IgG; eBioscience). Intracellular Foxp3 staining was performed by permeabilizing cells with phosphate-buffered saline (PBS) containing 0.1% saponin. For all staining, cells were concurrently incubated with anti-mouse CD16/32 (Fc block). Cell acquisition was performed using a FACSCalibur (BD Immunocytometry Systems), and analysis was performed using FlowJo (Treestar Inc., OR).

In vivo depletion of NK cells and macrophages. To examine the effect of NK cell depletion, animals were given anti-asialo GM-1 antibody (Wako Chemicals GmbH, Neuss, Germany; 30 μ l diluted to 200 μ l with PBS) intraperitoneally (i.p.) on days -1, +3, and +6 relative to infection with *P. yoelii*; this protocol was shown to lead to effective depletion of NK cells for at least 7 or 8 days post-treatment (results not shown). In separate experiments, macrophages were depleted by i.p. administration of 300 μ l of clodronate liposomes on days -2 and +3 relative to infection with *P. yoelii*. Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. It was encapsulated in liposomes as described earlier (57).

Quantitative reverse transcription-PCR. Whole spleen cells or purified CD4⁺ splenic T cells were obtained on selected days and snap-frozen in liquid nitrogen. RNA was extracted using RNAeasy mini kits (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. RNA was treated with DNase I, cDNA was reverse transcribed, and gene expression levels were determined by real-time PCR (TaqMan) using validated primer/probe sets for IFN- γ , IL-10, TGF- β , TNF- α , T-bet, GATA-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sets designed by Applied Biosystems, CA) and an ABI Prism 7000 sequence detection system (Applied Biosystems). Cytokine cDNA levels were normalized to levels of GAPDH.

Cytokine ELISA. Cytokine levels were determined by standard capture enzyme-linked immunosorbent assays (ELISAs). Rat anti-mouse IL-10 (JES5-2A5; rat IgG1; Mabtech, Sweden), rat anti-mouse IFN- γ (AN-18; rat IgG1; eBioscience), rat anti-mouse IL-12 (P40/P70) (C15.6; rat IgG1; eBioscience), and rat anti-mouse IL-2 (JES6-1A12; rat IgG2a eBioscience) antibodies, diluted in 0.5 M Tris-HCl buffer, pH 8.9, were used as capture reagents. Biotinylated rat anti-mouse IL-10 monoclonal antibody (Mab) (JES5-16E3; rat IgG2b; Mabtech), rat anti-mouse IFN- γ Mab (clone R4-6A2; rat IgG1; Mabtech), rat anti-mouse IL-12 (P40/P70) Mab (C17.8; rat IgG2a; eBioscience), and rat anti-mouse IL-2 (JES6-5H4; rat IgG2b; eBioscience) were used as detecting antibodies and were visualized using streptavidin-alkaline phosphatase (eBioscience) and *p*-nitrophenyl phosphate (Sigma Aldrich, United Kingdom). Absorbance was read at 405 nm using a MRX TC II microplate reader (Dynex Technologies Ltd., United Kingdom).

Statistical analysis. Statistical significance was calculated using Student's *t* test, unless otherwise stated, with results of *P* < 0.05 taken as significant.

RESULTS

C57BL/6 mice clear *P. yoelii* 17X (PyNL), but not *P. yoelii* 17XL (PyL) infection. Infection of C57BL/6 mice with 10⁴ PyL or PyNL pRBC led to patent parasitemia 3 days postinfection (p.i.). PyL infection resulted in a rapidly ascending parasitemia that approached 90% by 7 days p.i. (Fig. 1A) and was accompanied by acute anemia (Fig. 1B); infected mice became moribund on day 7 or 8 and were humanely killed (Fig. 1C). In contrast, during PyNL infection, parasitemia increased only very slowly, peaking at 30 to 50% on day 14 p.i. Although

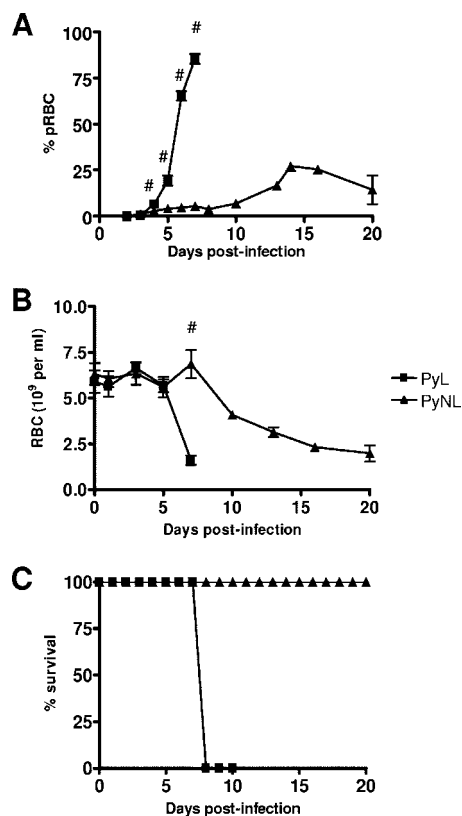


FIG. 1. Course of PyL and PyNL infections in C57BL/6 mice. C57BL/6 mice were infected i.v. with 10^4 lethal *P. yoelii* 17XL (PyL) or nonlethal *P. yoelii* 17X (PyNL) parasites and monitored for parasitemia (A), anemia (B), and survival (C). Groups consisted of four or five mice, and the results are representative of four independent experiments. Values that are significantly different ($P < 0.05$) in PyL- and PyNL-infected mice are indicated (#).

PyNL-infected mice eventually became severely anemic, mice eventually cleared the infection and recovered (data not shown). These observations are consistent with previous studies with these parasites in this mouse strain (6, 10, 36).

Failure to control PyL infection is not due to lack of CD4 T-cell responses. Although the very rapid progression of PyL infection has been linked to the erythrocyte invasion patterns of the parasite (18, 41), it is clear that the outcome of PyL infection is also influenced by the immune response of the host. In particular, triggering of early anti-inflammatory cytokine responses (IL-10 and TGF- β) (35, 36) and/or regulatory T-cell activity (15) may prevent infected animals from clearing the infection. The essential effector mechanisms that are inhibited by these early regulatory responses are not known but could be either innate or adaptive. We therefore began by determining whether differences in CD4⁺ T-cell responses during lethal and nonlethal *P. yoelii* infections could explain the observed differences in disease outcome. Spleen cells were obtained from PyL-infected and PyNL-infected and uninfected mice on days 1, 3, 5, and 7 p.i. and were analyzed immediately (ex vivo; Fig. 2) or were restimulated in vitro with the mitogen ConA (Fig. 3).

Significant activation of splenic lymphocytes was apparent as early as 3 days p.i. when more than 20% of freshly isolated

CD4⁺ T cells expressed the CD69 early activation marker (Fig. 2A). However, CD69 expression was transient, returning to baseline levels by day 5, and did not differ in PyL- and PyNL-infected animals. CD44 expression on CD4⁺ T cells increased more slowly and was maximal on day 7, but again, the expression levels did not differ in the PyL- and PyNL-infected mice (Fig. 2B). Transient upregulation of the high-affinity IL-2 receptor, CD25, occurred on effector (Foxp3⁻) cells on day 5 and was, again, similar in both groups of animals (Fig. 2C). We also calculated the total number of activated T cells per spleen and there was a suggestion that the total number of CD69⁺ (Fig. 2D) and CD44⁺ (Fig. 2E) CD4⁺ T cells was slightly higher in mice with nonlethal (PyNL) infections than in mice with lethal (PyL) infections, but reproducible and statistically significant differences, albeit minor, were observed only on day 5 p.i. Moreover, there were no significant differences in the numbers of CD25⁺ effector cells in the two groups of mice (Fig. 2F) and no evidence of skewing of the antigen specificity of the total T-cell pool (as determined by ex vivo analysis of CD69 and CD25 expression on CD4⁺ cells expressing 12 different T-cell receptor [TCR] V β chains [results not shown]).

As an additional test of the T-cell response, purified splenic CD4⁺ T cells collected at various time points p.i. were analyzed by real-time reverse transcription-PCR. No significant differences in the levels of transcripts for IFN- γ or TNF- α or for the Th1 and Th2 specific transcription factors T-bet and GATA-3, were observed between CD4⁺ cells isolated from lethal or nonlethal infection on either day 3, 5, or 7 p.i. (Fig. 2G).

Finally, to test for differences in T-cell effector function during the two infections, we assayed the ability of T cells from infected mice to respond to antigenic and mitogenic stimuli in vitro. One day p.i., splenic CD4⁺ T cells derived from both PyL- and PyNL-infected mice showed a modestly increased capacity both to proliferate (Fig. 3A) and to upregulate CD69 (Fig. 3B) in response to ConA compared to cells from uninfected mice. From 3 days p.i. onwards, and consistent with data from other murine malaria infections (34, 53), T cells from infected mice were markedly inhibited in their ability to upregulate CD69 or to proliferate in response to mitogen. The effect was polyclonal, as evidenced by inhibition of CD69 expression on cells expressing 12 different V β specificities (Fig. 3C), and not due to a decrease in the frequency of splenic CD4⁺ T cells (data not shown) but, importantly, was equally apparent in mice with either PyL or PyNL infection. No malaria-specific antigen recall response was observed by T cells derived from lethally or nonlethally infected mice at any time point examined (results not shown).

Thus, although marked changes in T-cell function are apparent during the course of both PyL and PyNL infections, the CD4⁺ T-cell response is remarkably similar in these two infections, with no significant delay, reduction in magnitude, or alteration in phenotype or function of the T-cell response in PyL-infected mice that might explain the increased virulence of the infection.

***P. yoelii*-induced T-cell hyporesponsiveness is both CD11b⁺ cell dependent and due to a T-cell intrinsic defect.** Although cells taken on day 1 p.i. were able to respond to ConA, responses in cells collected at later time points were markedly inhibited. To determine whether the lack of T-cell activation

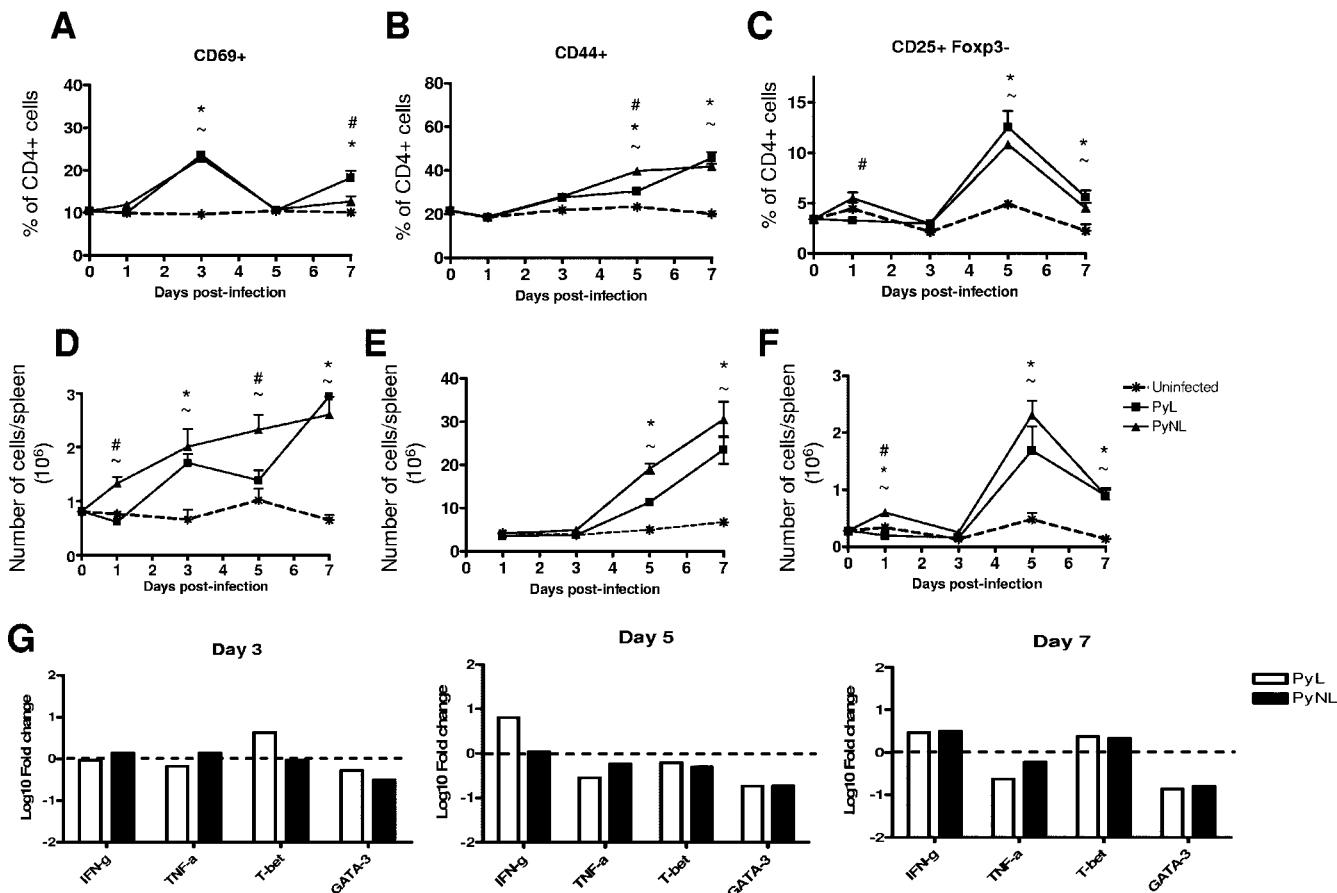


FIG. 2. Ex vivo splenic CD4 T-cell activation is comparable in PyL- and PyNL-infected mice. Mice were infected i.v. with PyL or PyNL parasites, and on selected days postinfection, the mice were sacrificed, and their spleens were removed to assess ex vivo T-cell activation. (A to F) The frequencies (A to C) and absolute numbers (D to F) of activated splenic CD4⁺ T cells were determined by expression of CD69 (A and D), CD44 (B and E), or CD25 (IL-2 receptor α chain) (C and F) in Foxp3⁻ cells. (G) On days 3, 5, and 7 p.i., CD4⁺ T cells were purified, and their phenotype was determined by real-time PCR (TaqMan). Expression levels were normalized to GAPDH and are presented as changes in the increase compared to CD4⁺ T cells from uninfected animals. Groups consisted of three to five mice, and the results are representative of three independent experiments. Symbols represent significant differences ($P < 0.05$) between groups: #, PyL-infected group versus PyNL-infected group; *, PyL-infected group versus uninfected group; ~, PyNL-infected group versus uninfected group. IFN-g, IFN- γ ; TNF-a, TNF- α .

was due to an inhibitory effect of antigen-presenting cells or to a T-cell intrinsic defect, we purified CD4⁺ T cells from the spleens of uninfected mice and PyL- and PyNL-infected mice 7 days p.i., labeled them with carboxyfluorescein succinimidyl ester (CFSE) to allow their subsequent identification (Fig. 4A), and cultured them with CD11b⁺ APCs purified from either uninfected or day 7 infected mice. The purified CD11b⁺ population was routinely 80 to 90% pure (Fig. 4B).

CD4⁺ T cells from uninfected animals were significantly impaired in their ability to upregulate CD25 following ConA stimulation when cocultured with CD11b⁺ APCs from *P. yoelii*-infected mice rather than CD11b⁺ cells from uninfected mice (Fig. 4C), suggesting that APCs derived from infected mice negatively regulate CD4⁺ T-cell activation. However, there was no difference in the ability of APCs from PyL- or PyNL-infected mice to support T-cell activation. In contrast, when cocultured with APCs from uninfected mice, ConA-induced CD25 upregulation was significantly impaired in CD4⁺ T cells from PyL-infected animals but not in CD4⁺ cells from PyNL-infected mice (Fig. 4C), indicating that there are defects

in both APCs and T cells from *P. yoelii*-infected animals and that the T-cell intrinsic defect is more marked in animals with lethal infections. Similar results were obtained for CD69 upregulation following ConA stimulation and also for T-cell activation by plate-bound anti-CD3/anti-CD28/IL-2, indicative of a generalized intrinsic defect in T-cell responsiveness (data not shown).

Since surface expression of CD25 and CD69 do not necessarily reflect the effector function of T cells, we also measured IFN- γ , IL-12, and IL-10 in the supernatants of ConA-stimulated APC-T-cell cocultures. CD4⁺ T cells from uninfected animals cocultured with CD11b⁺ cells from uninfected animals produced high levels of IL-2 (lower limit of detection, 0.08 ng/ml) following stimulation with ConA, but IL-2 production was almost completely ablated when the CD11b⁺ cells were derived from *P. yoelii*-infected animals (Fig. 4D). Furthermore, IL-2 production by CD4⁺ T cells from PyL-infected animals was significantly lower than that from CD4⁺ T cells of uninfected animals, even in the presence of CD11b⁺ cells from uninfected animals. In contrast, infection-derived CD11b⁺

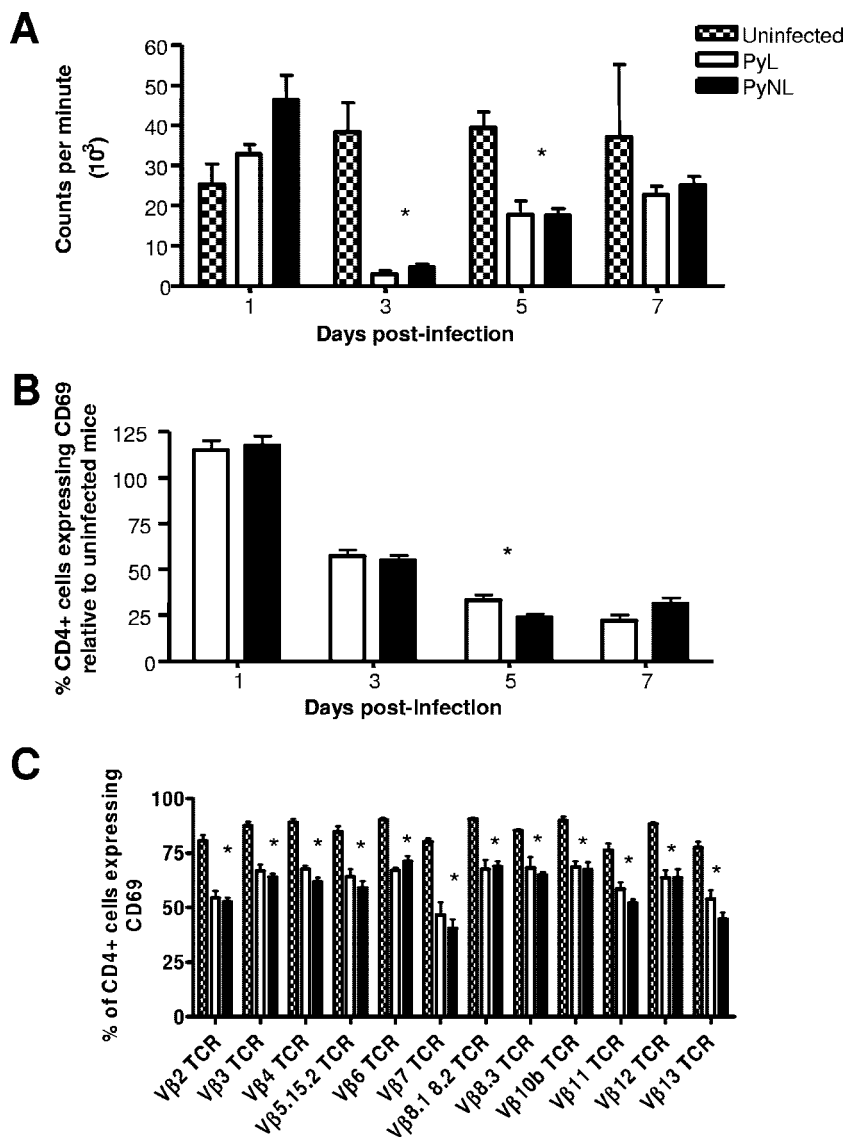


FIG. 3. Comparable responses of in vitro-restimulated splenic CD4 T cells from PyL- and PyNL-infected mice. On selected days postinfection, splenocytes were isolated and restimulated in vitro with ConA and analyzed after 72 h for proliferation (A) and after 18 h for CD69 upregulation on all CD4⁺ T cells (B) or CD4⁺ T cells expressing specific TCR V β genes (C). Groups consisted of three to five mice, and the results are representative of two independent experiments. Symbols represent significant differences ($P < 0.05$) between groups: *, infected (PyL and PyNL) versus uninfected groups in panels A and C; *, PyL-infected group versus PyNL-infected group in panel B.

cells were much more potent inducers of IFN- γ (lower limit of detection, 0.008 ng/ml) than CD11b⁺ cells from uninfected mice were, and CD4⁺ T cells from *P. yoelii*-infected mice produced more IFN- γ than those from uninfected animals (Fig. 4E). Infection-derived CD11b⁺ cells produced significantly less IL-12 (lower limit of detection, 0.08 ng/ml) than CD11b⁺ cells from uninfected mice (Fig. 4F), which was somewhat unexpected, given the ability of infection-derived CD11b⁺ cells to stimulate IFN- γ production. The highest levels of IL-12 were observed in cultures where both CD4⁺ and CD11b⁺ cells were derived from uninfected mice, suggesting that infection-derived CD4⁺ T cells may directly limit IL-12 production by CD11b⁺ cells.

Thus, defects in T-cell proliferation and IL-2 production

during acute blood-stage malaria infection do not necessarily reflect defects in T-cell effector function, and mice with lethal *P. yoelii* infections suffer from exponential parasite growth, despite having the capacity to produce high levels of T-cell-derived IFN- γ . It is likely that the IFN- γ response (and the CD11b⁺ IL-12 response) might be antagonized by IL-10, since T cells from PyL-infected mice produced significantly higher levels of IL-10 (lower limit of detection, 0.1 ng/ml) than T cells from PyNL-infected or uninfected animals did (Fig. 4G). This difference in IL-10 production was most marked when T cells were cultured with APCs from uninfected animals but was also apparent when T cells were cultured with APCs from infected animals. IL-10 production by APCs themselves appears to be minimal in this situation.

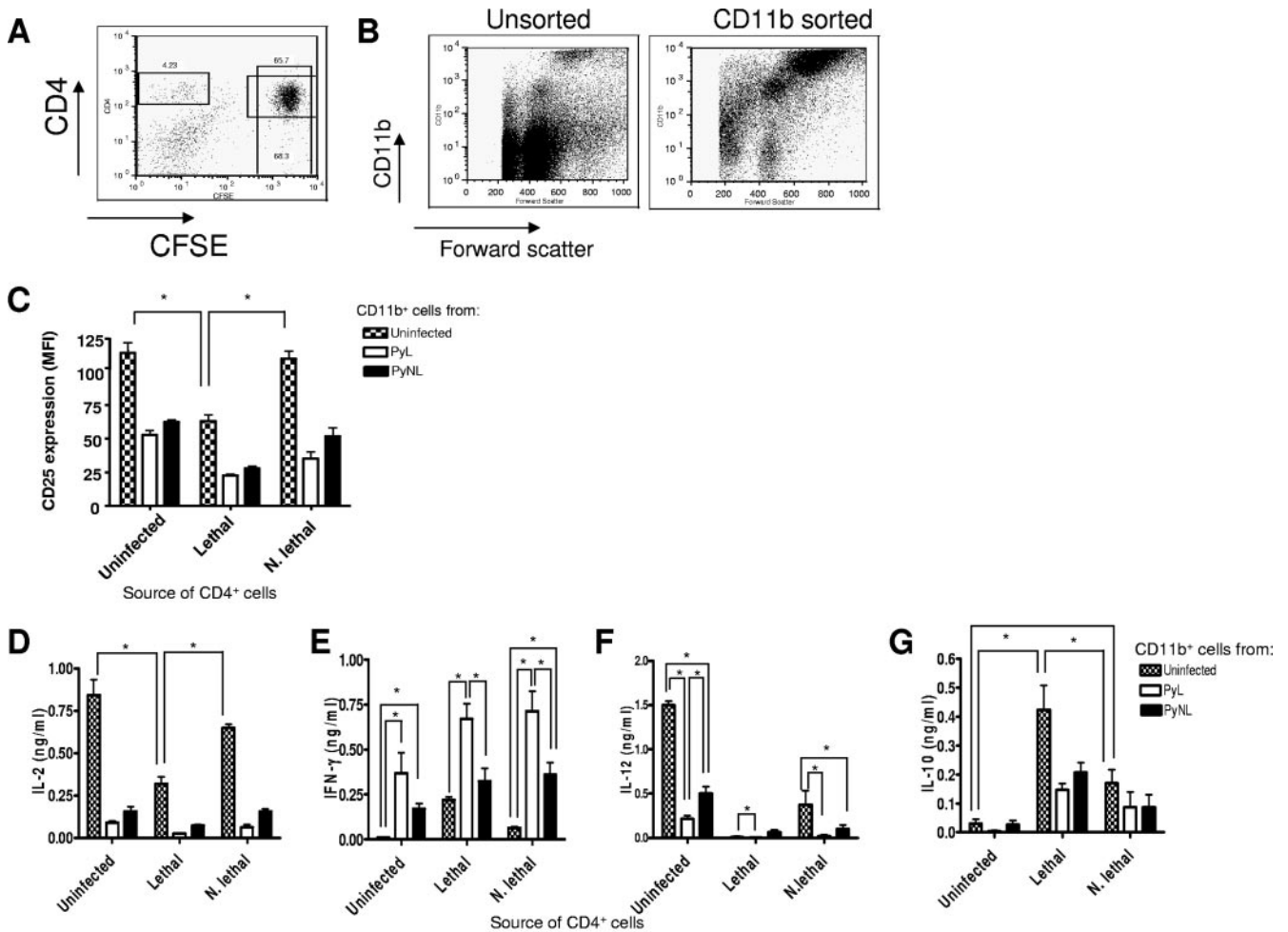


FIG. 4. *P. yoelii*-induced T-cell hyporesponsiveness is due to defects in both CD11b⁺ antigen-presenting cells and T cells. CD4⁺ T cells and CD11b⁺ cells were purified from PyL- and PyNL-infected mice (on day 7 p.i.) and uninfected control mice. CD4⁺ T cells were marked with CFSE, cocultured with CD11b⁺ cells from uninfected mice or from mice infected 7 days earlier with PyL or PyNL, and stimulated for 18 h with ConA. (A) Representative plot showing recovery of CFSE-labeled CD4⁺ T cells after coculture and ConA stimulation. (B) Representative plots showing the purity of unpurified (left) and purified (right) CD11b⁺ APCs. PE, phycoerythrin. (C) Activation of purified CD4⁺ T cells following ConA stimulation was determined by assessing CD25 expression on CFSE-labeled cells. MFI, mean fluorescence intensity. N, lethal, not lethal. (D to G) The levels of IL-2 (D), IFN- γ (E), IL-12 (F), and IL-10 (G) in the supernatants of T-cell-APC cocultures were assessed by ELISAs. The results are representative of two independent experiments with three to five mice per group. Significant differences ($P < 0.05$) between groups are indicated (*).

These data thus both confirm and significantly extend observations from the *P. yoelii*, *P. chabaudi*, and *P. berghei* infection models in which the primary defect appears to be at the level of the APC (26, 29, 37, 53). Clearly, by 7 days p.i., splenic CD4⁺ T cells from *P. yoelii*-infected mice have lost their ability to make and respond to IL-2 by proliferation, but they have enhanced capacity for production of both IFN- γ and IL-10, and both of these cytokines are more abundantly produced by T cells from PyL-infected mice than by T cells from PyNL-infected mice.

T cells do not contribute to control of parasitemia during early *P. yoelii* infection. The data presented thus far reveal small, but statistically significant, differences in CD4⁺ T-cell activation during lethal and nonlethal *P. yoelii* infections. While these data suggest that the inability to control lethal *P. yoelii* infection is not due to a failure to generate effective

T-cell immunity, this hypothesis was tested directly by comparing the course of infection in WT and RAG^{-/-} mice (Fig. 5).

The course of parasitemia in PyL-infected RAG^{-/-} mice was comparable to that in WT mice until day 6 p.i., but parasitemia was significantly higher in RAG^{-/-} mice than in WT mice on day 7 p.i. (90% versus 75%; Fig. 5A), but mice of both strains succumbed to infection on day 7 or 8 p.i. (Fig. 5B). Similar results were obtained using TCR- β ^{-/-} mice (data not shown). Thus, the inability to control lethal *P. yoelii* infection is only minimally affected by the absence of T cells and/or B cells with clonally rearranged antigen receptors. Moreover, and in agreement with previous studies in T- and B-cell-deficient animals (6, 12, 42), the course of PyNL infection in RAG^{-/-} mice did not differ either significantly or reproducibly from that in WT mice up to 10 days p.i., indicating that antigen-specific T cells do not contribute significantly to control of the primary

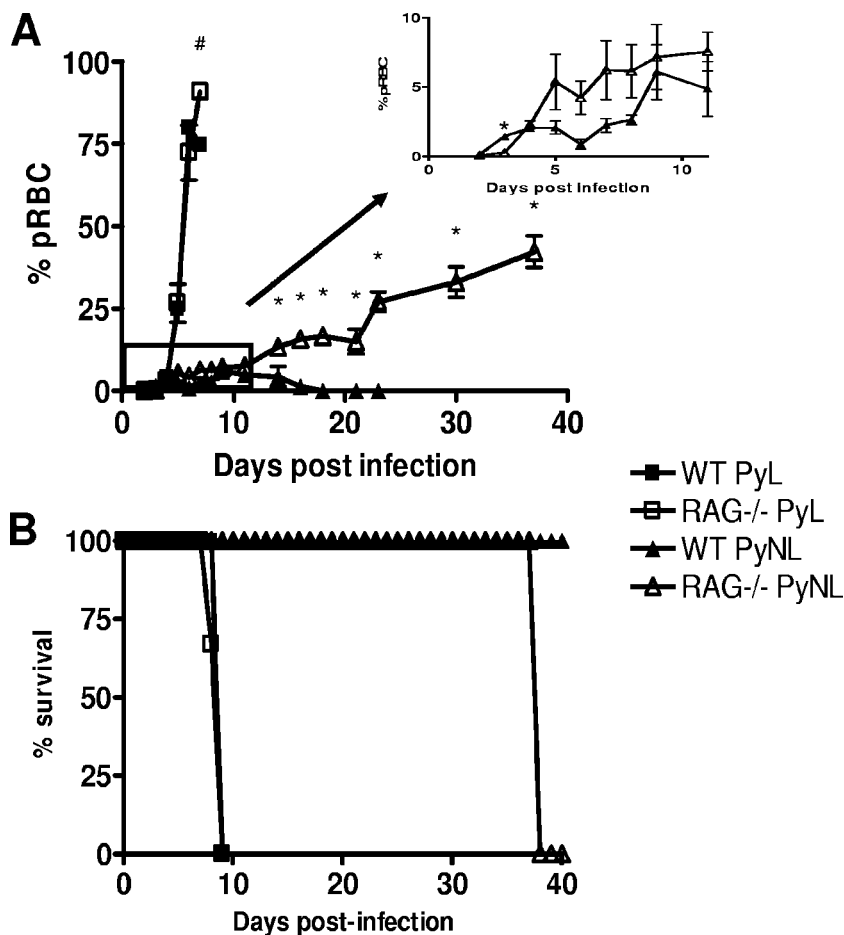


FIG. 5. Neither T nor B lymphocytes are required for the control of the primary wave of *P. yoelii* parasitemia. The course of parasitemia (A) and survival (B) were compared in PyL- and PyNL-infected C56BL/6 (WT) and RAG-1^{-/-} mice. Groups consisted of three to five mice, and the results are representative of four independent experiments. Symbols indicate significant differences ($P < 0.05$) between groups: #, PyL-infected RAG-1^{-/-} group versus WT group; *, PyNL-infected RAG-1^{-/-} group versus WT group.

wave of parasitemia in nonlethal *P. yoelii* infection. As previously shown, however, RAG^{-/-} mice were unable to control their infections over the long term with parasitemia rising steadily over a period of weeks (Fig. 5A), and the mice eventually succumbed to anemia approximately 35 to 40 days p.i. (Fig. 5B).

Taken together, these data indicate that adaptive immune responses have little, if any, role in controlling the first wave of PyL or PyNL parasitemia and thus that minor differences in CD4⁺ T-cell activation during the course of the two infections is unlikely to explain their differing outcomes. Nonetheless, adaptive T cells and/or B cells are critically important for long-term control and elimination of PyNL infections.

Innate (NK cell) responses partially control acute lethal, but not nonlethal, *P. yoelii* infection. It has been reported that NK cells are an important source of IFN- γ during *P. yoelii* and *P. chabaudi* infections and that depletion of NK cells increases the severity of malaria infection (6, 31). In apparent agreement with a protective role for NK cells during malaria infection, significantly increased NK cell activation (CD69 expression) was observed in peripheral blood of PyNL-infected mice (Fig. 6A) on day 1 p.i., whereas significant activation of pe-

ripheral blood NK cells was seen in PyL-infected mice only much later in infection (days 5 to 7 p.i.). Similarly, although splenic NK cells were activated in both PyL- and PyNL-infected mice as early as 1 day p.i., a higher proportion of splenic NK cells were activated in PyNL-infected mice than in PyL-infected mice (Fig. 6B).

To determine whether these differences in NK responses have any direct effect on the course of infection, WT and RAG^{-/-} mice were treated with anti-asialo GM-1 antibodies and infected with lethal or nonlethal *P. yoelii*. Experiments were performed with anti-asialo GM-1 antibody prior to infection to enable and optimize long-lasting effective depletion of NK cells for 7 or 8 days postadministration (results not shown). Anti-asialo GM-1 antibody administration does not affect the number of CD4⁺ and CD8⁺ T cells or macrophages (14). Anti-asialo GM-1 treatment of WT mice resulted in significantly more rapid growth of PyL from 5 days p.i. onwards, and antibody-treated mice generally succumbed to infection 1 day earlier than control mice (Fig. 6C and E). However, apart from a transient difference at 6 days p.i. (when parasitemia was in fact higher in control mice than in mice treated with anti-asialo GM-1), this effect was not seen in RAG^{-/-} mice (Fig. 6D and

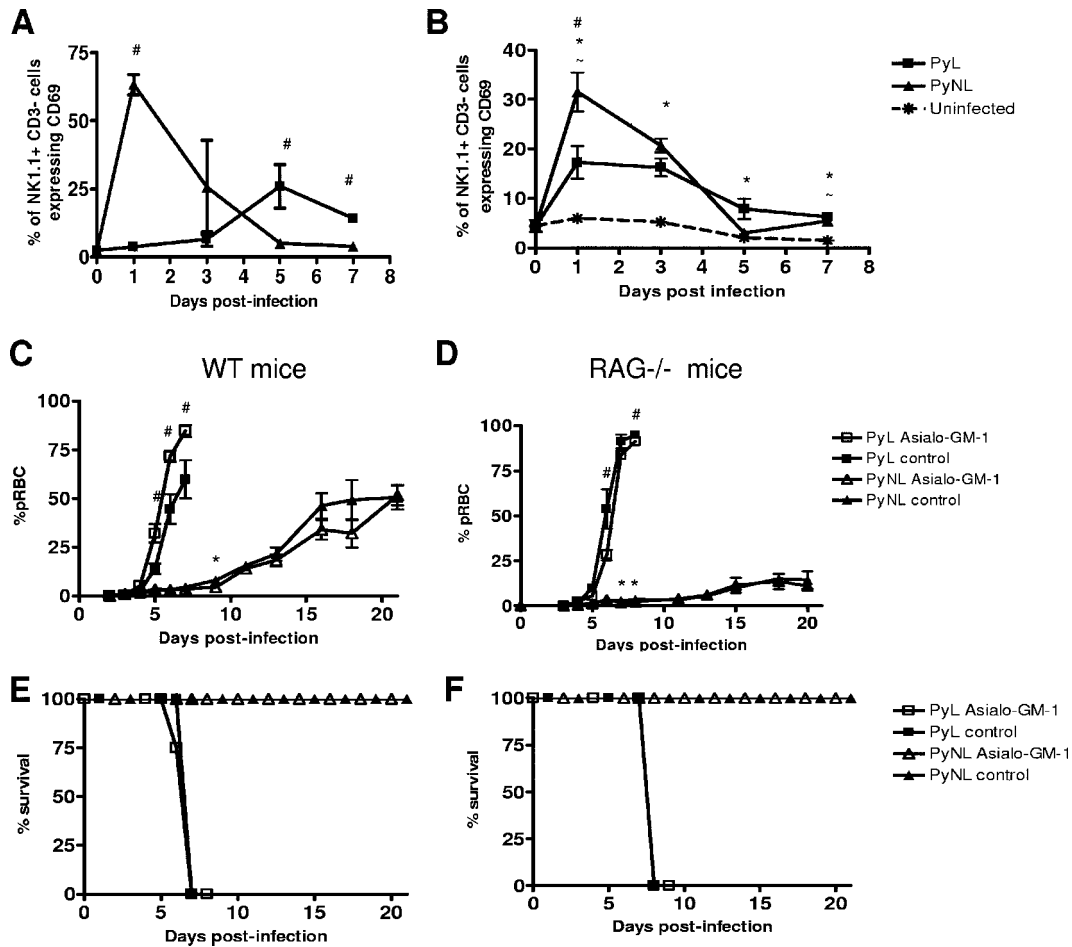


FIG. 6. Minimal role for NK cells in the control of the primary wave of *P. yoelii* parasitemia in C57BL/6 mice. (A and B) NK cell activation (CD69 expression on CD3⁺ NK1.1⁺ cells) in peripheral blood (A) and spleens (B) of PyL- and PyNL-infected mice on various days postinfection with PyL or PyNL. (C and D) Parasitemia (C and D) and survival (E and F) in WT (C and E) and RAG^{-/-} (D and F) mice treated i.p. on days -1, 3, and 6 relative to PyL or PyNL infection with asialo GM-1 antibody or PBS (control). Groups consisted of four or five mice, and the results are representative of two independent experiments. Symbols represent significant differences ($P < 0.05$) between groups as follows: in panels A and B, #, PyL-infected group versus PyNL-infected group; *, PyL-infected group versus uninfected group; ~, PyNL-infected group versus uninfected group; in panels C and D, #, PyL-infected, asialo GM-1 antibody-treated group versus control group; *, PyNL-infected, asialo GM-1 antibody-treated group versus control group.

F), suggesting that compensatory effector mechanisms develop in mice lacking T and B cells, which may obscure any contribution from NK cells.

In contrast, there was no evidence that asialo GM-1⁺ cells played any role in the control of PyNL infections, since the initial course of parasitemia was identical in treated and control, WT and RAG^{-/-}, mice and all mice survived for at least 3 weeks (Fig. 6C, E, D, and F).

IFN- γ production differs between PyL and PyNL infections but does not contribute to control of parasitemia. Given the overwhelming evidence for a crucial role for IFN- γ in controlling acute murine malaria infections (23, 25, 54), we considered the possibility that failure to produce IFN- γ in the initial few days of lethal *P. yoelii* infection might explain the very rapid parasite growth. In apparent support of this possibility, and consistent with patterns of NK activation, an early burst of IFN- γ was observed in the plasma of PyNL-infected (but not PyL-infected) mice on day 1 p.i. (Fig. 7A). Moreover, similar

levels of IFN- γ were seen 1 day p.i. in PyNL-infected WT and RAG^{-/-} mice (Fig. 7A), indicating that NK cells are very likely the principal source of the early IFN- γ response during PyNL infection. In contrast, a burst of IFN- γ was observed in plasma on day 5 p.i. in WT (but not RAG^{-/-}) PyL-infected (but not PyNL-infected) mice, indicating that at this later time point, and in agreement with the in vitro restimulation data (Fig. 4), T cells are the primary source of IFN- γ during PyL infection (Fig. 7A).

To determine whether these differing IFN- γ responses explained the differing course of lethal and nonlethal *P. yoelii* infections, the two infections were compared in WT and IFN- γ ^{-/-} mice. Given the lack of a major role for either NK cells or T cells in controlling acute *P. yoelii* 17XL infections (Fig. 5 and 6), it was not entirely surprising to find that parasite burdens did not differ significantly in IFN- γ ^{-/-} and WT mice during PyL infection (Fig. 7B) and that IFN- γ ^{-/-} mice generally succumbed to infection on the same day as WT mice with

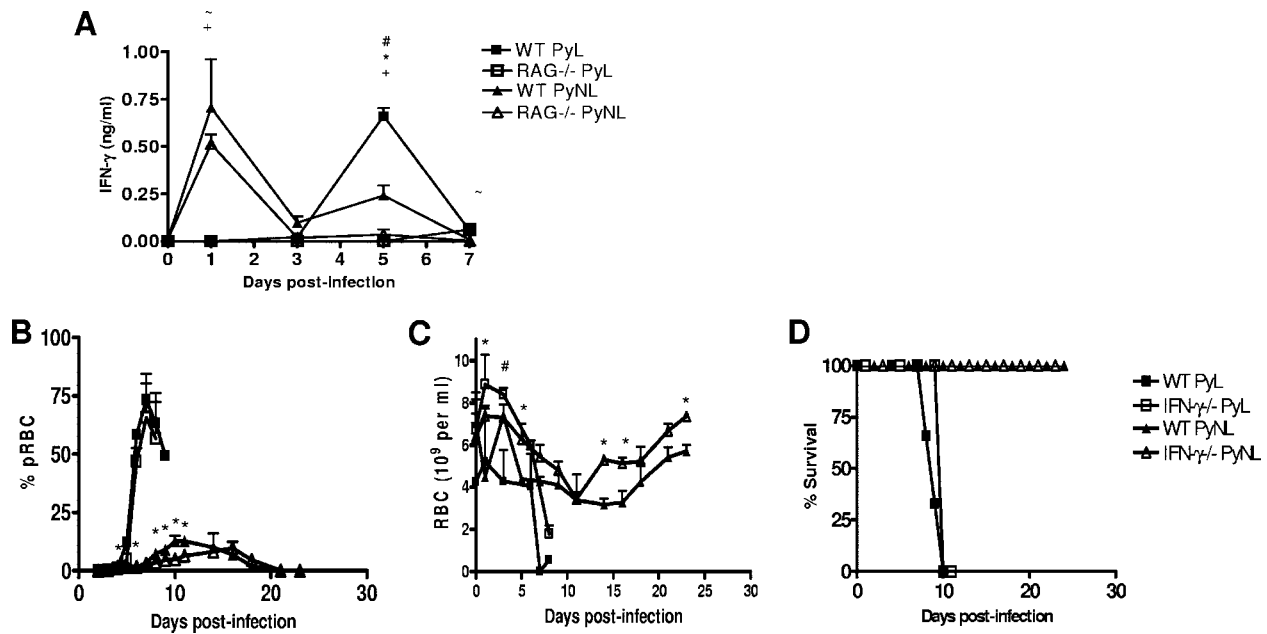


FIG. 7. Early T-cell-independent IFN- γ response differentiates PyL and PyNL infection but is not required for control of acute *P. yoelii* infection. (A) Plasma levels of IFN- γ in PyL- and PyNL-infected WT and RAG-1^{-/-} mice. (B to D) The course of parasitemia (B), anemia (C), and survival (D) in PyL- and PyNL-infected WT and IFN- γ ^{-/-} mice. Groups consisted of three to five mice, and the results are representative of three independent experiments. Symbols represent significant differences ($P < 0.05$) between groups: in panel A, #, PyL-infected RAG-1^{-/-} group versus WT group; *, PyNL-infected RAG-1^{-/-} group versus WT group; ~, PyL-infected RAG-1^{-/-} group versus PyNL-infected group; +, PyL-infected WT group versus PyNL-infected group; for panels B and C, *, PyNL-infected IFN- γ ^{-/-} group versus WT group.

comparable levels of anemia (Fig. 7C and D). However, given the essential role of IFN- γ in controlling nonlethal *P. chabaudi* infection (28, 31, 51, 56) and in preventing cerebral malaria in *P. berghei* K173 infection (30), it was rather unexpected to find that during PyNL infection, the course of infection was essentially identical in WT and IFN- γ ^{-/-} mice (Fig. 7B, C, and D). Thus, somewhat surprisingly, not only does IFN- γ production not explain the different outcomes of PyL and PyNL but IFN- γ production is also not required for effective control of *P. yoelii* infection.

Monocytes/macrophages are essential for parasite control during early acute lethal and nonlethal *P. yoelii* infections. Despite clear evidence that anti-inflammatory cytokines and/or regulatory T cells inhibit the clearance of PyL pRBC (15, 16, 21, 22, 35, 36), this study has not shown any role for T cells, NK cells, or IFN- γ in the control of acute *P. yoelii* infection. However, given the absence of MHC expression on the majority of red blood cells (19), it is probable that neither T cells nor NK cells play the terminal effector role in parasite killing. We therefore considered the possibility that immunosuppressive mediators may interact directly with phagocytic cells, such as macrophages (49, 50), that are believed to be the crucial final effectors that eliminate parasitized cells or free merozoites. To examine the importance of macrophages in the initial control of acute PyL and PyNL infections, mice were treated with clodronate liposomes (58). Administration of clodronate liposomes very effectively depleted F4-80⁺ cells for at least 7 days p.i. (Fig. 8A and B) but had minimal effects on T-cell, NK cell, or dendritic cell activation on day 2 postadministration (results not shown) when mice were infected with *P. yoelii*. Importantly, both PyL- and PyNL-infected, clodronate liposome-treated

mice experienced significantly more rapid parasite growth than did untreated mice (Fig. 8C). Moreover, clodronate-treated mice were significantly more anemic than control mice (Fig. 8D) and succumbed to PyL infection on average 1 day earlier (Fig. 8E).

DISCUSSION

In this study we have investigated the immunological basis of the differing virulence of two closely related strains of the malaria parasite *Plasmodium yoelii*, lethal *P. yoelii* 17XL (PyL) and nonlethal *P. yoelii* 17X (PyNL). By determining (for the first time) the relative importance of both innate and adaptive cellular responses to these parasites, we have identified a novel pathway for innate control of acute malaria infections in which pRBC are cleared by macrophages in a manner which appears to be independent of the "classical" lymphocyte-derived IFN- γ pathway of macrophage activation. Specifically, we observed no significant differences in the T-cell response to PyL or PyNL, and despite differences in the kinetics of the IFN- γ and NK responses to each of the two parasite strains, we found that the course of acute infection was essentially unaffected by the absence of T or B lymphocytes, NK cells, or IFN- γ . These data are somewhat unexpected, given the important roles that IFN- γ -producing NK cells and T lymphocytes play in other rodent malaria infections, such as *P. berghei* ANKA and *P. chabaudi chabaudi* (28, 30, 51), and highlight significant differences in the mechanisms of control of *P. yoelii* compared with other malaria infections. In contrast, depletion of macrophages with clodronate liposomes markedly reduced the ability of the mice to control acute PyL or PyNL infection. Taking these data

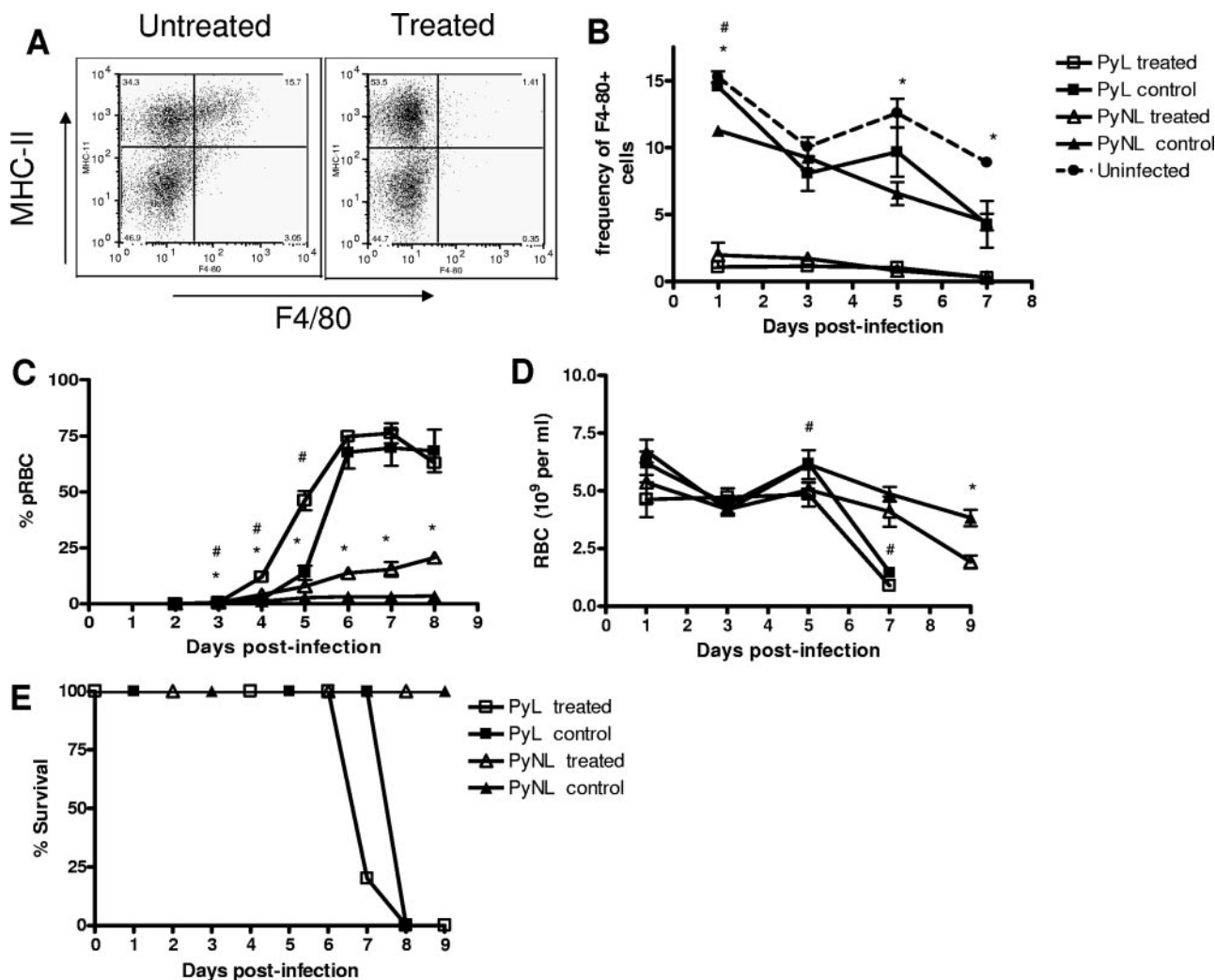


FIG. 8. Macrophages contribute to control of the primary wave of *P. yoelii* parasitemia. C57BL/6 mice were treated i.p. on days -2 and 3 relative to *P. yoelii* infection with 300 μ l of clodronate-liposome suspension. (A) Representative dot plots showing efficacy of splenic F4-80⁺ MHC class II⁺ macrophage depletion 3 days after clodronate liposome treatment (day 1 p.i.). (B) Efficacy of macrophage depletion (percentage of F4-80⁺ MHC class II⁺ splenocytes) over the initial 7 days of malaria infection. (C to E) Parasitemia (C), anemia (D), and mortality (E) in clodronate liposome-treated and untreated PyL- and PyNL-infected mice. Groups consisted of five mice, and the results are representative of two independent experiments. Symbols represent significant differences ($P < 0.05$) between groups: in panel B, #, untreated PyL-infected group versus untreated PyNL-infected group; *, untreated PyNL-infected group versus uninfected group; in panels C and D, #, PyL-infected, treated group versus control group; *, PyNL-infected, treated group versus control group.

together with previously published studies in which rapid induction of IL-10 or TGF- β and/or early activation of regulatory T cells were found to prevent control of PyL infections (15, 16, 22, 35, 36) suggests that these immunoregulatory pathways may act directly to suppress the effector functions of innately activated macrophages.

Somewhat surprisingly, effector T-cell activation, expansion, and function were very similar during lethal and nonlethal *P. yoelii* infections. Moreover, although T-cell hyporesponsiveness was observed during infection, it occurred to the same extent in PyL and PyNL infections and seemed to result from failure of CD11b⁺ APCs from infected animals to drive IL-2-dependent T-cell proliferation. These results are in agreement with previous studies with *P. yoelii* (26, 37) in which it was suggested that T-cell hyporesponsiveness during malaria infec-

tion was due to the acquisition of Toll-like receptor tolerance (26, 37) and mediated, by a soluble mediator that is not nitric oxide, prostaglandin E2, or TGF- β (26). We have confirmed that this downmodulation of T-cell activation is not mediated by TGF- β alone (data not shown), but we cannot yet rule out the possibility that TGF- β might act in combination with other regulatory molecules. Consequently, the extent of T-cell hyporesponsiveness and differences in T-cell function are unlikely to account for the differing virulence of the two infections. In agreement with this, the acute phases of PyL and PyNL infections were similar in wild-type and RAG^{-/-} mice. Thus, although adoptive transfer of specific effector T-cell clones can facilitate control of acute *P. yoelii* infection (2), in a natural primary infection, T cells are of limited importance during the early acute phase of disease. However, as previously shown

(6, 10, 42), T cells and/or B cells were critically required for resolution of nonlethal *P. yoelii* infection, as RAG^{-/-} mice were unable to resolve infection and succumbed with uncontrolled parasitemia.

In apparent agreement with the hypothesis that innate immune responses determine the initial course of malaria infection, we confirmed the presence of a very early (24 h) IFN- γ response in PyNL but not PyL infection (6, 10, 47) and, moreover, were able to correlate this with the kinetics of NK cell activation (peaking at 24 h p.i. for PyNL infection and 5 days p.i. for PyL infection). Nevertheless, despite the distinct differences in NK cell and IFN- γ responses evident during lethal and nonlethal *P. yoelii* infections, NK cell-depleted mice, although displaying slightly accelerated parasite numbers during lethal infection, did not display either increased ascending or peak parasite numbers during nonlethal infection, and IFN- γ ^{-/-} mice were as susceptible or resistant to lethal or nonlethal infection, respectively, as WT mice were. Although it has previously been reported that NK cells are required for optimal resistance to PyNL in F₁ hybrid BALB/c \times C57BL/6 mice and in CB17 SCID mice (6, 10), a recent study in RAG^{-/-} common γ -chain^{-/-} double knockout mice on a C57BL/6 background did not show a major role for NK cells during blood-stage PyNL infection (42). Additionally, a number of studies have provided conflicting reports on the importance of IFN- γ during *P. yoelii* infection. IFN- γ R^{-/-} 129 \times C57BL/6 mice cleared blood-stage nonlethal parasite infection with only a slight delay compared with WT mice (52), and recombinant IFN- γ treatment did not alter the course of PyNL infection (47). In contrast, IFN- γ ^{-/-} mice on a C57BL/6 \times 129 background were unable to clear PyNL infections (56). Moreover, recombinant IFN- γ protected SW, BALB/c, and CBA mice against PyL infection (47), and mice treated with anti-IFN- γ antibodies succumbed earlier to lethal *P. yoelii* infection but had parasite levels similar to those of control mice (21). These results suggest that the lack of an early NK/IFN- γ response in PyL-infected mice may impair parasite control. However, the parasite strain used in the study by Kobayashi et al. (21) was of intermediate virulence (killing control mice in 14 days rather than the 7 to 8 days typical of highly virulent strains), and the source of the IFN- γ (innate or adaptive) was not identified. The results in this study, taken together with the results in these previous studies, demonstrate that NK cells and IFN- γ signaling play a critical role in control of some—but not all—malaria infections with considerable variation between parasite strains/species and between different strains of mice. These data thus challenge a central dogma of malaria immunology (i.e., that IFN- γ is an absolutely essential mediator of protective immunity) and highlight the existence of alternative IFN- γ -independent pathways of resistance during malaria infection.

The lack of any major role for T and B cells, NK cells, or IFN- γ in controlling the initial wave of PyL or PyNL parasitemia raised the question as to whether innate immune responses play any role in limiting the primary wave of *P. yoelii* parasitemia. It is clear that virulent and avirulent strains of *P. yoelii* differ in their ability to invade mature red blood cells (60, 63), and this has recently been linked to differential copy number and expression patterns of genes putatively involved in erythrocyte invasion (1, 18, 41). Nevertheless, numerous studies from different laboratories have observed immunological

differences between the two infections, including disparate IFN- γ production, regulatory cytokine production, regulatory T-cell activation, and stromal cell activation (6, 10, 15, 16, 21, 22, 35, 36, 47, 62), indicating that the virulence of *P. yoelii* parasites is not simply due to their erythrocyte invasion capacity. Significantly, our observation that clodronate liposome depletion of macrophages (58) results in more rapid growth of both PyL and PyNL parasites and more rapid onset of anemia indicates that macrophages play a major role in limiting the acute phase of *P. yoelii* infection. This control is clearly suboptimal in PyL infection and might indeed be enhanced by IFN- γ (47) but is much more effective in PyNL infections—presumably because the more slowly growing parasite is more easily controlled by macrophage phagocytic and antiparasitic responses—and cannot be augmented by either endogenous or exogenous IFN- γ .

Macrophage-mediated control of blood-stage malaria infection has been well described for *P. chabaudi* infections: depletion of macrophages using silica renders normally resistant AS mice highly susceptible, mouse strains with defective macrophage responses are more susceptible to *P. chabaudi* (49, 50), and macrophages expressing heat shock protein 65 are reported to be essential for parasite control during nonlethal *P. yoelii* infection (65). Contrary to most assumptions, however, we have shown here that neither IFN- γ nor the major IFN- γ -producing subsets of lymphocytes (T cells and NK cells) are required for macrophages to become activated or to clear infected red blood cells during acute infection. Rather, our data suggest that *P. yoelii*-infected red blood cells can directly activate macrophages to carry out their effector functions and that these activated macrophages then mediate direct antiparasitic effects. The mechanisms by which macrophages kill or cripple blood-stage parasites are somewhat unclear; despite several studies reporting a role for reactive oxygen intermediates in parasite killing (7, 8, 9, 11), this respiratory burst does not seem to be essential, since gp91^{phox}^{-/-} mice lacking the NADPH oxidase system are no more susceptible to *P. yoelii*, *P. chabaudi*, or *P. berghei* infections than wild-type mice are (39).

In summary, we have shown that macrophages, but not NK cells, T or B lymphocytes, or IFN- γ , contribute to (partial) control of the primary wave of parasitemia in PyL- and PyNL-infected C57BL/6 mice. The molecular pathway(s) leading to macrophage activation by *P. yoelii* is not yet known, but it appears to be independent of IFN- γ and, at least in the case of PyNL, does not appear to be augmentable by exogenous IFN- γ (47). Taken together with the considerable body of evidence that regulatory cytokines and/or regulatory T cells can elevate the severity of acute *P. yoelii* infections (15, 35, 36), these data raise the intriguing possibility that an entirely innate pathway may exist in which parasite-induced IL-10 or TGF- β and/or regulatory T cells directly downregulate antiparasitic, macrophage-mediated effector mechanisms. This hypothesis is currently under investigation.

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