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In Vivo Regulation of Nitric Oxide Production by Tumor Necrosis Factor Alpha and Gamma Interferon, but Not by Interleukin-4, during Blood Stage Malaria in Mice

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We investigated whether gamma interferon (IFN-γ; a Th1 cytokine), tumor necrosis factor alpha (TNF-α), and interleukin-4 (IL-4; a Th2 cytokine) modulate nitric oxide (NO) production in vivo during blood stage infection with *Plasmodium chabaudi* AS. Treatment of resistant C57BL/6 mice, which resolve infection with *P. chabaudi* AS and produce increased levels of IFN-γ, TNF-α, and NO early during infection, with anti-IFN-γ plus anti-TNF-α monoclonal antibodies (MAbs) resulted in a reduction of both splenic inducible NO synthase mRNA and serum NO_3^- levels by 50 and 100%, respectively. Treatment with the anti-TNF-α MAb alone reduced only serum NO_3^- levels by 35%, and treatment with the anti-IFN-γ MAb alone had no effect on NO production by these mice during infection. Susceptible A/J mice, which succumb to infection with *P. chabaudi* AS and produce increased levels of IL-4 but low levels of IFN-γ, TNF-α, and NO early during infection, were treated with an anti-IL-4 MAb. The latter treatment had no effect on NO production by this mouse strain during infection. In addition, our results also demonstrate that treatment of resistant C57BL/6 mice with anti-IFN-γ plus anti-TNF-α MAbs affects, in addition to NO production, other traits of resistance to *P. chabaudi* AS malaria such as the peak level of parasitemia and the development of splenomegaly. Furthermore, the change in spleen weight was shown to be an IFN-γ-independent effect of TNF-α. Treatment of susceptible A/J mice during infection with an anti-IL-4 MAb had no effect on these markers of resistance. Thus, these results demonstrate that TNF-α and IFN-γ are critical in the regulation of NO production and other traits of resistance during *P. chabaudi* AS malaria in C57BL/6 mice. These data also indicate that treatment with an anti-IL-4 antibody alone is not able to induce NO production or confer resistance to A/J mice against *P. chabaudi* AS malaria.

Increased production of endogenous nitric oxide (NO) during infectious diseases such as listeriosis, leishmaniasis, and blood stage malaria, has been shown to correlate with protection against infection in mice (6, 18, 22, 36). Indeed, high levels of nitrate (NO_3^-), an oxidized form of NO, were detected in sera or urine of infected animals which cleared the infection, whereas treatment with inhibitors of NO synthesis resulted in either exacerbation of the disease or death. Recently, increased levels of NO_3^- have also been demonstrated in plasma of patients infected with the malaria parasite *Plasmodium falciparum* or *P. vivax* (8, 25). Moreover, the duration of coma due to cerebral malaria was shorter in children with relatively high NO_3^- plasma levels, which suggests that NO may have a protective role during this disease (8). The induction of NO synthesis during infection is thought to be mediated by the pathogens themselves or pathogen-secreted products in synergy with cytokines (reviewed in reference 24). For example, in vitro studies have demonstrated that bacterial lipopolysaccharide or *P. falciparum*-derived toxins act in synergy with the cytokine gamma interferon (IFN-γ) to induce the production of large quantities of NO by murine macrophages (10, 28). In vivo studies have shown that tumor necrosis factor alpha (TNF-α), in addition to IFN-γ, has a major role in up-regulating NO production during infections with *Toxoplasma gondii* (13) and *Francisella tularensis* (14). On the other hand, interleukin-4 (IL-4) has been demonstrated in vitro to inhibit the up-regulation of the inducible isoform of NO synthase (iNOS), the enzyme capable of producing high amounts of NO, by cytokine-activated macrophages (5).

Our laboratory has recently demonstrated that a Th1-associated increase in TNF-α and NO, early during infection, correlates with resistance to infection with blood stage *P. chabaudi* AS in mice (18, 19). Resistant C57BL/6 (B6) mice, which develop moderate levels of peak parasitemia and a marked splenomegaly on days 7 to 10 postinfection and clear the infection by 4 weeks, were shown to have increased IFN-γ (a Th1 cytokine), TNF-α, and iNOS mRNA levels in the spleen early during infection. In contrast, susceptible A/J (A) mice, which develop higher peak parasitemia levels and a less marked splenomegaly and succumb to infection, have high levels of IL-4 (a Th2 cytokine) mRNA and low levels of TNF-α and iNOS mRNA in the spleen early during infection. Consistent with these results, we detected high levels of IFN-γ protein and NO_3^- in serum recovered from B6 mice early during infection, whereas only low levels of IFN-γ and NO_3^- but high levels of IL-4 and IL-5 protein were found in sera recovered from A mice during the early phase of infection (19, 34, 35). Furthermore, treatment of resistant B6 mice with an anti-TNF-α polyclonal antibody or aminoguanidine, an iNOS inhibitor, resulted in increased mortality, indicating that both TNF-α and NO have a protective role in the early phase of blood stage *P. chabaudi* AS malaria (18, 19). In addition, treatment of susceptible A mice with human recombinant TNF-α protected these mice from an otherwise lethal infection (30).

In this study, we investigated whether IFN-γ, TNF-α, or IL-4

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modulates NO production and other traits of resistance, such as the peak parasitemia level and a marked splenomegaly, during blood stage infection with *P. chabaudi* AS. Our results demonstrate that TNF-α and IFN-γ, but not IL-4, are critical in the development of resistance to blood stage infection with *P. chabaudi* AS in vivo.

MATERIALS AND METHODS

Mice, parasite, and experimental infections. Mice, 8 to 12 weeks old, were age and sex matched in all experiments. B6 mice were obtained from Charles River (St. Constant, Quebec, Canada), and A mice were obtained from Jackson Laboratory (Bar Harbor, Maine). *P. chabaudi* AS was maintained in our laboratory as previously described (26), and experimental infections were initiated by intraperitoneal (i.p.) inoculation with 10^6 *P. chabaudi* AS-parasitized erythrocytes.

Treatment of mice with antibodies and recombinant molecules. Resistant B6 mice were treated i.p. with 0.2 ml of pyrogen-free saline containing 10^5 neutralizing units (protein content = 3.75 mg) of rat anti-murine TNF-α immunoglobulin G1 (IgG1) monoclonal antibody (MAb) (XT22 [2]; Upstate Biotechnology, Lake Placid, N.Y.) 6 h before and 4 days after *P. chabaudi* AS infection. The anti-TNF-α neutralizing titer (neutralizing units per milliliter) was determined as previously described (16) and is defined as the reciprocal of the highest anti-TNF-α dilution which, when added to an equal volume of test sample containing 20 cytotoxic units of recombinant TNF-α per ml, neutralizes 50% or more of the cytotoxic activity of recombinant TNF-α on L929 cells in the presence of actinomycin D. Control B6 mice were similarly treated with 0.2 ml of pyrogen-free saline containing an equivalent amount of normal rat serum IgG which was prepared as previously described (16). For neutralization of IFN-γ, resistant B6 mice were treated i.p. with 0.2 ml of pyrogen-free saline containing 1 mg of mouse anti-rat IFN-γ IgG1 MAb (DB-1 [37]; kindly provided by P. van der Meide, TNO Primate Centre, Rijswijk, The Netherlands) at the same time points during *P. chabaudi* AS infection as for the neutralization of TNF-α. DB-1 has been previously shown to abrogate resistance to *Leishmania major* in vivo (4). Control mice were similarly treated with 0.2 ml of pyrogen-free saline alone. For neutralization of both TNF-α and IFN-γ, resistant B6 mice were treated i.p. with 10^5 neutralizing units of XT22 and 1 mg of DB-1 as described above. Control mice were treated as described above for the neutralization of TNF-α. Susceptible A mice were treated i.p. with 0.4 mg of rat anti-murine IL-4 IgG1 MAb (11B.11 [15]; Biological Resources Branch, Biological Response Modifier Program, NCI-Frederick Cancer Research Facility, Frederick, Md.) 6 h before and 4 days after *P. chabaudi* AS infection. The dose of 11B.11 used in our study has been previously shown to increase resistance to murine listeriosis (15). Control mice were similarly treated with 0.2 ml of pyrogen-free saline containing 0.4 mg of normal rat serum IgG.

Determination of parasitemia, survival, and splenomegaly. Parasitemia was monitored during the course of infection at the times indicated, using previously described procedures (26). Survival of infected mice was assessed by twice-daily observation. Spleen weight, as a measure of splenomegaly, was determined on days 7 and 8 postinfection. Spleen weights from normal control mice were also determined. Because body weights among mice of the different treatment groups were similar (22 ± 1 g), we did not normalize spleen weight to body weight.

RT-PCR analysis of iNOS mRNA levels in spleens. Spleens of B6 and A mice, both normal and infected, were collected aseptically at the times indicated and immediately frozen in liquid nitrogen. Total RNA was isolated by a modification of the guanidine thiocyanate-CsCl method as previously described (19). The RNA yield of each sample was determined spectrophotometrically on the basis of the A_{260}, and the purity (ranging from 1.6 to 2.0) was assessed by the A_{260}/A_{280} ratio. iNOS mRNA levels were determined by semiquantitative reverse transcription-coupled PCR (RT-PCR) as described by Kramnik et al. (20). Briefly, 8-μl solutions containing 1 μg of total RNA and 1 μg of random primers (Pharmacia, Piscataway, N.J.) were heated (65°C, 10 min) to denature RNA and were then cooled on ice. Then 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Grand Island, N.Y.), 10 mM each deoxynucleoside triphosphate (dNTP) (Pharmacia), and 20 U of RNA guard (Pharmacia) in 12 μl of buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 0.1 mg of bovine serum albumin (Gibco BRL) per ml were added to the RNA samples, and the mixtures were incubated for 1 h at 38°C. The enzyme was inactivated by heating (95°C, 10 min), and 30 μl of RNase-free water was added to each sample. To compensate for uncalculable factors affecting the yield of PCR products (41), a competitive PCR standard for iNOS expression was generated as previously described (1, 11). For semiquantitative RT-PCR of iNOS mRNA, 4-μl aliquots of the reverse transcription products were amplified by using iNOS-specific primers in a final volume of 40 μl containing 1 U of *Taq* polymerase, 0.5 to 2 μM each primer, 40 fg of the competitive standard, and 0.2 mM dNTPs in PCR buffer. Each sample also contained 0.2 μl of [α-^{32}P]dCTP (10 μCi/ml; Amersham, Arlington Heights, Ill.). The reaction mixture was overlaid with 20 μl of mineral oil, and the DNA thermal cycler was programmed for 25 cycles at 94°C for 45 s, 62°C for 50 s, and 72°C for 1 min. Aliquots of 20 μl of the reaction mixture was then analyzed on a 4.5% polyacrylamide gel in Tris-borate-EDTA buffer. The gels were dried and exposed to X-ray film (Kodak Biomax-MR; Eastman Kodak Co., Rochester, N.Y.) for 48 h. The density of each band was determined by high-resolution optical densitometry (SciScan 5000; U.S. Biochemical, Cleveland, Ohio). As a control, 2-μl aliquots of all reverse transcription products were amplified in parallel by PCR, using sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (3). The dried gels were exposed to Kodak X-Omat AR X-ray film for 20 min.

NO_3^- concentration in serum. Blood was obtained from experimental mice by cardiac puncture at the times indicated, allowed to clot for 30 min at 4°C, and centrifuged at 13,800 × *g* for 3 min. Sera were collected and stored at −20°C until use. Serum NO_3^- levels were determined as described by Rockett et al. (27). Briefly, 30 μl of each sample was incubated for 20 min at room temperature with 5 μl of the enzyme, nitrate reductase (5 U/ml; Boehringer Mannheim, Laval, Quebec, Canada), and 15 μl of NADPH (1.25 mg/ml; Boehringer Mannheim). After incubation, 100 μl of Griess reagent and 100 μl of trichloroacetic acid (10% aqueous solution) were added. Protein precipitates were removed by centrifugation at 13,800 × *g* for 5 min, 100 μl of each supernatant, in duplicate, was transferred to a 96-well flat-bottom plate, and the A_{550} was measured with an enzyme-linked immunosorbent assay reader. NO_3^- concentrations were determined by using as a standard $NaNO_3$ diluted in pooled serum from uninfected control B6 or A mice, which was dialyzed against phosphate-buffered saline for 24 h. Concentrations of NO_3^- in the standard ranged from 12.3 to 1,000 μM. NO_3^- levels in the sera of experimental animals were calculated by using a software program kindly provided by K. A. Rockett (Australian National University, Canberra, Australia).

Statistical analysis. Statistical significance of differences in parasitemia, splenomegaly, iNOS mRNA levels, and NO_3^- levels in sera between control and antibody-treated mice was determined by Student's *t* test. A probability of less than 0.05 was considered significant.

RESULTS

Splenic iNOS mRNA levels in anti-TNF-α and anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. As shown in Fig. 1, treatment with either an anti-TNF-α or anti-IFN-γ MAb alone did not reduce iNOS expression, which was similar to iNOS expression in the spleens of control mice. However, iNOS expression in the spleen was significantly reduced (by 50%) when B6 mice were treated with both anti-TNF-α and anti-IFN-γ MAbs compared with expression in control mice (Fig. 1). These data, thus, demonstrate that treatment with both anti-TNF-α and anti-IFN-γ MAbs is required to significantly reduce iNOS expression in the spleens of resistant B6 mice during infection. This finding suggests that in the spleens of resistant B6 mice, TNF-α plus IFN-γ induce the expression of iNOS mRNA during the early phase of infection with *P. chabaudi* AS.

Serum NO_3^- levels in anti-TNF-α and anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. Figure 2 shows that treatment with an anti-TNF-α MAb alone significantly reduced (by approximately 50%) the accumulation of serum NO_3^-, whereas treatment with an anti-IFN-γ MAb alone did not affect the concentration of serum NO_3^- compared with levels in sera from control animals. Treatment with both anti-TNF-α and anti-IFN-γ MAbs completely abolished the accumulation of NO_3^- in sera compared with NO_3^- levels in sera obtained from control B6 mice on day 7 postinfection. Indeed, only basal NO_3^- levels (approximately 20 μM), similar to the levels in sera from normal, uninfected B6 mice, could be detected in sera collected from animals treated with anti-TNF-α and anti-IFN-γ MAbs (Fig. 2). Similar results were obtained when sera were collected on day 8 postinfection, coincident with peak parasitemia (data not shown). These data, thus, demonstrate that treatment with an anti-TNF-α MAb alone, but not with an anti-IFN-γ MAb alone, significantly reduces the accumulation of serum NO_3^-, whereas treatment with both anti-TNF-α and anti-IFN-γ MAbs completely abolishes the accumulation of NO_3^- in sera obtained from resistant B6 mice during infection. These results suggest that TNF-α, either alone or in synergy with IFN-γ, induces the systemic production of NO during the early phase of *P. chabaudi* AS malaria.

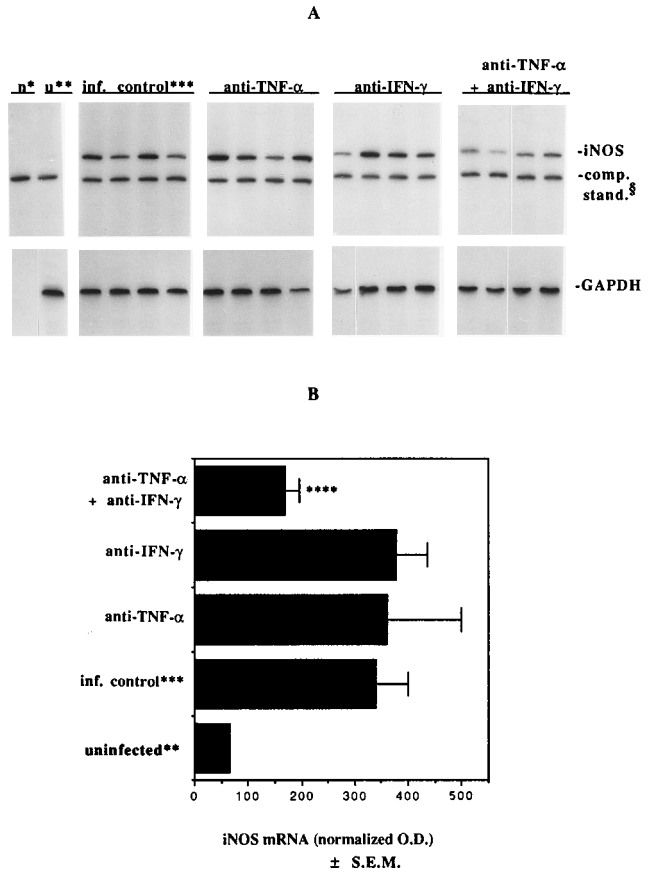

FIG. 1. Splenic iNOS mRNA levels of anti-TNF-α and/or anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. (A) Total RNA was purified from spleens recovered from an uninfected, control mouse (u**) and day 7-infected mice treated with normal rat serum IgG (inf. control***) or an anti-TNF-α and/or anti-IFN-γ MAb (*n* per treatment group = 4). Splenic iNOS mRNA expression per mouse was determined by RT-PCR. Aliquots of 40 fg of competitive standard (comp. stand.§) were coamplified with each of the cDNA samples. GAPDH mRNA levels were also determined by PCR for each sample. n*, negative control (no cDNA except for the competitive standard was added to the PCR mixture). The data shown are representative of two replicate experiments. (B) Scanning densitometry of the autoradiographs shown in panel A. The density of each band corresponding to iNOS mRNA expression was determined and normalized against the levels of the competitive standard, and the resulting values were then normalized against the bands corresponding to GAPDH expression. O.D., optical density; S.E.M., standard error of the mean; ****, $P < 0.05$ versus infected control mice.

Parasitemia, survival, and splenomegaly in anti-TNF-α and anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. As shown in Fig. 3, mice treated with either an anti-TNF-α or anti-IFN-γ MAb alone had a slight but not significant increase in the peak parasitemia levels compared with levels in control animals (46% ± 3% or 49% ± 4% versus 42% ± 3%). Treatment with both MAbs resulted in a peak parasitemia level significantly higher than that of control animals (58% ± 2% versus 42% ± 3%). However, 100% (30 of 30) of the B6 mice treated with either MAb alone or both together survived the infection. Figure 4 shows that mice treated with the anti-TNF-α MAb alone or in combination with the anti-IFN-γ MAb had a significant reduction in spleen weight (by 25%) compared with the spleen weights of control animals (242 ± 16 mg and 237 ± 10 mg versus 313 ± 24 mg). Taken together, these data demonstrate that treatment with both anti-TNF-α and anti-IFN-γ MAbs is required to significantly increase the peak parasitemia level of resistant B6 mice during infection. In contrast, survival of B6 mice during infection is not affected by either MAb alone or by both together. Furthermore, treatment with anti-TNF-α, but not anti-IFN-γ, partially but significantly decreases the development of splenomegaly in B6 mice during infection. These results, thus, suggest that the peak parasitemia level in B6 mice during *P. chabaudi* malaria is controlled by TNF-α in combination with IFN-γ. The development of splenomegaly during infection, on the other hand, appears to be partially regulated by TNF-α but not by IFN-γ.

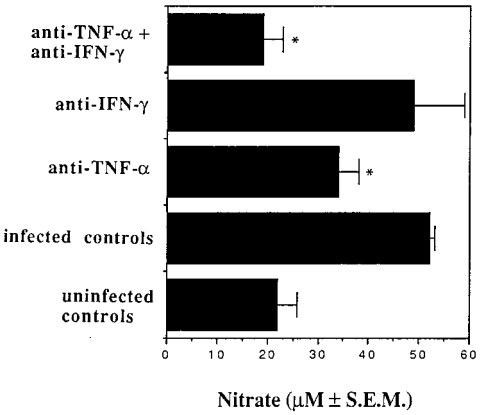

FIG. 2. Serum NO_3^- levels in anti-TNF-α and/or anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. Sera were obtained from uninfected control and day 7-infected mice treated with normal rat serum IgG (infected controls) or an anti-TNF-α and/or anti-IFN-γ MAb (*n* per treatment group = 4 to 6). Serum NO_3^- levels per mouse were determined by a modification of the Griess reaction. The data shown are representative of two replicate experiments. *, $P < 0.03$ versus infected control mice. S.E.M., standard error of the mean.

Splenic iNOS mRNA levels and serum NO_3^- levels in anti-IL-4 MAb-treated susceptible A mice during *P. chabaudi* AS infection. Our results show that treatment with an anti-IL-4 MAb did not affect iNOS mRNA expression in spleens (Fig. 5) and NO_3^- levels in sera (Table 1) of susceptible A mice on day 7 postinfection. Indeed, iNOS mRNA expression and serum NO_3^- levels in the anti-IL-4 MAb-treated mice were not increased compared with the low levels in control animals (Fig. 5 and Table 1). These data, thus, suggest that IL-4 by itself does not inhibit NO production by susceptible A mice during the early phase of *P. chabaudi* AS malaria.

Parasitemia, survival, and splenomegaly in anti-IL-4 MAb-treated susceptible A mice during *P. chabaudi* AS infection. Our results demonstrate that treatment with the anti-IL-4 MAb neither increased the survival rate (data not shown) nor decreased peak parasitemia levels (Table 1) compared with findings for control mice. Furthermore, treatment with the anti-IL-4 MAb did not increase spleen weights consistently compared with weights of control mice (Table 1). These data, thus, suggest that IL-4 by itself does not inhibit the development of resistance by susceptible A mice against infection to *P. chabaudi* AS.

DISCUSSION

We report here that TNF-α, either alone or in combination with IFN-γ, up-regulates NO synthesis, whereas IL-4 on its own does not appear to be involved in regulating NO production in vivo during blood stage infection with *P. chabaudi* AS. Indeed, treatment of infected B6 mice, which have been shown to produce increased levels of TNF-α, IFN-γ, and NO early during infection with *P. chabaudi* AS (18, 19), with anti-TNF-α plus anti-IFN-γ MAbs significantly reduced iNOS mRNA ex-

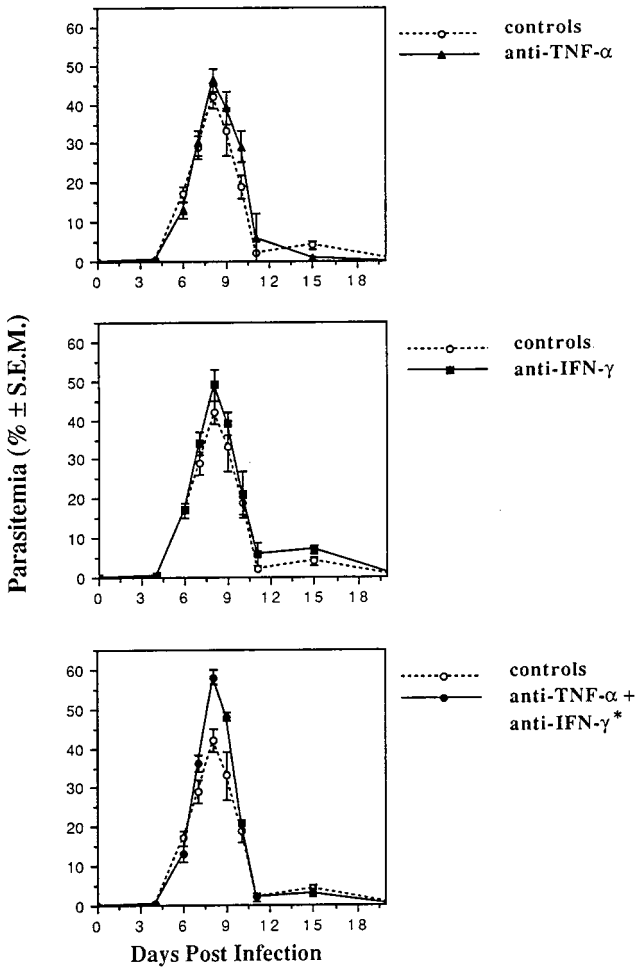

FIG. 3. Parasitemia in anti-TNF-α and/or anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. Parasitemia was determined at the times indicated on duplicate blood smears collected from mice treated with normal rat serum IgG (controls) or an anti-TNF-α and/or anti-IFN-γ MAb (*n* per treatment group = 4 to 6). The data shown are representative of two replicate experiments. *, $P < 0.002$ versus control mice. S.E.M., standard error of the mean.

pression in the spleen. Furthermore, treatment with the anti-TNF-α MAb alone or in combination with the anti-IFN-γ MAb significantly reduced NO_3^- levels in serum. In contrast, treatment of infected A mice, which have been shown to produce low levels of TNF-α, IFN-γ, and NO but high levels of IL-4 early during infection with *P. chabaudi* AS (18, 19), with an anti-IL-4 MAb alone did not affect iNOS mRNA expression in the spleen or NO_3^- levels in serum. These results are in agreement with recent data from our laboratory showing that treatment of susceptible A mice with recombinant IL-12, a cytokine with the ability to induce the development of Th1 cells (17), induces high serum levels of TNF-α, IFN-γ, and, interestingly, NO_3^- (33). Furthermore, treatment with recombinant IL-12 also induced spleen cells recovered from A mice during infection to produce high levels of IFN-γ in vitro. Interestingly, IL-12 treatment did not alter the production of IL-4 by these cells (33). Our results are also consistent with a recent study by von der Weid et al. (40) which demonstrated that resistant mice with a disrupted IL-4 gene still develop a functional Th2 response, which is required at a later stage of infection to completely resolve *P. chabaudi* AS malaria and clear the infection in a manner similar to that in their wild-type counterparts. Furthermore, Th1 clones specific for *P. chabaudi* AS have been shown to be protective in vivo by an NO-dependent mechanism (36). The results of these studies as well as the present data, thus, suggest that NO production during infection with *P. chabaudi* AS is regulated in vivo by Th1-associated cytokines IFN-γ and TNF-α and not by IL-4, a Th2 cytokine. It would be of interest to investigate if IL-10 and transforming growth factor β, other cytokines besides IL-4 suggested to inhibit NO production in vivo (12), are involved in the low production of NO by susceptible A mice. On the other hand, *Plasmodium*-derived toxins, which have been shown to induce NO production in vitro (28), do not appear to be major regulators of NO synthesis in vivo in our model. The parasite load, and thus the amount of parasite-derived toxins, in A mice is similar to or even greater than that in B6 mice 1 to 2 days before the peak of parasitemia, whereas the level of NO production at that time during infection is greater in B6 mice (18).

Our results also demonstrate that treatment of resistant B6 mice with anti-TNF-α plus anti-IFN-γ MAbs reduces splenic iNOS mRNA expression by only 50%, whereas serum NO_3^- levels are reduced by 100%. This finding may indicate that TNF-α in combination with IFN-γ affects NO synthesis both at the level of transcription and posttranscriptionally during infection with *P. chabaudi* AS. In other words, TNF-α in combination with IFN-γ may up-regulate transcription of the iNOS gene by 50% and may, in addition, be required for the stability and/or activity of iNOS protein. Treatment with anti-TNF-α plus anti-IFN-γ MAbs would then result in complete reduction of NO_3^- levels in serum but only partial reduction of iNOS mRNA levels. In this respect, it has been shown that transforming growth factor β, another pleiotropic cytokine produced during infection (29), accelerates the degradation of iNOS protein in mouse macrophages (39). On the other hand, NO_3^- levels in serum during infection with *P. chabaudi* AS may be derived from various organs which are differentially regulated with respect to NO synthesis as has been previously shown by Cunha et al. for a mouse model of sepsis (9). The latter study demonstrated that treatment with anti-TNF-α or anti-IL-1β decreased the level of iNOS enzyme activity in the heart but not in the lung. Thus, treatment with anti-TNF-α and anti-IFN-γ MAbs during *P. chabaudi* AS infection may completely abolish iNOS expression in major NO-synthesizing

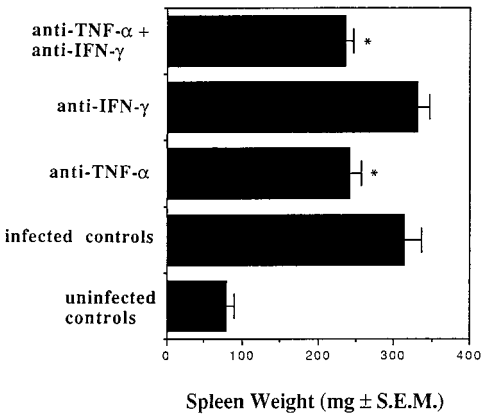

FIG. 4. Spleen weights of anti-TNF-α and/or anti-IFN-γ MAb-treated resistant B6 mice during *P. chabaudi* AS infection. Spleen weights were determined for uninfected control and day 7-infected mice treated with normal rat serum IgG (infected controls) or an anti-TNF-α and/or anti-IFN-γ MAb (*n* per treatment group = 4 to 6). The data shown are representative of two replicate experiments. *, $P < 0.05$ versus infected control mice. S.E.M., standard error of the mean.

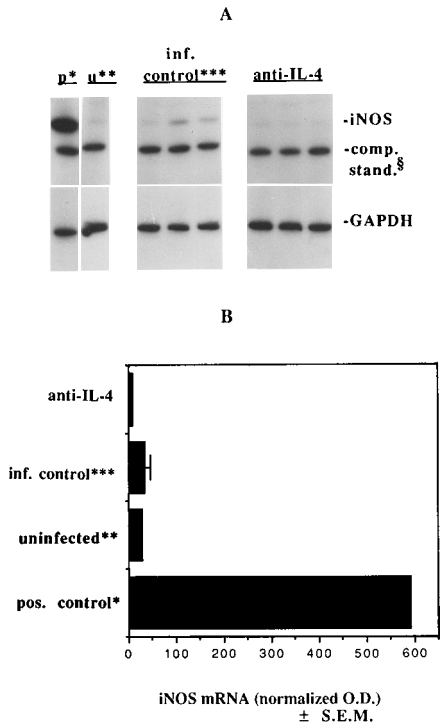

FIG. 5. Splenic iNOS mRNA levels of anti-IL-4 MAb-treated susceptible A mice during *P. chabaudi* AS infection. (A) Total RNA was purified from spleens recovered from an uninfected, control mouse (u**) and day 7-infected mice treated with normal rat serum IgG (inf. control***) or an anti-IL-4 MAb (*n* per treatment group = 3). Splenic iNOS mRNA expression per mouse was determined by RT-PCR. Aliquots of 40 fg of competitive standard (comp. stand.§) were coamplified with each of the cDNA samples. GAPDH mRNA levels were also determined by PCR for each sample. p*, positive control (splenic iNOS mRNA expression in resistant B6 mice on day 7 postinfection with *P. chabaudi* AS). The data shown are representative of two replicate experiments. (B) Scanning densitometry of the autoradiographs shown in panel A. The density of each band corresponding to iNOS mRNA expression was determined and normalized against the levels of the competitive standard, and the resulting values were then normalized against the bands corresponding to GAPDH expression. pos., positive; O.D., optical density; S.E.M., standard error of the mean.

organs such as the heart and other organs and only partially reduce iNOS expression in the spleen. This could then result in low and undetectable amounts of serum NO_3^- as we found in this study.

We report here that treatment of B6 mice with an anti-IFN-γ MAb alone results in a slight but not significant increase in the peak parasitemia level, whereas we have previously demonstrated that treatment of C57BL/10ScN mice with the same anti-IFN-γ antibody resulted in a significant increase in the peak parasitemia level (32). As it is well known that there is strain variation in the level of resistance to *P. chabaudi* AS malaria (31, 32), it is not surprising to find that during infection with *P. chabaudi* AS, the effect of treatment of different mouse strains with the same antibody is variable. We herein also report that treatment of B6 mice with an anti-TNF-α MAb alone does not affect survival, whereas we have recently shown that treatment of this mouse strain with a polyclonal anti-TNF-α antibody alone decreases survival (19). Considering the fact that specific regions of the TNF-α molecule have been shown to have specific biological properties (21), it appears that the polyclonal anti-TNF-α antibody which we used in our earlier study neutralizes several epitopes or biological properties of the TNF-α molecule which are, together, crucial for host survival, whereas the anti-TNF-α MAb which we used here affects one epitope or biological property of the molecule which, by itself, is not crucial for host survival. Furthermore, the present results also show that treatment of resistant B6 mice with anti-TNF-α plus anti-IFN-γ MAbs significantly increases peak parasitemia but does not alter survival of this mouse strain during infection with *P. chabaudi* AS. These results are in agreement with recent data from our laboratory which demonstrated that treatment of B6 mice with an anti-IL-12 antibody also increased peak parasitemia without affecting survival, presumably by suppressing the synthesis of TNF-α and IFN-γ (33). Furthermore, we have previously shown that treatment of *P. chabaudi* AS-infected B6 mice with the iNOS inhibitor aminoguanidine does not alter peak parasitemia but significantly decreases survival of these animals (18). These results are in contrast to those of a study by Taylor-Robinson et al. (36) in which thymectomized, *P. chabaudi*-infected NIH mice were treated with N^G-monomethyl-L-arginine in conjunction with T-cell transfers. Our findings, and the fact that treatment with either aminoguanidine or anti-TNF-α plus anti-IFN-γ MAbs inhibits NO synthesis, suggest first, that NO protects the host but is not involved in controlling the level of peak parasitemia and thus parasite killing, and second, that TNF-α in combination with IFN-γ induces one or more factors different from NO which are involved in parasite killing. In this regard, the existence of unidentified factors in serum which require the presence of both TNF-α and IFN-γ has previously been shown to mediate killing of the malaria parasite *P. cynomolgi* (23). Furthermore, the fact that treatment with an anti-IL-4 MAb did not affect the course of parasitemia and survival of infection with *P. chabaudi* AS is in agreement with a recent study, cited above, showing that mice with a disrupted IL-4 gene clear *P. chabaudi* AS infection in a manner similar to that of their wild-type counterparts (40).

Our results also demonstrate that TNF-α, but not IFN-γ or IL-4, is involved in the development of marked splenomegaly in resistant B6 mice during infection with *P. chabaudi* AS. Since TNF-α, but not IFN-γ and IL-4, has been shown to be chemotactic for monocytes (42) and to induce endothelial cells to express adhesion molecules favoring leukocyte emigration (38), it is tempting to speculate that TNF-α may be involved in the accumulation of leukocytes in the spleen and, thus, the development of splenomegaly. Moreover, a study by Campbell et al. (7), using a murine model of insulin-dependent diabetes mellitus, also suggests that TNF-α induces splenic enlargement.

Taken together, our results demonstrate that TNF-α and IFN-γ together affect different aspects of resistance to *P. chabaudi* AS malaria in vivo. These cytokines (i) induce high amounts of NO which are critical to survive the infection and (ii) are involved in controlling the peak level of parasitemia, which does not appear to depend on the presence of NO. TNF-α by itself also partially regulates the development of splenomegaly. In contrast, IL-4 alone, which is produced in high amounts by susceptible A mice early during infection,

TABLE 1. Serum NO_3^- levels, peak parasitemia levels, and spleen weights of anti-IL-4 MAb-treated susceptible A mice during *P. chabaudi* AS infection[a]

Treatment	Mean serum NO_3^- level (μM) ± SEM	Mean peak parasitemia level (%) ± SEM	Mean spleen wt (mg) ± SEM
Normal rat serum	19 ± 2	65 ± 3	165 ± 04
Anti-IL-4 MAb	21 ± 2	67 ± 3	175 ± 11

[a] Groups of five mice were used per treatment. The data shown are representative of two replicate experiments.

does not appear to be involved in the regulation of NO production, peak level of parasitemia, or splenomegaly. Protection against *P. chabaudi* AS malaria, thus, appears to be critically dependent on the production of increased levels of IFN-γ and TNF-α, which induce NO synthesis early during infection.

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REFERENCES

1. **Ahvazi, B. C., P. Jacobs, and M. M. Stevenson.** 1995. Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria. J. Leukocyte Biol. **58:**23–31.
2. **Appelberg, R., A. G. Castro, J. Pedrosa, R. A. Silva, I. A. Orme, and P. Minoprio.** 1994. Role of gamma interferon and tumor necrosis factor alpha during T-cell-independent and -dependent phases of *Mycobacterium avium* infection. Infect. Immun. **62:**3962–3971.
3. **Barrera, L. F., I. Kramnik, E. Skamene, and D. Radzioch.** 1994. Nitrite production by macrophages derived from BCG-resistant and -susceptible congenic mouse strains in response to IFN-γ and infection with BCG. Immunology **82:**457–464.
4. **Belosevic, M., D. S. Finbloom, P. H. Van der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFN-γ antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. J. Immunol. **143:**266–274.
5. **Bogdan, C., Y. Vodovotz, J. Paik, Q. Xie, and C. Nathan.** 1994. Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. J. Leukocyte Biol. **55:**227–233.
6. **Boockvar, K. S., D. L. Granger, R. M. Poston, M. Maybodi, M. K. Washington, J. B. Hibbs, Jr., and R. L. Kurlander.** 1994. Nitric oxide produced during murine listeriosis is protective. Infect. Immun. **62:**1089–1100.
7. **Campbell, I. L., L. Oxbrow, and L. C. Harrison.** 1991. Reduction in insulitis following administration of IFN-gamma and TNF-alpha in the NOD mouse. J. Autoimmunol. **4:**249–262.
8. **Cot, S., P. Ringwald, B. Mulder, P. Miailhes, J. Yap-Yap, A. K. Nüssler, and W. M. C. Eling.** 1994. Nitric oxide in cerebral malaria. J. Infect. Dis. **169:** 1417–1418.
9. **Cunha, F. Q., J. Assreuy, D. W. Moss, D. Rees, L. M. C. Leal, S. Moncada, M. Carrier, C. A. O'Donnell, and F. Y. Liew.** 1994. Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-α and IL-1-β. Immunology **81:**211–215.
10. **Ding, A., C. F. Nathan, and D. J. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. **141:**2407–2412.
11. **Förster, E.** 1994. An improved general method to generate internal standards for competitive PCR. BioTechniques **16:**18–20.
12. **Gazzinelli, R., I. P. Oswald, S. Hieny, S. L. James, and A. Sher.** 1992. The microbicidal activity of interferon-γ-treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-β. Eur. J. Immunol. **22:**2501–2506.
13. **Gazzinelli, R. T., I. Eltoum, T. A. Wynn, and A. Sher.** 1993. Acute cerebral toxoplasmosis is induced by *in vivo* neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J. Immunol. **151:**3672–3681.
14. **Green, S. J., C. A. Nacy, R. D. Schreiber, D. I. Granger, R. M. Crawford, M. S. Meltzer, and A. H. Fortier.** 1993. Neutralization of gamma interferon and tumor necrosis factor alpha blocks in vivo synthesis of nitrogen oxides from L-arginine and protection against *Francisella tularensis* infection in *Mycobacterium bovis* BCG-treated mice. Infect. Immun. **61:**689–698.
15. **Haak-Frendscho, M., J. F. Brown, Y. Iizawa, R. D. Wagner, and C. J. Czuprynski.** 1992. Administration of anti-IL-4 monoclonal antibody 11B11 increases the resistance of mice to *Listeria monocytogenes* infection. J. Immunol. **148:**3978–3985.
16. **Havell, E. A.** 1987. Production of tumor necrosis factor during murine listeriosis. J. Immunol. **139:**4225–4231.
17. **Hsieh, C., S. E. Macatonia, C. S. Tripp, S. E. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of Th1 CD4+ cells through IL-12 produced by *Listeria*-induced macrophages. Science **260:**547–549.
18. **Jacobs, P., D. Radzioch, and M. M. Stevenson.** Nitric oxide expression in the spleen, but not in the liver, correlates with resistance to blood-stage malaria in mice. J. Immunol., in press.
19. **Jacobs, P., D. Radzioch, and M. M. Stevenson.** A Th1 associated increase in TNF-α expression in the spleen correlates with resistance to blood-stage malaria in mice. Submitted for publication.
20. **Kramnik, I., E. Skamene, and D. Radzioch.** 1993. Assessment of lymphokine profiles in activated lymphocytes by semiquantitive PCR. J. Immunol. Methods **162:**143–153.
21. **Kumaratilake, L. M., D. A. Rathjen, P. Mack, F. Widmer, and V. Prasertsiriroj.** 1995. A synthetic tumor necrosis factor-α agonist peptide enhances human polymorphonuclear leukocyte-mediated killing of *Plasmodium falciparum* in vitro and suppresses *Plasmodium chabaudi* infection in mice. J. Clin. Invest. **95:**2315–2323.
22. **Liew, F. Y., S. Millott, C. Parkinson, R. M. J. Palmer, and S. Moncada.** 1990. Macrophage killing of *Leishmania* parasite *in vivo* is mediated by nitric oxide from L-arginine. J. Immunol. **144:**4794–4797.
23. **Naotunne, T. D. S., N. D. Karunaweera, G. Del Giudice, M. U. Kularatne, G. E. Grau, R. Carter, and K. N. Mendis.** 1991. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. J. Exp. Med. **173:**523–529.
24. **Nathan, C., and Q. Xie.** 1994. Regulation of biosynthesis of nitric oxide. J. Biol. Chem. **269:**13725–13728.
25. **Nüssler, A. K., W. M. C. Eling, and P. Kremsner.** 1994. Patients with *Plasmodium falciparum* malaria and *Plasmodium vivax* malaria show increased nitrite and nitrate plasma levels. J. Infect. Dis. **169:**1418–1419.
26. **Podoba, J. E., and M. M. Stevenson.** 1991. $CD4^+$ and $CD8^+$ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. Infect. Immun. **59:**51–58.
27. **Rockett, K. A., M. M. Awburn, E. J. Rockett, W. B. Cowden, and I. A. Clark.** 1994. Possible role of nitric oxide in malarial immunosuppression. Parasite Immunol. **16:**243–249.
28. **Rockett, K. A., D. Kwiatkowski, C. A. W. Bate, M. M. Awburn, E. J. Rockett, and I. A. Clark.** *In vitro* induction of nitric oxide by an extract of *Plasmodium falciparum*. Submitted for publication.
29. **Silva, J. S., D. R. Twardzik, and S. G. Reed.** 1991. Regulation of *Trypanosoma cruzi* infections *in vitro* and *in vivo* by transforming growth factor β (TGF-β). J. Exp. Med. **174:**539–545.
30. **Stevenson, M. M., and E. Ghadirian.** 1989. Human recombinant tumor necrosis factor alpha protects susceptible A/J mice against lethal *Plasmodium chabaudi* AS infection. Infect. Immun. **57:**3936–3939.
31. **Stevenson, M. M., J. J. Lyanga, and E. Skamene.** 1982. Murine malaria: genetic control of resistance to *Plasmodium chabaudi*. Infect. Immun. **38:** 80–88.
32. **Stevenson, M. M., M. Tam, M. Belosevic, P. van der Meide, and J. Podoba.** 1990. Role of endogenous gamma interferon in host response to infection with blood-stage *Plasmodium chabaudi* AS. Infect. Immun. **58:**3225–3232.
33. **Stevenson, M. M., M. Tam, S. F. Wolf, and A. Sher.** 1995. IL-12 induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via an NO-dependent mechanism. J. Immunol. **155:**2545–2556.
34. **Stevenson, M. M., and M.-F. Tam.** 1993. Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice. Clin. Exp. Immunol. **91:**77–83.
35. **Stevenson, M. M., and M.-F. Tam.** Unpublished data.
36. **Taylor-Robinson, A. W., R. S. Phillips, A. Severn, S. Moncada, and F. Y. Liew.** 1993. The role of Th1 and Th2 cells in a rodent malaria infection. Science **260:**1931–1934.
37. **van der Meide, P. H., M. Dubbeld, K. Vijverberg, T. Kos, and H. Schellekens.** 1986. The purification and characterization of rat gamma interferon by use of two monoclonal antibodies. J. Gen. Virol. **67:**1058–1071.
38. **Vassalli P.** 1992. The pathophysiology of tumor necrosis factors. Annu. Rev. Immunol. **10:**411–452.
39. **Vodovotz, Y., C. Bogdan, J. Paik, Q. Xie, and C. Nathan.** 1993. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. J. Exp. Med. **178:**605–614.
40. **von der Weid, T., M. Kopf, G. Köhler, and J. Langhorne.** 1994. The immune response to *Plasmodium chabaudi* malaria in interleukin-4-deficient mice. Eur. J. Immunol. **24:**2285–2293.
41. **Wang, A. M., M. V. Doyle, and D. F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86:**9717–9721.
42. **Wang, J. M., S. Walter, and A. Mantovani.** 1990. Re-evaluation of the chemotactic activity of tumour necrosis factor for monocytes. Immunology **71:**364–367.

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