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THE PROTECTIVE ROLE OF TUMOR NECROSIS FACTOR-alpha AND NITRIC OXIDE DURING BLOOD-STAGE INFECTION WITH *PLASMODIUM CHABAUDI* AS IN MICE

by

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A Thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Institute of Parasitology and Centre for the Study of Host Resistance
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To Mies, Doon, Muis and Merel.

ABSTRACT

The kinetics of production and role of tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) during the early phase of blood-stage infection with *Plasmodium chabaudi* AS were investigated using two inbred strains of mice which differ in the level of resistance to this parasite. Analysis of the *in vivo* expression of TNF-α and inducible nitric oxide synthase (iNOS) revealed that, early during infection, resistant C57BL/6 mice, which clear the infection by 4 weeks, have higher levels of TNF-α and iNOS mRNA in the spleen and TNF-α mRNA in the liver than susceptible A/J mice which succumb to the disease 10 days after initiation of infection. Moreover, resistant mice expressed high levels of IFN-γ (a Th1 marker) and low levels of IL-4 (a Th2 marker) mRNA in the spleen, whereas susceptible A/J mice had low levels of IFN-γ but high levels of IL-4 mRNA in the spleen early during infection. Increased levels of NO_3^- were detected in serum of resistant C57BL/6 mice only at the time of peak parasitemia. Furthermore, treatment of resistant C57BL/6 mice with anti-IFN-γ and anti-TNF-α monoclonal antibody demonstrated that TNF-α, either alone or in synergy with IFN-γ, plays a major role in the up-regulation of NO production during *P. chabaudi* AS malaria. Moreover, treatment with the iNOS inhibitor aminoguanidine, eliminated resistance of these mice to infection with *P. chabaudi* AS without affecting parasitemia, suggesting that NO may not be involved in parasite killing *in vivo*. Taken together, these results demonstrate that a Th1-associated increase in TNF-α early during infection, as occurs in resistant mice, leads to the up-regulation of NO production which is crucial for survival of the host. On the other hand, our results also suggest that a Th2 response, as occurs in susceptible mice, does not result in protective levels of TNF-α and NO. However, susceptible A/J mice were found to express high levels of TNF-α and iNOS mRNA in the liver and had excessive serum levels of TNF-α protein later during infection just before death occurred. Thus, the role of TNF-α and NO during malaria appears to depend not only on the amount of these molecules produced, but also on the timing and the site of their production.

RÉSUMÉ

La cinétique de production et le role du tumor necrosis factor-alpha (TNF-α) et de l'azote nitrique (NO) au cours de la phase précose de la phase sanguine de l'infection avec *Plasmodium chabaudi* AS ont été étudiés chez 2 lignées cosanguines de souris qui se distinguent par leur niveau de résistance à ce parasite. Les souris C57BL/6 sont considérées comme résistantes car elles contrôlent l'infection par *P. chabaudi* AS en 4 semaines, alors que les souris A/J sont plus sensibles à l'infection puisqu'elles succombent 10 jours après l'inoculation du parasite. Nos résultats ont indiqué que, tôt après l'infection, les souris résistantes C57Bl/6 avaient des niveaux plus élevés d'ARNm codant pour le TNF-α dans la rate et le foie et pour le iNOS dans la rate comparativement aux souris sensibles A/J. De plus, les cellules spléniques prélevées de souris résistantes exprimaient des niveaux élevés d'ARNm codant pour l'IFN-γ (un marqueur Th1) et des quantités faibles d'ARNm codant pour l'IL-4 (un marqueur Th2), alors que les cellules spléniques de souris sensibles exprimaient des niveaux faibles d'ARNm codant pour l'IFN-γ et des niveaux élevés d'ARNm codant pour l'IL-4 au cours de l'infection. Des concentrations accrues de $NO3^-$ ont été détectées dans le sérum seulement des souris résistantes au moment du pic de parasitémie. Le traitement des souris résistantes avec des anticorps monoclonaux dirigés contre l'IFN-γ ou le TNF-α a démontré que le TNF-α, seul ou en synergie avec l'IFN-γ, stimulait la production du NO au cours de l'infection causée par *P. chabaudi* AS. Nous avons également observé qu'un traitement avec l'aminoguanidine, un inhibiteur, du iNOS, annhilait la résistance des souris C57BL/6 à l'infection causée par *P. chabaudi* AS sans toutefois affecter le niveau de parasitémie ce qui suggère que le NO n'est peut être pas directement toxique pour le parasite in vivo. Dans l'ensemble, ces résultats démontrent que l'augmentation de TNF-α, associée à une réponse de type Th1 chez les souris résistantes durant la phase précoce de l'infection stimule la production de NO qui est essentielle à la survie de l'hôte. Par ailleurs, nos résultats suggèrent également que la réponse Th2 détectée chez les souris sensibles n'induit pas la

production de quantités suffisantes de TNF-α et de NO pour conférer une protection chez l'hôte. Par contre, des niveaux excessifs de TNF-α dans le sérum et une forte expression d'ARNm codant pour le TNF-α et le iNOS dans les cellules hépatiques ont été détectés chez les souris sensibles durant la phase tardive de l'infection un peu avant la mort. Le rôle du TNF-α et du NO au cours de la malaria semble donc être associé non seulement à la quantité de ces molécules produites, mais également à la cinétique et au site de production.

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TABLE OF CONTENTS

Page

CHAPTER 1: GENERAL INTRODUCTION

INFECTION WITH *PLASMODIA*

TUMOR NECROSIS FACTOR-alpha

NITRIC OXIDE

CHAPTER 2: A Th1 ASSOCIATED INCREASE IN TNF-α EXPRESSION IN THE SPLEEN CORRELATES WITH RESISTANCE TO BLOOD-STAGE MALARIA IN MICE

CHAPTER 3: NITRIC OXIDE IN THE SPLEEN, BUT NOT IN THE LIVER, CORRELATES WITH RESISTANCE TO BLOOD-STAGE MALARIA IN MICE

CHAPTER 4: *IN VIVO* REGULATION OF NITRIC OXIDE PRODUCTION BY TNF-α AND IFN-γ, BUT NOT IL-4, DURING BLOOD-STAGE MALARIA IN MICE

CHAPTER 5: GENERAL DISCUSSION AND CLAIMS TO ORIGINALITY

LIST OF FIGURES

Page

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

Page

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

A	A/J
AG	aminoguanidine
B6	C57BL/6
BSA	bovine serum albumin
cDNA	complementary DNA
cpm	counts per minute
dNTP	deoxynucleotidetriphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FAD	flavin adenine dinucleotide
Fc	fraction crystalizable (of an antibody)
FCS	fetal calf serum
FMN	flavin mononucleotide
HBSS	Hank's balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
hu rTNF	human recombinant tumor necrosis factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
mAb	monoclonal antibody
MHC	major histocompatibility complex
mo rTNF	mouse recombinant tumor necrosis factor

NADPH	nicotinamide adenine dinucleotide phosphate reduced
NK	natural killer cell
iNOS	inducible nitric oxide synthase
NO_2^-	nitrite
NO_3^-	nitrate
O.D.	optical density
LPS	lipopolysaccharide
p	probability
PBS	phosphate-buffered saline
PRBC	parasitized red blood cells
ROI	reactive oxygen intermediates
RT PCR	reverse transcription polymerase chain reaction
S.E.M.	standard error of the mean
SSC	standard saline citrate
TCA	trichloroacetic acid
TGF	transforming growth factor
Th cell	T helper cell
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Tris	tris(hydroxymethyl)aminomethane

PREFACE

In accordance with the regulation of the Faculty of Graduate Studies and Research concerning thesis preparation and approved by the Institute of Parasitology, manuscripts submitted for publication have been incorporated into this thesis. The following is quoted from "Guidelines for Thesis Preparation":

"Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis."

"If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated."

"The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list."

"Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis."

"In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis."

This thesis consists of three manuscripts (Chapters 2, 3 and 4), each with its own Abstract, Introduction, Materials and Methods, Results and Discussion. The General Abstract, General Introduction (Chapter 1) and General Discussion (Chapter 5) relate to the combined research studies presented in this thesis. References appear at the end of each Chapter.

The research work presented in this thesis was performed by the author under supervision and guidance of Dr. Mary M. Stevenson at the Centre for the Study of Host Resistance, Montreal General Hospital Research Institute. The author received technical guidance provided by Miss MiFong Tam, senior technician in Dr. Stevenson's laboratory, and by Dr. Igor Kramnik and Luis Barrera, postdoctoral fellow and Ph.D. student, respectively, in Dr. Danuta Radzioch's laboratory at the Centre for the Study of Host Resistance. Much of the research work was also performed in Dr. Radzioch's laboratory. The author and Drs. Stevenson and Radzioch are coauthors of all three manuscripts presented. The manuscripts appearing in Chapters 2, 3 and 4 have been submitted for publication*.

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*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 to Immunology.

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. Submitted to Journal of Immunology.

Jacobs, P., D. Radzioch, and M. M. Stevenson. *In vivo* regulation of nitric oxide production by TNF-α and IFN-γ, but not IL-4, during blood-stage malaria in mice. Submitted to Infection and Immunity.

CHAPTER 1

GENERAL INTRODUCTION

INFECTION WITH *PLASMODIA*

Introduction to- and life cycle of *Plasmodia.*

Plasmodia are protozoan parasites which live in vertebrate tissues and are transmitted by an insect vector. A total of about 155 species of *Plasmodium* parasitize a wide variety of vertebrates such as reptiles, birds, rodents, bats, monkeys and humans. Only 4 species, however, infect humans: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* (1). The insect vectors of the 4 *Plasmodia* infecting humans are mosquitoes of the genus *Anopheles* (2). In the vertebrate host, *Plasmodia* may cause malaria. Human malaria is one of the most devastating diseases of mankind. It is estimated that malaria threatens about 40% of the world's population and kills about 1.5 million people each year (3). Resistance of the mosquito vector to insecticides and of *Plasmodia* to anti-malarial drugs are exacerbating the current situation (4). Malaria is nowadays a problem of people living in subtropical and tropical Africa, Asia and America. Eradication has eliminated malaria from the United States, Cuba, Europe, Israel, Australia and Japan (5).

The life cycle of *Plasmodia* is complex and involves an asexual reproduction phase which occurs in the vertebrate host and a sexual phase which takes place in the mosquito vector. Infection in the vertebrate host is initiated by the injection of sporozoites into the bloodstream during feeding by infected mosquitoes. The sporozoites then quickly migrate to the liver, where they invade hepatocytes. Inside the hepatocytes, they mature and divide asexually by schizogony to form merozoites. Rupture of the hepatocytes releases large numbers of free merozoites into the bloodstream. The merozoites then invade erythrocytes, mature and divide by schizogony to form a variable number, depending upon the species of *Plasmodium*, of new merozoites. Rupture of the infected red cells releases the new merozoites which, in turn, invade other erythrocytes. This erythrocytic cycle tends to become synchronous in untreated, human malaria infections and is associated with the typical pattern of fever seen during this disease. Thus, rupture of infected red cells and subsequent release of new merozoites tends to occur every 48 or 72 hours, depending upon the species of *Plasmodium*, and coincides with fever spikes. The merozoites proliferate in the blood until death of the host or until they are controlled or terminated by the host's immune response or, in the case of humans, by anti-malarial drugs. However, during the erythrocytic cycle, a small proportion of merozoites also differentiate into male and female gametocytes which, after full maturation, await ingestion by a mosquito. When male and female gametocytes are ingested by a mosquito during a blood meal, they become extracellular following erythrocyte lysis. Inside the gut of the mosquito, sperm-like male gametes are formed and fertilization of the female gametes occurs. Zygotes transform into ookinetes which encyst on the outer surface of the mosquito midgut wall and release, after maturation, many thousands of sporozoites which invade the salivary glands of the mosquito. Infectious sporozoites are injected into the vertebrate host during the next bloodmeal (1, 5, 6).

Blood-stage malaria in man: pathology

The erythrocytic cycle, or blood-stage phase of the parasite's life cycle, is responsible for the symptoms and pathology of malaria. Uncomplicated malaria is characterised by high fever associated with headache, vomiting, nausea, muscular pain, weakness and malaise. These non-specific, flu-like symptoms can be caused by all four species of *Plasmodium* infecting humans. However, infection with one species, *P. falciparum*, can result in severe and/or lethal disease in about 2% of patients (7). The disease is then characterised by one or a combination of the following life-threatening features: a diffuse encephalopathy associated with loss of consciousness (known as cerebral malaria), severe anemia, hypoglycemia, renal failure and pulmonary oedema (5, 8-9). The reason why 2% of patients develop severe and lethal disease, in contrast to the 98% of patients who experience flu-like malaria, depends on both the parasite and the host. Thus, parasite factors such as the size of the initial inoculum of sporozoites and virulence of different *P. falciparum* strains, and host factors such as genetic background, immune status, nutritional status and sociological situation of the host all play an important role in determining survival or death during infection with *P. falciparum* (7). The analysis of the role of the host's immune response, associated with its genetic background, in determining resistance to malaria forms the basis of this thesis.

Blood-stage malaria in man: protective immunity

It is generally assumed that the acquisition of protective immunity to blood-stage malaria is slow and only develops after many years of exposure (5, 6, 8, 10, 11). Indeed, the incidence of parasitemia is most common in young children and declines steadily with age in areas where malaria is highly endemic (6, 10). Mortality from malaria is also most common in young children (< 5 years old) living in malaria-endemic areas (6, 10). Children older than 5 years from these areas may still have high blood parasitemia levels but the disease becomes largely asymptomatic (6, 10). Thus, an anti-disease or clinical immunity, distinct from an anti-parasite immunity, appears to develop in these children (11). With progressing age, an anti-parasite immunity leading to very low parasitemia levels accompanied by rare or very mild clinical symptoms may then develop (12, 13). Complete sterile immunity is uncommon and protection to malaria is rapidly lost when an individual is no longer exposed to reinfection (5). In regions where there is very low or seasonal exposure to malaria parasites, clinical and/or anti-parasite immunity usually is never acquired and the disease affects all age groups (8). Furthermore, protective immunity to blood-stage malaria is species-specific. For example, immunity to *P. falciparum* malaria does not protect against infection with *P. vivax* (5). Immunity against *P. falciparum* malaria is also believed to be strain-specific (6, 14). However, a recent study indicated that antibody-mediated protection against *P. falciparum* malaria was not strain-specific (15).

Both antibody-dependent and antibody-independent immune effector mechanisms appear to be involved in protective immunity to blood-stage human malaria. The potential of antibodies to confer protection against malaria is apparent from the fact that infants born to immune mothers are protected by maternally derived antibodies during the first 6 months of life (16). Passive transfer of whole immune serum or purified immunoglobulins has also been shown to protect children against malaria (17, 18). Furthermore, *in vitro* studies have demonstrated that antibodies can 1) block the invasion of merozoites into red blood cells (19); 2) opsonize parasites for phagocytosis (20) or antibody-dependent cellular inhibition of parasite growth (10); 3) agglutinate, and thus immobilize, merozoites (21); and 4) block the binding of parasitized red blood cells to capillary endothelium which may facilitate clearance of these parasitized cells from the circulation (22). *In vivo* studies have shown that antibody-mediated protection against *P. falciparum* malaria is achieved by the interaction of antibodies with effector cells rather than by the inhibition of merozoite invasion or mobility (10, 23). Moreover, protection against blood-stage *P. falciparum* in African children has been shown to correlate with the production of malaria-specific, cytophilic antibodies (24). Indeed, protected individuals had predominately serum IgG1 and IgG3, two cytophilic isotypes, whereas non-cytophilic IgG2 and/or IgM predominated in serum of non-protected individuals (24).

CD8+ T cells are not believed to play a role as cytolytic effectors during blood-stage malaria, since mature red blood cells do not express MHC class I molecules (25). However, CD8+ cells may have a suppressor role during *P. falciparum* malaria as has been previously suggested (23). On the other hand, CD4 + T cells are thought to fulfill two major functions during an immune response to blood-stage malaria parasites, first, providing help for antibody production, and second, producing cytokines which activate non-lymphoid effector cells such as monocytes/macrophages and neutrophils to phagocytose parasitized erythrocytes and/or kill malaria parasites (23, 26). Evidence for the T dependency of anti-malarial antibodies was obtained in co-culture experiments in which autologous T and B cells collected from *P. falciparum* immune donors were stimulated *in vitro* with parasite antigens. This T-B interaction resulted in a strictly T cell dependent secretion of *P. falciparum*-specific antibodies (27). Moreover, there is a good correlation between interleukin-4 (IL-4, a Th2 cytokine) production by in *vitro* activated T cells and the serum levels of antibodies specific for the *P. falciparum* antigens used for T cell activation (28). Evidence for the protective role of T cell-derived cytokines in relation to monocyte/macrophage activation has been obtained by the observations that *P. falciparum*-protected individuals have higher serum interferon-gamma (IFN-γ, a Th1 cytokine) levels than nonprotected individuals (29) and that increased levels of neopterin, a low molecular weight compound derived from guanosinetriphosphate and produced in high quantities by IFN-γ-stimulated macrophages (30), are present in serum and urine of patients with uncomplicated *P. falciparum* malaria (31, 32). Furthermore, the level of IFN-γ production by in *vitro* activated T cells collected from individuals previously infected with *P. falciparum* has been shown not to correlate with the levels of anti-malarial antibodies in the donors' plasma (28). This observation suggests that IFN-γ may be involved in cell-mediated protection rather than antibody-mediated protection. The concept that CD4+ Th1 cells, which upon activation secrete IL-2 and IFN-γ, up-regulate cell-mediated immune responses and down-regulate Th2-type responses whereas CD4+ Th2 cells, which upon activation secrete IL-4, IL-5, IL-6 and IL-10, provide help for antibody production and down-regulate Th1-type responses is well established during infection in mice (33), and, thus, may also be true during human malaria (23). Furthermore, *in vitro* studies have shown that IFN-γ increases phagocytosis of *P. falciparum* by human macrophages (34) and induces these cells to secrete reactive oxygen intermediates (ROI) which leads to the death of non-phagocytised *P. falciparum* parasites inside erythrocytes (35). Neutrophils are also able to phagocytose human malaria parasites and generate toxic ROI upon cytokine stimulation *in vitro* (36). In addition to macrophages and neutrophils, natural killer (NK) cells and γ/δ T cells have also been suggested to act as effector cells against blood-stage malaria (37, 38). The mechanism of parasite killing by these effector cells *in vivo* is uncertain. In the case of macrophages and neutrophils, these cells may first phagocytose and then kill the parasites by ROI or lysosomal enzymes, and/or, they may secrete effector molecules which kill parasites inside erythrocytes in the bloodstream. Indeed, an as yet unidentified, non-immunoglobulin factor present in human serum from malarious areas in Sudan has been shown to induce intraerythrocytic death of *Plasmodium falciparum in vitro* (39). A role for cell-mediated immune responses in the generation of this serum factor, and thus in killing of *Plasmodia*, has been hypothesized (40). The monokine, tumor necrosis factor-alpha (TNF-α), secreted mainly by activated macrophages, and nitric oxide (NO), a short-lived gas produced in high quantities by many cell types upon activation, are thought, besides ROI, to act as effector, killer agents against human malaria parasites. Because the topic of the present thesis concerns the protective role of both TNF-α and NO during malaria *in vivo*, I will present a more detailed literature review regarding these molecules in relation to malaria later in this introductory chapter. The rationale for focusing in this thesis on TNF-α and NO, and not ROI, will also be discussed later in this chapter.

Splenomegaly is a common symptom of malaria infection (5). Moreover, it is generally accepted that the spleen plays a crucial role in host defence against malaria. Splenectomy in humans can lead to a significant recrudescence of a previously subpatent parasitemia (41). A recent case report study in Thailand indicated that splenectomy in humans delays the clearance of infection with *P. falciparum* (42). Furthermore, a study by Ho et al (43) demonstrated that the spleen *in vivo* clears *P. falciparum* parasites from the blood stream of patients. In the latter study, activated macrophages in the spleen were suggested to be the effector cells (43). Enhanced clearance has also been shown to correlate with the degree of splenomegaly (44). T cells, in addition to macrophages, have been suggested to migrate to the spleen during malaria (reviewed by 6) and the spleen has also been shown to be the major site of antimalarial antibody production (45). Thus, during malaria, the spleen appears to be the site of cellular activation and interactions, and clearance of parasitized cells.

Little is known about the role of the liver in blood stage malaria. Autopsy of fatal cases of *P. falciparum* malaria has demonstrated that Kupffer cells and free sinusoidal macrophages phagocytose parasitized erythrocytes, an observation which suggests that the liver, in addition to the spleen, may be involved in the clearance of malaria parasites from the blood stream (46). The liver may also be involved in the synthesis of acute phase proteins and complement factors. However, the importance of either complement or acute phase proteins in protection against malaria is not well known (37, 46). Thus, the production of TNF-α and NO in the spleen as well as the liver during *P. chabaudi* AS malaria is the focus of chapters 2 and 3.

With the exception of genes affecting invasion or survival of *Plasmodia* in red blood cells (reviewed in 26 and 47), little is known about genes which may be involved in the regulation of immune responses during human blood-stage malaria. No consistent MHC class II restriction of any particular anti-malaria immune responses has been demonstrated in human populations (23). For example, an investigation of approximately 500 African donors typed for MHC class II showed no correlation between the MHC types and *in vitro* proliferation or IFN-γ secretion of their malaria-antigen activated T cells (48). On the other hand, a study with monozygotic twins living under similar environmental conditions in a malaria-endemic region of Africa suggested that non-MHC gene(s) regulate both antibody and T cell responses to defined *P. falciparum* antigens (49). It is presently not known whether these putative genes controlling the immune response to malaria are expressed at the level of B or T cells or antigen presenting cells, such as macrophages. I will discuss the contribution of the genetic background of the host to the regulation of the immune response against blood-stage malaria in chapter 5.

Blood-stage *P. chabaudi* AS: a murine malaria model.

It is often not possible to conduct particular experiments in humans in order to investigate the interaction of the malaria parasite and its host. Therefore, various simian, avian and rodent models have been developed (50). Simian models, either with their natural parasites or adapted human parasites, and rodent models with parasites isolated from African wild rodents are most commonly studied (50). Rodents, in particular mice, offer the advantages that they are relatively easy to maintain and are immunologically and genetically well characterized. More important, however, is that the general means by which parasites are recognized and eliminated by the immune system are probably similar in mouse and man. On the other hand, several aspects of the interaction between humans and *Plasmodia* are unique (10), thus caution is always required in order to extrapolate from experimental models to humans in the study of malarial immunity.

Four species of *Plasmodium* are commonly used in mouse malaria models: *P. berghei*, *P. yoelii*, *P. vinckei* and *P. chabaudi*. *P. berghei* and *P. vinckei* invariably cause lethal infections in mice. In contrast, infections with *P. yoelii* and *P. chabaudi* are generally self-resolving, although death can occur in certain mouse strains when infected with certain parasite strains (51). For example, the *P. chabaudi* strain used in the experimental studies conducted in this thesis, that is, *P. chabaudi* AS, has been shown to cause a self-resolving infection in resistant C57BL/6 (B6) strain mice, whereas a lethal infection is seen in susceptible A/J (A) strain mice (52). This model, thus, allows one to study the role of the host's immune system and/or genetic background in relation to resistance against malaria. Furthermore, *P. chabaudi* AS, which infects mature red blood cells in contrast to *P. yoelii* which tends to invade only reticulocytes, is considered a good representative of human *P. falciparum*, which infects all, mature and immature, red cells (50, 51, 53, 54).

Experimental infections with blood stage *P. chabaudi* AS are initiated by direct inoculation of parasitized erythrocytes, taken from a previously infected mouse or a frozen stock of infected red cells, into mice, thereby bypassing the liver stage of the parasite's life cycle. The course of infection is monitored by quantitating the percentage of parasitized erythrocytes in appropriately stained blood smears taken at different times after infection. Few parasites are found in the prepatent period following infection. Thereafter, a rapid rise in parasitemia is observed. Fatal infections, as seen in A mice, are characterized by high peak parasitemias with subsequent death of the host. Self-resolving infections, as seen in B6 mice, tend to have more moderate levels of peak parasitemia which is subsequently controlled in these mice. Recrudescences of parasitemia, although at much lower peak levels, may then occur several weeks after infection and the mice are immune to challenge infections.

The immune response during infection with *P. chabaudi* AS in mice has several similarities with the above described immune response against *P. falciparum* in humans. Indeed, although the kinetics and relative importance of different anti-malarial immune mechanisms may still be very different between human and mice, both antibody-dependent and antibody-independent immune effector mechanisms appear to be involved in protective immunity to both blood-stage *P. chabaudi* AS and *P. falciparum* malaria. Experiments using transfer of purified T cells or *in vivo* depletion of T cells by antibody treatment have indicated that αβ TCR+, CD4+ T cells are essential for control of blood stage *P. chabaudi* AS infection (55, 56). Moreover, CD4+ T cells, in contrast to CD8+ and γδ TCR+ T cells which appear to have a limited role in the control of *P. chabaudi* AS infections (reviewed in 57), are thought to play a major role in the regulation of both antibody-independent and antibody-dependent immune effector mechanisms during *P. chabaudi* AS malaria. Indeed, previous studies by Langhorne et al (58) and our laboratory (59) have demonstrated that, during infection with *P. chabaudi* AS, parasite-specific, inflammatory-type CD4+ Th 1 cells predominate during the early or acute phase of infection, followed by the appearance, during the late phase of infection, of Th 2 cells which are capable of providing help for antibody production. Moreover, a study by Taylor-Robinson et al (53) has demonstrated that, during infection with *P. chabaudi* AS, a parasite-specific CD4+ Th 1 clone, derived from mice during acute infection and capable of producing IFN-γ and IL-2, protects NIH mice by a NO-dependent, and thus presumably cellular mechanism, whereas a parasite-specific CD4+ Th 2 clone, derived from mice during a secondary infection and capable of producing IL-4, protects these mice by enhancing the production of specific IgG1 antibody. Thus, an antibody-independent response during the early phase followed by an antibody-dependent response during the late phase of a primary infection with *P. chabaudi* AS appears to confer protection against the disease. In contrast, a strong Th2 response early during infection, as has been observed in susceptible A mice (59), may lead to a severe and lethal course of infection with *P. chabaudi* AS. Thus, host protective mechanisms appear to be divergent in resistant and susceptible mice during the early, acute phase of infection. This finding may shed some light on why *P. falciparum* is severe in some patients and not in others. In chapters 2, 3 and 4 of this thesis, the *in vivo* production and role of TNF-α and NO, which are used as markers for a cell-mediated immune response and may act as effector, anti-parasitic agents, are investigated during the early, acute phase of infection with *P. chabaudi* AS in both resistant B6 and susceptible A mice. The reason that the role of ROI as potential effector molecules was not investigated is because a study by Cavacini et al (60) demonstrated that P/J strain mice, which have defects in their capacity to produce high levels of ROI during infection, resolved an infection with *P. chabaudi adami* as effectively as BALB/c control mice. These results, thus, suggest that other molecules, besides ROI, can be involved in protection against infection with *P. chabaudi* AS.

Also similar to human *P. falciparum* malaria, is the fact that the spleen appears to be important for the resolution of infection with *P. chabaudi* AS. Indeed, previous studies in our laboratory have shown that splenectomized B6 mice are unable to control and clear the malaria parasites (61, 62). Moreover, the spleen has been shown to be required for the expression of both T cell-dependent, cell-mediated (63) and antibody-dependent (62) immune responses against murine malaria. Furthermore, macrophages have been shown to play a crucial role in resistance to *P. chabaudi* AS. Indeed, treatment, early during infection, of otherwise susceptible A mice with the liposome-encapsulated, macrophage-activating agent muramyl dipeptide glycerol dipalmitate resulted in decreased parasitemia and increased survival, whereas treatment of otherwise resistant B6 mice with macrophage-depleting silica resulted in death of the host (64). Taken together, these results suggest that macrophages in the spleen may play an important role in resistance to *P. chabaudi* AS malaria. Also, in the absence of the spleen, the liver has been shown to be a site of immune activation during murine malaria (65, 66), and it has been suggested that the liver may have an important role in recovery from blood-stage malaria (62, 65, 66). Kupffer cells or macrophages infiltrating the liver during infection with *P. chabaudi* AS may thus, similar to macrophages in the spleen, be involved in immune phagocytosis of free merozoites and/or parasitized red cells and in the production of parasiticidal molecules such as TNF-α, NO and ROI.

As in human malaria, little is known about the genes which may be involved in the regulation of immune responses during *P. chabaudi* AS blood-stage malaria. Previous studies in our laboratory have indicated that resistance to *P. chabaudi* AS, as seen in B6 mice and characterized by prolonged survival, low peak parasitemia and marked splenomegaly, is controlled by a major, single, dominant, non H-2 linked gene (52, 67). Although the genetic mechanism is not known, the phenotype of susceptible mice, such as A mice, is expressed as high peak parasitemia levels, reduced increase in spleen weight, and death due to infection (52, 67). Furthermore, it was also demonstrated that expression of resistance is influenced by the sex of the host, that is, female mice are more resistant than male mice. Minor genes may also contribute to the level of resistance to *P. chabaudi* AS (52). Moreover, the phenotypic expression of the resistance gene appears to occur early during the course of infection with *P. chabaudi* AS. In this regard, it has been suggested that the expression of the resistance gene relates to controlling parasite multiplication (67). However, similar to the situation in human malaria, it is currently not known if the resistance gene is expressed at the level of the antigen presenting cell, T or B cell.

2. TUMOR NECROSIS FACTOR-alpha

Role, structure, production, regulation and action on cells

TNF-α was initially described as a protein that could be induced by treating mice with bacille Calmette-Guérin (BCG) followed by lipopolysaccharide (LPS) and causes necrosis of some mouse tumors (68). It was subsequently shown that the same molecule was responsible for an elevation in plasma triglycerides accompanied by anorexia and cachexia in *Trypanosoma brucei*-infected rabbits and was an essential mediator in septic shock induced by LPS injection in mice (69). Currently, it is known that TNF-α is a multipotent molecule involved in either protective inflammatory processes and immune responses or in the pathogenesis of inflammation and infection which may lead to organ dysfunction and death (reviewed in 70, 71). Indeed, TNF-α has been demonstrated to have a protective role during bacterial (72, 73) and parasitic (74, 75) infections. In contrast, TNF-α has been implicated in pathogenesis during sepsis (69), infectious diseases such as malaria (76), and a wide variety of autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis (reviewed in 77). The paradoxical role of TNF-α, that is, protection versus pathology, is thought to depend mainly on the amount of TNF-α produced (70, 71, 76). Low and moderate amounts of TNF-α are believed to induce the local production of a well-orchestrated cascade of regulatory molecules resulting in a protective immune response, whereas excessive amounts of TNF-α are thought to induce systemic and massive release of regulatory molecules resulting in immunopathological events. Moreover, it is often assumed that, in addition to the amount of TNF-α produced, the time period over which its production is sustained, the tissues where it is produced, and the presence of cytokines regulating its production are involved in determining the role of TNF-α (76, 78, 79). The importance of these factors in determining the role of TNF-α during infection with *P. chabaudi* AS is the focus of chapter 2 of this thesis.

Soluble TNF-α is biologically active as a trimer with a total molecular mass of 52 kDa (80). However, TNF-α can also exist as an unprocessed, membrane-bound form with a molecular mass of 26 kDa (81). The membrane-bound form of TNF-α appears not only to serve as the precursor of the monomeric secretory component but has also been shown to be able to mediate macrophage activation and cytotoxicity against TNF-α-sensitive targets (81-83).

Macrophages and monocytes are the major, but not the only cells, able to synthesize TNF-α. T and B lymphocytes, mast cells, polymorphonuclear cells, keratinocytes, astrocytes, microglial cells, Kupffer cells, smooth muscle cells and some tumor cells can also produce TNF-α (reviewed in 70). TNF-α is synthesized following cell activation. LPS is the most powerful TNF-α-inducer, however, viral, fungal, mycobacterial and parasite antigens, C5a anaphylatoxin, IL-1, IL-2, GM-CSF, CSF-1 and, in an autocrine manner, TNF-α itself may also trigger TNF-α synthesis (reviewed in 70, 71). Moreover, *in vitro* studies have indicated that TNF-α synthesis can be regulated. For example, IFN-γ, which can induce an increase in TNF-α mRNA by itself, has been shown to be a potent amplifier of LPS-induced TNF-α protein release (84, 85). In contrast, glucocorticoids, IL-4, IL-6 and IL-10 have been shown to be able to suppress the production of TNF-α by LPS and IFN-γ at both the level of TNF-α gene transcription and TNF-α mRNA translation (84, 86-88). Furthermore, a recent study has demonstrated that TGF-β1 is also able to inhibit TNF-α synthesis by a post-transcriptional mechanism (89). Thus, during infection *in vivo*, TNF-α may be produced by various cell types and its production appears to be tightly regulated by other cytokines.

TNF-α exerts its activities through clustering of TNF receptors which are present on nearly all cell types except erythrocytes and unstimulated T lymphocytes (80). There are two types of receptors, TNF-R1 or p55 and TNF-R2 or p75, which can be differentiated by their size, affinity for TNF-α and recognition by monoclonal antibodies (80, 90). Morover, the two receptors are thought to trigger different intracellular pathways and have distinct roles. Studies using mice with a disrupted TNF-R1 gene have shown that TNF-R1 mediates both protective immunity against *Listeria monocytogenes* and the lethal effect of LPS (91, 92). A study with TNF-R2-deficient mice indicated that TNF-R2 is involved in transducing the lethal effect by LPS (93). However, the specific signaling pathways responsible for the harmful and/or beneficial effects following the clustering of TNF receptors by TNF-α are currently not well known.

Since nearly all cell types bear TNF receptors, it is not surprising that TNF-α exerts pleiotropic activities (70, 80, 84). Moreover, many of the bioactivities of TNF-α are shared by other cytokines, in particular, IL-1 (94). The specific effects of TNF-α on monocytes/macrophages, lymphocytes, polymorphonuclear leukocytes, endothelial cells, fibroblasts, nervous tissue cells, adipocytes, muscle cells, keratinocytes and tumor cell lines *in vitro* have been recently reviewed by Vassalli (70). I will discuss some of these effects in relation to malaria in the following section.

Tumor necrosis factor-alpha and blood stage malaria

Soluble malaria-parasite antigens which are released into the circulation during the rupture of infected red blood cells are able to induce the production of TNF-α *in vivo* (95). Moreover, these antigens, derived from both human and mouse malaria-parasites, are shown to act directly on macrophages for the release of TNF-α *in vitro* (95, 96). Furthermore, these TNF-α-inducing molecules possess LPS-like properties, as shown by their reactivity with *Limulus* amoebocyte lysate; and the major component for the induction of TNF-α has been shown to be a phospholipid (97, 98). In mice, these antigens also elicit the production of T-independent antibodies that block their ability to induce TNF-α (99). Because, as I will discuss further, TNF-α is believed to be responsible for many of the clinical symptoms of malaria, it has been proposed that these T-independent antibodies mediate the anti-disease immunity observed in *Plasmodium*-infected children and that these antibody-inducing antigens could be used for the generation of an anti-disease vaccine (11).

Evidence that TNF-α is indeed produced during human malaria stems from the fact that high plasma levels of TNF-α are detected in patients with uncomplicated *P. falciparum* or *P. vivax* malaria (100, 101). Moreover, it was found that plasma levels during severe, non-fatal, and severe, fatal malaria were two and ten times higher, respectively, compared to plasma levels during uncomplicated *P. falciparum* malaria (100). These findings, thus, demonstrate that TNF-α production is increased during malaria and that excessive levels of production may contribute to pathology or death due to malaria.

The role of TNF-α in pathology due to malaria has received, understandably so, much attention. General, non-specific symptoms of malaria, such as fever and chills, occur coincident with the time when infected erythrocytes burst and TNF-α-inducing parasite antigens appear in the blood stream (101). It is also known that TNF-α has pyrogenic properties and a recent study indicated that treatment with anti-TNF-α antibody reduced fever in patients infected with *P. falciparum* (102, 103). These data, thus, demonstrate that TNF-α is involved in the induction of fever during malaria. However, fever has been suggested to limit parasite multiplication and may thus have a protective rather than a pathologic effect (104). Potentially more harmful is the fact that TNF-α is able to up-regulate the expression of ICAM-1, a known adhesion receptor for *P. falciparum*-infected erythrocytes, on endothelial cells which may lead to sequestration of parasitized red cells in the brain and the development of cerebral malaria (8, 70, 105).

Treatment with anti-TNF-α antibody was also shown to prevent the development of a murine form of cerebral malaria caused by *P. berghei* ANKA in CBA mice (106). Furthermore, Clark et al (107) suggested that TNF-α also could induce endothelial cells to produce NO during malaria, which could then mediate certain clinical signs seen in cerebral malaria, such as coma and increased intracranial pressure. TNF-α may also play a role in the development of anemia during malaria. Mice infected with *P. vinckei* and treated with human recombinant TNF-α were shown to experience increased erythrophagocytosis and dyserythropoiesis indicating that TNF-α contributes to anemia (108). Moreover, a recent study demonstrated that transgenic mice carrying a constitutively expressed human TNF-α gene and infected with *P. yoelli* or *P. chabaudi* AS developed severe anemia which was probably caused by macrophage activation leading to enhanced erythrophagocytosis and not by the direct inhibition of erythropoeiesis by TNF-α (109). TNF-α has also been suggested to induce hypoglycemia during malaria (76). However, it appears unlikely that TNF-α is a major player in the development of hypoglycemia since treatment with anti-TNF-α antibody did not prevent hypoglycemia in mice infected with *P. yoelli* (110). TNF-α, through its ability to up-regulate NO production, may also mediate immunosuppression which is commonly observed during malaria (reviewed in 6). Indeed, *in vitro* studies by Rockett et al (111) and in our laboratory (112) have shown that NO is able to suppress lymphoproliferation during murine malaria.

TNF-α was also shown to have a protective role during malaria since treatment with human recombinant TNF-α could suppress infection with *P. chabaudi adami* in CBA mice and, more strikingly, rescue susceptible A/J mice from an otherwise lethal infection with *P. chabaudi* AS (75, 113). TNF-α is thought to function as a mediator of cell-mediated parasite killing (78). Indeed, TNF-α by itself does not kill malaria parasites directly since recombinant TNF-α has no effect on *P. falciparum* and *P. yoelli in vitro* (114, 115). Instead, *in vitro* studies have shown that TNF-α increases killing of *P. falciparum* by neutrophils (36, 116). In the latter studies, TNF-α was thought to increase the expression of Fc and complement receptors leading to a more efficient phagocytosis of malaria parasites which requires antibody and complement. TNF-α, usually in synergy with IFN-γ, is also known to act as a powerful activator of monocytes/macrophages (70). Activated macrophages demonstrate an increase in phagocytic activity and are able to produce reactive oxygen and nitrogen intermediates (70). These intermediates may then kill malaria parasites in the bloodstream passing through macrophage-rich, blood-filtering organs, such as spleen and liver. Indeed, TNF-α-containing sera from BCG and LPS-treated animals and malaria patients from Sudan have been shown to kill *P. falciparum in vitro* (40). These findings may indicate that TNF-α is involved in the induction of a component that kills malaria parasites. The exact nature of this factor induced by TNF-α has not been characterized but could include peroxidized fatty acids derived from reactive oxygen intermediates and lipids, as proposed by Rockett et al (117), or NO. The role of TNF-α in the induction of NO early during infection with *P. chabaudi* AS was investigated in the experimental studies presented in chapter 4.

3. NITRIC OXIDE

Structure, role, production, regulation and action on cells

NO is an inorganic, gaseous radical with a short half-life measured in seconds (118). It is one of the smallest (30 Da) biologically active metabolites and has been shown to be produced by slime molds, insects, horseshoe crabs, mollusks, chickens, mice, rats, cows and humans (119). Although the specificity of NO's interactions is minimal, the molecule is involved in both delicate biological processes such as neurotransmission, peristalsis and vasodilatation, and the destruction of microorganisms and tumor cells (119, 120). However, production of NO during infectious diseases and non-infectious disorders such as diabetes and stroke may result in tissue damage (119). Similar to TNF-α, the biological role of NO appears to be determined by its level. It is believed that pulses of small amounts of NO regulate delicate, physiological processes described above whereas sustained and high levels of NO can act in a destructive and/or pathologic manner (119, 121).

NO is produced from L-arginine by oxidation of a guanidino nitrogen, with L-citrulline as a co-product. The reaction is catalysed by NO synthases (NOS) and requires NADPH, FAD, FMN, tetrahydrobiopterin, calmodulin, and heme (122). Although there are 3 genes corresponding to 3 different NOS isoforms, the enzymes can be divided in 2 functional phenotypes. The first type, denoted as cNOS, is triggered by $Ca2^+$-elevating agonists such as bradykinin, produces transient and small amounts of NO, is found in neurons, skeletal muscle, neutrophils, pancreatic islets, endometrium, epithelia and endothelial cells, and regulates the delicate, homeostatic processes described above. The second type, denoted as iNOS, is induced by a wide variety of microbes or microbial products and numerous cytokines, produces sustained and high levels of NO, is found in macrophages, fibroblasts, hepatocytes, endothelial cells, neurons, neutrophils, smooth muscle cells, cardiac myosites, epithelia, chondrocytes, keratinocytes and some tumor cells, and appears to play an important role during host defense. Excessive production of NO by iNOS may result in host pathology (119-121, 123). Because of its role in host defense, the following paragraphs relate to iNOS.

Interferons, proinflammatory cytokines such as TNF-α and IL-1, microbes and microbial products, are able to induce NO synthesis by many cell types (119, 121, 124). IFN-γ and LPS alone induce transcription of iNOS in murine macrophages resulting in low levels of NO (125-127). Maximal transcriptional induction of iNOS leading to high levels of NO is reached when macrophages are activated with IFN-γ in synergy with LPS (125, 126). The requirement of IFN-γ for the production of high NO levels is demonstrated by the fact that macrophages recovered from BCG-infected mice with a disrupted IFN-γ gene were unable to produce NO upon stimulation with LPS, whereas addition of exogenous IFN-γ could reverse this effect (128). Furthermore, the synergistic effect between IFN-γ and microbes or microbial products such as LPS is thought to be mediated by TNF-α which acts in an autocrine manner to up-regulate NO production. Indeed, addition of anti-TNF-α antibody was shown to reduce NO production by IFN-γ-stimulated macrophages infected with intracellular pathogens *in vitro* (129, 130). Moreover, recent studies have demonstrated that treatment with anti-IFN-γ or anti-TNF-α antibody resulted in decreased NO production in BCG-infected mice and decreased iNOS mRNA expression in the brain of *Toxoplasma gondii*-infected mice *in vivo* (131, 132). On the other hand, cytokines such as IL-4 and IL-10, which are mainly produced by CD4+ Th2 cells, and TGF-β, which is produced by activated macrophages, have been shown to suppress NO synthesis (89, 133, 134). IL-4 appears to reduce both iNOS transcription and its enzymatic activity whereas, in addition to down-regulating iNOS transcription, TGF-β is able to lead to the destabilization of iNOS mRNA and the degradation of iNOS protein (89, 134, 135). IL-10, on the other hand, has been shown to block TNF-α production, an important positive regulator of NO synthesis, at the level of both mRNA and protein synthesis (133). The *in vivo* regulation of iNOS by TNF-α, IFN-γ and IL-4 early during blood-stage infection with *P. chabaudi* AS is the subject in chapter 4.

In biological systems, NO reacts with oxygen (O_2), superoxide (O_2^-), transition metals such as iron (Fe) and copper (Cu), thiols (components containing chemically reactive sulfur [S]), DNA and tyrosine residues (118, 136). Under oxygen-rich conditions, NO may also interact with itself to form other reactive nitrogen intermediates which ultimately decompose to nitrites (NO_2^-) and nitrates (NO_3^-) (123). The latter molecules can be more easily detected in culture medium and body fluids and are often used as quantitative indicators of NO production.

The precise molecular mechanisms by which high levels of NO protect the host during infection appear to be complex and are not well known. NO has been demonstrated to be able to bind and inhibit the activity of numerous metal- and thiol-containing enzymes (reviewed in 136). Therefore, it has frequently been proposed that NO inactivates vital enzymes and damages DNA of pathogens resulting in inhibition of replication and/or death of the microorganisms (121, 137, 138). Moreover, numerous and diverse pathogens have been shown to be susceptible to NO *in vitro* (123). On the other hand, NO appears able to protect against damage mediated by reactive oxygen intermediates. Indeed, Wink et al (139) demonstrated *in vitro* that NO protects against hydrogen peroxide-mediated cytotoxicity. These authors suggested that NO, through its metal-binding property, may block the formation of extremely toxic hydroxyl radicals which are formed from the metal-mediated reduction of hydrogen peroxide. Moreover, recent evidence suggests that peroxynitrite, which is generated from NO and O_2^-, does not appear to induce cytotoxicity as has been previously suggested (140). Furthermore, treatment with L-NMMA, an iNOS inhibitor, was also shown to increase oxygen radical-mediated liver injury in mice infected with *Corynebacterium parvum* and treated with LPS (141). This observation suggests that, *in vivo*, NO also protects against oxygen stress-mediated pathology. Thus, an intriguing and subtle interplay appears to exist between reactive oxygen and nitrogen intermediates which, if well-orchestrated, results in moribund pathogens and relatively undamaged host cells.

Nitric oxide and blood stage malaria

Recently, it was demonstrated that two preparations of *P. falciparum* extract were able to induce, in the presence of IFN-γ, the production of NO by the RAW 164.7 mouse macrophage cell line *in vitro* (142). In this study, the levels of NO induced by malaria-parasite antigens and IFN-γ were as high as the levels induced by LPS and IFN-γ and were not dependent on the presence of TNF-α which was, in addition to NO, produced by the stimulated macrophages. Evidence for the production of NO *in vivo* during malaria was demonstrated by the finding that patients infected with *P. falciparum* or *P. vivax* have increased plasma levels of NO (143, 144). Furthermore, *in vitro* studies have shown that reactive nitrogen intermediates, in particular nitrite and nitrate ions and nitrosothiol derivates of cysteine and glutathione, were toxic for *P. falciparum* which suggests that NO has a parasiticidal role during malaria (145). In this regard, a study by Taylor-Robinson et al (53) demonstrated that a Th1 CD4+ T cell clone specific for *P. chabaudi* AS could protect T cell-depleted NIH mice against infection with *P. chabaudi* AS by an NO-dependent mechanism. In the latter study, treatment with L-NMMA not only abolished the protective effect of the Th1 clone but also resulted in increased peak parasitemia suggesting that NO is involved in parasite killing *in vivo*. On the other hand, the results of a study by Kremsner et al (146) indicated that, during infection with *P. vinckei* in BALB/c mice, NO may not be involved in controlling parasitemia but may protect against oxygen radical-induced tissue damage. Furthermore, and as already mentioned above, NO may also mediate immunosuppression (111, 112) and may play a role during the pathogenesis of cerebral malaria (107). The production and role of NO during infection with *P. chabaudi* AS is the main theme of chapter 3.

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CHAPTER 2

A Th1 ASSOCIATED INCREASE IN TNF-α EXPRESSION IN THE SPLEEN CORRELATES WITH RESISTANCE TO BLOOD-STAGE MALARIA IN MICE.

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Abstract

We investigated the kinetics of tissue-specific mRNA expression and systemic production of TNF-α and of splenic mRNA expression of IFN-γ and IL-4, cytokines that may regulate TNF-α production, during the early phase of blood-stage infection with *Plasmodium chabaudi* AS. Northern blot analysis revealed that resistant C57BL/6 mice, which clear the infection by 4 weeks, had higher levels of TNF-α mRNA in the spleen and liver early during infection compared to susceptible A/J mice, which succumb to the disease 10 days after initiation of infection. Treatment of resistant mice with a polyclonal anti-TNF-α antibody confirmed the protective role of TNF-α early during the course of infection. Furthermore, resistant C57BL/6 mice also expressed high levels of IFN-γ (a Th1 marker) and low levels of IL-4 mRNA (a Th2 marker) in the spleen, whereas susceptible A/J mice had low levels of IFN-γ but high levels of IL-4 mRNA in the spleen early during infection. On the other hand, susceptible A/J mice expressed high levels of TNF-α mRNA in the liver and had high serum levels of TNF-α protein, as measured by ELISA, later during infection just before death occured. These results, thus, demonstrate that a Th-1 associated increase in TNF-α mRNA expression in the spleen early during infection correlates with resistance to *P. chabaudi* AS, whereas increased TNF-α mRNA levels in the liver and excessive levels of the TNF-α protein in serum later during infection correlates with susceptibility. Thus, the role of TNF-α during malaria appears to depend on the timing and site of its expression and the presence of cytokines regulating its production.

Introduction

Tumor necrosis factor-alpha (TNF-α) is a protein produced during many infectious diseases, including malaria (reviewed in Ref. 1). Blood-stage malaria parasites have been shown to release endotoxin-like antigens which induce the production of TNF-α *in vivo* (2). High levels of TNF-α have been demonstrated in the blood of malaria patients infected with *Plasmodium falciparum* or *P. vivax* (3, 4). The biological effects of TNF-α during malaria infection are presumed to be both pathogenic and protective. Treatment of patients with anti-TNF antibody has indicated that TNF-α is involved in the induction of high malarial fever (5). Furthermore, studies in murine malaria models have shown that TNF-α may mediate the two major causes of mortality associated with malaria, anemia and cerebral malaria (6-8). In contrast, repeated injections of recombinant TNF-α into malaria-infected mice has been shown to reduce parasitemia and protect against lethal infection (9). It is often assumed that the protective versus pathogenic role of TNF-α during malaria depends on the quantity of TNF-α produced, the time period over which its production is sustained, the tissue where it is produced and the presence of cytokines regulating its production (10, 11). Whereas most studies have focused on the quantity of TNF-α produced (3-5), only one *in vivo* study has demonstrated that increased expression of TNF-α mRNA in the brain correlated with susceptibility to murine cerebral malaria (11). The same authors also suggested that, in the brain, a Th1-type response may up-regulate, whereas a Th2-type response may down-regulate, TNF-α production (11).

Our laboratory has recently demonstrated that elimination of blood-stage *P. chabaudi* AS by resistant B6 mice occurs by sequential activation of CD4+ Th1 cells followed by activation of Th2 cells (12). In contrast, induction of a strong Th2 response early in infection, as observed in susceptible A mice, has been suggested to lead to fulminant parasitemia and a lethal course of malaria (12). Furthermore, we demonstrated that treatment with human recombinant TNF-α (hu rTNF) early during infection protects susceptible A mice from an otherwise lethal infection (13). Thus, A mice treated with hu rTNF-α experience a similar course of infection as resistant B6 mice which is characterized by moderate levels of peak parasitemia on days 7-10 post infection and clearance of infection by 4 weeks. Treatment of resistant B6 mice with the same dose of hu rTNF-α, however, had no effect suggesting that this mouse strain produces sufficient amounts of TNF-α. Treatment with a 5-fold higher dose of hu rTNF-α resulted in death of B6 mice within hours. We also demonstrated that an intact spleen is required to resolve *P. chabaudi* AS malaria (14). Others have suggested that the liver, in addition to the spleen, may have a protective role during blood-stage malaria (15).

In the present study, we investigated the kinetics of tissue-specific and systemic production of TNF-α and of Th1 and Th2 cytokines that may regulate its production *in vivo*. More specifically, we determined in both mouse strains during the first 2 weeks post infection: 1) TNF-α mRNA levels in spleen and liver, 2) serum levels of TNF-α protein, and 3) IFN-γ (a Th1 marker) and IL-4 (a Th2 marker) mRNA levels in spleen. In addition, we investigated the protective role of TNF-α in resistant B6 hosts by treating this mouse strain with a polyclonal anti-TNF antibody. Our results confirm that TNF-α has a protective role and indicate that increased levels of TNF-α in the spleen and the liver early during infection are required in order to survive infection with *P. chabaudi* AS. In contrast, increased TNF-α levels in the liver and serum later during infection appear to have a deleterious effect on the outcome of this disease. Furthermore, a Th1 response sustains a high expression of TNF-α in the spleen, whereas a Th2 response down-regulates the expression of TNF-α.

Materials and Methods

Mice, parasite and experimental infections. B6 and A strain mice, 8-14 weeks old, were age- and sex-matched in all experiments. Mice were bred at the animal facility of the Montreal General Hospital Research Institute from breeding pairs obtained from Jackson Laboratories, Bar Harbor, ME. *P. chabaudi* AS was maintained as previously described (16). Mice were infected intraperitoneally with 10^6 *P. chabaudi* AS parasitized red blood cells (PRBC) and parasitemia during the subsequent course of infection was monitored at the the times indicated using previously described procedures (12).

Anti-TNF-α antibody treatment. Rabbit anti-recombinant murine TNF-α IgG was kindly provided by Dr. E. A. Havell (Trudeau Institute, Saranac Lake, NY). The production and purification of these antibodies, and their capacity to specifically neutralize TNF-α has been previously described (17). Resistant B6 mice were treated intraperitoneally with 0.2 ml pyrogen-free saline containing 10^4 neutralizing units anti-TNF-α antibody (protein content = 2.4 mg), 6 hours prior to and 4 days after *P. chabaudi* AS infection. Control mice were similarly treated with 0.2 ml pyrogen-free saline containing an equivalent amount of normal rabbit serum IgG which was prepared in the same way as the anti-TNF-α antibodies.

mRNA levels in spleen and liver. Spleens and livers 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 described by Chirgwin *et al* (18). The RNA yield of each sample was determined spectrophotometrically on the basis of the A_{260} and the purity (ranging from 1.5 to 1.8) assessed by the A_{260}:A_{280} ratio. mRNA levels were determined by Northern blot analysis (19). Briefly, total RNA samples (20 µg) were separated on a 1.2% agarose gel containing 2.2 M formaldehyde, blot transferred to a nylon membrane (Schleicher & Schuell, Keene, NH) and UV cross-linked (UV Stratalinker 1800, Stratagene, La Jolla, CA). The blots were prehybridized for 8 hours, hybridized overnight with a specific DNA probe, labeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL; specific activity: 10^9 cpm/µg DNA) and washed. Hybridization was detected by autoradiography with Kodak X-Omat-AR film (Rochester, NY). mRNA levels were quantified by high resolution optical densitometry (SciScan 5000, United States Biochemical, Cleveland, OH) and normalized to 18 S rRNA. A 1.9 kb fragment of rRNA, which recognized 18 S rRNA, was kindly provided by Dr. N. Arnheim and has been previously described in detail (20). A 1.3 kb fragment of the murine TNF-α gene was kindly provided by Dr. A. Cerami (Picower Institute for Medical Research, Manhasset, NY). Fragments of the murine IFN-γ (1.4 kb) and IL-4 (0.6 kb) genes were kindly provided by Dr. Ken-ichi Arai (DNAX, Palo Alto, CA). TNF-α, IFN-γ and IL-4 mRNA and 18 S rRNA migrate with mobilities of 1.3, 1.4, 0.6 and 1.9 kb, respectively.

TNF-α protein in serum. Blood from experimental mice was obtained by cardiac puncture at times indicated, allowed to clot for 30 min at 4°C and centrifuged at 13,800 x g for 3 min. Sera were collected and stored at -20°C until use. A modification of a double-sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine TNF-α protein in serum. Briefly, 96-well plates (Immunolon II, Dynatech, Chantilly, VA) were coated overnight at 4°C with 50 µl of hamster anti-murine TNF-α monoclonal antibody (2 µg/ml; Genzyme, Boston, MA) per well. The wells were washed and incubated overnight at 4°C with either 50 µl serum samples or, as a positive control and standard, 50 µl of murine recombinant TNF-α (Genzyme) diluted in 10% normal mouse serum (2-fold serial dilution ranging from 1.6 ng/ml to 12.5 pg/ml). After washing, the wells were coated with 150 µl of skim milk powder (5% weight/volume) for 20 min at room temperature. Subsequently, the wells were washed and incubated for 2 hours at room temperature with 50 µl rabbit polyclonal anti-murine TNF-α (1:1800 dilution) which was prepared and purified by standard procedures (21). After washing, the wells were incubated for 1 hour with 50 µl goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (1:3000 dilution; Bio-Rad, Richmond, CA). The wells were washed and 50 µl of enzyme substrate (ABTS, Boehringer Mannheim, Laval, Quebec) was added for 10-20 min. The A_{405} was determined using an ELISA reader. The levels of TNF-α in the serum samples (pg/ml) were calculated from the standard curve.

Statistical analysis. Statistical significance of differences in mortality between control and experimental groups receiving anti-TNF-α antibody was determined using a non-parametric Kruskal-Wallis test. Statistical significance of differences in TNF-α, IFN-γ and IL-4 mRNA levels, and TNF-α serum protein levels between normal and infected animals as well as between B6 and A mice was determined by Student's t-test. A probability of less than 0.05 was considered significant.

Results

Anti-TNF-α antibody treatment of resistant mice We have previously demonstrated that treatment with hu rTNF-α could protect susceptible A mice against an otherwise lethal infection with *P. chabaudi* AS (13). In order to confirm the protective role of TNF-α during infection, we treated resistant B6 mice with anti-TNF-α antibody, 6 hours prior to and 4 days post infection, and parasitemia and survival were monitored. A similar treatment schedule using the same antibodies has been shown previously to eliminate the resistance of B6 mice to infection with *Toxoplasma gondii* (22). As a control, infected B6 mice were treated with normal rabbit serum. Figure 1A shows that 50% (5/10) of anti-TNF-α antibody treated mice died at, or 1 to 2 days later than, the peak of parasitemia which occured on day 7 post infection. In contrast, 100% (10/10) of the control mice treated with normal rabbit serum survived. However, depletion of TNF-α did not significantly alter peak parasitemia level (Fig. 1B). The mean peak parasitemia level (± S. E. M.) for the experimental mice was 46.2% (± 6.0) versus 47.3 % (± 2.5) for control mice. These data demonstrate that treatment of resistant B6 mice with anti-TNF-α antibody results in significantly increased mortality but does not alter the peak parasitemia level. These observations, thus, confirm that endogenous TNF-α has a protective role during *P. chabaudi* AS malaria and suggest that TNF-α is not involved in controlling the course of parasitemia.

TNF-α mRNA kinetics in spleen and liver. To determine if differences in TNF-α expression in the tissues during infection with blood-stage *P. chabaudi* AS contribute to resistance, total RNA was extracted from spleens and livers harvested from B6 and A mice at various times during the first 2 weeks post infection and TNF-α mRNA levels determined by Northern blot analysis. Tissue expression of TNF-α mRNA was also examined in normal, uninfected control mice of each strain. As shown in Figure 2, significantly increased levels of TNF-α mRNA were found in the spleens of resistant B6 mice as early as day 3 post infection compared to basal levels in the spleens of uninfected, control mice on day 0. TNF-α levels remained increased through day 7 post infection, when parasitemia ranged from 20 to 30% PRBC. TNF-α levels returned to basal levels on day 9 post infection when the peak of parasitemia (40% PRBC) occurred and remained at this level until day 14 post infection when the experiment was terminated. In contrast, TNF-α expression in the spleens of susceptible A mice was not significantly increased over that of the basal level in the spleens of normal, control animals until day 5 post infection when parasitemia was approximately 10%. TNF-α mRNA levels returned to basal levels on day 7 in susceptible hosts and remained at this level through day 9 post infection when peak parasitemia levels of > 50% PRBC occurred. As previously reported (13), death of 100% of susceptible A littermates occurred between days 9 to 12 post infection. Similar to TNF-α expression in the spleens of B6 mice, TNF-α mRNA levels in the livers of B6 mice were significantly increased in comparison to basal levels of control mice early during infection, that is, on day 5 and 7 post infection, and returned to basal levels later on during infection (Fig. 3). In contrast to the moderate increase in TNF-α mRNA expression in the spleens of A mice on day 5 post infection, liver TNF-α mRNA levels in susceptible A mice were significantly increased in comparison to basal levels of control mice later during infection, that is, reaching its highest level on day 9 post infection coincident with the peak of parasitemia (Fig. 3). Although the exact time course varied from experiment to experiment, significant differences in the level as well as the timing of TNF-α expression in spleens and livers of resistant B6 versus susceptible A mice were consistently observed. These results, thus, demonstrate that early during acute infection, that is, during the ascending phase of parasitemia, resistant B6 mice have much higher levels of TNF-α mRNA in the spleen and liver than susceptible A mice. In contrast, susceptible A mice have significantly increased levels of TNF-α mRNA in the liver later during infection at the time of peak parasitemia just before death occurs. This suggests that tissue-specific production of TNF-α in spleen and liver early during infection confers protection, whereas production of TNF-α in the liver later during infection has a deleterious effect on the outcome of this disease.

TNF-α protein kinetics in serum. In order to determine the *in vivo* systemic production of TNF-α during infection with *P. chabaudi* AS, levels of TNF-α protein were determined by ELISA in sera collected from B6 and A mice at different times during infection. TNF-α protein was not detectable in the sera collected from uninfected mice of either strain (day 0) or infected mice of either strain on day 3 post infection (Table 1). Low levels of TNF-α protein (< 20 pg/ml) were found in only 33% of mice of either strain on day 5-6 post infection when parasitemia ranged from 16 to 34%. The highest level of TNF-α protein in the serum of either mouse strain was apparent on day 7-10 post infection coincident with the peak of parasitemia. More important, however, is that during this period, firstly, more A than B6 mice (80% versus 44%, respectively) had detectable TNF-α protein levels in serum, and secondly, the average TNF-α protein level detected in A mice was 3 times higher than in B6 mice (117 versus 38 pg/ml, respectively). As described above, 100% of susceptible A mice died between days 9 to 12 post infection. Low levels of TNF-α protein (< 20 pg/ml) could be detected in serum of B6 mice on day 14 post infection when only 1% of peripheral RBC were infected. These data, thus, indicate that, at peak parasitemia, higher levels of TNF-α protein levels are present in the serum of susceptible A mice than of resistant B6 mice. This suggests that high systemic production of TNF-α protein late during infection may have a deleterious effect on the outcome of *P. chabaudi* AS malaria.

IFN-γ (Th1) versus IL-4 (Th2) mRNA kinetics in spleen. Previous results from our laboratory have shown that *ex vivo,* in response to malaria antigen within 1 week of infection, spleen cells isolated from resistant B6 produce high levels of IFN-γ (a Th1 cytokine) and low levels of IL-5 (a Th2 cytokine) (12). In contrast, spleen cells from susceptible A mice produce high levels of IL-5 and low levels of IFN-γ within the first week of infection (12). Others have shown that increased levels of TNF-α mRNA in the brain of *P. berghei* ANKA-infected mice are associated with a Th1-like response (11). To determine if, in the spleen, a Th1 versus a Th2 response correlates with a high versus a low expression of TNF-α, respectively, we determined by Northern blot analysis the kinetics of *in vivo* expression of IFN-γ and of another Th2 cytokine, IL-4, mRNAs in the spleen of both B6 and A mice during the first 2 weeks post infection. Tissue expression of IFN-γ and IL-4 was also examined in normal, uninfected control mice of each strain. Figure 4 shows that significantly increased levels of IFN-γ mRNA were found in the spleens of resistant B6 mice from day 3 through day 9 post infection compared to basal levels in the spleen of uninfected, control mice on day 0. A maximal increase in IFN-γ expression occurred on day 7 post infection several days before peak parasitemia, when parasitemia ranged from 20 to 30% PRBC. IFN-γ mRNA levels returned to basal levels on day 14 post infection when the experiment was terminated. In contrast, a significant increase in IFN-γ mRNA expression in the spleen of susceptible A mice compared to the basal level in the spleen of normal, control animals only occured on day 5 post infection when parasitemia was approximately 10%. Moreover, the maximal IFN-γ mRNA level in the spleens of susceptible A mice on day 5 post infection was significantly lower than the IFN-γ mRNA levels in the spleens of resistant B6 mice on days 5 and 7 post infection. In contrast to IFN-γ expression, the highest levels of IL-4 mRNA were found in the spleens of A mice (Fig. 5). Significantly increased levels of IL-4 mRNA in the spleens of A mice were found on days 5 and 7 post infection compared to basal levels in the spleens of control mice. A significant increase in IL-4 expression in the spleens of B6 mice was found on day 7 post infection compared to basal levels in the spleens of control mice. However, the maximal expression of IL-4 in the spleen of resistant B6 mice on day 7 post infection was 2 times lower than the maximal expression of IL-4 in the spleens of susceptible A mice on day 5 post infection. These data indicate that, early during infection, resistant B6 mice have high levels of IFN-γ mRNA and low levels of IL-4 mRNA, whereas susceptible A mice have low levels of IFN-γ but high levels of IL-4 mRNA. These results, thus, suggest that, in the spleen, a Th1 response sustains a high expression of TNF-α, whereas a Th2 response down-regulates the expression of TNF-α.

Discussion

We report here that a Th1-associated increase in TNF-α in the spleen early during infection correlates with protection to blood-stage *P. chabaudi* AS malaria. Indeed, we found significantly higher IFN-γ (a Th1 marker) and TNF-α mRNA levels in the spleens of resistant B6 mice than susceptible A mice within the first week post infection. In contrast, higher IL-4 (a Th2 marker) mRNA levels were found in the spleens of susceptible A mice than resistant B6 mice during the same period. A similar correlation between increased TNF-α mRNA levels and high IFN-γ versus low IL-4 expression has recently been demonstrated in the brain of *P. berghei* ANKA-infected mice (11). Our results are also in agreement with previous results from our laboratory indicating that spleen cells recovered from resistant B6 mice early during infection and stimulated with malaria antigen released high levels of IFN-γ and low levels of IL-5 (a Th2 marker), whereas similarly stimulated cells in the spleen recovered from susceptible A mice produced high levels of IL-5 and low levels of IFN-γ (12). Furthermore, studies using a model of sepsis with mice lacking the IFN-γ receptor have shown that IFN-γ has an important role in the up-regulation of TNF-α production *in vivo* (23). In contrast, IL-4 has been demonstrated to down-regulate TNF-α mRNA levels in human monocytes *in vitro* (24). Our results, thus, suggest that parasite antigen stimulated-cells in the spleen of resistant B6 mice produce high levels of IFN-γ which upregulate the production of TNF-α, whereas T cells in the spleen of susceptible A mice produce high levels of IL-4 which inhibit the early production of

TNF-α. We do not yet know the reason why a Th1 versus Th2 type of response develops in the spleen of B6 versus A mice, respectively. We are currently investigating if there is differential production of IL-12, a cytokine with the ability to induce the development of Th1 cells (25), since we observed that treatment with murine r IL-12 protects A mice against *P. chabaudi* AS (Stevenson *et al.*, in prep.).

Macrophages in the spleen of B6 mice may be the major TNF-α producing cells *in vivo*. Previous studies in our laboratory have shown that macrophages recovered from the spleen of B6 mice during the first week post infection and stimulated with malaria antigen or PRBC are able to produce high quantities of TNF-α protein *in vitro* (26). Moreover, similarly stimulated macrophages recovered from the spleen of susceptible A mice also produce high levels of TNF-α (26). These and our present findings indicate, firstly, that macrophages recovered from the spleen of A mice and separated from a suppressive, Th2-type splenic environment are capable of producing high amounts of TNF-α, and secondly, that the observed differences in TNF-α expression *in vivo* between B6 and A mice are probably due to immunoregulatory cytokines and not to the direct TNF-α-inducing effect of malaria antigens on macrophages (2).

We also report here that treatment of resistant B6 mice early during infection with anti-TNF-α antibody results in 50% mortality but has no effect on the course of parasitemia. This result is partially in agreement with previous results from our laboratory showing that treatment of A mice during the early course of infection with hu rTNF-α results in 100% survival but decreased parasitemia (13). It thus appears that TNF-α has a protective role early during infection but that its role may involve more besides the inhibition of parasite growth. TNF-α has no direct parasiticidal effect *in vitro* (9), but has a crucial role in modulating the initial phase of the cell-mediated immune response *in vivo* (27). In this respect, we have recently observed that resistance to infection with *P. chabaudi* AS correlates with increased levels of nitric oxide, a molecule produced during cell-mediated immune responses (28), and suggested that NO is not involved in parasite killing but may protect the host against oxygen radical-mediated tissue damage during blood stage malaria (Jacobs *et al.*, submitted). We are currently investigating the hypothesis that the protective role of TNF-α in the spleen *in vivo* involves the induction of nitric oxide, which likewise has a protective role in this organ.

Our present results also demonstrate that TNF-α expression is not only increased in the spleen but also in the liver of resistant B6 mice early during infection. Liver macrophages recovered from mice during acute malaria have been shown to have an increased capacity to secrete TNF-α *in vitro* (29). The liver, in addition to the spleen, may thus have a protective role during malaria infection as has been suggested previously (15). However, we could not demonstrate an increase in NO production in the liver of resistant B6 mice (Jacobs *et al*, submitted). This observation may suggest that, in contrast to what has been suggested for liver stage malaria (30), the protective role of TNF-α in the liver during blood-stage malaria may not involve the induction of nitric oxide. TNF-α in the liver may induce the production of acute-phase proteins (31). Little is known, however, about the precise role of these proteins during malaria (31) and previous results from our laboratory have shown that, early during infection, susceptible A mice have higher levels of serum amyloid P-component, a major acute phase protein in mice, than resistant B6 mice (32). Furthermore, the data presented here also demonstrate that both increased TNF-α expression in the liver and high TNF-α protein levels in serum are detectable in susceptible A mice, but not resistant B6 mice, later during infection coincident with the time A mice begin to succumb to infection. Thus, this high systemic production of TNF-α by A mice late during infection seems to correlate with pathology and death due to malaria. Moreover, it has been suggested that TNF-α induces liver damage in mice during *P. berghei* malaria (33) and endotoxemia *in vivo* (34). Furthermore, high amounts of TNF-α in the sera of malaria patients correlate with severe and fatal disease (3). It has been suggested that this excessive and harmful production of TNF-α is the result of a massive release of TNF-α-inducing malaria antigens during schizogeny (10, 31). The data presented here suggest that excessive production of TNF-α, as seen only in susceptible A mice, may be a consequence of the host's initial Th2 immune response which is unable to limit parasite multiplication, as demonstrated by a high peak parasitemia, resulting in a massive release of malaria antigens later on during infection.

Taken together, our results suggest that TNF-α has a protective role during blood-stage *P. chabaudi* AS malaria when it is produced in the spleen and the liver early during infection. Moreover, a Th1 type response in the spleen appears to sustain a high expression of TNF-α, whereas a Th2 response appears to down-regulate TNF-α expression. In contrast, increased TNF-α mRNA levels in the liver and excessive levels of the protein in serum later during infection appear to have a deleterious effect on the outcome of this disease. In conclusion, the paradoxical role of TNF-α, that is, protection versus pathology during malaria, may, thus, not only depend on the amount of TNF-α released but also on the timing and site of its expression, and the presence of other cytokines regulating its production. A better understanding of the kinetics and site of TNF-α production *in vivo* will be necessary before TNF-α-modulating agents are considered for treatment of malaria.

Acknowledgements

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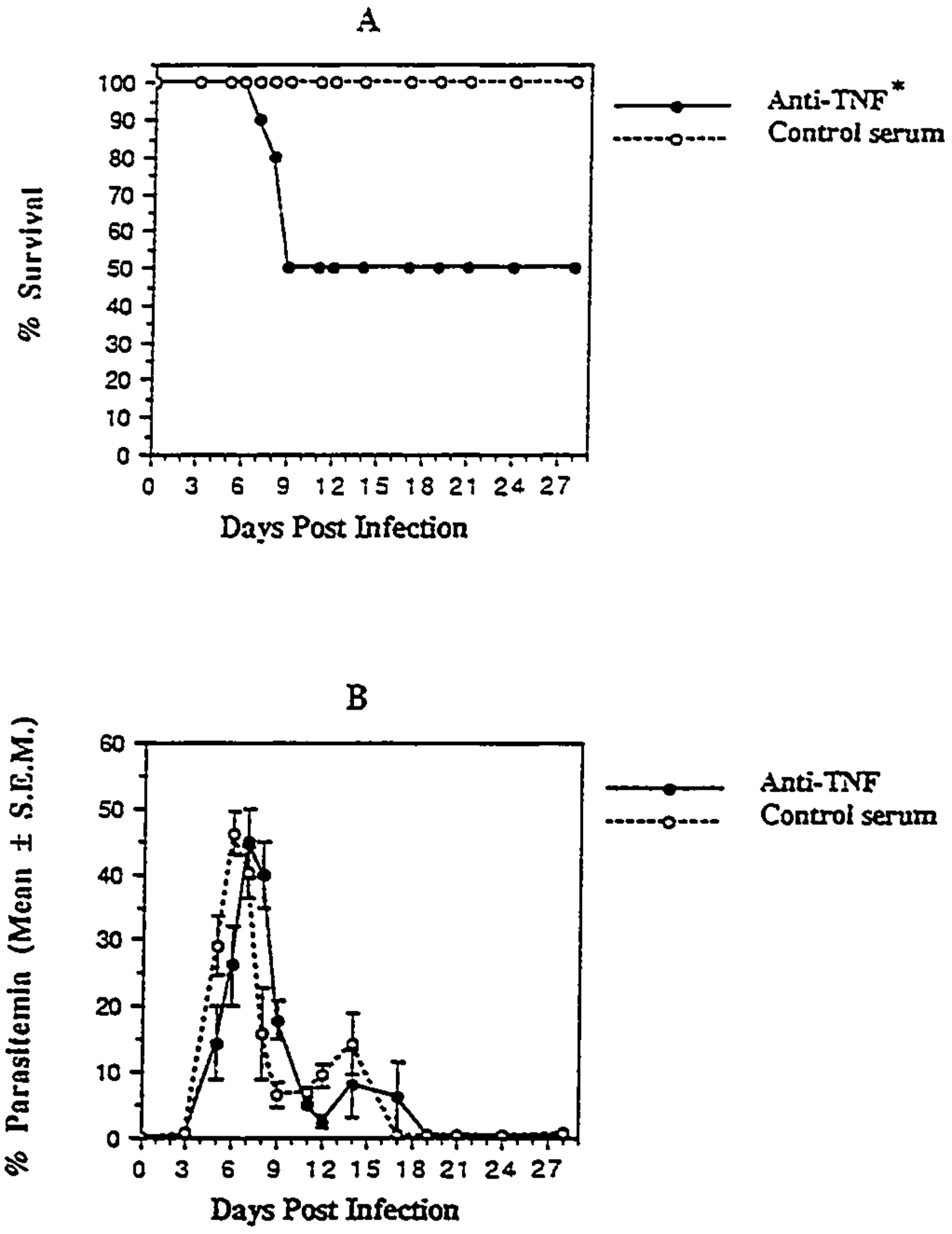
Figure 1. Effect of treatment with polyclonal anti-TNF-α antibody on host response to *P. chabaudi* AS. Groups of 5 mice (resistant B6 strain) were treated intraperitoneally with 10^4 neutralizing units rabbit anti-murine TNF-α antibody (protein content = 2.4 mg), 6 hours prior to and 4 days after *P. chabaudi* AS infection and **(A)** survival and **(B)** parasitemia were monitored. Control mice (n=5) were similarly treated with 2.4 mg normal rabbit serum proteins. The data shown are pooled from two replicate experiments. $^* p < 0.02$ versus control mice.


A
% Survival
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Days Post Infection
Anti-TNF*
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B
% Parasitemia (Mean ± S.E.M.)
60
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0 3 6 9 12 15 18 21 24 27
Days Post Infection
Anti-TNF
Control serum

Figure 2. Kinetics of TNF-α mRNA levels in the spleens of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. **(A)** Total RNA was purified from spleens recovered from 1-4 mice per time point and TNF-α mRNA expression in individual spleens was determined by Northern blot analysis. 18 S rRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of two replicate experiments. **(B)** Scanning densitometry of the autoradiographs shown in panel A. The density of the bands corresponding to TNF-α mRNA expression was determined and normalized to the levels of 18 S rRNA expression. O.D.: Optical Density. * $p < 0.04$ versus control mice on day 0, ** $p < 0.04$ versus A mice on day 3 post infection, *** $p < 0.01$ versus A mice on day 7 post infection, • control mice were pooled for statistical analysis.

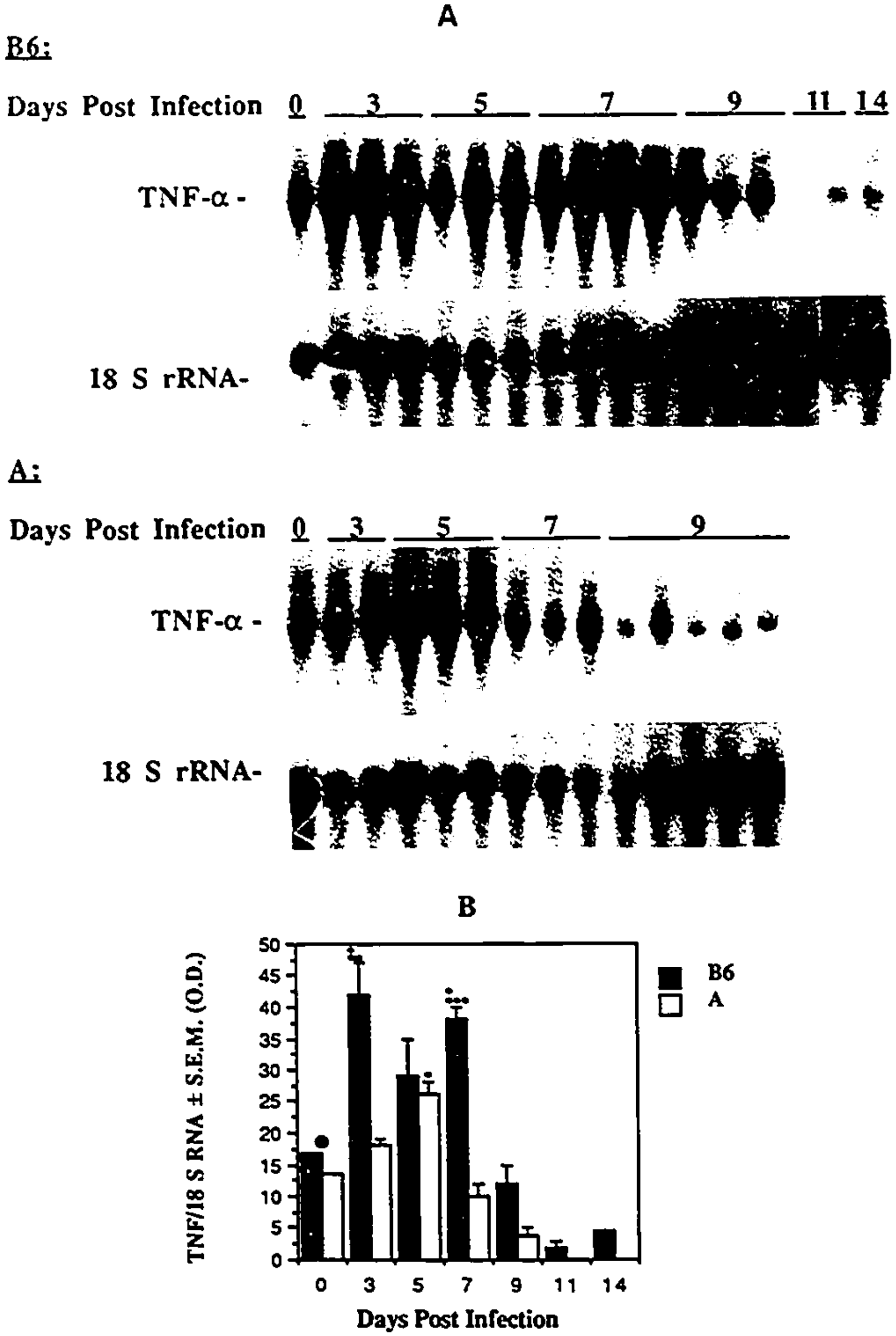
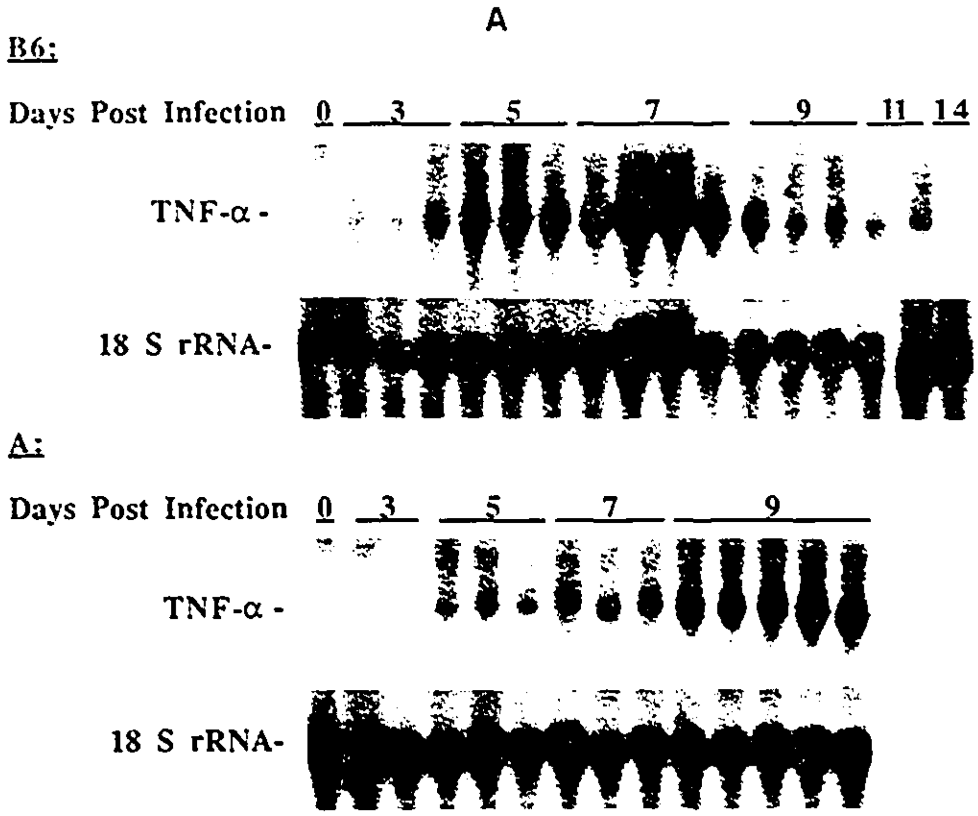

A
B6:
Days Post Infection 0 3 5 7 9 11 14
TNF-α -
18 S rRNA-
A:
Days Post Infection 0 3 5 7 9
TNF-α -
18 S rRNA-
B
TNF/18 S RNA ± S.E.M. (O.D.)
50
45
40
35
30
25
20
15
10
5
0
0 3 5 7 9 11 14
Days Post Infection
B6
A

Figure 3. Kinetics of TNF-α mRNA levels in the livers of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. (A) Total RNA was purified from livers recovered from 1-5 mice per time point and TNF-α mRNA expression in individual livers was determined by Northern blot analysis. 18 S rRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of two replicate experiments. (B) Scanning densitometry of the autoradiographs shown in panel A. The density of the bands corresponding to TNF-α mRNA expression was determined and normalized to the levels of 18 S rRNA expression. O.D.: Optical Density . * $p < 0.04$ versus control mice on day 0, ** $p < 0.01$ versus A mice on day 5 post infection, *** $p < 0.01$ versus A mice on day 7 post infection, • control mice were pooled for statistical analysis.


A
B6:
Days Post Infection
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3
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TNF-α -
18 S rRNA-
A:
Days Post Infection
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7
9
TNF-α -
18 S rRNA-

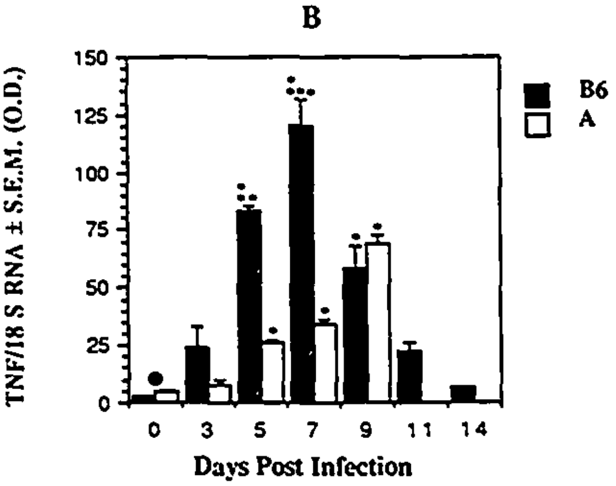
B
TNF/18 S RNA ± S.E.M. (O.D.)
150
125
100
75
50
25
0
0
3
5
7
9
11
14
Days Post Infection
■ B6
□ A

Figure 4. Kinetics of IFN-γ mRNA levels in the spleens of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. **(A)** Total RNA was purified from spleens recovered from 1-3 mice per time point and IFN-γ mRNA expression in individual spleens was determined by Northern blot analysis. 18 S rRNA levels were also determined by Northern blot analysis for each sample. **(B)** Scanning densitometry of the autoradiographs shown in panel A. The density of the bands corresponding to IFN-γ mRNA expression was determined and normalized to the levels of 18 S rRNA expression. O.D.: Optical Density. * $p < 0.04$ versus control mice on day 0, ** $p < 0.03$ versus A mice on day 3 post infection, *** $p < 0.05$ versus A mice on day 7 post infection.

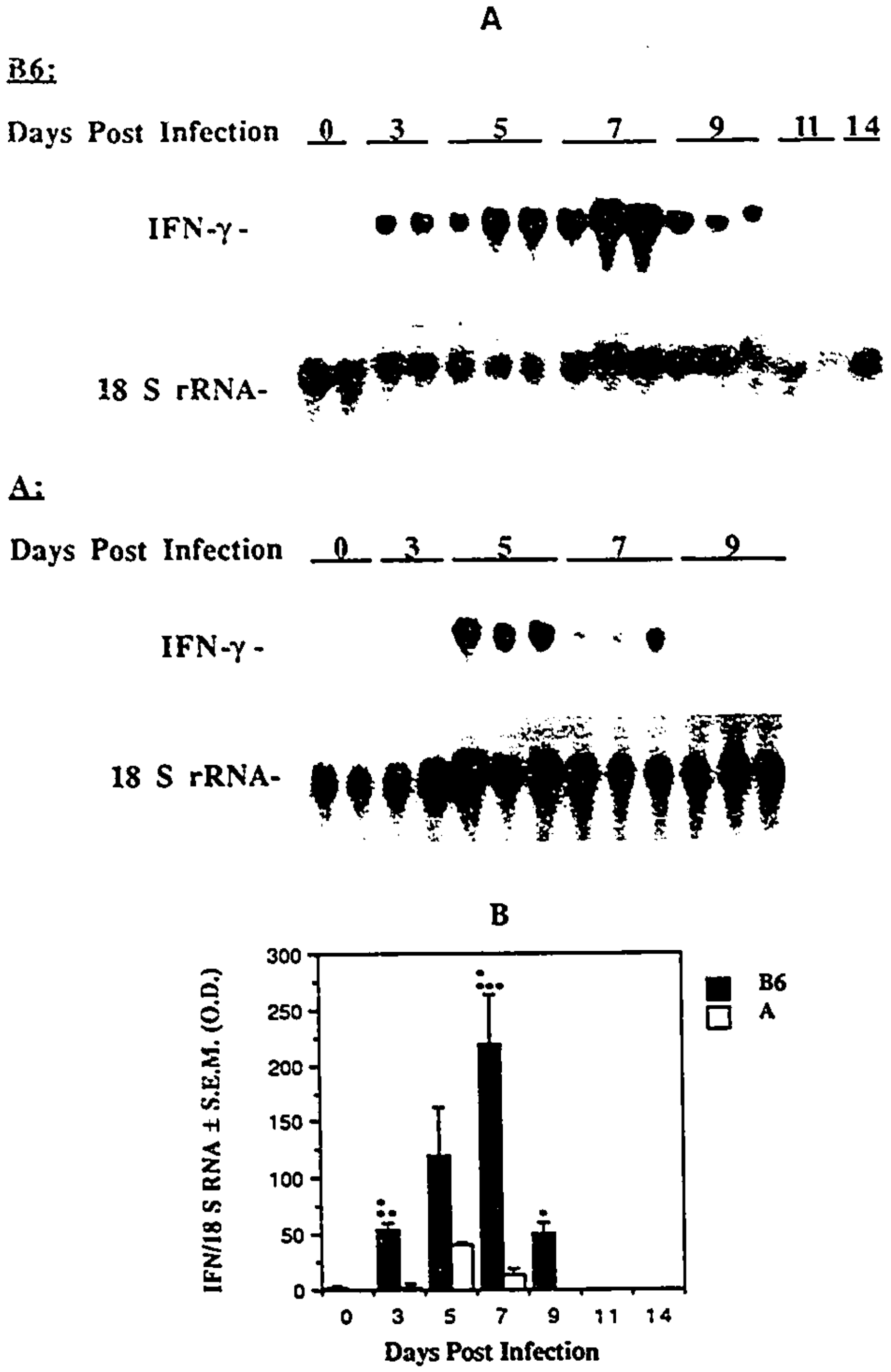
A
B6:
Days Post Infection 0 3 5 7 9 11 14
IFN-γ -
18 S rRNA-
A:
Days Post Infection 0 3 5 7 9
IFN-γ -
18 S rRNA-
B
IFN/18 S RNA ± S.E.M. (O.D.)
300
250
200
150
100
50
0
B6
A
0 3 5 7 9 11 14
Days Post Infection

Figure 5. Kinetics of IL-4 mRNA levels in the spleens of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. (A) Total RNA was purified from spleens recovered from 1-3 mice per time point and IL-4 mRNA expression in individual spleens was determined by Northern blot analysis. 18 S rRNA levels were also determined by Northern blot analysis for each sample. (B) Scanning densitometry of the autoradiographs shown in panel A. The density of the bands corresponding to IL-4 mRNA expression was determined and normalized to the levels of 18 S rRNA expression. O.D.: Optical Density. * $p < 0.02$ versus control mice on day 0, ** $p < 0.01$ versus B6 mice on day 5 post infection.

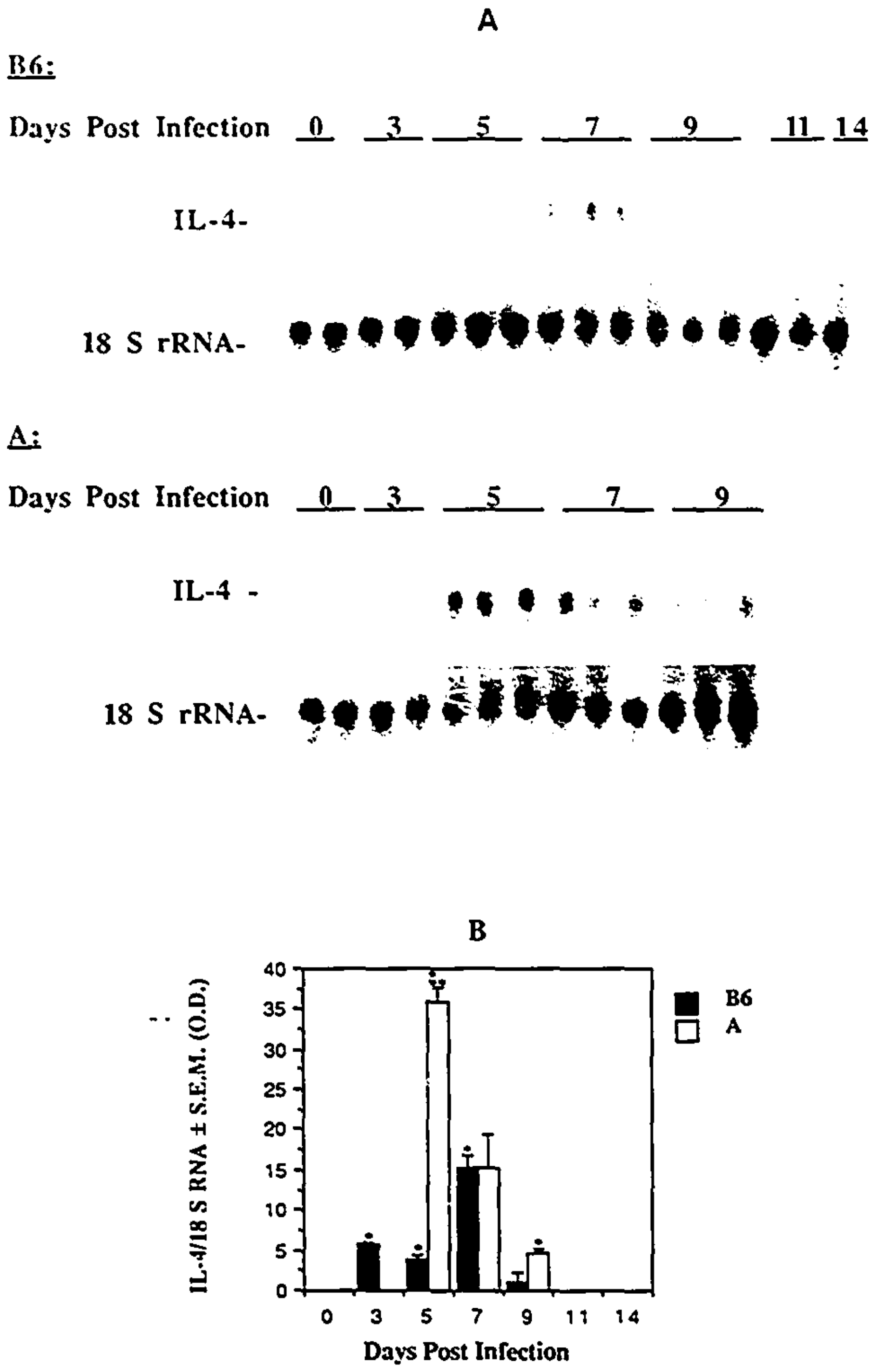
A
B6:
Days Post Infection 0 3 5 7 9 11 14
IL-4-
18 S rRNA-
A:
Days Post Infection 0 3 5 7 9
IL-4 -
18 S rRNA-
B
IL-4/18 S RNA ± S.E.M. (O.D.)
0
5
10
15
20
25
30
35
40
0 3 5 7 9 11 14
Days Post Infection
B6
A

TABLE 1.

Kinetics of TNF-α protein in serum of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS [a].

Days Post-Infection	Parasitemia (% ± S. E. M.)		Prevalence of serum-TNF-α positive mice (%)		TNF-α level (pg/ml ± S. E. M.)	
	B6	A	B6	A	B6	A
0	0	0	0	0	0	0
3	4 ± 4	5 ± 2	0	0	0	0
5-6	16 ± 1	34 ± 0	33	33	≤ 20	≤ 20
7-10	38 ± 4	45 ± 3	44	80	38 ± 18	117 ± 32[b]
14	1 ± 0	-	33	-	≤ 20	-

[a] Blood from groups of 3-8 A and B6 mice was obtained by cardiac puncture at different times post infection and TNF-α protein was determined by a modification of a double sandwich ELISA. The data shown are representative of two replicate experiments. [b] $p < 0.04$ versus B6 mice on days 7-10 post infection.

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CONNECTING STATEMENT

Studies in Chapter 2 demonstrate that a Th1 associated increase in TNF-α production early during infection correlates with resistance to blood-stage *P. chabaudi* AS malaria. As a first step to test the hypothesis that the protective role of TNF-α involves the up-regulation of NO production, the following study analyzes the kinetics of production and role of nitric oxide in both resistant and susceptible mice during the early phase of infection.

CHAPTER 3

Nitric Oxide Expression in the Spleen, But Not in the Liver, Correlates With Resistance to Blood-Stage Malaria in Mice[1]

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Abstract

The production and role of nitric oxide during the early phase of blood-stage infection with *Plasmodium chabaudi* AS was analyzed using two inbred strains of mice which differ in the level of resistance to this parasite. Northern blot analysis of *in vivo* expression of inducible nitric oxide synthase (iNOS) revealed that, early during infection, resistant C57BL/6 mice, which clear the infection by 4 weeks, have higher levels of iNOS mRNA in the spleen than susceptible A/J mice. In contrast, susceptible A/J mice have significantly increased levels of iNOS mRNA in the liver later during infection at the time these mice succumb to infection. Furthermore, both splenic and peritoneal macrophages recovered from resistant C57BL/6 mice produce significantly higher levels of NO_2^- *in vitro* in response to LPS than similarly stimulated macrophages from susceptible A/J mice. Increased levels of NO_3^- were only detected in serum of resistant C57BL/6 mice at the time of peak parasitemia. Treatment with the iNOS inhibitor, aminoguanidine, reduced NO_3^- levels in serum of C57BL/6 mice and eliminated resistance of these mice to *P. chabaudi* AS malaria without affecting parasitemia. These results, thus, demonstrate that the ability to produce high amounts of NO early during infection with blood-stage *P. chabaudi* AS correlates with resistance but that NO may not be involved in parasite killing. Moreover, the tissue site of NO production, that is, spleen versus liver, appears to be critical and correlates with resistance versus susceptibility to *P. chabaudi* AS malaria, respectively.

Introduction

Nitric oxide (NO), when produced in large quantities by the inducible isoform of the enzyme nitric oxide synthase (iNOS), has the potential to kill a variety of pathogens (reviewed in Ref. 1). The induction of the NO pathway by cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), and microbes or microbial products has been shown in a variety of cell types including murine macrophages (2), endothelial cells (3) and hepatocytes (4). Human hepatocytes, respiratory epithelia and macrophages also express functional iNOS (reviewed in Ref. 5). Evidence that cytokine-induced NO synthesis is protective *in vivo* has been demonstrated by injection of iNOS inhibitors, or anti-IFN-γ and anti-TNF-α antibody, in mice during experimental leishmaniasis (6), tularemia (7), listeriosis (8), and blood-stage malaria (9). The fact that these treatments led to an increase in the number of microorganisms, and in some cases, to exacerbation of clinical symptoms, suggests that NO has an important role in protection against infection. In contrast, Clark and his colleagues (10-12) have proposed that high quantities of NO produced during malaria may contribute to some pathologic features of the disease such as hypotension, immunosuppression and cerebral malaria.

Our laboratory has previously demonstrated that the level of resistance to blood-stage infection with the rodent malaria parasite, *Plasmodium chabaudi* AS, varies among inbred strains of mice (13, 14). Resistant C57BL/6 (B6) mice develop moderate levels of peak parasitemia on days 8-10 post infection, clear the infection by 4 weeks and are immune to challenge infection. In contrast, susceptible A/J (A) mice experience significantly higher levels of peak parasitemia and subsequently 100% succumb to infection. Thus, host protective mechanisms appear to be divergent in resistant and susceptible hosts during the early, acute phase of infection. Indeed, control and elimination of the parasite during acute infection has been shown to occur in resistant B6 hosts by activation of $CD4^+$ Th1 cells (15, 16). In contrast, induction of a strong Th2 response early in infection has been observed in susceptible A mice and suggested to lead to a severe and lethal course of acute blood-stage malaria (15). In addition to CD4$^+$ Th1 cells, macrophages and an intact spleen are required to resolve *P. chabaudi* AS malaria (17-20). Others have suggested that the liver as well as the spleen plays a protective role during blood-stage malaria, in particular during *P. yoelii* infection (21).

These observations, and the fact that adoptive transfer of a Th1 clone has been shown to protect against blood-stage *P. chabaudi* AS by an NO-dependent mechanism (9), led us to dissect the role of NO during primary infection with this parasite. Few studies have actually measured the level of NO, or its oxidized forms, nitrite (NO_2^-) and nitrate (NO_3^-), produced during blood-stage malaria and none have identified the tissue site of its production or documented the time period over which NO production is sustained during infection (9, 12, 22). Therefore, we have compared the following parameters during the course of infection in resistant B6 and susceptible A mice: 1) iNOS mRNA levels in spleen and liver; 2) NO_2^- production in vitro by splenic and peritoneal macrophages; and 3) NO_3^- concentrations in serum. The effect of treatment with the NO inhibitor, aminoguanidine, on the course and outcome of infection in resistant B6 hosts was also examined. Our results demonstrate that the level, the timing and, most importantly, the tissue site of NO production during infection correlates with resistance versus susceptibility to blood-stage *P. chabaudi* AS malaria.

Materials and Methods

Mice, parasite and experimental infections. B6 and A strain mice, 8-14 weeks old, were age- and sex-matched in all experiments. Mice were bred at the animal facility of the Montreal General Hospital Research Institute from breeding pairs obtained from Jackson Laboratories, Bar Harbor, ME. *Plasmodium chabaudi* AS was maintained as previously described (23). Mice were infected intraperitoneally with 10^6 *P. chabaudi* AS parasitized red blood cells (PRBC) and parasitemia during the subsequent course of infection was monitored at the times indicated using previously described procedures (15).

iNOS mRNA levels. Spleens and livers 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 described by Chirgwin et al (24). Briefly, frozen tissue was transferred to 7 ml of guanidine thiocyanate solution [4 M guanidine thiocyanate (Gibco BRL, Grand Island, NY)], 25 mM sodium citrate (Sigma, St Louis, MO), 0.1 M β-mercaptoethanol (Sigma), 0.5% N-lauroylsarcosine (Sigma) and 200 µl Antifoam A (Sigma), and homogenized at maximum speed for 2 min with a polytron homogenizer (Brinkman, Kinematica, Switzerland). The homogenates were centrifuged at 550 g for 15 min and the supernatants were layered onto a 5 ml CsCl/EDTA (5.7 M/1 mM; pH 7.0) cushion and centrifuged at 30,000 rpm for 20 h at 22° C in a fixed-angle rotor. The pellets were resuspended in 500 µl TE (10 mM Tris-HCl/1 mM EDTA; pH 7.4) at 65° C and incubated with 500 µl water-saturated phenol:chloroform: isoamylalcohol (25:24:1 by volume) for 15 min at 65° C. The phases were separated by centrifugation at 13,800 g for 3 min. RNA in the aqueous phase was precipitated with 45 µl sodium acetate (3 M; pH 4.8) and 1 ml 95% ethanol at -20° C overnight. RNA was sedimented by centrifugation at 13,800 g for 20 min at 4° C, washed twice with cold 70% ethanol, vacuum-dried for 3 min, and dissolved in 100 µl RNase-free water (5 Prime -3 Prime, Boulder, CO). The RNA yield of each sample was determined spectrophotometrically on the basis of the A_{260} and the RNA purity (ranging from 1.5 to

1.8) assessed by the A_{260}:A_{280} ratio. iNOS mRNA levels were determined by Northern Blot analysis (25). Total RNA samples (20 µg) were separated for approximately 3 h at 60 V on 1.2% agarose gel containing 2.2 M formaldehyde. Separated RNA was blot transferred to a nylon membrane (Schleicher & Schuell, Keene, NH) and UV cross-linked (UV Stratalinker 1800, Stratagene, La Jolla, CA). The blots were prehybridized at 42° C for 14 h with 20 ml hybridization buffer containing denatured salmon sperm DNA (0.15 mg/ml, Sigma). A specific DNA probe, labeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL; specific activity: approximately 10^9 cpm/µg DNA) by nick translation (Gibco-BRL), was added to the hybridization buffer and allowed to hybridize for 14 h. The hybridized blots were washed 3 times for 20 min at 42° C with 150 ml 10x SSC (Sigma)/ 0.1% SDS (Sigma) and 3 times at 55° C with 150 ml 0.1% SSC/ 0.1% SDS. Hybridization was detected by autoradiography with Kodak X-Omat-AR film (Rochester, NY). mRNA levels were quantified by high resolution optical densitometry (SciScan 5000, United States Biochemical, Cleveland, OH) and normalized to 18 S rRNA. A 1.9 kb fragment of rRNA, which recognizes 18 S rRNA, was kindly provided by Dr. N. Arnheim and was previously described in detail (26). A 2.3 kb fragment of the macrophage iNOS gene (27) was kindly provided by Dr. S. Snyder (Johns Hopkins University, Baltimore, MD). iNOS mRNA and 18 S rRNA migrated with mobilities of 3.6 and 1.9 kb, respectively.

NO_2^- production by peritoneal and splenic macrophages. Resident peritoneal macrophages were harvested from normal or infected B6 and A mice, at the times indicated, by peritoneal lavage with 10 ml medium consisting of RPMI 1640 (Gibco BRL) supplemented with 10% heat inactivated FCS (Hyclone, Logan, UT), 2% HEPES buffer (Gibco BRL) and 0.12% gentamicin (Schering, Pointe Claire, Quebec). Spleens were also aseptically removed and pressed through a sterile, stainless-steel mesh. Spleen cells were suspended in 10 ml medium, centrifuged at 350 g for 10 min and erythrocytes were lysed with cold 0.17 M NH_4Cl. Membrane debris was removed by filtering the cell suspensions through sterile gauze and the cells were then washed twice with HBSS (Gibco BRL). Total and differential counts were determined on peritoneal and spleen cell suspensions. The viability of spleen cells was determined by trypan blue exclusion and was always > 90%. Cells were adjusted to 10^6 macrophages per ml, and aliquots of 100 µl of each cell suspension, in triplicate, were added to 96-well plates (Flow, McLean, VA). Macrophages were allowed to adhere for 2 h at 37° C in 5% CO_2. Nonadherent cells were removed by washing 3 times with warm HBSS. Macrophage monolayers were stimulated with 0.001, 0.01, 0.1, 1 and 10 µg/ml of *Escherichia coli* 0127:B8 LPS (Difco, Detroit, MI) or incubated with medium alone as controls. Since NO_2^- production by splenic or peritoneal macrophages in response to different doses of LPS was similar, data have been pooled for each mouse strain. Supernatants were collected 20 h later and immediately assayed for NO_2^- by the Griess reaction (28). Briefly, 50 µl of each supernatant were incubated with 50 µl Griess reagent [equal volumes of 1% sulfanilamide (Sigma) and 0.1% naphtylethylenediamine dihydrochloride (Sigma) in 2.5% H_3PO_4] for 5 min at room temperature and the A_{550} was measured with an ELISA reader. NO_2^- concentration was determined using $NaNO_2$ as a standard. Results are expressed as NO_2^- production (µM) per 10^5 macrophages.

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 using the method described by Rockett *et al.* (12). Briefly, 30 µl of each sample were incubated for 20 min at room temperature with 5 µl of the enzyme nitrate reductase (5 U/ml; Boehringer Mannheim, Laval) and 15 µl NADPH (1.25 mg/ml; Boehringer Mannheim); after incubation, 100 µl of Griess reagent and 100 µl of TCA (10% aqueous solution) were added. Protein precipitates were removed by centrifugation at 13,800 g for 5 min and 100 µl of each supernatant, in duplicate, were transferred to a 96 well, flat-bottom plate and the A_{550} was measured using an ELISA reader. NO_3^-

concentrations were determined using as a standard, $NaNO_3$, diluted in pooled serum from uninfected control B6 or A mice which was dialysed against PBS for 24 h. Concentrations of NO_3^- in the standard ranged from 12.3 to 1000 μM. NO_3^- levels in the sera of experimental animals were calculated using a software program kindly provided by Dr. K.A. Rockett (Australian National University, Canberra).

Treatment with aminoguanidine. B6 mice were injected i.p. twice daily with 1, 5 or 9 mg aminoguanidine hemisulfate salt (Sigma) diluted in 0.2 ml pyrogen-free saline. Mice were treated with aminoguanidine beginning on day 3 post infection and daily for 10 days or beginning on day 5-6 post infection and daily for 4-5 days. Control mice were treated similarly with pyrogen-free saline. Normal, uninfected B6 mice were treated with 1, 9, 15 or 20 mg aminoguanidine for a minimum period of 10 days in order to control for toxicological properties, in terms of weight-loss or death, of aminoguanidine.

Statistical analysis. Statistical significance of differences in mortality between control and experimental groups receiving aminoguanidine was determined using a non-parametric Kruskal-Wallis test. Statistical significance of differences in parasitemia and NO_3^- levels in serum between control and aminoguanidine treated mice was determined by Student's t-test. The same t-test was used to determine statistical significance of differences in iNOS mRNA levels, NO_2^- production *in vitro* and NO_3^- levels in serum between normal and infected animals as well as between B6 and A mice. A probability of less than 0.05 was considered significant.

Results

iNOS mRNA levels in spleen and liver of resistant and susceptible mice during *P. chabaudi* AS infection. To determine if differences in iNOS expression in the tissues during infection with blood-stage *P. chabaudi* AS contribute to resistance, total RNA was extracted from spleens and livers harvested from B6 and A mice at various times during the first 2 weeks post infection and iNOS mRNA levels determined by Northern blot analysis. Tissue expression of iNOS mRNA was also examined in normal, uninfected control mice of each strain. As shown in Figure 1, small increases in iNOS mRNA levels were found in the spleens of resistant B6 mice as early as day 3 post infection compared to basal levels in the spleens of uninfected, control mice on day 0. The peak increase in iNOS expression, which was 6-fold normal, occurred in the spleen of B6 hosts on day 7 post infection several days before the peak parasitemia, when parasitemia ranged from 20 to 30% PRBC. Thereafter, iNOS levels returned to basal levels through day 14 post infection when the experiment was terminated. In contrast, iNOS expression in the spleens of susceptible A mice was not significantly increased over that of the basal level in the spleens of normal, control animals until day 5 post infection when there was a 2-fold increase in iNOS mRNA levels. At this time, there were approximately 10% PRBC in the peripheral blood. iNOS mRNA levels returned to basal levels on day 7 in susceptible A hosts and remained at this level through day 9 post infection when peak parasitemia levels of >50% PRBC occurred. Death of susceptible A mice usually occurs between days 9 to 12 post infection. In contrast to iNOS expression in the spleen, liver iNOS mRNA levels were significantly increased in comparison to basal levels of control mice only in susceptible A mice on day 9 post infection coincident with the peak of parasitemia (Figure 2). Although the exact time course varied from experiment to experiment, significant differences in the peak level as well as the timing of iNOS expression in the spleens and livers of resistant B6 versus susceptible A mice were consistently observed. These results demonstrate that early during acute infection, that is, during the ascending phase of parasitemia, resistant B6 mice have higher levels of iNOS transcripts in the spleen than susceptible A mice. On the other hand, susceptible A mice have significantly increased levels of iNOS mRNA in the liver later during infection at the time of peak parasitemia just before death occurs.

NO_2^- production by macrophages from resistant and susceptible mice during *P. chabaudi* AS infection. Previous results from our laboratory have demonstrated that splenic as well as peritoneal macrophages from susceptible A mice exhibit defects in activation during blood-stage *P. chabaudi* AS infection (18). Since activated macrophages are known to be a major source of cytokine-inducible NO, the ability of splenic and resident peritoneal macrophages to produce NO_2^- was assessed in vitro. Macrophages were recovered from B6 and A mice at various times during *P. chabaudi* AS infection as well as from normal, uninfected mice of both strains. Since splenic macrophages recovered from infected mice of either mouse strain produced only low levels of NO_2^- spontaneously (data not shown), cells were stimulated in vitro with LPS. It should be pointed out that the response of macrophages from infected mice in terms of the quantity of NO_2^- produced was not dependent upon the dose of LPS used.

Splenic macrophages recovered from infected B6 mice produced significantly higher than normal levels of NO_2^- in response to LPS as early as 3 days post infection (Figure 3A). Levels of NO_2^- produced by splenic macrophages from these resistant hosts gradually increased and peaked on day 10 post infection, coincident with the peak of parasitemia, when more than 20 µM NO_2^- were produced. High NO_2^- production persisted throughout the entire course of infection. Significantly increased production of NO_2^- by macrophages recovered from infected B6 mice compared to similarly stimulated macrophages prepared from uninfected mice was apparent at all times during infection with the exception of day 21. Splenic macrophages recovered from A mice on days 5 and 7 post infection also produced significantly higher NO_2^- levels in response to LPS than cells from uninfected A mice. However, on day 7 post infection, splenic macrophages recovered from resistant B6 mice produced approximately 5 times more NO_2^- compared to splenic macrophages from A mice, an observation consistent with significantly higher levels of iNOS expression in the spleens of resistant B6 hosts at this time.

Following stimulation with LPS, resident peritoneal macrophages recovered from infected B6 mice at all times during infection through 28 days produced significantly higher NO_2^- levels than cells recovered from uninfected control mice (Figure 3B). In addition, cells recovered from infected B6 mice produced significantly more NO_2^- than resident peritoneal macrophages from infected A mice early during infection. Similar to splenic macrophages, this difference was apparent on day 3 as well as on day 7 post infection when peritoneal macrophages from B6 mice produced 5 to 8 times more NO_2^- than cells from A mice. No difference in NO_2^- levels between peritoneal macrophages from B6 versus A was detected on day 5 post infection when macrophages from A mice produced significantly higher than normal levels of NO_2^-. With the exception of day 5, macrophages recovered from infected A mice produced levels of NO_2^- similar to macrophages from uninfected control mice. Similar to the observation described above for splenic macrophages, unstimulated peritoneal macrophages from infected B6 and A mice produced only background levels of NO_2^- (data not shown). Taken together, these results demonstrate that, early during infection with *P. chabaudi* AS, both splenic and resident peritoneal macrophages recovered from resistant B6 mice produce significantly higher levels of NO_2^- *in vitro* in response to LPS than similarly stimulated macrophages from susceptible A mice. Moreover, NO_2^- production by both macrophage populations from B6 mice is sustained throughout almost the entire course of infection.

Increased serum NO_3^- correlates with resistance to *P. chabaudi* AS. To determine if differences in NO production are apparent between resistant and susceptible hosts in vivo during infection with *P. chabaudi* AS, levels of NO_3^-, a stable end-product of NO, were determined in serum of B6 and A mice at different times during infection. The level of NO_3^- in the sera collected from uninfected mice of either strain was approximately 25 µM (Figure 4). On day 8 post infection which, in this experiment corresponded to the peak parasitemia, there was a 5-fold increase in NO_3^- in the sera of B6 mice compared to uninfected control mice. No increase in NO_3^- could be detected in sera of A mice at any time through 8 days post infection. Thus, the observation that, during infection with *P. chabaudi* AS, NO production is increased only in resistant B6 mice at peak parasitemia suggests a role for this molecule in protection against blood-stage malaria.

Effect of treatment with aminoguanidine on host response to *P. chabaudi* AS. To determine if the ability to produce high levels NO plays a protective role during *P. chabaudi* AS malaria, B6 mice were treated with aminoguanidine, a selective inhibitor of iNOS (29), and survival and parasitemia were monitored (Figure 5). As a control, infected B6 mice were treated with pyrogen-free saline. Uninfected B6 mice were also treated with different doses of aminoguanidine to control for side-effects of this product. None of the uninfected control mice, treated twice daily for 10 days with 1, 5, 9 or 15 mg aminoguanidine, experienced any weight loss (data not shown) or died (Figure 5A). However, 100% (3/3) of normal mice treated with 20 mg aminoguanidine died within 2-3 hours. Therefore, these data demonstrate that intraperitoneal administration of up to 15 mg aminoguanidine, twice daily for 10 days, has no apparent side effects, in terms of weight loss or death, in normal B6 mice. In contrast, treatment of *P. chabaudi* AS infected B6 mice with 5 or 9 mg aminoguanidine, twice daily beginning on day 3 post infection, resulted in 20 and 80% mortality, respectively (Figure 5A). Similar treatment with either dose of aminoguanidine but beginning on days 5 to 6 post infection also resulted in 80 to 100% mortality (data not shown). Treatment of infected mice with 1 mg aminoguanidine (data not shown) or saline alone resulted in 100% survival to infection. Infected mice treated with either 5 or 9 mg aminoguanidine died at, or 1 to 2 days after, the peak of parasitemia. Surprisingly, aminoguanidine treatment did not alter parasitemia (Figure 5B). That is, the course of infection of the aminoguanidine treated animals was similar to that of the control mice.

To confirm that aminoguanidine treatment inhibits NO production in vivo, B6 mice were treated twice daily with 5 and 9 mg aminoguanidine, starting on days 5 to 6 post *P. chabaudi* AS infection, and NO_3^- levels measured in the sera at the peak of parasitemia. Sera collected from infected, pyrogen-free saline-treated, control mice at peak parasitemia and uninfected mice were also examined. As a positive control, NO_3^- levels in the sera of normal B6 mice 20 hours after intraperitoneal treatment with 20 µg LPS were also determined. Whereas infected, control mice had high serum NO_3^- levels, infected mice treated with 5 or 9 mg aminoguanidine had 4 to 5 times lower NO_3^- levels which were comparable to basal NO_3^- levels of uninfected mice (Table I). Similarly, aminoguanidine treatment significantly reduced NO_3^- levels in the sera of LPS treated B6 mice. Taken together, these data thus demonstrate that treatment with aminoguanidine significantly reduces NO production by B6 mice during infection and eliminates resistance of these mice to *P. chabaudi* AS malaria but does not alter the course of parasitemia.

Discussion

We report here that, early during primary blood-stage *P. chabaudi* AS infection, resistant B6 mice produce significantly higher amounts of NO compared to susceptible A mice as measured by iNOS gene expression, macrophage production of NO_2^- in vitro and serum levels of NO_3^-. The highest iNOS mRNA levels were found in the spleens of resistant B6 mice on day 7 post infection, when parasitemia ranged from 20 to 30%, whereas no iNOS transcripts could be detected in the liver of these mice during infection. In contrast, a small, albeit significant, increase in iNOS mRNA levels was detected in the spleen of susceptible A mice on day 5 post infection, when parasitemia was approximately 10%, whereas in the liver of these mice, increased amounts of iNOS transcripts were found on day 9 post infection, coincident with peak parasitemia. These data suggest that, early during infection, high NO production in the spleen, but not in the liver, has a protective effect on the outcome of *P. chabaudi* AS malaria. Moreover, the high iNOS expression in the spleen of B6 mice correlated with increased NO_3^- levels in the serum of these mice 1 to 2 days later. We can not exclude, however, the possibility that during infection with *P. chabaudi* AS other tissues in addition to the spleen, such as the endothelial lining of the vasculature which has been shown to be involved in NO-mediated killing of *Schistosoma mansoni* (3), may also produce high amounts of NO.

The results presented here also demonstrate that splenic macrophages recovered from resistant B6 mice produce significantly more NO_2^- than splenic macrophages recovered from susceptible A mice on days 3 and 7 post infection. These findings are consistent with the data on iNOS expression in the spleen. However, it is possible that other cell types in addition to macrophages produce NO in vivo in the spleen during infection. In this context, Taylor-Robinson et al (30) recently demonstrated that a $CD4^+$ Th1 clone, derived from the spleen early during infection with *P. chabaudi*, produces large amounts of NO.

Interestingly, the amount of NO produced by resident peritoneal and splenic macrophages recovered from mice of either strain during infection was not dependent upon the dose of LPS. This may indicate, considering that optimal NO production by murine macrophages requires a priming signal by IFN-γ plus a second signal, such as LPS (31), that resident peritoneal and splenic macrophages from resistant B6 mice are highly primed in vivo during infection, and following stimulation in vitro with even small amounts of LPS (<0.1 μg), are capable of producing high amounts of NO. In contrast, macrophages recovered from A mice are either not primed or are suppressed and, thus, are unable to produce increased amounts of NO. These findings are in agreement with previous data from our laboratory indicating that, during infection, resident peritoneal and splenic macrophages from B6 mice are in a higher state of activation than macrophages from A mice, that is, a higher percentage of macrophages from B6 mice express Ia antigen and produce more oxygen metabolites in vitro than macrophages from A mice (18). Furthermore, it is well documented that the Th1 cytokine IFN-γ, in conjunction with microbial products, leads to macrophage activation (1), whereas the Th2 cytokines, IL-4 and IL-10, down-regulate macrophage microbicidal activity and cytokine production (32-34). We recently demonstrated that spleen cells from B6 mice produce high levels of IFN-γ in vitro within the first week of infection, whereas, during the same period, spleen cells from A mice produce low levels of IFN-γ but high levels of IL-4 and IL-10 (16, and unpublished observations). We also observed that addition of murine recombinant IL-4 or IL-10 to macrophages recovered from *P. chabaudi* AS infected B6 mice diminished the ability of these cells to produce NO, whereas addition of recombinant IFN-γ increased NO production by macrophages recovered from infected A mice (unpublished observations). Therefore, a Th1 response in the spleen early during infection with *P. chabaudi* AS leads to macrophage activation and NO production as observed in resistant B6 mice, whereas a Th2 response appears to suppress splenic macrophage NO production and results in severe and lethal disease as observed in susceptible A mice.

The in vivo role of NO in protection against blood-stage *P. chabaudi* AS was investigated directly by treating resistant B6 mice with the iNOS inhibitor, aminoguanidine. This treatment resulted in serum NO_3^- levels similar to those of uninfected control mice and, more strikingly, in lethal infection with ≥ 80% mortality. Although NO_2^- and NO_3^- have been shown to kill blood-stages of *P. falciparum* in vitro (35), aminoguanidine treatment did not result in an increase in parasitemia, suggesting that NO is not involved in parasite killing. These findings are consistent with the results of a recent study by Rockett *et al.* (12) in which *P. chabaudi*-infected mice were treated with L-NMMA and no increase in parasitemia was observed. These results as well as ours, however, appear to conflict with the results of another study which demonstrated a marked increase in parasitemia in *P. chabaudi*-infected mice receiving L-NMMA (9). It should be noted, however, that the protocol used in our study, that is, treatment of immunocompetent mice with aminoguanidine, may not be comparable with the protocol used in the latter study (9) in which thymectomized mice were treated with L-NMMA in conjunction with T cell transfers. Moreover, although aminoguanidine has been shown to be as potent as L-NMMA as an inhibitor of iNOS, it is 10 to 100-fold less potent as an inhibitor of the constitutive isoforms of the enzyme (29), which are present in endothelial cells and cerebellar neurons, and are involved in maintaining vascular tone and neurotransmission, respectively (5). We were, however, still concerned with the high doses of aminoguanidine required in the studies reported here compared to other studies. For example, a dose of aminoguanidine as low as 1 mg, administered twice daily for 8 days, was found to be effective in abolishing resistance to *Listeria monocytogenes* (36) while a dose of 1.5 mg, administered daily for 3 days by gastric instillation, resulted in elimination of protective immunity to challenge with *Plasmodium berghei* sporozoites (37). Recently, daily, intraperitoneal administration of 8 mg aminoguanidine for more than 10 days was shown to ameliorate experimental autoimmune encephalomyelitis (38). Furthermore, in this study, the LD_{50} for aminoguanidine was found to be 32 mg per day and normal SJL control mice, treated with 8 mg aminoguanidine once daily for more than 3 weeks, did not show any clinical abnormalities and experienced no weight loss. Similarly, none of the normal, non-infected control B6 mice in our study died or experienced any weight loss after treatment twice daily for 10 days with a dose as high as 15 mg aminoguanidine. A single intraperitoneal dose of 20 mg aminoguanidine was, however, lethal. Although we did not observe any side effects following twice daily administration of 5-9 mg aminoguanidine treatment, we still can not exclude that aminoguanidine exerts effects other than iNOS inhibition, such as binding to reactive aldehydes formed during oxidative stress (39). This may have some relevance considering that oxidative stress occurs during malaria infection (40) and that highly toxic reactive aldehydes are produced (41). Nevertheless, our results clearly demonstrate that treatment with aminoguanidine decreases serum NO_3^- levels and leads to lethal infection without affecting parasitemia.

Increased NO production during infection may be protective by a mechanism other than being parasiticidal. Kremsner *et al.* (42) demonstrated that mice pretreated with L-NMMA and infected with *P. vinckei* and treated with chloroquine plus IFN-γ exhibited higher mortality and more pronounced liver and kidney lesions. These authors suggested that NO was protective either by directly inactivating tissue-damaging oxygen radicals or by preventing hypoxic tissue damage by inhibiting leukocyte adhesion to endothelium and/or increasing vasodilatation. In a separate study, it was found that treatment of

Corynebacterium parvum-infected and LPS-treated mice with L-NMMA increased liver damage induced by oxygen radicals (43). Direct evidence that NO can prevent cytotoxicity resulting from oxygen radicals was shown in vitro (44). During *P. chabaudi* AS infection, NO may thus act as a tissue protective molecule.

Recent evidence suggests that NO may also play a crucial role in protection against blood-stage malaria in humans. It has been demonstrated that patients infected with *P. falciparum* or *P. vivax* have increased plasma levels of NO (45,46). Moreover, it was shown that the duration of coma due to cerebral malaria was shorter in children with relatively high NO plasma levels which may indicate that NO has a protective role in cerebral malaria (45). Interestingly, there was no significant correlation in this study between NO levels and parasitemia.

The moderate increase in iNOS mRNA levels in the liver of A mice at peak parasitemia, which corresponds to the time these mice begin to succumb to infection, suggests that NO production in the liver later during infection may have a deleterious rather than a protective effect on the outcome of this disease. In an attempt to inhibit NO production late during infection, we treated A mice twice daily with 5 mg aminoguanidine starting 1 day prior to peak parasitemia. However, this treatment regimen did not rescue these mice from death and had no effect on parasitemia (data not shown). These results, thus, suggest that increased expression of iNOS in the liver of susceptible A mice correlates with, but does not on its own, cause death due to *P. chabaudi* AS malaria. Similar to these findings, Harbrecht et al (47) recently demonstrated that increased NO synthesis correlates with, but does not cause, liver injury during endotoxemia in BALB/c mice. Moreover, these authors showed that the increase in NO production was due to an increase in LPS-induced TNF-α. Interestingly, high expression of TNF-α mRNA in the liver and high serum levels of TNF-α were detectable in susceptible A, but not resistant B6, mice during peak parasitemia just before death (Jacobs, P. and Stevenson, M.M., Manuscript submitted).

Taken together, our results demonstrate that resistance to *P. chabaudi* AS correlates with the ability to produce high amounts of NO early during infection. However, the tissue site of NO production appears to be critical. High iNOS mRNA levels in the spleen during the ascending phase of parasitemia correlate with resistance, whereas increased iNOS mRNA levels in the liver around peak parasitemia correlate with susceptibility. In resistant hosts, reduction of NO production by treatment with the iNOS inhibitor aminoguanidine abolishes resistance without affecting parasitemia, suggesting that NO is not involved in parasite killing. Rather, our data suggest that NO may protect the host against oxygen radical-mediated tissue damage during blood-stage malaria. The precise regulatory mechanisms of iNOS expression in vivo and the physiological role of NO in different organs during lethal and non-lethal infection with *P. chabaudi* AS are, however, not yet known. Our murine malaria model in resistant and susceptible hosts may, thus, provide an excellent tool to explore the role of NO in order to design future anti-malarial strategies.

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Figure 1. Kinetics of iNOS mRNA levels in the spleen of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. **(A)** Total RNA was purified from spleens recovered from 1-4 mice per time point and iNOS mRNA expression in individual spleens was determined by Northern blot analysis. 18 S rRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of two replicate experiments. **(B)** Scanning densitometry of the autoradiographs shown in panel A. The density of the bands corresponding to iNOS mRNA expression was determined and normalized against the bands corresponding to 18 S rRNA expression. O. D. = Optical Density. * $p < 0.04$ versus control mice on day 0, ** $p < 0.03$ versus A mice on day 5, *** control A and B mice were pooled for statistical analysis.

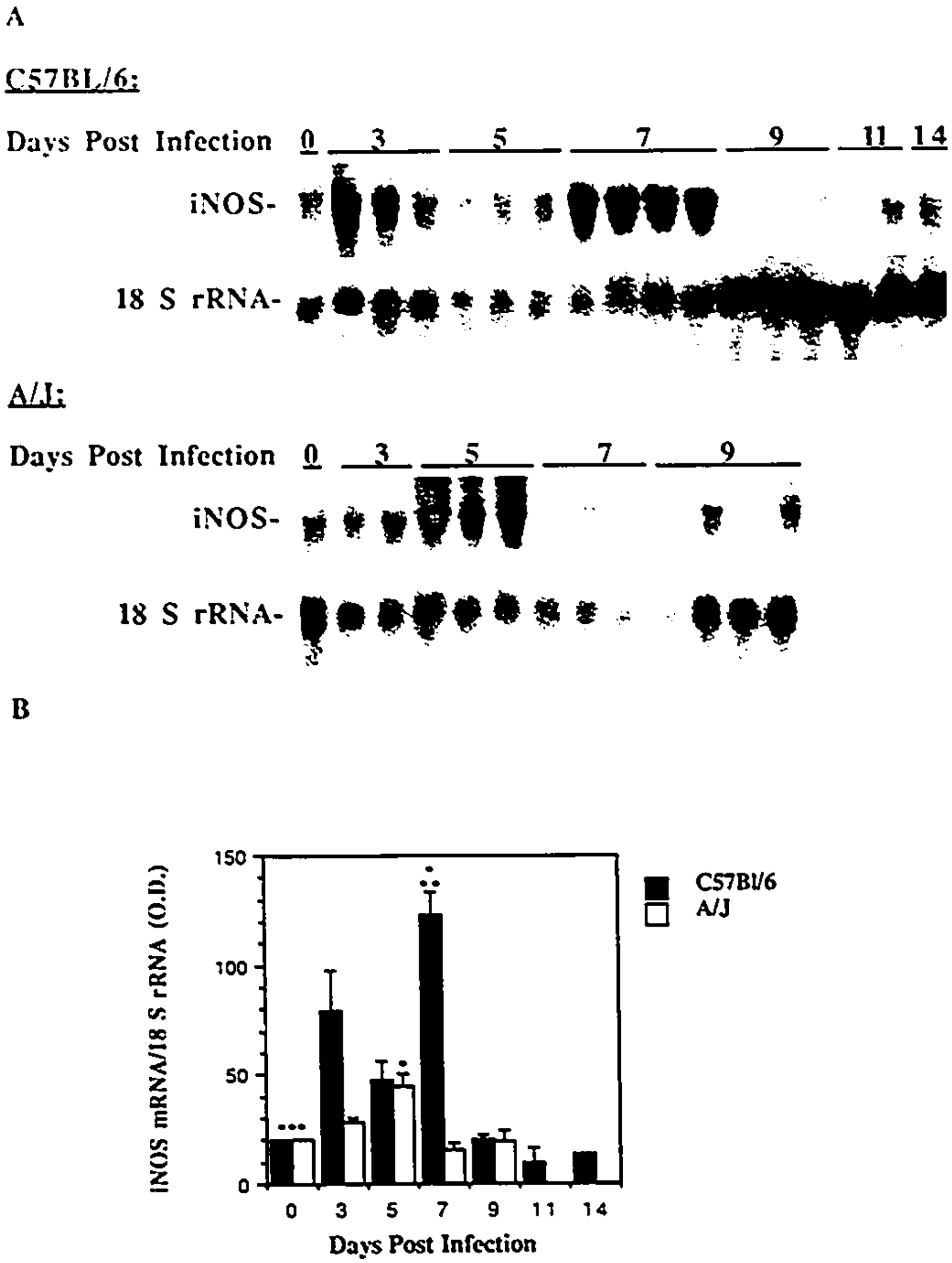

A
C57BL/6:
Days Post Infection 0 3 5 7 9 11 14
iNOS-
18 S rRNA-
A/J:
Days Post Infection 0 3 5 7 9
iNOS-
18 S rRNA-
B
iNOS mRNA/18 S rRNA (O.D.)
150
100
50
0
0 3 5 7 9 11 14
Days Post Infection
C57Bl/6
A/J

Figure 2. Kinetics of iNOS mRNA levels in the liver of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. **(A)** Total RNA was purified from livers recovered from 1-5 mice per time point and iNOS mRNA expression in individual livers was determined by Northern blot analysis. 18 S rRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of two replicate experiments. **(B)** Scanning densitometry of the autoradiographs shown in panel A. The density of the bands corresponding to iNOS mRNA expression was determined and normalized against the bands corresponding to 18 S rRNA expression. O. D. = Optical Density. * $p < 0.01$ versus control mice on day 0, ** control A and B mice were pooled for statistical analysis.

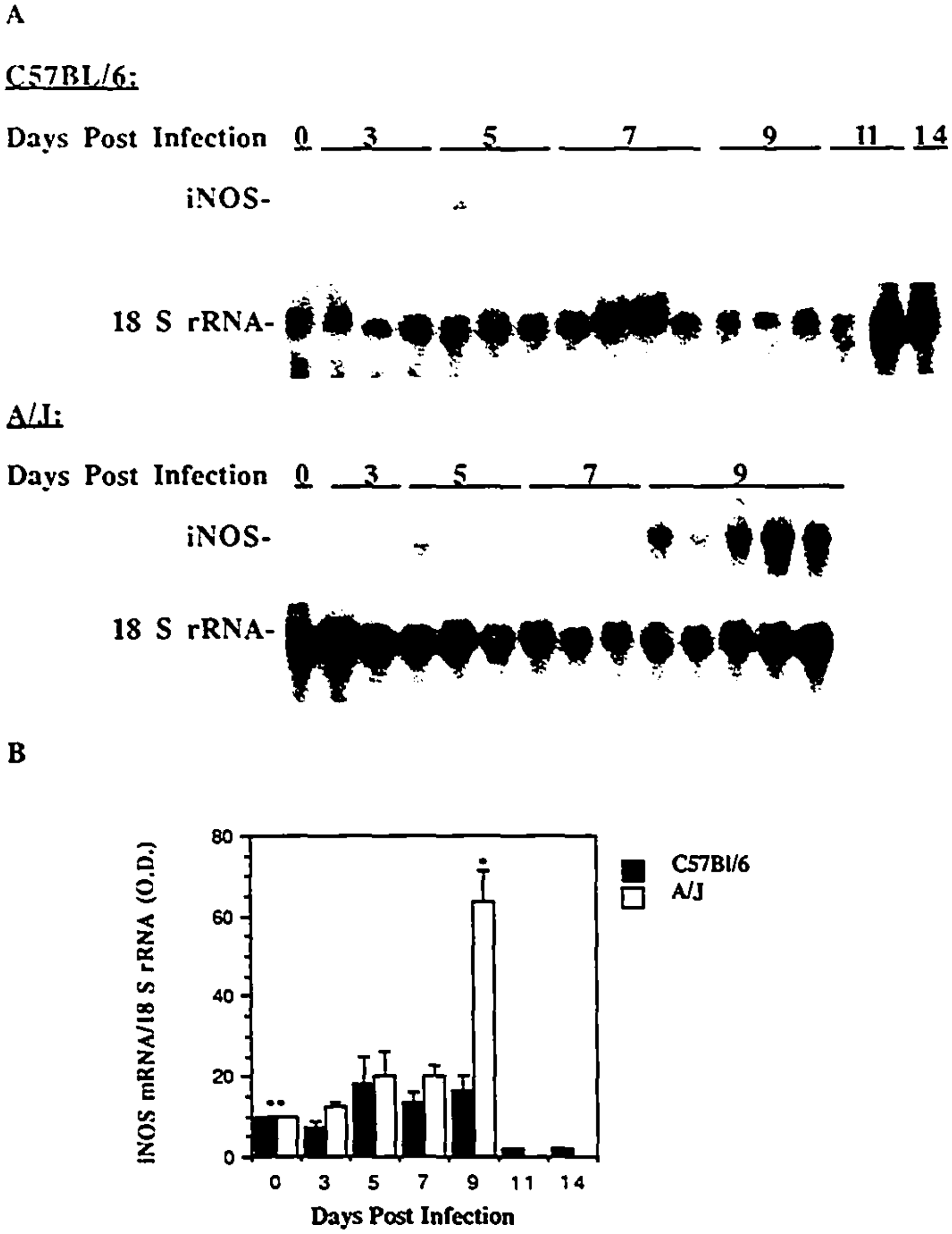

A
C57BL/6:
Days Post Infection 0 3 5 7 9 11 14
iNOS-
18 S rRNA-
A/J:
Days Post Infection 0 3 5 7 9
iNOS-
18 S rRNA-
B
80
60
40
20
0
iNOS mRNA/18 S rRNA (O.D.)
**
*
C57Bl/6
A/J
0 3 5 7 9 11 14
Days Post Infection

Figure 3. Kinetics of NO_2^- production *in vitro* by **(A)** splenic and **(B)** resident peritoneal macrophages from resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. Supernatants were collected from macrophage monolayers prepared from 5 individual mice per time point, stimulated for 20 h with 5 different doses of LPS (ranging from 1ng to 10 µg/ml) and NO_2^- levels were determined by the Griess reaction. Since NO_2^- production per time point in response to various doses LPS was similar, data for all 5 doses have been pooled. The data shown are representative of three replicate experiments. * $p < 0.05$ versus control mice on day 0, ** $p < 0.01$ versus A mice on day 7.

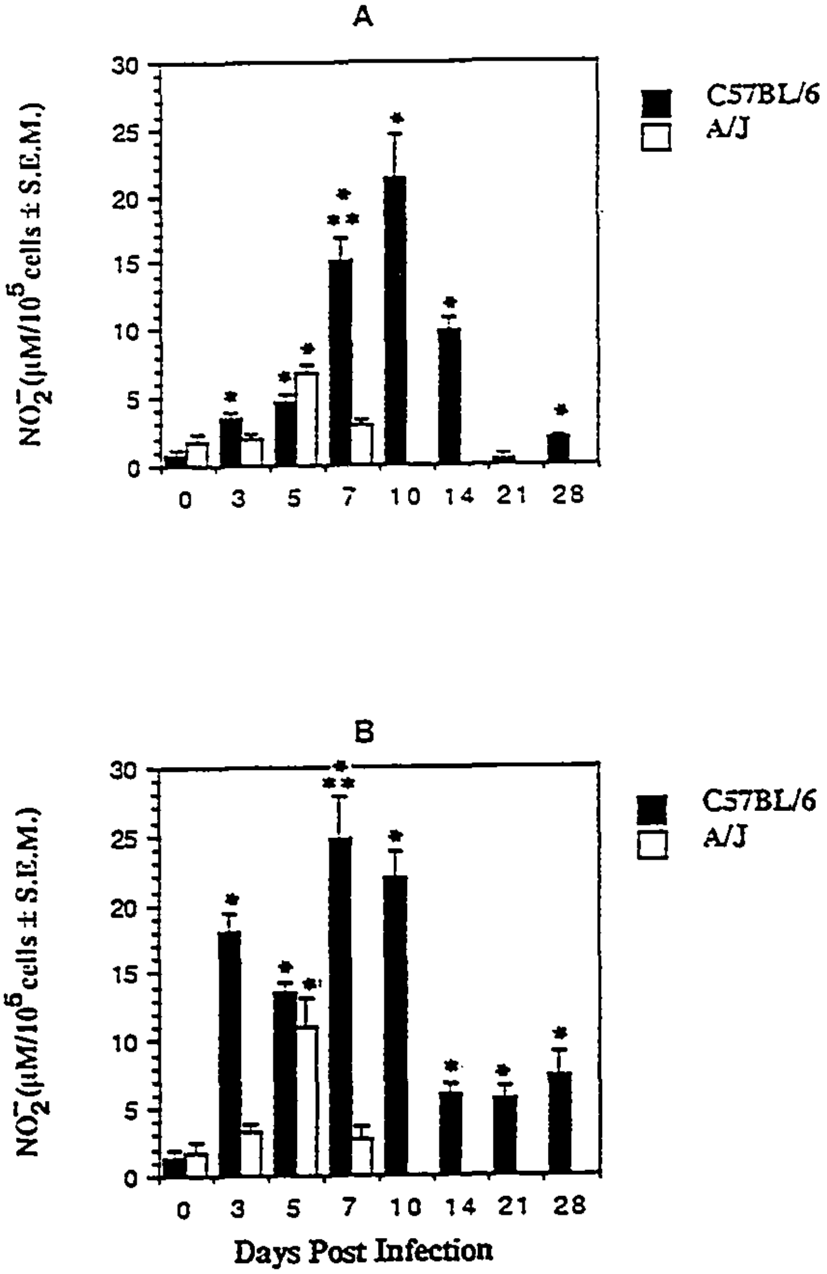

A
NO$_2^-$(µM/10^{5}cells ± S.E.M.)
0
5
10
15
20
25
30
C57BL/6
A/J
0
3
5
7
10
14
21
28
B
NO$_2^-$(µM/10^{5}cells ± S.E.M.)
0
5
10
15
20
25
30
C57BL/6
A/J
0
3
5
7
10
14
21
28
Days Post Infection

Figure 4. Kinetics of NO_3^- levels in serum of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. Serum was obtained from 4-6 individual mice per time point and NO_3^- levels were determined by a modification of the Griess reaction. Data shown are pooled from 2 replicate experiments. * $p < 0.04$ versus control mice on day 0.

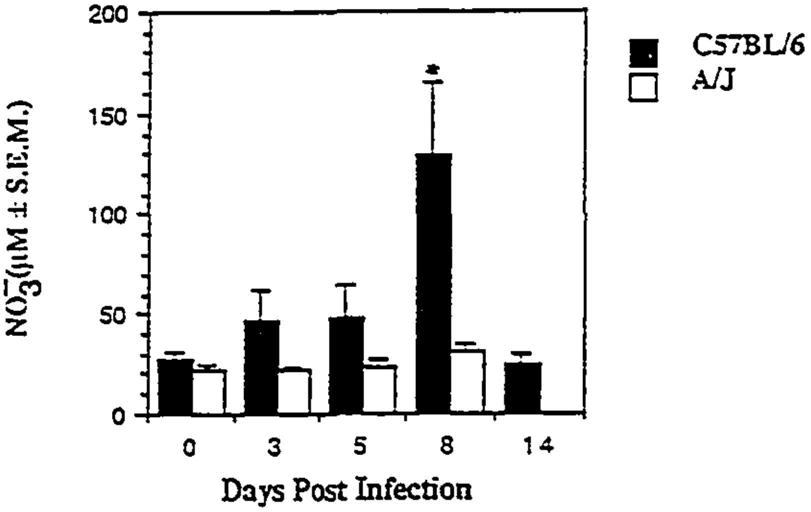
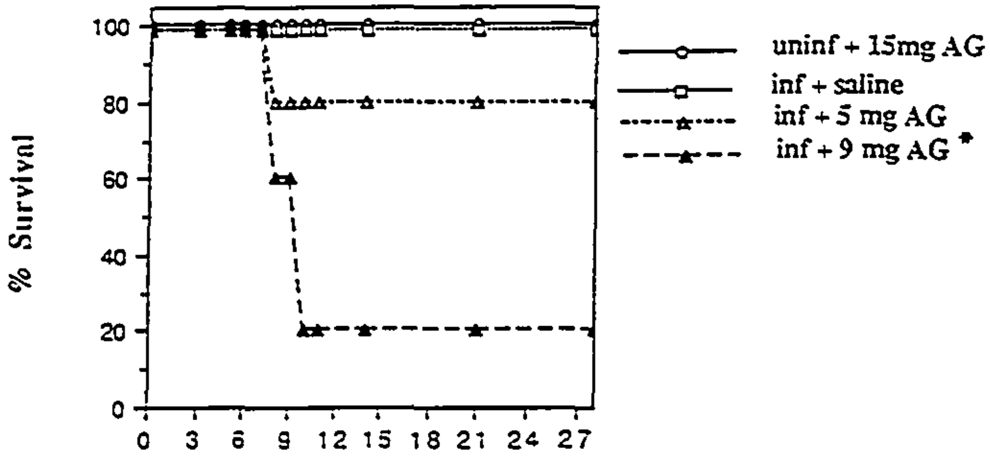

200
150
100
50
0
NO_3^- (μM ± S.E.M.)
0
3
5
8
14
Days Post Infection
*
■ C57BL/6
□ A/J

Figure 5. Effect of treatment with aminoguanidine (AG) on host response to *P. chabaudi* AS. Groups of 5 mice (resistant C57BL/6 strain) were treated twice daily with different doses of aminoguanidine and (A) survival and (B) parasitemia were monitored. The data shown are representative of three replicate experiments. [a] uninf: uninfected, [b] inf: infected with 10^6 PRBC, * $p < 0.02$ versus control mice (= inf + saline), ** parasitemia of mice treated with 5 mg AG, which is similar to parasitemia of mice treated with 9 mg AG, is not shown.

A


% Survival
100
80
60
40
20
0
0
3
6
9
12
15
18
21
24
27
uninf + 15mg AG
inf + saline
inf + 5 mg AG
inf + 9 mg AG *

B

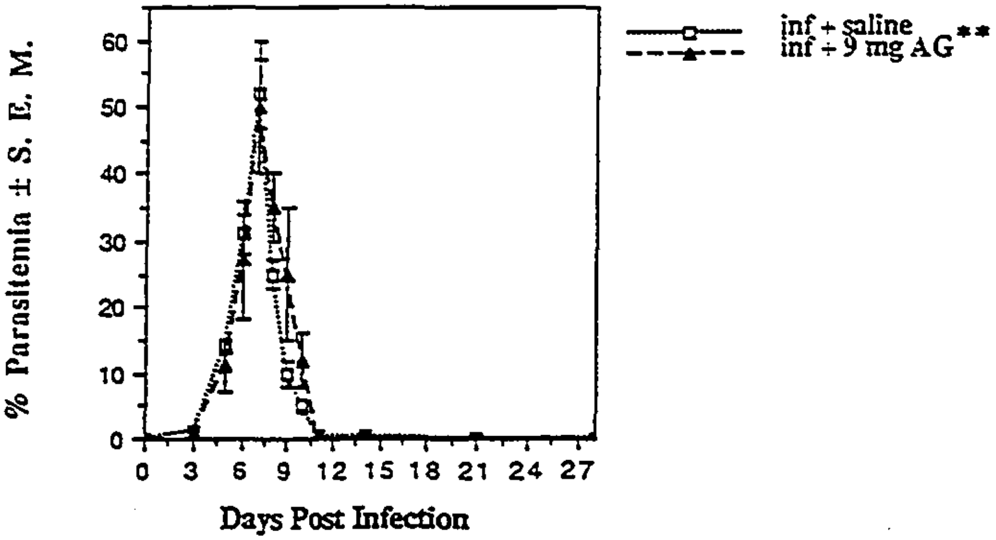

% Parasitemia ± S. E. M.
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
27
Days Post Infection
inf + saline
inf + 9 mg AG **

TABLE I.

Treatment with aminoguanidine inhibits the accumulation of NO_3^- in serum during infection with *P. chabaudi* AS.

Treatment[a]	NO_3^- (μM + S.E.M)
uninfected + saline	18 ± 3
P. chabaudi AS + saline	86 ± 24
P. chabaudi AS + 5 mg aminoguanidine	15 ± 2[c]
P. chabaudi AS + 9 mg aminoguanidine	19 ± 3[c]
uninfected + LPS[b]	1281 ± 417
uninfected + LPS[b] + 9 mg aminoguanidine	36 ± 11[c]

[a] Groups of 4-6 B6 mice were used per treatment. The data shown are representative of two replicate experiments.

[b] As a positive control, mice were treated with 20 μg LPS 20 hours before sera were collected.

[c] $p < 0.04$ versus without aminoguanidine treatment.

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CONNECTING STATEMENT

Studies in Chapters 2 and 3 demonstrate that a Th1 associated increase in TNF-α production and high levels of nitric oxide early during infection correlate with resistance to blood-stage *P. chabaudi* AS malaria, whereas a Th2 response appears to correlate with susceptibility to the disease. The following study investigates if IFN-γ (a Th1 cytokine), TNF-α and IL-4 (a Th2 cytokine) are involved in the regulation of nitric oxide early during infection.

CHAPTER 4

IN VIVO REGULATION OF NITRIC OXIDE PRODUCTION BY TNF-α AND IFN-γ, BUT NOT IL-4, DURING BLOOD-STAGE MALARIA IN MICE

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Running title: Cytokines Regulate Nitric Oxide Synthesis during Malaria

Abstract

We investigated whether IFN-γ (a Th1 cytokine), TNF-α and IL-4 (a Th2 cytokine) modulate nitric oxide (NO) production *in vivo* during blood-stage infection with *P. 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 and anti-TNF-α monoclonal antibody resulted in a reduction of both splenic iNOS mRNA and serum NO_3^- levels by 50 and 100%, respectively. Treatment with anti-TNF-α alone reduced only serum NO_3^- levels by 35% and treatment with anti-IFN-γ 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 anti-IL-4 monoclonal antibody or mouse recombinant TNF-α. The latter treatments 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-TNF-α and/or IFN-γ antibody 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 which were shown to be controlled by IFN-γ in synergy with TNF-α and TNF-α alone, respectively. Treatment of susceptible A/J mice with anti-IL-4 antibody or mouse recombinant TNF-α had no effect on these resistance markers during infection with *P. chabaudi* AS. Thus, these results demonstrate that TNF-α, either alone or in synergy with IFN-γ, plays a major role 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 anti-IL-4 antibody or mouse recombinant TNF-α is not able to induce NO production or confer resistance to A/J mice against *P. chabaudi* AS malaria.

Introduction

Increased production of endogenous nitric oxide during infectious diseases such as listeriosis, leishmaniasis and blood-stage malaria, has been shown to correlate with protection against infection in mice (1-4). 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 either resulted in exacerbation of the disease or death. Recently, increased levels of NO_3^- have also been demonstrated in plasma of patients infected with the malaria parasites, *Plasmodium falciparum* or *P. vivax* (5, 6). 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 (5). 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 Ref. 7). For example, *in vitro* studies have demonstrated that bacterial lipopolysaccharide or *P. falciparum*-derived toxins synergize with the cytokine interferon-gamma (IFN-γ) to induce the production of large quantities of NO by murine macrophages (8, 9). *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* (10) and *Francisella tularensis* (11). On the other hand, interleukin-4 has been demonstrated *in vitro* to inhibit the up-regulation of the inducible isoform of nitric oxide synthase (iNOS), the enzyme capable of producing high amounts of NO, by cytokine-activated macrophages (12).

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 (4, 13). Resistant C57BL/6 (B6) mice, which develop moderate levels of peak parasitemia on days 7-10 post infection 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 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 serum recovered from A mice during the early phase of infection (13, 14 and unpublished observation). Furthermore, treatment of resistant B6 mice with anti-TNF-α 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 (4, 13). In addition, treatment of susceptible A mice with human recombinant TNF-α (hu rTNF-α) protected these mice from an otherwise lethal infection (15). Previous studies in our laboratory have also shown that resistant B6 mice have, in addition to having a 100% survival rate and lower peak parasitemia, a more marked splenomegaly than susceptible A mice during the early phase of *P. chabaudi* AS malaria (16).

In the present study, we investigated whether IFN-γ, TNF-α or IL-4 modulate NO production *in vivo* during blood-stage infection with *P. chabaudi* AS. More specifically, we treated resistant B6 mice with anti-IFN-γ and/or anti-TNF-α antibody and determined iNOS expression in the spleen and NO_3^- accumulation in serum during the early phase of infection with this parasite. Susceptible A mice were treated with anti-IL-4 or mouse recombinant TNF-α (mo rTNF-α) and iNOS expression in the spleen and NO_3^- accumulation in serum were determined as for B6 mice. In addition to the effect on NO production, we also determined if these treatments affected other traits of resistance to *P. chabaudi* AS malaria such as survival, the course of parasitemia as well as the development of splenomegaly. Our results demonstrate that TNF-α, either alone or in synergy with IFN-γ, is a major factor involved in up-regulating NO production during blood-stage *P. chabaudi* AS malaria in resistant B6 mice. Treatment of susceptible hosts with anti-IL-4, however, had no effect on NO production. Our experiments also indicate that TNF-α, but not IL-4, are involved in the regulation of other traits of resistance to *P. chabaudi* AS malaria such as the peak parasitemia level and splenomegaly. Thus, TNF-α, either alone or in synergy with IFN-γ, plays a major role in the development of resistance to blood-stage infection with *P. chabaudi* AS *in vivo*.

Materials and Methods

Mice, parasite and experimental infections. Mice, 8-12 weeks old, were age- and sex-matched in all experiments. B6 mice were obtained from Charles River (St. Constant, Quebec) and A mice were obtained from Jackson Laboratories (Bar Harbor, ME). *P. chabaudi* AS was maintained in our laboratory as previously described (17) and experimental infections were initiated by intraperitoneal inoculation with 10^6 *P. chabaudi* AS parasitized red blood cells (PRBC).

Treatment of mice with antibodies and recombinant molecules. Resistant B6 mice were treated intraperitoneally (i. p.) with 0.2 ml pyrogen-free saline containing 10^5 neutralizing units (protein content = 3.75 mg) rat anti-murine TNF-α IgG1 monoclonal antibody (mAb) [XT22 (18); Upstate Biotechnology, Lake Placid, NY], 6 hours prior to and 4 days after *P. chabaudi* AS infection. The anti-TNF-α neutralizing titer (neutralizing units/ml) was determined as previously described (19) 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 rTNF-α/ml, neutralizes 50% or more of the cytotoxic activity of rTNF-α on L929 cells in the presence of actinomycin D. Control B6 mice were similarly treated with 0.2 ml pyrogen-free saline containing an equivalent amount of normal rat serum IgG which was prepared according to previously described standard procedures (19). For neutralization of IFN-γ, resistant B6 mice were treated i. p. with 0.2 ml pyrogen-free saline containing 1 mg of mouse anti-rat IFN-γ IgG1 mAb [DB-1 (20); kindly provided by Dr. 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.

Control mice were similarly treated with 0.2 ml 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 XT22 and 1 mg 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 rat anti-murine IL-4 IgG1 mAb [11B.11 (21); Biological Resources Branch, BRMP, NCI-Frederick Cancer Research Facility, Frederick, MD 21701-1013)], 6 hours prior to 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 (21). Control mice were similarly treated with 0.2 ml pyrogen-free saline containing 0.4 mg of normal rat serum IgG. A separate group of susceptible A mice were treated intravenously (i. v.) with 0.2 ml pyrogen-free saline containing 10^2, 10^3, 10^4 or 10^5 units of mo rTNF-α (Genzyme, Cambridge, MA), 6 hours prior to and on days 3 and 5 after *P. chabaudi* AS infection. Control A mice were inoculated i. v. with 0.2 ml pyrogen-free saline alone.

Determination of parasitemia, survival and splenomegaly. Parasitemia was monitored during the course of infection at the times indicated using previously described procedures (17). Survival of infected mice was assessed by twice daily observation. Spleen weight, as a measure of splenomegaly, was determined on days 7 and 8 post infection. Spleen weight from normal control mice was also determined. Because body weight among mice of the different treatment groups was similar (22 ± 1 g), we did not normalize spleen to body weight.

RT-PCR analysis of iNOS mRNA levels in spleen. 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 (13). 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) assessed by the A_{260}:A_{280} ratio. iNOS mRNA levels were determined by semiquantitative RT-PCR as described by Kramnik *et al* (22). Briefly, 8 μl solutions containing 1 μg of total RNA and 1 μg of random primers (Pharmacia, Piscataway, NJ) were heated (65° C, 10 min) to denature RNA and were then cooled on ice. 200 U of M-MLV reverse transcriptase (Gibco BRL, Grand Island, NY), 10 mM of each 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/ml bovine serum albumin (Gibco BRL) were added to the RNA samples and the mixtures were incubated for 1 hour at 38° C. The enzyme was inactivated by heating (95° C, 10 min) and 30 μl RNase free water were added to each sample. In order to compensate for uncalculable factors affecting the yield of PCR products (23), a competitive PCR standard for semiquantitative detection of iNOS expression was generated based on the method by Förster (24). Briefly, a 527 bp fragment of the macrophage iNOS gene (25), kindly provided by Dr. S. Snyder (Johns Hopkins University, Baltimore, MD) was amplified using iNOS specific primers (sense primer: 5'-TGGATTTGGAGCAGAAGTGC-3'; antisense primer: 5'-CTTTGTTACAGCTTCCAGCC-3') in a final volume of 40 μl containing 1 U of Taq polymerase (Bio/Can, Mississauga, Ontario), 0.5-2 μM of each primer and 0.2 mM of deoxynucleotidetriphosphates (dNTPs) in PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100). The reaction mixture was overlaid with 20 μl of mineral oil (Sigma, St-Louis, MO) and the DNA was amplified by 26 cycles of 94° C for 1 min, 54° for 1 min and 72° C for 2 min in a thermal cycler (Perkin Elmer, Norwalk, CT). The size of the PCR product was confirmed by 1.2% agarose gel electrophoresis and the DNA was purified using a PCR product purification kit (Qiagen, Chatworth, CA). Aliquots of 50 ng of the PCR product served as a template for the amplification of a 410 bp fragment using the same sense primer and PCR conditions as described above, but using an antisense 32 bp internal linker primer (5'-CTTCCAGCCTGGTCCTCTGGTCAAACTCTTGG-3') matching a 22 bp sequence 140 bp downstream of the original position of the antisense primer. In addition to the 22 matching bases, the primer had a tail of 10 bases at the 5' end identical to the first 10 bases at the 3' end of the original antisense primer. The 410 bp PCR product was purified and its size confirmed as described above. An aliquot of 150 ng of the 410 bp fragment was then re-amplified with the original iNOS specific sense and antisense primers using the same PCR conditions described above. The resulting 420 bp PCR product was run on a 1.2% agarose gel, the band was cut out, purified by spinning through a 0.22 µM cellulose acetate filter (Costar, Cambridge, MA) for 30 min and used as a competitive standard. The quantity of the standard DNA was determined using DNA Dipstick (Invitrogen, San Diego, CA) and the standard was calibrated. A 40 fg aliquot of the standard gave a similar strength signal as the iNOS positive spleen samples and fell into the linear range of the standard calibration curve. For semiquantitative RT-PCR of iNOS mRNA, 4 µl aliquots of the reverse transcription products were amplified using iNOS specific primers in a final volume of 40 µl containing 1 U Taq polymerase, 0.5-2 µM of each primer, 40 fg of the competitive standard and 0.2 mM dNTPs in PCR buffer. Each sample also contained 0.2 µl [α-^{32}P]dCTP (10 µCi/ml, Amersham, Arlington Heights, IL). The reaction mixture was overlaid with 20 µl mineral oil and the DNA thermal cycler was programmed for 25 cycles at 94° C for 45 sec, 62° C for 50 sec 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, Rochester, NY) for 48 hours. The density of the bands was determined by high resolution optical densitometry (SciScan 5000, US Biochemical, Cleveland, OH). As a control, 2 µl aliquots of all reverse transcription products were amplified in parallel by PCR using sense and antisense primers for GAPDH (26). The dried gels were exposed to Kodak X-Omat-AR (Rochester) 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 x g for 3 min. Sera were collected and stored at -20° C until use. Serum NO_3^- levels were determined using the method described by Rockett *et al* (27). Briefly, 30 µl of each sample were incubated for 20 min at room temperature with 5 µl of the enzyme, nitrate reductase (5 U/ml; Boehringer Mannheim, Laval), and 15 µl 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 x g for 5 min and 100 µl of each supernatant, in duplicate, were transferred to a 96-well, flat-bottom plate and the A_{550} was measured using an ELISA reader. NO_3^- concentrations were determined using as a standard, $NaNO_3$, diluted in pooled serum from uninfected control B6 or A mice which was dialysed against PBS for 24 h. Concentrations of NO_3^- in the standard ranged from 12.3 to 1000 µM. NO_3^- levels in the sera of experimental animals were calculated using a software program kindly provided by Dr. K. A. Rockett (Australian National University, Canberra).

Statistical analysis. Statistical significance of differences in parasitemia, splenomegaly, iNOS mRNA levels and NO_3^- levels in serum between control and antibody or mo rTNF-α-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. We have previously demonstrated that resistant B6 mice express increased levels of TNF-α, IFN-γ and iNOS mRNA in the spleen early during infection with *P. chabaudi* AS (4, 13). The highest levels of these mRNA's were found on day 7 post infection when parasitemia ranged from 20 to 30%, coincident with 1 day prior to peak parasitemia. In order to investigate the role of TNF-α and IFN-γ in the up-regulation of NO production during blood-stage malaria, we treated resistant B6 mice with neutralizing anti-TNF-α and/or anti-IFN-γ mAb and determined iNOS mRNA expression in the spleens on day 7 post infection by RT-PCR. Expression of iNOS mRNA was determined similarly in the spleens of control mice. As shown in Figure 1, 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 to control mice. Treatment with either anti-TNF-α or anti-IFN-γ mAb alone, however, did not reduce iNOS expression which was similar to iNOS expression in the spleen of control mice (Figure 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 spleen of resistant B6 mice during infection. This suggests that, in the spleen of resistant B6 mice, TNF-α synergizes with IFN-γ to induce the expression of iNOS mRNA during the early phase of infection with *P. chabaudi* AS.

Serum $NO3^-$ levels in anti-TNF-α and anti-IFN-γ mAb-treated resistant B6 mice during *P. chabaudi* AS infection. Previous results from our laboratory demonstrated that, during *P. chabaudi* AS malaria, resistant B6 mice have increased serum levels of $NO3^-$ on day 8 post infection coincident with peak parasitemia (4). To determine if treatment of resistant B6 mice with anti-TNF-α and/or anti-IFN-γ mAb affects systemic production of NO during infection, we treated these mice with mAb as described and determined the levels of $NO3^-$ in sera collected on days 7 and 8 post infection. $NO3^-$ levels were also determined in sera collected from control mice. Figure 2 shows that treatment with both anti-TNF-α and anti-IFN-γ mAbs completely abolished the accumulation of $NO3^-$ levels in serum compared to $NO3^-$ levels in serum obtained from control B6 mice on day 7 post infection. Indeed, only basal $NO3^-$ levels (approximatily 20 µM), similar to the levels in serum from normal, uninfected B6 mice, could be detected in serum collected from animals treated with anti-TNF-α and anti-IFN-γ mAbs. Treatment with anti-TNF-α mAb also significantly reduced, although not as dramatically, the accumulation of serum $NO3^-$ levels, whereas treatment with anti-IFN-γ mAb alone did not affect the concentration of serum $NO3^-$ compared to serum from control animals. Similar results were obtained when serum was collected on day 8 post infection, coincident with peak parasitemia (data not shown). These data, thus, demonstrate that treatment with anti-TNF-α mAb, but not anti-IFN-γ mAb, significantly reduces the accumulation of serum NO_3^-, whereas treatment with both anti-TNF-α and anti-IFN-γ 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.

Parasitemia, survival and splenomegaly in anti-TNF-α and anti-IFN-γ mAb-treated resistant B6 mice during *P. chabaudi* AS infection. Previous results from our laboratory indicated that treatment of resistant C57BL/10ScN mice with two different anti-IFN-γ mAbs (R4-6A2 and DB-1) significantly increased the level of peak parasitemia but did not affect survival (28). In contrast, we recently demonstrated that treatment of resistant B6 mice with polyclonal anti-TNF-α antibody did not alter the level of peak parasitemia but resulted in decreased survival (13). We herein investigated if treatment of resistant B6 mice with anti-TNF-α and/or anti-IFN-γ mAb affected three previously described (16) markers of resistance to infection with *P. chabaudi* AS, namely, 1) a high survival rate, 2) a relatively low peak parasitemia, and 3) a marked splenomegaly. As shown in Figure 3, mice treated with either anti-TNF-α or anti-IFN-γ mAb alone resulted in a slight, but not significant increase in the peak parasitemia levels compared to control animals (46 ± 3% and 49 ± 4% versus 42 ± 3 %, respectively). However, treatment with both mAbs resulted in a significantly higher peak parasitemia level than that of control animals (58 ± 2% versus 42 ± 3 %, respectively). Furthermore, 100% (30/30) of the B6 mice treated with either mAb alone or both together survived the infection. Figure 4 shows that mice treated with anti-TNF-α mAb alone or in combination with anti-IFN-γ mAb had a significant reduction in spleen weight (by 25%) compared to the spleen weights of control animals (242 ± 16 mg and 237 ± 10 mg versus 313 ± 24 mg, respectively). 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 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 peak parasitemia level in B6 mice during *P. chabaudi* malaria is controlled by TNF-α in synergy with IFN-γ. The development of splenomegaly during infection, on the other hand, appears to be partially induced by TNF-α, but not by IFN-γ.

Splenic iNOS mRNA levels and serum NO_3^- levels in anti-IL-4 mAb or mo rTNF-α-treated susceptible A mice during *P. chabaudi* AS infection. We have previously demonstrated that susceptible A mice produce high amounts of IL-4 mRNA on day 5 post infection and suggested that this Th2 cytokine, in addition to other Th2 cytokines such as IL-10, may suppress NO production in this host during infection (4, 13). We also demonstrated that i.v. treatment with 10^5 units hu rTNF-α rescued A mice from an otherwise lethal *P. chabaudi* AS infection and suggested that TNF-α may induce the production of protective amounts of NO (4, 13, 15). In order to determine if IL-4 and TNF-α are able to suppress and induce, respectively, NO production *in vivo* during *P. chabaudi* AS malaria, we treated susceptible A mice with either anti-IL-4 mAb or with 10^5 units mo rTNF-α and determined iNOS mRNA expression in the spleen and NO_3^- levels in serum on days 7 and 8 post infection. We also examined iNOS mRNA expression in the spleen and NO_3^- levels in sera of control mice. Our results indicate that treatment with anti-IL-4 mAb or mo rTNF-α did not affect iNOS mRNA expression in the spleen (Figure 5) and NO_3^- levels in serum (Table 1) of susceptible A mice on day 7 post infection. Indeed, iNOS mRNA expression and serum NO_3^- levels in the anti-IL-4 mAb or mo rTNF-α-treated mice were not increased compared to the low levels of control animals (Figure 5 and Table 1). Similar results were obtained when iNOS mRNA expression and serum NO_3^- levels of mo rTNF-α-treated animals were examined on day 8 post infection, coincident with peak parasitemia, or when A mice were treated with 10^4 units mo rTNF-α (data not shown). 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. Furthermore, these results also demonstrate that mo rTNF-α by itself is not able to induce NO production by A mice during infection with *P. chabaudi* AS.

Parasitemia, survival and splenomegaly in anti-IL-4 mAb or mo rTNF-α-treated susceptible A mice during *P. chabaudi* AS infection. Previous results from our laboratory had already demonstrated that treatment with hu rTNF-α increased the survival rate and significantly decreased peak parasitemia levels of susceptible A mice during infection (15). Here, we investigated if treatment with anti-IL-4 mAb or mo rTNF-α, as described above, results in increased resistance of otherwise susceptible A mice to *P. chabaudi* AS malaria. We therefore examined the effect of these treatments on the markers of resistance to infection with *P. chabaudi* AS described above. Our results demonstrate that treatment with either anti-IL-4 mAb or mo rTNF-α neither increased the survival rate (data not shown) or decreased peak parasitemia levels (Table 1) compared to control mice. Similar results were obtained when A mice were treated with 10^2, 10^3, or 10^4 units mo rTNF-α (data not shown). Furthermore, treatment with either anti-IL-4 mAb or mo rTNF-α did not increase spleen weight consistently compared to control mice (Table 1). A significant increase in spleen weight was observed on day 7 when mice were treated with mo rTNF-α (Table 1) but this was not the case when spleen weight was measured on day 8 post infection (data not shown). 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 and demonstrate that treatment with mo rTNF-α, in contrast to treatment with hu rTNF-α, is not able to confer resistance to A mice against *P. chabaudi* AS malaria.

Discussion

We report here that TNF-α, either alone or in synergy 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 (4, 13), with anti-TNF-α and anti-IFN-γ mAbs significantly reduced iNOS mRNA expression in the spleen. Furthermore, treatment with anti-TNF-α mAb alone or in combination with 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 (4, 13), with anti-IL-4 mAb alone did not affect iNOS mRNA expression in the spleen nor 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 (29), induces high serum levels of TNF-α, IFN-γ and, interestingly, NO_3^- (30). 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 (30). Our results are also consistent with a recent study by von der Weid *et al* (31) which indicated that mice with a disrupted IL-4 gene develop a functional Th1 response and clear a primary infection with *P. chabaudi* AS in a manner similar to 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 (32). 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 TNF-α and not by IL-4. It would be of interest to investigate if IL-10 and TGF-β, other cytokines besides IL-4 suggested to inhibit NO production *in vivo* (33), 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* (9), 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, is similar to or even greater in A compared to B6 mice 1-

2 days before the peak of parasitemia whereas the level of NO production at that time during infection is greater in B6 mice.

Our results also demonstrate that treatment of resistant B6 mice with anti-TNF-α and anti-IFN-γ reduces splenic iNOS mRNA expression by only 50% whereas serum $NO3^-$ levels are reduced by 100%. This finding may indicate that TNF-α in synergy with IFN-γ affects NO synthesis both at the level of transcription as well as post-transcriptionally during infection with *P. chabaudi* AS. In other words, TNF-α in synergy 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 and anti-IFN-γ mAbs would then result in complete reduction of $NO3^-$ levels in serum but only partial reduction of iNOS mRNA levels. In this respect, it has been shown that TGF-β, another pleiotropic cytokine produced during infection (34), accelerates the degradation of iNOS protein in mouse macrophages (35). On the other hand, $NO3^-$ 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* using a mouse model of sepsis (36). 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-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 $NO3^-$ as we found in the present study.

To our surprise, treatment of susceptible A mice with mo rTNF-α did not increase the level of iNOS mRNA in the spleen or $NO3^-$ levels in serum during infection with *P. chabaudi* AS. Moreover, this treatment did not decrease the level of peak parasitemia nor did it increase the survival rate of susceptible A mice during infection. These findings contrast sharply with previous results from our laboratory which demonstrated that treatment with similar doses of hu rTNF-α significantly decreased the level of peak parasitemia and rescued susceptible A mice from an otherwise lethal infection with *P. chabaudi* AS. The biological effect of mo rTNF-α versus hu rTNF-α in A mice during *P. chabaudi* AS malaria is thus remarkably different. In this regard, it has been shown that mo rTNF-α binds to both mouse TNF receptors [TNFR1 (p55) and TNFR2 (p75), Reviewed in 37] whereas hu rTNF-α binds only to mouse TNFR1 (p55) (38). It is thus tempting to suggest that ligation of TNFR1 (p55), in contrast to ligation of both TNFR1 (p55) and TNFR2 (p75), confers protection in A mice against *P. chabaudi* AS malaria. We also observed that treatment of A mice with mo rTNF-α, and thus presumably ligation of both TNFR1 (p55) and TNFR2 (p75), resulted in earlier and increased iNOS mRNA expression in the liver, concomitant with severe liver necrosis (39). Moreover, some mo rTNF-α-treated animals died earlier compared to untreated A mice during infection with *P. chabaudi* AS (39). Taken together, these findings are consistent with results from Rothe *et al* (40) and Erickson *et al* (41) which showed that mice deficient in TNFR1 (p55) are more susceptible to infection with *Listeria monocytogenes* while mice deficient in TNFR2 (p75) have a dramatic decrease in TNF-induced tissue necrosis and death. It would be of interest to investigate whether there is a differential expression of the two TNF receptors between resistant B6 and susceptible A mice during infection with *P. chabaudi* AS.

Our results also show that treatment of resistant B6 mice with anti-TNF-α and 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 indicated that treatment of B6 mice with anti-IL-12 antibody also increases peak parasitemia without affecting survival, presumably by suppressing the synthesis of TNF-α and IFN-γ (30). 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 (4). These findings, and the fact that treatment with both aminoguanidine or anti-

TNF-α and anti-IFN-γ mAbs inhibits NO synthesis, suggest, firstly, that NO protects the host but is not involved in controlling the level of peak parasitemia and thus parasite killing, and secondly, that TNF-α in synergy with IFN-γ induces one or more factor(s) different from NO which are involved in parasite killing. In this regard, the existence of unidentified factor(s) in serum which require the presence of both TNF-α and IFN-γ have previously been shown to mediate killing of the malaria parasite *P. cynomolgi* (42). Furthermore, the fact that treatment with anti-IL-4 mAb did not affect the course of parasitemia and survival to infection with *P. chabaudi* AS is in agreement with a recent study, already cited above, showing that mice with a disrupted IL-4 gene clear *P. chabaudi* AS-infection in a similar manner as their wild-type counterparts (31).

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 (43) and to induce endothelial cells to express adhesion molecules favoring leukocyte emigration (44), 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, the results of other studies also suggest that TNF-α induces splenic enlargement (45).

Taken together, our results demonstrate that TNF-α, either alone or in synergy with IFN-γ, affects different aspects of resistance to *P. chabaudi* AS malaria *in vivo*: 1) it induces high amounts of NO which are critical to survive the infection, 2) it is involved in controlling the peak level of parasitemia which does not appear to depend on the presence of NO, and, 3) it partially regulates the development of splenomegaly. In contrast, IL-4 alone which is produced in high amounts by susceptible A mice during infection, 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, depends on the production of increased levels of Th1-associated TNF-α which induces NO-synthesis early during infection.

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Figure 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 (= u **) mouse and day 7 infected mice treated with normal rat serum IgG (= infected controls ***) or anti-TNF-α and/or anti-IFN-γ mAb (n per treatment group = 4). Splenic iNOS mRNA expression per individual mouse was determined by RT-PCR. Aliquots of 40 fg of competitive standard$^{\$}$ were co-amplified with each of the cDNA samples. GAPDH mRNA levels were also determined by PCR for each sample. n* = negative control (that is, 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 the bands 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. **** $p < 0.05$ versus infected control mice.

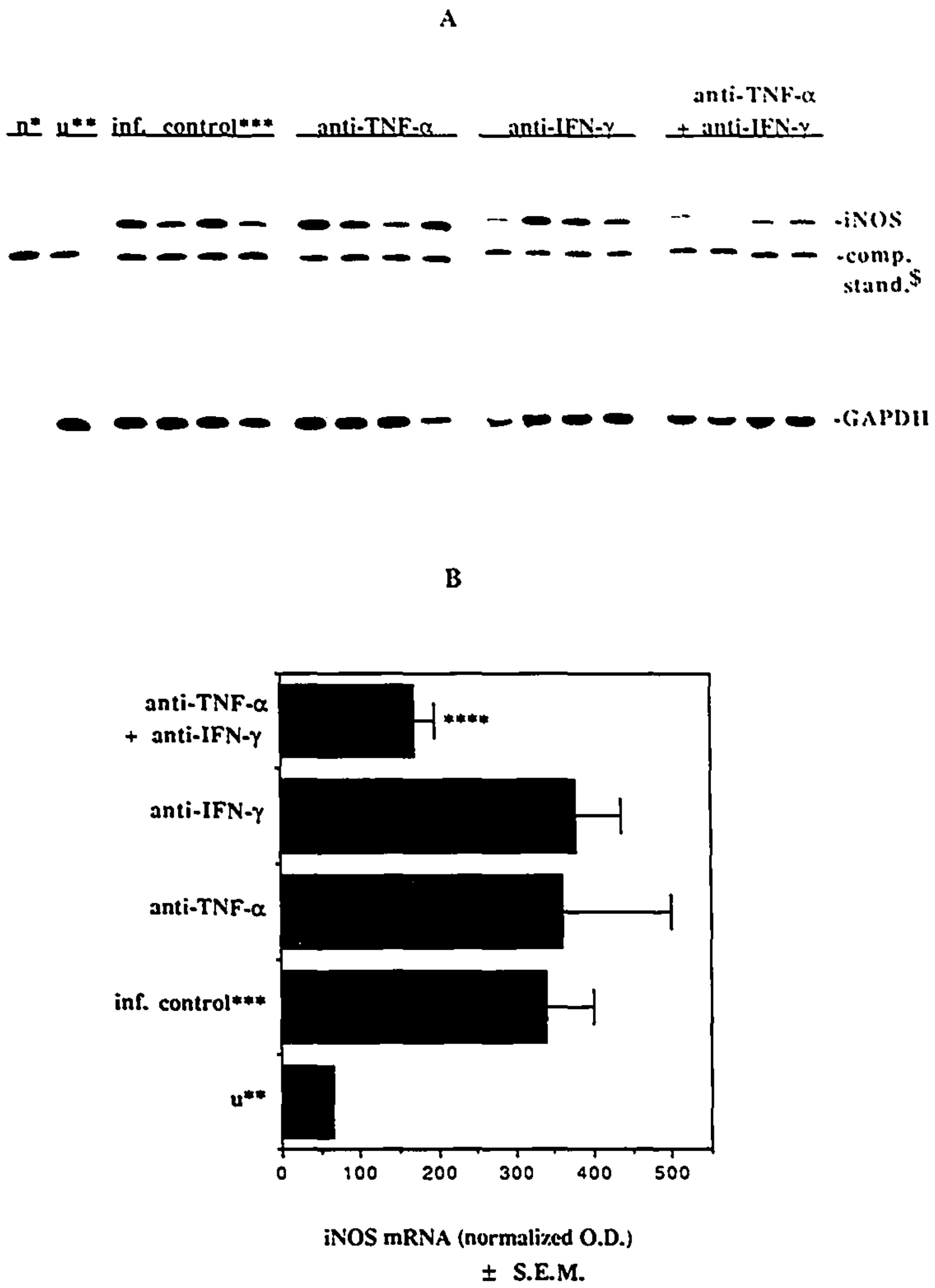

A
n*
u**
inf. control***
anti-TNF-α
anti-IFN-γ
anti-TNF-α
+ anti-IFN-γ
-iNOS
-comp. stand.$
-GAPDH
B
anti-TNF-α
+ anti-IFN-γ
****
anti-IFN-γ
anti-TNF-α
inf. control***
u**
0
100
200
300
400
500
iNOS mRNA (normalized O.D.)
± S.E.M.

Figure 2.

Serum NO_3^- levels in anti-TNF-α and/or anti-IFN-γ mAb-treated resistant B6 mice during *P. chabaudi* AS infection. Serum was obtained from uninfected control and day 7 infected mice treated with normal rat serum IgG (= infected controls) or anti-TNF-α and/or anti-IFN-γ mAb (n per treatment group = 4-6). Serum NO_3^- levels per individual 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.

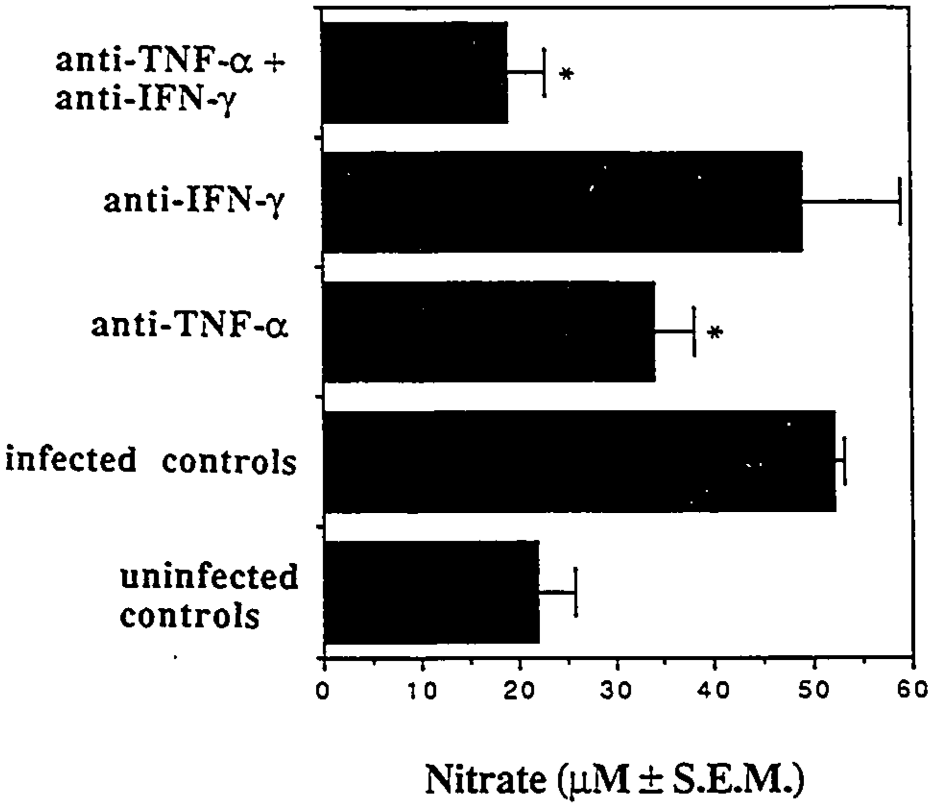

anti-TNF-α + anti-IFN-γ
*
anti-IFN-γ
anti-TNF-α
*
infected controls
uninfected controls
0
10
20
30
40
50
60
Nitrate (μM ± S.E.M.)

Figure 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 anti-TNF-α and/or anti-IFN-γ mAb (n per treatment group = 4-6). The data shown are representative of two replicate experiments. * $p < 0.002$ versus control mice.

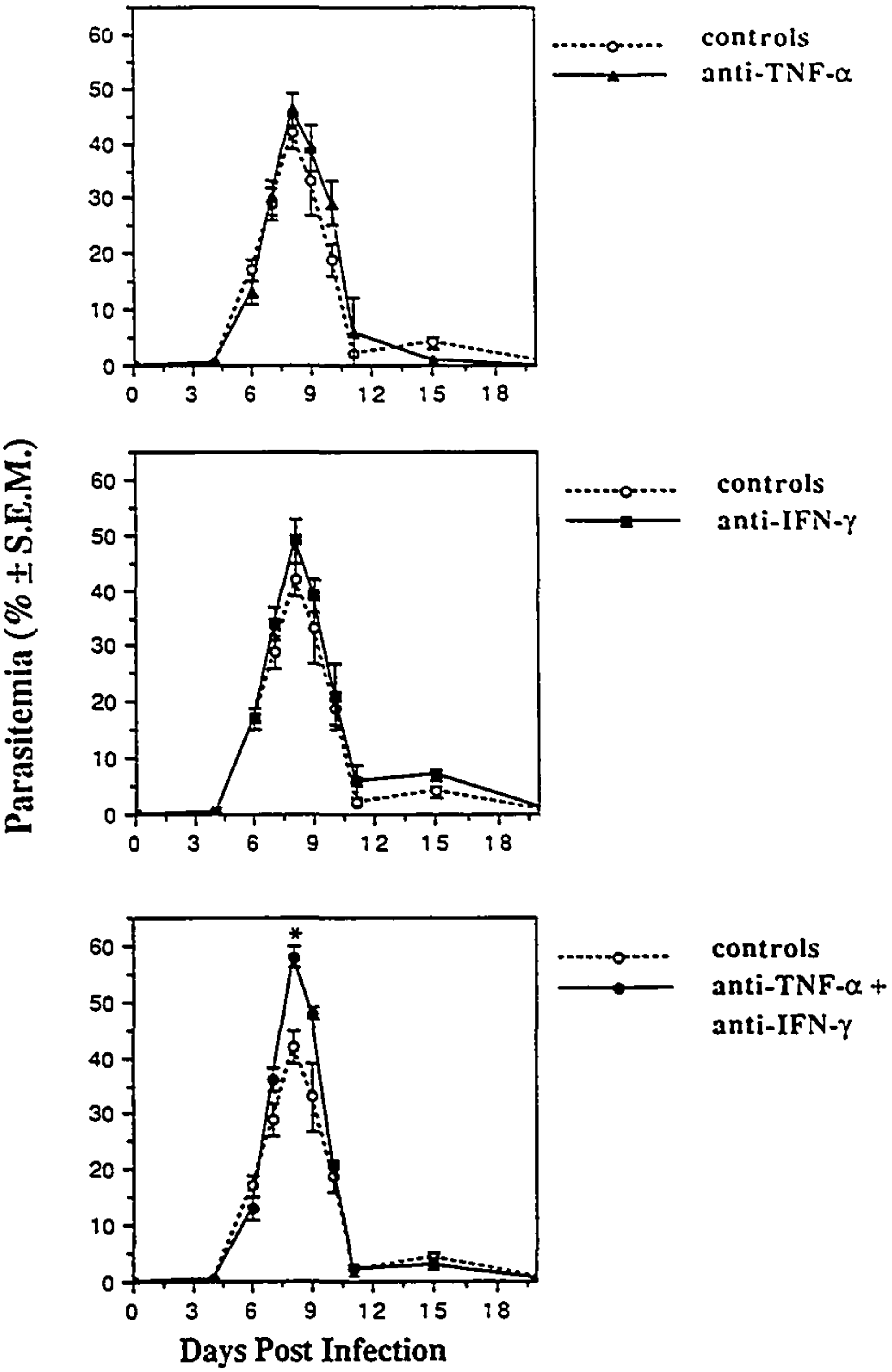
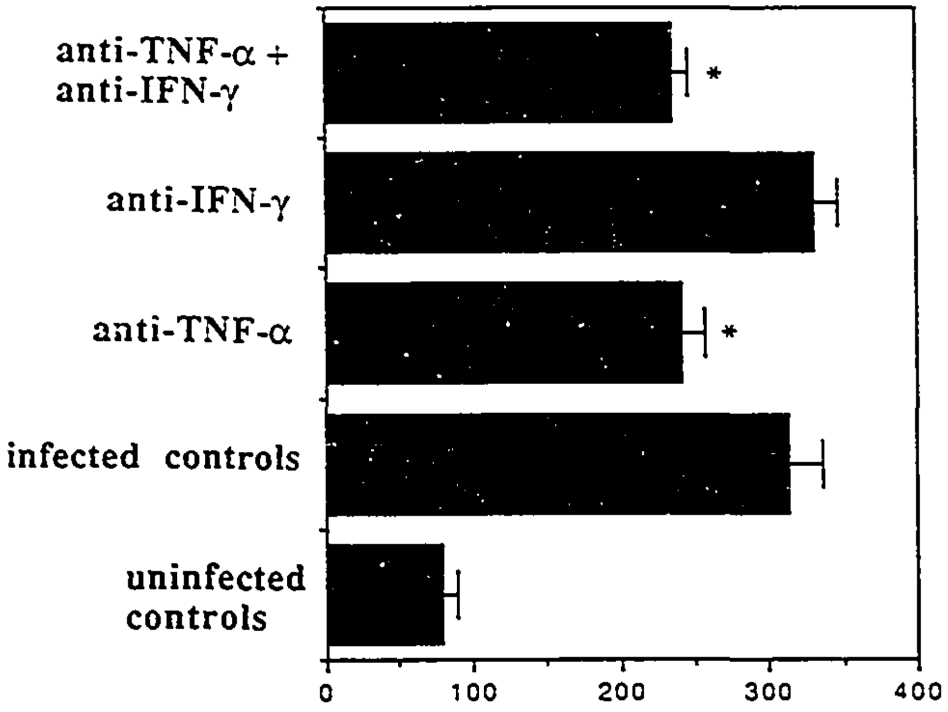

Parasitemia (% ± S.E.M.)
Days Post Infection
controls
anti-TNF-α
controls
anti-IFN-γ
controls
anti-TNF-α +
anti-IFN-γ
*
0
10
20
30
40
50
60
0
3
6
9
12
15
18

Figure 4.

Spleen weight of anti-TNF-α and/or anti-IFN-γ mAb-treated resistant B6 mice during *P. chabaudi* AS infection. Spleen weight was determined for uninfected control and day 7 infected mice treated with normal rat serum IgG (= infected controls) or anti-TNF-α and/or anti-IFN-γ mAb (n per treatment group = 4-6). The data shown are representative of two replicate experiments. * $p < 0.05$ versus infected control mice.



Spleen Weight (mg ± S.E.M.)

Figure 5.

Splenic iNOS mRNA levels of anti-IL-4 mAb or mo rTNF-α -treated susceptible A mice during *P. chabaudi* AS infection. **(A)** Total RNA was purified from spleens recovered from an uninfected, control (= u **) mouse and day 7 infected mice treated with normal rat serum IgG (= infected controls ***) or anti-IL-4 mAb or mo rTNF-α (n per treatment group = 3). Splenic iNOS mRNA expression per individual mouse was determined by RT-PCR. Aliquots of 40 fg of competitive standard$^{\$}$ were co-amplified with each of the cDNA samples. GAPDH mRNA levels were also determined by PCR for each sample. p* = positive control (that is, splenic iNOS mRNA expression in resistant B6 mice on day 7 post infection 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 the bands 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.

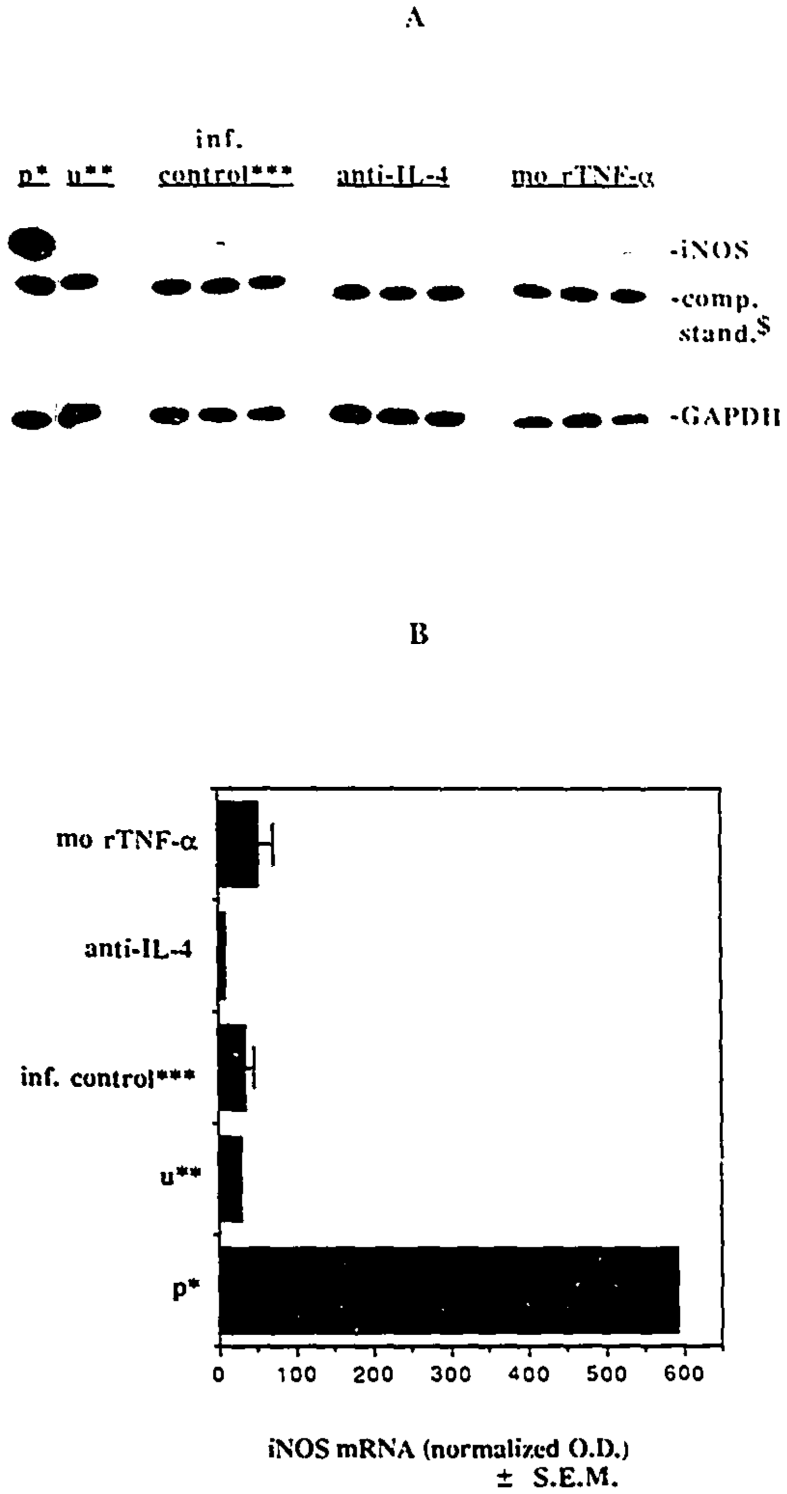

A
inf.
p*
u**
control***
anti-IL-4
mo rTNF-α
-iNOS
-comp. stand.$
-GAPDH
B
mo rTNF-α
anti-IL-4
inf. control***
u**
p*
0
100
200
300
400
500
600
iNOS mRNA (normalized O.D.) ± S.E.M.

TABLE I

Serum NO_3^- levels, peak parasitemia levels and spleen weights of anti-IL-4 mAb or mo rTNF-α-treated susceptible A mice during *P. chabaudi* AS infection.

Treatment	Serum NO_3^- (µM ± S.E.M.)	Peak Parasitemia (% ± S.E.M.)	Spleen Weight (mg ± S.E.M.)
normal rat serum[a]	19 ± 2	65 ± 3	165 ± 04
anti-IL-4 mAb	21 ± 2	67 ± 3	175 ± 11
mo r TNF-α	16 ± 2	58 ± 9	221 ± 22[b]

[a] i.v. treatment of control mice with 0.2 ml pyrogen-free saline gave similar results as treatment with normal rat serum.

[b] $p < 0.05$ versus control mice.

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CHAPTER 5

GENERAL DISCUSSION AND CLAIMS TO ORIGINALITY

Previous results from our laboratory have demonstrated that resistance to blood-stage *P. chabaudi* AS has a genetic basis. Indeed, segregation analysis of backcross and F2 progeny derived from susceptible and resistant strains of mice has shown that a major, dominant, autosomal, non-H-2-linked gene or a closely linked set of genes controls resistance to blood-stage *P. chabaudi* AS (1). Analysis of recombinant inbred strains derived from susceptible A and resistant B6 confirmed this genetic control of resistance (2). Furthermore, the phenotypic expression of this gene occurs early during the course of infection and, thus, may relate to the control of parasite multiplication (2). In this regard, more recent *in vitro* studies showed that there were marked qualitative differences in the production of cytokines, which are known to regulate mechanisms potentially involved in controlling parasite multiplication, between resistant B6 and susceptible A mice early during the course of infection. Indeed, splenocytes recovered from resistant B6 mice early during infection were shown to produce high levels of the Th1 cytokine IFN-γ whereas splenocytes recovered from susceptible A mice at the same time during infection produced high levels of the Th2 cytokines IL-4, IL-5 and IL-10 (3 and unpublished observation). These data, in addition to previous results demonstrating that CD4+ T cells, macrophages and an intact spleen are required to resolve *P. chabaudi* AS malaria (4-6), suggest that IFN-γ-mediated macrophage activation in the spleen plays a major role in resistance to infection with *P. chabaudi* AS. Moreover, TNF-α, a cytokine mainly produced by macrophages, was also shown to have a dominant role in determining resistance to this parasite (7).

The studies presented in this thesis suggest that, early during infection with blood-stage *P. chabaudi* AS, a Th1 response, as occurs in the spleen of resistant B6 but not susceptible A mice, leads to increased TNF-α production. TNF-α alone, or in synergy with IFN-γ, was shown to affect peak parasitemia levels, splenomegaly and the up-regulation of NO. Moreover, the production of NO was demonstrated to be crucial in order to survive *P. chabaudi* AS malaria. However, despite the fact that NO derivates have been shown to kill malaria parasites *in vitro* (8), NO did not appear to be involved in parasite killing *in vivo.* The present studies also suggest that a Th2 response early during the course of infection, as occurs in susceptible A mice, down-regulates the production of protective amounts of TNF-α and NO. Indeed, high levels of IL-4, but low levels of IFN-γ, TNF-α and NO are found in these mice early during infection. Moreover, *in vitro* studies have previously shown that IL-4 and IL-10 down-regulate the production of NO and TNF-α by macrophages, respectively (9, 10). Furthermore, the high peak parasitemia levels seen in susceptible A mice later during infection appear, in addition to provoking a high degree of anemia (11), to trigger the production of excessive and pathologic levels of TNF-α which ultimately results in death. The increased levels of iNOS mRNA in the liver of susceptible A mice observed at the time of death may also be induced by the high TNF-α levels in this organ.

In the final section of this thesis, I would like to address some issues which demand further clarification.

The *P. chabaudi* AS resistance gene

As mentioned above, a gene which appears to be expressed early during infection and may be involved in controlling parasite multiplication determines resistance to blood-stage infection with *P. chabaudi* AS. An early event which relates to controlling parasite multiplication and resistance to *P. chabaudi* AS malaria, is the differential production of T cell derived cytokines, that is, the production of Th1 versus Th2 cytokines, between resistant and susceptible mice. In other words, the resistance gene may relate to the expression of an early and protective Th1 response as seen in resistant B6 mice. Interestingly, IL-12, a cytokine produced by antigen presenting cells such as macrophages and B cells, has been shown to promote the development and proliferation of Th1 cells, partially through enhancing the production of IFN-γ (12-14). Moreover, a recent study from our laboratory demonstrated that treatment of A mice with recombinant IL-12 could induce the development of resistance to *P. chabaudi* AS in an otherwise susceptible host via a Th1 response involving IFN-γ, TNF-α and NO (15). Thus, the resistance gene may be expressed earlier than the period in which Th1 cytokines are produced and its function may relate to the induction of the Th1 response. In other words, the resistance gene may be expressed at the level of the antigen presenting cell and may relate to the production of IL-12 or at the level of cells, which produce Th1-promoting IFN-γ in response to IL-12, such as NK cells (16). It might thus be of interest to investigate the role of NK cells in resistance to blood-stage *P. chabaudi* AS. In addition, mapping the chromosomal location of the resistance gene and determining the cell type expressing it will be needed to confirm the above hypotheses. The demonstration of the existence of a human homologue of this resistance gene could be a next step.

Tissue-specific production of TNF-α and NO during infection with *P. chabaudi* AS

I demonstrated that resistance to blood-stage *P. chabaudi* AS malaria correlates with high levels of TNF-α -and iNOS mRNA in the spleen and increased levels of TNF-α mRNA in the liver early during the course of infection. I hereby assumed that bioactive TNF-α and iNOS protein is indeed produced in these organs. Justification for this assumption is based on the following facts: 1) TNF-α protein and NO_3^-, an oxidized product resulting from iNOS activity, are present in serum collected from resistant mice early during infection; 2) although the induction of untranslated pools of TNF-α mRNA has been shown *in vitro* (17), LPS-like *Plasmodia* antigens are known to induce the secretion of TNF-α protein *in vivo* (18) and LPS has been demonstrated to both up-regulate TNF-α gene transcription and translation *in vitro* and *in vivo* (19-21); and 3) there is little evidence for the existence of untranslated pools of iNOS mRNA (22). Furthermore, and as I discussed before, we can not exclude that both TNF-α and NO are produced in organs other than spleen and liver during infection with *P. chabaudi* AS. In this regard, LPS administration to transgenic mice bearing a reporter gene construct in which the TNF coding sequence was replaced by a chloramphenicol acetyltransferase (CAT) coding sequence induced CAT activity in kidney, heart, islets of Langerhans, spleen, lung, fallopian tubes and uterus (23).

Furthermore, I demonstrated that macrophages in the spleen recovered from *P. chabaudi* AS infected B6 mice are able to produce high levels of NO upon stimulation with LPS. Previous results from our laboratory showed that, following LPS stimulation, these cells also produce high levels of TNF-α (24). These data, thus, suggest that macrophages in the spleen may represent the cellular source of both TNF-α and NO *in vivo*. However, many other cell types in the spleen such as T, B and endothelial cells are able to produce TNF-α and/or NO (20, 25). Moreover, the procedure which is used in our studies to separate macrophages from the rest of the spleen, based on the ability of macrophages to adhere to plastic, appears to isolate only macrophages from the spleen's white pulp and not from the red pulp or marginal zone (26). It is thus possible that, in addition to cells other than macrophages, red pulp and marginal zone macrophages produce high amounts of TNF-α and NO *in vivo* during infection with *P. chabaudi* AS. Furthermore, preliminary *in situ* hybridization studies in our laboratory indicate that, in the liver of susceptible A mice, both hepatocytes and Kupffer cells produce NO during infection with *P. chabaudi* AS (unpublished observations). Kupffer cells are also known to produce TNF-α (20).

I also presented data which suggest that TNF-α and IFN-γ are required to up-regulate NO production in the spleen of resistant B6 mice early during infection with *P. chabaudi* AS. Although high levels of TNF-α mRNA were also found in the liver of these animals, high iNOS expression in this organ could not be detected. A lack of high local production of IFN-γ or other stimuli required to induce iNOS expression in the liver, but not in the spleen, may explain the latter findings. Indeed, it is known that the requirements for induction of iNOS differ depending upon the cell-type; for example, IL-1 can synergize with TNF-α to increase the production of NO by hepatocytes whereas IL-1 has no effect on macrophages for the secretion of NO (27, 28). Furthermore, the moderate increase in iNOS mRNA levels in the liver of susceptible A mice later during infection, coincident with peak parasitemia, may be the result of the presence of excessive levels of parasite antigen in these mice which, via the massive induction of TNF-α and perhaps other inflammatory mediators such as IL-1, up-regulates iNOS expression.

Role of TNF-α and NO during infection with *P. chabaudi* AS

A major objective of this thesis was to try to understand how TNF-α and NO mediate protection during *P. chabaudi* AS malaria. My results demonstrate that TNF-α, when produced early during infection, is involved in controlling parasite multiplication, splenomegaly, and NO production. All three of these TNF-α-induced effects may contribute to resistance against *P. chabaudi* AS malaria as follows: 1) controlling parasite multiplication, perhaps through the induction of reactive oxygen intermediates, may prevent the development of high levels of peak parasitemia, severe anemia and death, as seen in susceptible A mice; 2) as the spleen is believed to be required for the expression of an effective immune response during *P. chabaudi* AS malaria (29), a marked splenomegaly may contribute quantitatively to this protective response; and 3) NO may protect host tissue against damage by reactive oxygen intermediates as I will discuss further in more detail. Except for the induction of splenomegaly, TNF-α was also shown to exert these protective effects in synergy with IFN-γ. Moreover, I demonstrated that increased IFN-γ production associates with, and may even be required for the induction of, high levels of TNF-α. In contrast, excessive production of TNF-α late during infection, as seen in susceptible A mice, which appears to occur independently of IFN-γ, may aggravate anemia by enhancing erythrophagocytosis, as recently suggested by Taverne et al (30). Excessive TNF-α production may also be involved in hypoglycemia and liver damage as discussed before. However, I treated susceptible A mice with pentoxifylline, a drug shown to prevent TNF-α synthesis (31), late during infection and was not able to prevent these mice from dying during *P. chabaudi* AS malaria. These data, thus, suggest that TNF-α may contribute to, but is not the major cause of death in this malaria model. These findings are also fully in agreement with studies by Yap et al (11) which demonstrated that anemia is the principal cause of death of susceptible A mice during infection with *P. chabaudi* AS. Furthermore, in addition to IL-12, both TNF-α and IFN-γ are thought to be involved in the generation of Th1 cells (12). Thus, it appears that the following sequence of events is necessary for the development of resistance to *P. chabaudi* AS as occurs in resistant hosts, such as B6 mice. First, during the very first day(s) following infection with *P. chabaudi* AS, IL-12 in synergy with TNF-α up-regulates the production of IFN-γ which leads to the proliferation of Th1 cells. Second, soon after, that is, 1-3 days before peak parasitemia, IFN-γ produced by Th1 cells up-regulates TNF-α and both molecules synergize to induce reactive oxygen intermediates and NO. However, if excessive levels of malaria-antigen induce excessive levels of TNF-α late during infection, that is, at or 1-2 days after peak parasitemia, there is exacerbation of disease, as occurs in susceptible hosts, such as A mice.

The studies wherein resistant B6 mice were treated with aminoguanidine, a specific iNOS inhibitor (32), demonstrated that inhibition of NO production does not affect peak parasitemia. This observation suggests that NO may not be involved in controlling parasite multiplication. However, NO was shown to be crucial for survival of the host and I suggested that NO protects host cells, in addition to other known anti-oxidant systems (33), against damage from reactive oxygen intermediates (ROI). Thus, I propose that instead of NO, ROI, although they can damage host cells, are produced to either directly kill malaria parasites, as has been previously demonstrated *in vivo* (34), or be involved in indirect killing through the formation of peroxidized fatty acids which were also shown to be lethal for malaria parasites *in vitro* (35). The role of TNF-α and IFN-γ in controlling parasite multiplication, as mentioned above, may thus be mediated by the induction of ROI. However, I can not exclude that, in some cases, NO is involved in parasite killing *in vivo* because, as it appears that there is a subtle interplay between NO and ROI (36), it would be not surprising that there is also a high degree of redundancy between both species of reactive radicals. In other words, NO may act as a killer molecule when only low levels of ROI are produced, as may be the case in the study of Cavacini et al (37) in which P/J mice resolved an infection with *P. chabaudi* adami and the study by Taylor-Robinson et al (38) in which a Th1 clone transferred protection to T-cell depleted *P. chabaudi* AS-infected mice. Alternatively, NO may detoxify ROI when the latter are produced in large amounts, as may be the case in our study using immunologically intact mice. Furthermore, NO has also been shown to suppress lymphoproliferation during infection with *P. chabaudi* AS (39, 40). The latter findings may demonstrate another mechanism through which NO protects the host from a potentially harmfull, cell-mediated, inflammatory-type response. The production of NO in the liver of susceptible A mice late during *P. chabaudi* AS malaria, as seen in our study, may be a fruitless attempt to protect this organ against TNF-α-mediated damage. Furthermore, high levels of NO may, similar to ROI, also damage host cells (36). Little is known, however, about mechanisms which protect cells against high levels of NO (36).

Taken together, it appears that the *P. chabaudi* AS resistance gene is expressed early and correlates with a complex pathway of TNF-α-mediated events which leads to the control of parasite multiplication concomitant with NO-mediated host protection. Susceptibility, on the other hand, is characterised by the lack of early, TNF-α-mediated events which results in high parasitemia levels concomitant with the late production of excessive amounts of TNF-α, pathology and death. The fact that, during human malaria infections, increased NO production also appears to correlate with protection (41) whereas high serum TNF-α levels correlate with pathology and death (42), underscores the relevance of my findings in the understanding of human immune responses to blood-stage malaria.

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CLAIMS TO ORIGINALITY

Chapter 2 demonstrates that the role of TNF-α during *P. chabaudi* AS malaria may depend on the timing and site of its expression and the presence of cytokines regulating its production.

(1) Resistant C57BL/6 mice have higher levels of TNF-α mRNA in the spleen and liver early during infection compared to susceptible A/J mice.
(2) The high levels of TNF-α mRNA in the spleen of resistant mice correlate with increased levels of IFN-γ mRNA and low levels of IL-4 mRNA. On the other hand, the low levels of TNF-α mRNA in the spleen of susceptible mice correlate with increased levels of IL-4 mRNA and low levels of IFN-γ mRNA.
(3) Susceptible A/J mice express higher levels of TNF-α mRNA in the liver and have higher serum levels of TNF-α protein later during infection compared to resistant C57BL/6 mice.
(4) Neutralization of TNF-α in resistant C57BL/6 mice during infection results in increased mortality but has no effect on peak parasitemia level.

Chapter 3 demonstrates that the ability to produce high amounts of NO correlates with resistance to *P. chabaudi* AS malaria and that the site of its expression is critical.

(1) Both splenic and peritoneal macrophages recovered from resistant C57BL/6 mice during infection produce higher levels of NO_2^- *in vitro* in response to LPS than similarly stimulated macrophages from susceptible A/J mice.
(2) Resistant C57BL/6 mice have, during infection with *P. chabaudi* AS, higher levels of splenic iNOS mRNA and serum NO_3^- *in vivo* compared to susceptible A/J mice.

(3) Inhibition of NO production by resistant C57BL/6 mice during infection results in increased mortality but has no effect on peak parasitemia level.

(4) Susceptible A/J mice have a moderate increase in iNOS mRNA levels in the liver later during infection.

Chapter 4 demonstrates that TNF-α and IFN-γ, but not IL-4, play a major role in the regulation of NO production and other traits of resistance during *P. chabaudi* AS malaria.

(1) Neutralization of TNF-α and IFN-γ in resistant C57BL/6 mice during infection results in reduced NO production and increased peak levels of parasitemia.

(2) Neutralization of TNF-α alone in these mice also results, although to a lesser extent compared to neutralization of both TNF-α and IFN-γ, in reduced NO production and reduces the development of splenomegaly.

(3) Neutralization of IFN-γ alone in resistant C57BL/6 mice during infection slightly increases peak parasitemia levels but has no effect on NO production and the development of splenomegaly.

(4) Neutralization of IL-4 alone in susceptible A/J mice during infection has no effect on NO production, peak levels of parasitemia and the development of splenomegaly.