MECHANISM(S) OF INTERLEUKIN-12-INDUCED PROTECTION AGAINST EARLY
MURINE BLOOD-STAGE *P. CHABAUDI* AS MALARIA

by

Hakeem Sam

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

Department of Experimental Medicine and Centre for the Study of Host Resistance
McGill University, Montreal, Quebec, Canada

Oct 1998

Canada

Dedication

To my parents and brothers and sisters in the extended Sam family

Abstract

Previous studies suggest that IL-12, the potent Th1-inducing cytokine, IFN-γ, TNF-α and nitric oxide (NO) contribute to resistance against blood-stage malaria. Early Th1 responses correlate with resistance, whereas predominantly Th2 responses are associated with susceptibility. In the present studies, the requirements for endogenous IL-12 and its role in host defense against blood-stage *P. chabaudi* AS malaria were investigated. Our results reveal, for the first time, significant differences in the kinetics of endogenous IL-12 p70 synthesis and splenic IL-12R β1 and β2 mRNA expression between resistant B6 and susceptible A/J mice that correlate with the polarization of Th responses observed in these hosts during early blood-stage malaria. The spleen was found to be the major source of systemic IL-12 in infected B6 hosts. In addition, significant differences were observed between acutely infected B6 and A/J hosts, on a per cell basis, in IL-12 p70 release by splenic macrophages in vitro. Importantly, these differences correlated with greater malaria parasite-induced IFN-γ synthesis in vitro by spleen cells from infected B6 mice. Furthermore, systemic IL-12 production and Th1 responses were found to be unimpaired in *P. chabaudi AS* infected mice deficient in TNFR compared to wild type controls. However, LPS, but not PRBC, -induced NO synthesis by splenic macrophages was significantly reduced in infected TNFR deficient hosts. Finally, compared to controls receiving chloroquine (CQ) alone, the mechanism(s) of combined low dose IL-12 and CQ therapy for malaria-infected A/J mice was found to involve increased splenocyte expression of IL-12R β1 and β2 and IFN-γ mRNA, together with enhanced parasite antigen-induced synthesis of IFN-γ by spleen cells in vitro. This novel IL-12 and CQ therapeutic strategy resulted in significantly reduced parasitemia, enhanced survival, and was effective against established blood-stage malaria. Taken together, IL-12 appears to be a key member of a network of early-acting cytokines whose actions have important consequences for the development of host adaptive immunity to blood-stage malaria.

Résumé

Des études antérieures ont montré que IL-12, une cytokine inductrice des cellules Th1, IFN-γ, TNF-α et l'oxyde nitreux (NO) contribuent à la résistance de la malaria lors du stage sanguin. Une réponse Th1 précoce et prédominante amène une résistance à la maladie alors qu'une réponse Th2 prédominante amène plutôt une susceptibilité. Dans ce projet de recherche, la nécessité de IL-12 endogène et son rôle dans la défense de l'hôte contre le pathogène *P. chabaudi* AS causant la malaria lors du stage sanguin ont été étudié. Nos résultats révèlent pour la première fois qu'il y a des différences significatives dans la cinétique de la synthèse de IL-12 p70 endogène et dans l'expression de l'ARNm de la rate de IL-12R β1 et β2 entre les souris résistantes B6 et susceptibles A/J et que ces différences correspondent à la polarisation d'une réponse Th observée lors des stages sanguins précoces de la malaria. Il a également été observé que la rate est le site majeur de production de IL-12 systémique dans des hôtes B6 infectés. De plus, des différences significatives *in vitro* dans le largage de IL-12 p70 par des macrophages de la rate ont été observé entre les hôtes B6 et A/J. Ces différences correspondent à une synthèse accrue *in vitro* de IFN-γ induite par le parasite de la malaria dans les cellules de la rate provenant des souris B6 infectées. Par ailleurs, la production systémique de IL-12 et la réponse Th1 n'étaient pas affectées chez des souris déficientes en TNFR et infectées par *P. chabaudi* AS lorsque comparées à des témoins sauvages. Cependant, l'induction de la synthèse de NO par LPS et non par PRBC chez les macrophages de la rate était réduite de façon significative chez des hôtes infectés et déficients en TNFR. Finalement, il a été observé qu'une faible dose de IL-12 combiné à une thérapie à la chloroquinine (CQ) chez des souris A/J infectées par la malaria a amené une expression accrue de l'ARNm IL-12 β1, β2 et IFN-γ chez les splénocytes ainsi qu'une synthèse *in vitro* plus élevée de IFN-γ, cytokine induite par les antigènes du parasite, par les cellules de la rate lorsque comparé à des témoins n'ayant reçus que de la CQ. Cette stratégie thérapeutique utilisant l'IL-12 et la CQ a amené une baisse significative de la présence du parasite, une augmentation de la survie des souris et a été efficace à contrer le stage sanguin de la malaria. Ces résultats combinés semblent montrer que IL-12 serait un membre clé d'un réseau de cytokines agissant précocement et dont l'action a des conséquences importantes dans le développement d'une immunité adaptée de l'hôte contre le stage sanguin de la malaria.

Acknowledgements

I would like to express my sincere gratitude to my research supervisor Dr. Mary M. Stevenson for the constant support, advice, help and encouragement, without which this work would not be possible.

I would also like to gratefully acknowledge past and present members of my thesis committee, Drs R. Blostein, G. Matlashewski, F. Gervais, S. Mahanty, E. Schurr, W. Lapp, and J. Trasler for their advice, invaluable suggestions and constructive criticisms.

Many thanks to Drs D. Radzioch, D. Malo, F. Gervais, R. Blostein, E. Schurr and W. Lapp for welcoming me to your laboratories to seek help and advice whenever necessary. I wish to thank all the members of these laboratories, there is just not enough space to name you all, for all of your help, support and friendship. Special thanks to Krikor Kichian and Sophie Clermont for helping me to get going with RT-PCR and Southerns. My sincere thanks also goes to Claude Lachance for the French translation of the thesis abstract.

I wish to thank all the past and present members of Dr. Stevenson's laboratory for your help, support and friendship. The technical assistance of Mifong Tam and Juliette Goodlet is gratefully acknowledged, and the useful suggestions and constructive criticisms of Dr. K. Mohan are particularly appreciated.

I would like to express sincere thanks to Dr. Alicja Zobel-Brown for the constant encouragement, support and friendship.

I am most grateful for the special MD-Ph.D. studentship support from MRC Canada.

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List of Abbreviations

BFU-E	burst forming unit-erythroid
C57BL/6	B6
cDNA	complementary DNA
Con A	concanavalin A
CQ	chloroquine
ELISA	enzyme-linked immunoabsorbent assay
HRPO	horseradish peroxidase
i. p.	intraperitoneal
i. v.	intravenous
IFN	interferon
IL-	interleukin-
IL-12R	IL-12 receptor
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
mAb	monoclonal antibody
mpAg	malaria parasite antigen
O. D.	optical density
p	probability
p.i.	post infection
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PHA	phorbol acid
PRBC	parasitized red blood cell
rIL-12	recombinant IL-12
RT-PCR	reverse transcriptase - polymerase chain reaction
s.c.	subcutaneous
SEM	standard error of the mean
TNF	tumor necrosis factor
TNFR	TNF receptor

Statement of Thesis Office

According to the "Guidelines for Thesis Preparation", issued by the Faculty of Graduate Studies and Research, Department of Experimental Medicine, the text of the five indented paragraphs below is included in this thesis in order to inform the External Examiner of Faculty Regulations:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. 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, and introduction which clearly states the rationale and objectives of the study, a review of 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 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.

Statement of Originality

The following aspects of this thesis are considered to be original contributions to knowledge.

Chapter 1. The comprehensive review on the role of IL-12 in host resistance to malaria in this general introduction was not previously published.

Chapter 2. The experimental results presented in this chapter are the first to demonstrate significant differences in endogenous IL-12 synthesis between resistant and susceptible mouse strains during early blood-stage *P. chabaudi* AS malaria. The spleen is shown to be the major source of systemic IL-12 production in resistant B6 hosts. In addition, this is the first study of in vivo IL-12R β1 and β2 mRNA expression in the spleens of malaria-infected mice. Significant differences between resistant B6 and susceptible A/J in the kinetics of up-regulation splenic of IL-12R β2 mRNA levels in following malaria infection were not previously reported. Primers, probes and optimal cycling conditions for RT-PCR analysis of IL-12R mRNA were designed by the author, based on the published full length cDNA sequences for the two receptors obtained from Genebank.

Chapter 3. The experimental results presented in this chapter represent the first analysis of IL-12 production by splenic macrophages during early blood-stage *P. chabaudi* AS malaria. Differences between resistant B6 and susceptible A/J mice in secretion of biologically active p70, but not p40, by these cells in vitro were not previously reported. Spontaneous, LPS- and PRBC -induced release of IL-12 p70 by splenic macrophages were significantly higher in malaria-infected B6 compared to A/J mice.

Chapter 4. My contribution of experimental results to this chapter addresses the role of cytokine or cytokine receptor-mediated mechanism(s) in cure of *P. chabaudi* AS

infected A/J mice treated with combined low dose IL-12 and chloroquine therapy. This novel therapeutic strategy resulted in substantially higher splenocyte mRNA expression for IL-12R β1 and β2 in treated versus untreated controls. Downstream of IL-12 signaling, splenocyte IFN-γ mRNA expression and malaria parasite-specific induction of IFN-γ synthesis by spleen cells in vitro were also higher in treated mice. These results were not previously reported.

Chapter 5. The experimental results presented in this chapter represent the first analysis of endogenous IL-12 production and in vitro Th1 cytokine responses by spleen cells from malaria-infected mice genetically deficient in both p55 and p75 TNFR. Moreover, the analysis of splenic IL-12R β1, IL-12R β2, and IFN-γ mRNA expression in these mice are the first of its kind. Evidence is presented in this chapter that demonstrates unimpaired IL-12 production in vivo, splenic IL-12R β1 and β2 mRNA expression, and IFN-γ synthesis by spleen cells from malaria-infected mice deficient in TNFR compared to wild type controls.

Preface

Thesis Organization and Authorship

The manuscript-based format is used in this thesis. **Chapter 1** consists of a general introduction and comprehensive literature review, followed by 4 experimental **chapters 2-5**. **Chapter 6** consists of a general discussion and summary that integrates the combined research studies presented in this thesis. **Connecting statements** are included as **prefaces to Chapters 3, 4 and 5.**

Chapter 2 is accepted for publication in "The Journal of Immunology". **Chapters 3** is accepted for publication in "Clinical and Experimental Immunology". **Chapter 5** is in preparation for submission. The research work presented in these chapters were 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. Dr. Stevenson is the co-author of these chapters.

Chapter 4 is accepted for publication in "Infection and Immunity". The first author of this chapter is Dr. K. Mohan, a post-doctoral fellow in Dr. Stevenson's laboratory. Hakeem Sam and Dr. Stevenson are co-authors of this chapter. Mr. Sam and Dr. K. Mohan contributed 40% and 60%, respectively, to the experimental work presented in this chapter. Mr. Sam was responsible for the design of primers, probes and RT-PCR conditions for assessing IL-12R β1 and β2 mRNA expression, as well as cytokine and cytokine receptor responses following combined low dose IL-12 and chloroquine therapy for established blood-stage malaria.

Chapter One

GENERAL INTRODUCTION

INFECTION WITH MALARIA

Introduction

Malaria poses a major health threat to the lives of millions who live in endemic areas. Over 300 million people are infected annually, resulting in a mortality rate of 2 million per year with most of the casualties representing children under 5 years of age (1, 2). Malaria is caused by the hemoprotozoan *plasmodium* parasite. Of a total of 123 *Plasmodia* species, there are four that infect man (3). These are *P. ovale*, *P. vivax*, *P. malariae* and *P. falciparum*. *P. vivax* invades only reticulocytes, whereas *P. falciparum* invades erythrocytes at all stages of developmental maturation (2). Of these two, it is the infection caused by the latter that is responsible for most of the mortality and morbidity attributed to this disease. Four rodent malaria species infect inbred mouse strains, namely *P. chabaudi*, *P. yoelii*, *P. vinkei*, and *P. berghei* (2). Murine and simian models of malaria have been extremely useful in the study of the host immune response against this disease.

Traditionally, the most effective form of therapy available to patients suffering from malaria has been chemotherapy with available quinine or non-quinine based drugs. Unfortunately, the emergence of drug-resistant forms of the parasite has raised fears about possible epidemic outbreaks that could have disastrous consequences. These concerns have fueled the search for alternative forms of prophylactic and therapeutic management of this deadly disease. Recently, hope for a protective vaccine against malaria was heightened by the discovery of a putative novel malaria vaccine, designated SPf66 by its discoverer Manuel Elkin Patarroyo (4, 5). Results of efficacy trials in humans, however, were disappointing and revealed no significant benefit of the drug over placebo (4). A better understanding of the immunologic

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interactions between the host and the malaria parasite may lead to the development of effective immunotherapeutic strategies in the near future.

Life cycle of *Plasmodia*

The life cycle of *Plasmodia* is complex and has previously been reviewed (3, 6). The typical life cycle of *Plasmodia* is shown in Figure 1 for *P. falciparum*. Briefly, infection in a mammalian host is initiated by the bite of an infected female anopheles mosquito during blood-feeding. The mosquito vector injects sporozoites, stored in its salivary glands, which migrate via the host lymphatics and blood vessels to the liver where hepatocytes are infected. Within the hepatocytes, the parasite undergoes a series of cycles of asexual replication. This replicative phase is referred to as exoerythrocytic schizogeny and constitutes the liver stage of infection that lasts a period of about two weeks. Infected hepatocytes reveal no signs of inflammation and there are no symptoms experienced by the host at this stage of the disease. Several days later, infected hepatocytes burst, releasing thousands of merozoites into the host blood-stream. Released merozoites invade the host erythrocytes and within these cells, the parasite undergoes a second stage of asexual replication. This second replicative process is termed erythrocytic schizogeny. After numerous replicative cycles, infected erythrocytes, now containing a heavy parasite burden, rupture and release parasite toxins and abundant merozoites into the host blood-stream. The released merozoites infect new erythrocytes and thus propagate blood-stage malaria. Within the erythrocyte, a number of merozoites undergo sexual differentiation to form gametocytes, which are infective to an uninfected mosquito during blood feeding. The gametocytes are involved in a sexual replicative phase of the parasite that occurs in the

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Figure 1

MAN

Immature schizont → Mature schizont

Erythrocytic schizogony

Late trophozoite

Ring form

Penetration of red blood cells

Microgametocyte

Merozoites

Macrogametocyte

Merozoites

Hepatic schizogony

Anopheles mosquitoes

Mature schizont

Sporogonic phase

Hypnozoite

Macrogametocyte

Penetration of parenchymal cells

Sporozoites injected by mosquito

Oocyst containing sporozoites

Oocyst on stomach wall

Exflagellating microgametocyte

Ookinete

LIFE CYCLE of *PLASMODIUM* spp.

Adapted and redrawn from NCDC

web address: www.cdfound.to.it/html/pf1c.htm

gut of the mosquito. This sexual replication results in the formation of sporozoites that are infective to a mammalian host, thus completing the life cycle of the parasite.

All of the symptoms of malaria, typically pyrexia and general malaise, are associated with the blood-stage infection. Severe pathophysiologic complications of blood-stage malaria include anemia, renal failure, hypoglycemia, and cerebral malaria (7). Anemia is a major cause of morbidity and mortality in severe human malaria (2, 7-9). The development of cerebral malaria may involve the capacity of the parasite to modify the surface cytoadherence properties of infected erythrocytes. The net result is that infected erythrocytes have surfaces that readily adhere to the intima of endothelial cerebral vessels, as well as to the surface of nearby uninfected erythrocytes. Effectively, host erythrocytes form clumps or rosettes that occlude the lumen of cerebral vessels, leading to cerebral anoxia. An extremely severe complication during quinine therapy of malaria is blackwater fever, characterized by brisk hemolytic anemia and renal failure. This complication is possibly mediated by the formation of antibodies to quinine (2).

Plasmodium chabaudi AS: Murine model of blood-stage malaria

Rodent models have been extremely useful tools for addressing specific aspects of the host immune response against malaria. Reagents for depletion or characterization of specific cell types, as well as for cytokines are readily available. Moreover, the recent availability of selectively gene-targetted mutant mice has further enhanced the utility of these rodent models for murine malaria studies. *P. chabaudi* strain AS is a recognized rodent model of blood-stage malaria that is well established in our laboratory (10). This mouse model has been described as most akin to *P. falciparum* infection in humans (11). In both murine and human hosts, the parasite

invades erythrocytes of all stages and partial sequestration of the parasite occurs predominantly in the liver. In addition, recovery from the acute primary parasitemia is followed by one or more patent recrudescences. Moreover, *P. chabaudi* AS harbors many antigens analogous to those of *P. falciparum*. In contrast to murine malaria infections, however, in which acute parasitemias peak at levels greater than 30%, *P. falciparum* infection in humans results in parasitemias that rarely exceed 10% (12, 13).

The genetic difference in innate resistance to blood-stage *P chabaudi* AS infection between two inbred mouse strains, C57BL/6 (B6) and A/J, forms the basis of the murine malaria model used in our studies. Blood-stage disease is initiated directly by i.p. injection with 10^6 malaria-parasitized erythrocytes (PRBC), effectively bypassing the initial liver-stage infection observed in humans after a bite by a mosquito vector (14, 15). There is usually a prepatent period of 2-3 days. After this period, evidence of blood-stage malaria can be established on the basis of microscopic examination of infected erythrocytes on blood smears stained by a hematologic stain such as Diff-Quik or Giemsa. Resistant B6 mice exhibit a typical course of infection characterized by moderate primary peak parasitemia (30-40%) and anemia, marked splenomegaly, and clearance of the infection by 4 weeks post infection (16). The acute primary parasitemia in resistant B6 mice is usually reduced to subpatent levels by day 14. However, low level parasite recrudescence appears thereafter until parasitemia is reduced to undetectable levels by 4 weeks post infection. In contrast, A/J mice have a course of infection marked by fulminant primary peak parasitemia (50-60%) and severe anemia, moderate splenomegaly and the infection is fatal in 100% of these mice by 10-12 days post infection (16). In both mouse strains, malaria-induced splenomegaly is accompanied by drastic histologic changes in the architecture

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of the spleen (17). Spleens from susceptible A/J mice show a greater severity in depletion of metallophilic macrophages of the splenic marginal zone (17).

The spleen is a major site for the development of anti-plasmodial mechanisms during blood-stage malaria. In rats infected with *P. berghei*, resolution of acute malaria was found to be spleen-dependent (18). In *P. chabaudi* AS infection, splenectomized B6 mice were able to control the acute infection but could not resolve the infection and reduce parasitemia to subpatent levels (19). Unlike normal controls, splenectomized mice exhibited persistent and patent parasitemia. Interestingly, splenectomized mice could be protected when reconstituted with spleen cells from immune mice. Moreover, this protection provided by adoptive transfer of spleen cells from immune to splenectomized mice was shown to be B-cell dependent.

Available evidence suggests that the spleen also plays a crucial role in extramedullary erythropoiesis during blood-stage malaria (10). Anemia is one of the pathophysiological hallmarks of blood-stage malaria and may be an important factor in death due to this infection (7, 9). The induction of anemia is due, not only to the massive destruction of infected host erythrocytes and removal by erythrophagocytosis by splenic phagocytes, but also, to the induction of ineffective erythropoiesis. Measurement of erythropoietic activity in vivo by ^{59}Fe incorporation indicates a significantly higher erythropoietic activity and mobilization of erythroid progenitor cells in spleens of resistant B6 compared to susceptible A/J mice during the period of malaria-induced anemia (10). In contrast, erythropoietic activity in the bone marrow was suppressed in both mouse strains. Thus, in the resistant B6 mouse, the burst of splenic erythropoiesis may be in part responsible for maintaining the malaria-induced anemia at moderate and tolerable levels. Susceptible A/J mice are severely anemic just prior to death. However, about 80% of these mice could be rescued by blood transfusion given 2-3 days during peak parasitemia (10).

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HOST IMMUNITY AGAINST BLOOD-STAGE MALARIA

Genetic factors

The outcome of infection with blood-stage malaria is determined by multiple factors including the sex and genetic background of the host. Studies in humans suggest that the immune response to *P. falciparum* is genetically determined. Anti-malarial antibody responses in monozygotic and dizygotic twins, compared to age, sex and HLA class II-matched siblings, revealed the greatest concordance in monozygotes (20).

In rodents, resistance to *P. chabaudi* AS malaria showed marked heterogeneity among inbred mouse strains, manifested phenotypically by differences in parasitemia, erythropoietic responses, survival and degree of splenomegaly (15, 21). Analysis of strain variation in the level of resistance to blood-stage malaria, using AXB/BXA recombinant inbred mouse strains derived from susceptible A/J and resistant B6 progenitors, suggested that resistance was controlled by a single non-H-2-linked gene or closely-linked genes (15). Moreover, control of resistance by this gene appears to be influenced by the gender of the host. Female mice were more resistant than their male counterparts (21). Genetic loci involved in controlling parasitemia were recently mapped to regions on mouse chromosomes 8 and 9 (22, 23). Candidate genes found at these loci include the class-A scavenger receptor, the cytokine IL-15, haptoglobin, two retinol-binding proteins (*RBP1* and *2*), and several erythrocyte antigens including glycophorin A and erythrocyte protein 1.

Evidence from the measurement of circulating concentrations of testosterone in male and female H-2 congenic mouse strains, or from testosterone treatment of female animals, suggests that the male hormone may contribute to *P. chabaudi* AS malaria

susceptibility in mice (24, 25). The most susceptible animals were males, testosterone-treated females, and testosterone-treated castrated males. Hormonal factors have also been shown to affect host resistance to other protozoan parasite infections. For example, the outcome of *Nematospiroides dubins* is dependent on the gender of the host (26).

T cell heterogeneity and role in resistance to malaria

Initial evidence implicating T cells in immunity to malaria came from experiments conducted in thymectomized animals, which were shown to be more susceptible to *P. berghei* or *P. yoelii* than intact controls (27). Since these initial observations, much has been learned about the specific roles of various subtypes of T cells, including α/β TCR CD4+ T helper and CD8+ cytotoxic T cells, and $\gamma\delta$ TCR T cells, in host defense against blood-stage malaria.

CD4+ T helper cells

It is currently well established that α/β TCR CD4+ T helper cells are essential for the development of protective immunity against blood-stage malaria (28, 29). Mice with a significantly reduced function of the CD4+ T cell compartment, as in athymic nude mice, thymectomized mice, or in animals CD4+ T cell-depleted by mAb treatment, were unable to clear and resolve infection with malaria (28, 30, 31). It is also well recognized that CD4+ T helper cells play a crucial role, not only in the generation of effective cell mediated immunity, but also, in enhancing an effective humoral response mediated by B cells against malaria. Our understanding of the role of CD4+ T helper cells in host defense against malaria and other infectious diseases

has been greatly advanced by the discovery that activated CD4+ T helper cells can be subdivided on the basis of patterns of cytokine secretion.

Using techniques for the in vitro propagation of antigen specific T cell clones, two distinct subsets of CD4+ T helper cells have been described. Activated T helper type 1 (Th1) cells secrete predominantly IL-2, IFN-γ and TNF-β, cytokines involved in macrophage activation for phagocytic activity and cytokine secretion. In addition, Th1 cells mediate delayed type hypersensitivity (DTH), and provide B cell help for the production of IgG2a antibodies. On the other hand, activated T helper type 2 (Th2) cells secrete mainly IL-4, IL-5, IL-6 and IL-10, and provide B cell help for IgG1 and IgE synthesis (32-34). Although initially described as a Th2 product, current evidence suggests that IL-10 may also be secreted by Th1 cells and by macrophages (35). Both Th1 and Th2 cells appear to originate from a common precursor cell designated Th0. As expected, Th0 cells have a mixed Th1/Th2 cytokine secretion, with IL-2 as the predominant cytokine secreted. An interesting observation is that the development of Th1 or Th2 cells appears to be modulated by the same cytokines secreted by these cells. IFN-γ secreted by Th1 cells promotes Th1 cell development, in cooperation with IL-12, a potent Th1-inducing cytokine secreted predominantly by macrophages. In addition, IFN-γ inhibits Th2 cell development. In contrast, IL-4 promotes Th2 cell development, but inhibits Th1 cell development, suggesting cross-regulatory mechanisms for Th1 vs. Th2 cell development (36).

Which type of Th response is predominantly evoked during a parasite infection can be an important determinant of the balance between host resistance and susceptibility. The first clear evidence of the in vivo relevance of this Th1/Th2 dichotomy in relation to an infectious disease was reported in murine leishmaniasis (37). This disease, characterized by cutaneous and visceral lesions, is caused by infection with *Leishmania major*. A strong Th1 response was shown to correlate with

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spontaneous healing of the cutaneous lesions, whereas a strong Th2 response led to disseminated visceral disease (38-41). The situation is reversed in certain murine worm infections. For example, a strong Th2 response was protective in mice infected with the nematode *Heligmosomoides polygurus* (42).

Unlike other infectious diseases, a unique feature of protective immunity against malaria is that neither a Th1 nor Th2 response alone is sufficient for an effective host defense. Evidence from rodent models suggests that the sequential activation of a strong Th1 response, followed by an equally robust Th2 response is required for protective immunity against malaria (43). Using limiting dilution analysis of splenic lymphocytes from *P. chabaudi chabaudi*-infected mice, Langhorne and co-workers (44) showed that during the early, acute phase of infection, activated CD4+ T cells were mainly of the Th1 type, while later on in the infection, activated CD4+ T cells were predominantly of the Th2 type.

These findings were later corroborated by Taylor-Robinson and Phillips (45) who showed that *P. chabaudi*-specific CD4+ T helper cell clones derived from spleens of infected mice during acute infection were of the Th1 type, whereas clones derived from reinfected mice were Th2. These investigators further showed that adoptive transfer of *P. chabaudi*-specific Th1 or Th2 cell clones into naive mice CD4 depleted by thymectomy and treated with rat monoclonal antibody specific for mouse CD4 protected these mice against *P. chabaudi* infection (11, 46). Moreover, Th1 clones protected by nitric oxide (NO)-dependent mechanisms, whereas Th2 clones protected via IgG1-dependent mechanisms.

In addition, our laboratory has demonstrated that during acute blood-stage *P. chabaudi* AS malaria, resistant B6 mice mount Th1 responses, whereas early, predominantly Th2 responses are associated with susceptibility in A/J hosts (43). As

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described later in this chapter, administration of the potent Th1-inducing cytokine, IL-12, during the first five days of infection was found to rescue 75% of susceptible A/J mice from a normally lethal course of infection with blood-stage *P. chabaudi* AS malaria (16). These results raised the hypothesis that IL-12 production might be deficient in susceptible A/J compared to their resistant B6 counterparts during the first few days of *P. chabaudi* AS infection. This hypothesis is addressed in **Chapter 2** of this thesis, wherein the analysis of in vivo IL-12 production in *P. chabaudi* AS infected B6 and A/J mice during early blood-stage malaria is the main focus.

CD8+ cytotoxic and γδ T cells

Whereas ample evidence exists in support of the critical role of CD4+ T helper cells in host defense against blood-stage malaria, the roles of CD8+ cytotoxic and γδ T cells are less clearly defined. Using adoptive transfer experiments, Vinetz and co-workers (47) reported that CD4+, but not CD8+, T cells conferred protection against blood-stage *P. yoelii*. A protective role of CD8+ T cells in *P. chabaudi adami* infected mice has been suggested for the resolution of the later stages of infection (48). Depletion of CD8+ T cells by mAb treatment had no effect on the course of primary parasitemia or on primary peak parasitemia in B6 mice infected with *P. chabaudi* AS (31). However, CD8+ T-cell depletion in these mice led to two recrudescent parasitemias and required greater than five weeks to resolve the infection, compared to four weeks in control animals, suggesting a possible role of CD8+ cytotoxic T cells in parasite resolution during the late stages of blood-stage malaria. In contrast, recent experiments conducted in β2-microglobulin gene knockout mice that express no functional CD8+ T cells suggest that these cells may not be required for protection

against either blood-stage or liver stage malaria. Wild type and knockout animals similarly resolved infections when tested with *P. yoelii 17X, P. chabaudi* AS, or *P. chabaudi adami* (49). As with all knockout animals, one cannot rule out the possibility of compensatory mechanisms.

Studies by other investigators suggest that CD8+ cytotoxic T cells play a role in the resolution of liver stage malaria. When mice were immunized with a crude antigen preparation from *P. falciparum* and then infected with *P. yoelii*, the majority of *P. falciparum* reactive T cells that developed in these animals were of the CD8+ phenotype (50). Moreover, adoptive transfer of a CD8+ cytotoxic T-cell clone that recognizes the sporozoite surface protein 2 protected one hundred percent of *P. yoelii* infected mice against liver stage malaria (51).

It is presently unclear what the exact role γδ T cells might be in host defense against malaria. Mice genetically deficient in γδ T cells could control and reduce a primary infection of *P. chabaudi chabaudi* AS, with a slight delay in the time of clearance of the acute phase of infection, and significantly higher recrudescent parasitemias compared with control intact mice. CD4- CD8- γδ T cells expand in the spleens of mice during non-lethal blood-stage malaria (52). In acutely infected *P. falciparum* malaria patients, marked increases in circulating γδ T cells were observed (53). This increase was both in the relative proportions, as well as the absolute numbers of γδ T cells. Resolution or pathogenesis of malaria may be mediated by cytokines secreted by γδ T cells. Evidence for the capacity of γδ T cells to secrete cytokines was demonstrated in a murine model of influenza pneumonia, where activated γδ T cells were capable of producing IL-2, IFN-γ, TNF-β, GM-CSF, and IL-10 (54).

Role of Th1 (IFN-γ and IL-2) vs. Th2 (IL-10 and IL-4) cytokines

As mentioned previously, both Th1 and Th2 responses contribute to protective immunity against malaria. The effector functions of activated T cells is largely mediated by key cytokines released by these cells following cognate interactions with costimulatory signals and parasite antigens expressed by antigen presenting cells (APCs) in the context of MHC molecules. Studies in humans and animal models of malaria suggest important and critical roles for IFN-γ and IL-2 on the one hand, and IL-10 and IL-4 on the other, in modulating complete recovery of the host from blood-stage infection.

Activated Th1 cells are an important source of IL-2 and IFN-γ. Studies in mice infected with blood-stage *P. chabaudi* AS suggest that NK cells may also contribute significantly to IFN-γ production (55). In response to *P. falciparum* antigens, T cells isolated from adult Gambian patients infected with malaria produced IL-2 and IFN-γ in vitro, suggesting specific priming and activation of these cells in vivo by the on-going infection (56). Moreover, patients acutely infected with *P. falciparum* had detectable serum IFN-γ, in contrast to sera obtained from uninfected patients (57). In addition, IL-2 secretion by Con A-stimulated spleen cells was significantly elevated by day 1 post infection in mice infected with lethal (17XL) and non-lethal *P. yoelii* strain (17XNL), or with *P. berghei* (58). Serum IL-2 levels in these malaria-infected mice were also elevated during the early stages of infection, followed by a decrease that correlated with an increase in a serum IL-2 inhibitor (59). Molecular studies later revealed the identity of the serum IL-2 inhibitor as the IL-2 receptor (60). Taken together, these studies suggested the importance of IL-2 and IFN-γ in the immune response to malaria, but did not address whether their roles were pathological or protective.

Evidence for a role in protective immunity against malaria has been demonstrated for IFN–γ. Treatment in vivo with recombinant IFN–γ was protective in mice infected with *P. chabaudi adami*, *P. vinkei*, and lethal *P. yoelii* strain 17XL (61, 62). In *P. chabaudi* AS infected mice, in vivo treatment with monoclonal antibodies against IFN–γ led to a significant increase in primary peak parasitemia (63, 64). In addition, 77% of IFN-γR deficient mice succumbed to *P. chabaudi chabaudi* infection compared to 100% survival in wild type mice (65).

Although an inflammatory response seems beneficial to the host for controlling acute malaria infection, this protection often comes at a price: the risk of inflammation-induced pathology (37). Overproduction of IFN–γ may lead to a vigorous and uncontrolled inflammatory response resulting in host tissue damage, possibly mediated by reactive oxygen species derived from activated monocyte/macrophages and neutrophils. Moreover, proinflammatory cytokines, most notably TNF–α, are potent endogenous pyrogens and their release at excessive levels could contribute to the severe fever and chills that is often the resounding complaint of malaria patients. As such, IFN–γ overproduction has been implicated in the development of cerebral malaria, likely involving the up-regulation of TNF receptors (66), and in mortality resulting from infection with a virulent strain of *P. berghei* (67). Both TNF–α and IFN–γ may play roles in the evolution of dyserythropoeitic anemia (2).

Unlike IFN–γ, IL-10 is an anti-inflammatory cytokine that can inhibit monocyte/macrophage activation and cytokine release. Therefore, IL-10 may be required in modulating the early Th1-associated inflammatory response mounted by the host at an optimal level, thus avoiding the pathological consequences of excessive inflammatory responses. In female, but not male, mice genetically deficient in IL-10, *P. chabaudi* infection resulted in increased Th1 responses and 56% mortality,

compared with 100% survival in wild type controls (68). It has also been proposed that IL-10 may be the essential factor that helps in the eventual switch from a Th1 to Th2 response required for sterile protection against murine blood-stage malaria (69). Exogenous IL-10 was found to inhibit in vitro production of TNF–α, IL-1β and IL-6 by peripheral blood mononuclear cells (PBMC) from patients acutely infected with *P. falciparum* (70). Hence, deficient IL-10 levels could result in excessive secretion of proinflammatory cytokines like TNF-α. This is exactly what was found in Ghanaian children suffering from *P. falciparum* malaria (71). IL-10 concentrations were significantly lower, whereas TNF–α levels were significantly higher in children with complicated versus uncomplicated *P. falciparum* malaria.

In contrast to IL-10, gene-targetted IL-4 knockout mice were able to control and clear a primary *P. chabaudi* AS infection no differently from control mice (72). However, recrudescent parasitemias were significantly higher in knockout compared to wild type animals, suggesting a role for IL-4 in complete parasite clearance. Surprisingly, Th2 responses, for example IgE production, were somewhat delayed but otherwise not significantly impaired in IL-4 knockout mice. This finding may reflect the redundancy of function of Th2 type cytokines. IL-13 shares many biological activities with IL-4, and may be compensating for many of the actions of IL-4 in IL-4-deficient animals (73).

Role of B cells and antibodies

Both humoral and cell-mediated immunity contribute to host protective mechanisms against malaria (28, 48). In Thai patients with *P. falciparum* malaria, intravenous injection of pooled IgG from hyperimmune Africans led to faster clearance of asexual parasitemias and reduction in malaria symptoms, suggesting an

important protective role of malaria-specific antibodies. No sterile immunity could be achieved in these IgG-treated patients, as indicated by parasite recrudescence later on after the transferred antibodies had disappeared (74).

Mice depleted of B cells by anti-μ antibody treatment since birth were able to control acute *P. chabaudi* AS infection, but could not reduce parasitemia to subpatent levels (69). Persistent parasitemia was detectable in B-cell depleted mice for up to 75 days. In contrast, control mice completely cleared the infection by day 28 post infection. Moreover, B cell deficient mice did not mount a significant Th2 response during the later stages of infection, as indicated by the failure of splenic CD4+ T cells to produce significant levels of IL-4 and IL-10. Over a 50-day observation period, there was a sustained production of IFN-γ and IL-2 by splenic CD4+ T cells recovered from B-cell deficient mice, suggesting failure to switch from a Th1 to Th2 response was accompanied by an inability to eliminate the parasite. Additional studies in B-cell knockout mice have corroborated these initial results (75). B-cell knockout mice were capable of controlling an acute *P. chabaudi* AS infection and reducing the initial peak parasitemia to low levels. After this initial period, a chronic and persistent infection ensued in B-cell knockout mice, and was unresolved for up to 120 days post infection (75). Taken together, these studies demonstrate that B cells play a critical role in parasite elimination during the later stages of erythrocytic-stage *P. chabaudi* AS malaria. B cells may be important, not only as sources of opsonizing antibodies, but also, in cognate interactions with CD4+ T cells that help in the Th1 to Th2 switch in response to the malaria parasite.

Role of phagocytic cells and their secreted products

It has been observed that, at least in vitro, both macrophages and neutrophils have phagocytic activity against malaria-infected erythrocytes that may be enhanced by IFN-γ (76). When activated, both cell types may be a source of H_2O_2 and other reactive oxygen and nitrogen species. In vitro, intraerythrocytic stages of both human and murine malaria were found to be susceptible to destruction by H_2O_2 (77-79). Activated monocyte/macrophages release a number of proinflammatory cytokines including IL-1, TNF-α, and IL-6, molecules that may be beneficial or detrimental to host protective immune responses (80). Moreover, monocyte/macrophages secrete the non-cytokine, but important, immunomodulatory molecule, nitric oxide (NO). The roles of TNF-α and NO in host immunity against malaria have been the subject of intense investigation by various laboratories, including ours, during the past decade (81-83).

TNF-α was initially identified as a serum factor in *M. bovis* (strain BCG) infected mice treated with LPS (84), and as cachectin, owing to its ability to induce severe wasting in trypanosome-infected rabbits (85). TNF-α and cachetin were found to be identical following molecular cloning and sequencing (86). At low concentrations, TNF-α was associated with inhibition of *P. falciparum* parasite growth by mechanisms involving lipid peroxidation and production of prostaglandin derivatives (87). Recently, using Northern blot analysis, Jacobs and colleagues in our laboratory (81) demonstrated that an early Th1-associated increase in splenic mRNA expression of TNF-α correlates with resistance to blood-stage *P. chabaudi* AS in C57BL/6 mice.

On the other hand, excessive or inappropriate production of TNF-α may be of disastrous consequences to the host and may, in fact, reflect difficulties in controlling parasite growth (88). Children with cerebral malaria had significantly higher

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circulating TNF–α than those with uncomplicated *P. falciparum* infection (89). The role of TNF–α in the pathogenesis of cerebral malaria may be related to its ability to up-regulate cell adhesion molecule expression on endothelial cells. Modification of the cyto-adherence properties of endothelial cells of cerebral blood vessels has been implicated in rosette formation and the development of cerebral anoxia (90). TNF–α also contributes to pyrexia, hypoglycemia, and dyserythropoietic responses (90).

In response to parasitic infections, NO is synthesized by activated monocyte/macrophages from the amino acid precursor L-arginine, under catalysis of the enzyme inducible nitric oxide synthase (iNOS) (91-93). IFN–γ is a potent activator of macrophage phagocytic activity and release of reactive oxygen and nitrogen intermediates (79). Activated Th1 cells may also be an important source of NO secretion (94). Trace amounts of NO, catalyzed by constitutive nitric oxide synthase (cNOS), are also released under normal physiological conditions by endothelial and neuronal cells, where NO plays an important role as a vasodilator and neurotransmitter, respectively (95). In vitro, reactive nitrogen intermediates mediate killing of erythrocytic stage *P. falciparum* growth by monocytes (96). Reactive nitrogen intermediates also mediated killing of cultured *P. falciparum* gametocytes by white blood cells (97). In addition, L-arginine-dependent mechanisms have been implicated in the destruction of hepatic stage *P. yoelii* and *P. berghei* parasites (98, 99). Taken together, these studies suggested that, at least in vitro, NO might be an important mediator of parasite killing by activated host effector cells.

Aminoguanidine is a selective inhibitor of iNOS (100) and, thus, has been used to investigate the role of iNOS-induced NO production in vivo against malaria. Treatment of resistant B6 mice with a course of aminoguanidine significantly increased mortality in these mice following infection with blood-stage *P. chabaudi* AS (82). Interestingly, resistance in C57BL mice was abolished by aminoguanidine

treatment without any significant effect on the course of parasitemia, suggesting that in vivo, NO may not be involved in parasite destruction. It has been suggested that NO may protect host cells via its actions as a scavenger of reactive oxygen intermediates (101). During acute blood-stage *P. chabaudi AS* malaria, splenic adherent macrophages recovered from resistant B6, compared with susceptible A/J, mice released significantly higher levels of NO in vitro following LPS stimulation (82).

INTERLEUKIN-12 (IL-12)

Discovery and Characterization

The discovery of IL-12 was foreshadowed by a report in 1989 by Nakamura and co-workers (102) of an unidentified endotoxin-induced serum factor, purified as a 70 kDa molecule, that induced IFN-γ production by macrophage-depleted splenocytes. In the same year, IL-12, designated as NK cell stimulatory factor (NKSF), was purified by Kobayashi and co-workers (103), as a 70-75 kDa glycoprotein, from supernatants of a phorbol-12,13-dibutyrate (PDBu) -induced, Epstein-Barr virus (EBV)-transformed lymphoblastoid B cell line, RPMI 8866. The purified molecule had potent actions on NK and T cells, in particular, the induction of IFN-γ production, NK cell-mediated cytotoxicity, and enhanced T cell responses to mitogens. IL-12 was later purified from supernatants of an EBV-transformed B cell line (NC37) and described as cytotoxic lymphocyte maturation factor (CLMF), based on its synergistic actions with IL-2 in the generation of lymphokine-activated killer (LAK) cells (104).

Molecular cloning of IL-12 genes and characterization of the recombinant protein finally revealed that NKSF and CLMF are indeed the same molecule, and

hence, the name IL-12 was adopted (105-107). To clone human IL-12 genes, cDNA was synthesized from cellular RNA isolated from PDBu-induced RPMI 8866, the EBV-transformed human B cell line, ligated to EcoRI adaptors and then to lamda zap phage vectors, and subcloned into mammalian plasmid expression vectors. Cos cells were transfected with these vectors in order to express recombinant IL-12 proteins. Oligonucleotide probes were synthesized from tryptic peptides prepared from purified NKSF. These probes were subsequently used to screen a library of recombinant phage clones. A number of positive clones were selected and sequenced in order to obtain the full-length cDNA of IL-12 genes. By using human IL-12 probes to screen a mouse genomic cDNA library under low stringency conditions, murine IL-12 genes were also cloned (107, 108). IL-12 is encoded by two genes, p40 and p35, which are localized on separate chromosomes, as revealed by fluorescence in situ hybridization (FISH) studies. In humans, p40 and p35 are found on chromosomes 5 and 3 respectively, and on chromosomes 11 and 6 respectively, in the mouse (108).

The promoter regions of both the murine and human IL-12 p40 genes contain elements such as a TATA box, NF-kB and NF-IL6 consensus sequences (108). Nonetheless, there are notable differences such as the presence of the regulatory elements *ets* and AP-1 consensus sequences in the human, but not the murine, IL-12 p40 gene promoter. These differences might reflect differences in the regulation of the murine and human IL-12 genes. In addition to NF-kB and NF-IL6 consensus sequences, the promoter region of the murine p35 gene also contains gamma activation sequence motifs (GAS sequences). GAS sequences were originally thought to be a specific target of IFN-γ. However, it is currently believed that these sequences are not a unique target of IFN-γ. In fact, multiple cytokines and growth factors exert their effects on target cells by a mechanism involving GAS motifs.

IL-12 possesses a unique disulphide-linked heterodimeric structure composed of two subunits, 40 (p40) and 35 (p35) kDa encoded, respectively, by the p40 and p35 genes (107). Together, the two subunits make up a 70-75 (p70) kDa molecule that is the biologically active form of IL-12. Murine IL-12 is biologically active against human target cells, but the human IL-12 is inactive against murine target cells (107). Amino acid homology analysis indicates that the p40 subunit is more conserved than the p35 subunit (70% versus 60%) between murine and human IL-12 (107). Therefore, the inability of human IL-12 to be active against murine target cells may be a reflection of evolutionary divergence in the p35 subunit. Several amino acid sequence homologies are shared among IL-12 p35, IL-6 and GM-CSF (109). Moreover, the amino acid sequence of p40 bears homology to those of the extracellular portion of ciliary neurotrophic factor receptor alpha (CNTF-Rα), G-CSF-R, and IL-6R (110).

Cellular Sources and Regulation of IL-12 Secretion

Production of biologically active IL-12 p70 requires that both p40 and p35 genes be expressed in the same cell type (106). The p35 gene appears to be ubiquitously expressed in cells of both lymphoid and non-lymphoid origins, whereas expression of the p40 gene tends to be restricted to cells of lympho-hematopoeitic lineage (105). By Northern blot analysis, using full-length murine p40 and p35 cDNA as probes, p35 mRNA was detectable in cells or tissues from lung, brain and spleen. In contrast, p40 mRNA was detectable in cells or tissues from the spleen and thymus, but not the kidney, brain, liver or lung (105). It was previously thought that p40 mRNA expression is highly up-regulated following activation of IL-12 producing cells, whereas p35 mRNA expression is constitutive (108). However, there is

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evidence to suggest that the induction of mRNA expression of both genes can be up-regulated, although compared to p35, the p40 gene appears to be much more highly regulated (111, 112). Secretion of the free p40 heavy chain vs. secretion of IL-12 heterodimer seems to be differentially regulated, and the p40 chain is often produced in large (10-100-fold) excess compared to the biologically active heterodimer. Under these conditions, free p40 heavy chains can dimerize to form p40-p40 homodimers, which, at least in vitro, and following systemic treatment of mice with recombinant p40 homodimers, have been demonstrated to be capable of antagonizing the biological actions of IL-12 p70 (113). At the present time, however, the role of p40 homodimers as a natural antagonist of IL-12 activity under physiological conditions is unclear (114, 115).

IL-12 synthesis has been described in various cell types, including neutrophils (116, 117), dendritic cells (118), and microglia (119). Monocyte/macrophages are generally regarded, however, as the major physiological producers of IL-12 (120). The analysis of IL-12 production in vitro by splenic macrophages recovered from resistant B6 and susceptible A/J mice acutely infected with *P. chabaudi* AS malaria is the main subject of **Chapter 3**. Although IL-12 was originally isolated from an EBV-transformed human B cell line, it is presently unclear whether or not normal B cells constitute an endogenous source of IL-12 (121). Induction of IL-12 mRNA expression and protein production occurs in IL-12 producing cells following infection or stimulation with numerous parasites, bacteria and other microbial products (122-126). Cytokines play an important role as modulators of IL-12 production. IFN-γ can synergize with microbial products for the induction of IL-12 p40 and p70 release and may also play an important role in modulating the quantities of p40 vs. p70 released by monocyte/macrophages (127-129). IL-10 is a potent inhibitor of IL-12 production by human peripheral blood mononuclear cells and mouse peritoneal

exudate cells (111, 128). Other negative modulators of IL-12 production include IL-4, IL-13 and TGF-β (128). The regulation of monocyte IL-12 production by IL-4 and IL-13 is complex, however. Addition of IL-4 or IL-13 to PBMC cultures inhibited production of IL-12, TNF-α, IL-10, and IL-1β induced by LPS or *Staphylococcus aureus* added simultaneously with the cytokines. However, pre-treatment of PBMC with IL-4 or IL-13 for greater than 20 h enhanced LPS or *Staphyloccus*-induced IL-12 production by several fold (130).

IL-12 Receptor (IL-12R) and Signal Transduction

Initial studies of IL-12R were based on flow cytometric analysis of IL-12 binding to specific IL-12 receptors on various cell types. The results of these studies indicated that activated NK, CD4+ and CD8+ T cells were the major cell types that express high affinity IL-12R (131, 132). Initial equilibrium binding studies, using radiolabelled human IL-12 (^{125}I-huIL-12), suggested that activated human T cells express a single affinity class of IL-12R (133). However, further detailed studies, using ^{125}I-huIL-12 of lower radio-specific activity and analysis of equilibrium binding data by the method of Scatchard revealed three affinity classes of IL-12 binding sites on activated human T and NK cells. Based on the apparent dissociation constants of 5-20 and 50-200 pM, and 2-6 nM, these affinity classes were, respectively, designated low, medium and high (134). Similarly, three affinity classes of IL-12 binding sites were identified on Con A-activated mouse splenocytes using ^{125}I-mouse IL-12 (^{125}I-mo IL-12) (134, 135).

Using a non-neutralizing anti-IL-12R monoclonal antibody (2-4E6) that is capable of precipitating the complex of IL-12R/^{125}I-huIL-12 from cell lysates, a component of human IL-12R, later designated IL-12R β1, was first cloned by

screening a library of COS cells transfected with PHA-activated human lymphoblast cDNA (136). The isolated cDNA of the human IL-12R β1 subunit encoded a type I transmembrane protein 662 amino acids long, consisting of a cytoplasmic region of 91 amino acids, a transmembrane region of 30 amino acids and an extracellular portion of 516 amino acids. The protein belonged to the cytokine receptor superfamily, bearing strong structural homology to gp 130 (136). The cDNA encoding the mouse homologue of IL-12R β1 was isolated from Con A-activated lymphoblast cDNA by cross-hybridization (137). RT-PCR analysis of mRNA transcripts in COS cells transfected with IL-12R β1 cDNA revealed that, in mouse but not human cells, there exists an alternative form of mRNA transcript in which the transmembrane region has been deleted. However, this alternative form of IL-12R β1 was not secreted, suggesting the use of alternative anchor sites. Both forms of mouse IL-12R β1 were expressed at the surface of transfected COS cells.

The cloned cDNAs for both mouse and human IL-12R β1 encoded for only IL-12 binding sites of low affinity on transfected COS cells. This inability to reconstitute high affinity IL-12 binding sites predicted the existence of other components of the IL-12R complex (135). Indeed, cDNAs for an additional component of both human and mouse IL-12R complex were successfully cloned and designated IL-12R β2 (138). When expressed in COS cells, cDNAs for IL-12R β2 encoded a β-type subunit responsible for the low affinity class of IL-12 binding sites. Coexpression of cDNAs for both IL-12R β1 and β2 in a single COS cell, however, lead to the formation of both high affinity and low affinity binding sites.

Both IL-12R β subunits belong to the hematopoietin cytokine receptor superfamily (135). Members of this receptor superfamily are single-pass transmembrane glycoproteins that possess a number of sequence and structural motifs in their extracellular regions, in particular, four conserved cysteine residues and a

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WSXSW motif. The receptors for growth hormone (GH), prolactin (PRL) and IL-2 all belong to the hematopoietin receptor family. The cytoplasmic domain of these receptors is devoid of intrinsic tyrosine kinase activity. Therefore, functional activity of these cytokines requires coupling to cytoplasmic protein tyrosine kinases of the Janus family (JAKs). There are four known JAK kinases, namely JAK1, JAK2 and TYK2, which are ubiquitously expressed, and JAK3, which is expressed predominantly in activated lymphoid and myeloid cells (139). In addition to JAKs, signal transduction and the induction of gene expression by hematopoietin receptor is believed to involve the activation of a family of transcription factors designated STATs, or signal transducers and activators of transcription. IL-12 utilizes JAK2 and TYK2, whereas IL-2 utilizes JAK1 and JAK3 (139). STAT4 appears to be uniquely activated by IL-12, although evidence suggests that IL-12 can also activate STAT1 and STAT3 (140).

Biological Actions of IL-12

Effects on cytokine production

IL-12 induces IFN-γ production, in a dose-dependent fashion, by resting and activated NK and T cells (103). Both CD4+ and CD8+ T cells are induced by IL-12 to produce IFN-γ (141). IL-12 also strongly synergizes with other stimuli, such as IL-2 and phorbol diesters, to induce IFN-γ synthesis by NK and T cells. These effects of IL-12 on IFN-γ production appear to be largely independent of IL-2 since mAb treatment against IL-2 only partially block the IL-12 induced IFN-γ production (141). The mechanism of synergy between IL-12 and IL-2 on IFN-γ production appears to be post-translational and involves a greater that 2-fold increase in the half-life of IFN-γ mRNA in cells treated with the combination of IL-12 and IL-2 compared to

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cells treated with either cytokine alone (142). IL-12 is capable of inducing the production of other cytokines by NK and T cells, including GM-CSF, TNF–α, and IL-8 (143).

Treatment of normal B6 mice with murine rIL-12 resulted in increased serum levels of IFN–γ (144). Moreover, liver lymphoid cells and splenocytes from these IL-12-treated mice were found capable of spontaneously secreting IFN–γ in vitro in the absence of any added stimulant. In contrast, cells from vehicle-treated mice secreted no IFN–γ. Other cytokines can function as co-factors for IL-12-induced IFN–γ synthesis. For example, TNF–α alone did not induce IFN–γ synthesis, but when added to IL-12, TNF–α was found to be an important co-factor for IL-12-induced IFN–γ production by NK cells from mice with severe combined immunodeficiency (SCID) (145, 146). In addition, production of IFN–γ by SCID mouse splenocytes stimulated with the combination of IL-12 and TNF–α was completely ablated by simultaneous treatment with mAbs against IL-1β, but not IL-1α, suggesting an important role of IL-1β in IL-12-induced IFN–γ production by NK cells in this model (147). In contrast, IL-10 was a potent inhibitor of IL-12-induced IFN–γ production by NK cells (111).

Effects on cellular cytotoxicity

IL-12 treatment enhances the cytotoxic activity of peripheral blood lymphocytes (PBL) and resting or activated NK cells against a variety of target cells, including various tumors and virus-infected cells (143). IL-12 is particularly potent in its actions on cellular cytotoxicity, since IL-12 concentrations as low as 0.4 pM effectively enhanced NK cell cytotoxic activity (103). Treatment of normal B6 mice with a single dose of rIL-12 (1 μg i.p.) was shown to enhance cytotoxic lymphocyte

(CTL) and NK cell lytic responses in the spleens and livers of IL-12 treated compared to vehicle-treated controls (144).

Role in the induction of Th1 responses

IL-12 induces the development of Th1 cells from undifferentiated Th0 precursors (148-150). In contrast, IL-4 promotes Th0 cell development towards a Th2 phenotype (36). In an accessory cell-free system in vitro, naïve CD4+T cells were cultured in the presence of anti-CD3 mAbs and IL-12, IL-4 or both (151, 152). Cells were restimulated after 6 days with anti-CD3 mAbs alone. IL-12 treatment directly induced IFN–γ production by CD4+ T cells. In addition, following anti-CD3 restimulation cells previously cultured in the presence of IL-12 and anti-CD3 produced high levels of IFN–γ and minimal levels of IL-4. In contrast, cells previously cultured with IL-4 and anti-CD3 produced substantial quantities of IL-4 and low levels of IFN–γ. When comparable levels of IL-12 and IL-4 were present during the primary stimulation, predominantly IL-4 but minimal IFN–γ production was observed upon secondary restimulation with anti-CD3. These results suggested that when both cytokines were present, the effects of IL-4 on Th2 cell development were largely dominant over IL-12-induced Th1 cell development. It appears, however, that once Th1 cell development is established by exposure to IL-12, IL-4 is ineffective in inducing Th2 cell development (36).

IFN–γ acts as a co-stimulus with IL-12 in favoring Th1 cell development (153). IFN–γ may also contribute to Th1 cell development by providing inhibitory signals for Th2 cell development. However, IFN–γ by itself does not induce Th1 cell development (150, 154). Results of several studies in normal, parasite or viral-infected animals have largely confirmed the capacity of rIL-12 treatment to induce

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strong Th1 responses in vivo. Evidence from B6 mice genetically deficient in both the p40 and p35 genes for IL-12 have also demonstrated the crucial role of IL-12 in the development of Th1 cell responses (155, 156). Compared to wild-type controls, IL-12 deficient mice had substantially impaired Th1 responses, evident by reduced levels of LPS-induced serum IFN–γ levels in vivo and keyhole limpet hemocyanin (KLH)-induced IFN–γ synthesis in vitro by lymph node cells from mice previously immunized with KLH in complete Freund's adjuvant (CFA). In contrast, KLH-induced secretion of IL-4 by lymph node cells was enhanced in IL-12 deficient mice. DTH responses were also significantly reduced in IL-12 deficient compared to wild type controls (155).

Effects on erythropoiesis

IL-12 synergizes with the combination of steel factor and IL-3 to induce formation of mixed erythroid and myeloid colonies (157-159). In the presence of NK cells, IL-12 indirectly inhibits formation of erythropoietic colonies by IL-3 and GM-CSF through IL-12-induced IFN–γ and TNF–α. Therefore, IL-12 exerts dual enhancing and indirect inhibiting actions on erythropoiesis. IL-12 treatment of normal B6 mice resulted in massive splenomegaly that was attributed largely to the induction of extramedullary hematopoiesis rather than proliferation of mature lymphoid cells. In contrast to the spleen, erythropoietic activity in the bone marrow was suppressed by IL-12 treatment (144).

Effects on cell proliferation

IL-12 enhances proliferation of Con A-activated mouse lymphoblasts or human PHA-activated lymphoblasts (107). IL-12 induces proliferation of human

PHA-activated lymphoblasts of both CD4+ and CD8+ subsets. Unlike IL-2, however, IL-12 causes little or no proliferation of resting T or NK cells (160). IL-12 enhances proliferation of mouse Th1, but not Th0 or Th2, clones in response to IL-2, IL-7 or IL-15 (161). It has been demonstrated that loss of responsiveness of Th2 cells to IL-12-induced proliferation is due to the inability to phosphorylate, rather than the absence of, STAT4 in response to IL-12 stimulation (162). The inability to phosphorylate STAT4 in response to IL-12 stimulation may be related to the fact that Th2 cells appear to lose mRNA expression for the IL-12R β2, which is required together with IL-12R β1 high affinity interaction between IL-12 and its receptor (163, 164). Results of IL-12R β1 and β2 mRNA expression by splenocytes under various experimental conditions are presented in **Chapters 2, 4 and 6** of this thesis.

Other biological activities

IL-12 has important actions on antibody isotype and subclass synthesis. Treatment of CBA/J mice with IL-12 in combination with protein antigens adsorbed to alum strongly enhanced synthesis of antigen-specific and complement-fixing IgG2a, 2b, and IgG3 subclasses, whereas IgE synthesis was suppressed (165). These effects of IL-12 appeared to be partially IFN–γ independent. IL-12 also regulates the expression of cell surface molecules. The expression of CD2, CD11a, CD54, and CD56 on NK cells is up-regulated by IL-12 (166). The expression of a number of cytokine receptors, including those for IL-12, IL-2 (CD25), and the p75 TNFR, is also modulated by IL-12 (132, 167).

Shared and distinct biological activities with interferon-gamma-inducing factor

(IGIF)

IGIF was initially isolated as a 18.3 kDa factor from the livers of mice previously injected with heat-killed *Propionibacterium acnes* and subsequently challenged with LPS to induce shock (168). Structural analysis suggested that IGIF belongs to the IL-1 family of cytokines (169). Cloning and functional characterization of recombinant human and mouse IGIF revealed biological activities of IGIF distinct from the IL-1 family and suggested the designation of this novel cytokine as IL-18 (170, 171). IGIF is synthesized as an inactive precursor (pro-IGIF). Activation of pro-IGIF requires cleavage by the interleukin-1β converting enzyme (ICE) (172, 173). The major cellular sources of IGIF are Kuppfer cells, macrophage-like cells in the liver, and activated macrophages (170, 174, 175).

Recombinant IGIF is a potent inducer of IFN-γ production in established Th1 cells in the presence of anti-CD3 mAb. In the presence of anti-CD3 mAb, rIGIF was more potent than IL-12 for IFN-γ production by Th1 cells (170). Despite similar actions on IFN-γ production, there is evidence to suggest that IGIF and IL-12 induce IFN-γ production by anti-CD3-stimulated Th1 cells by independent mechanisms. Treatment with mAb against IL-12 does not inhibit IGIF-induced IFN-γ production. Conversely, addition of anti-IGIF mAb does not inhibit IFN-γ production by IL-12 (170). Like IL-1α and IL-1β, IGIF synergizes with IL-12 for IFN-γ induction by NK cells. Unlike IL-1α or 1β, but like IL-12, IGIF alone can induce IFN-γ production by NK cells (175). In addition, both IGIF and IL-12 induce NK cell cytolytic activity (171) and act as co-stimulatory factors for Con A or IL-2-induced proliferation of Th1, but not Th2, clones in the presence of antigen and anti-CD3 mAb (176).

Under certain conditions, both IGIF and IL-12 can contribute to the development of liver pathology. Administration of neutralizing anti-IGIF antiserum

prevented liver necrosis and bleeding in *P. acnes* treated BALB/c mice challenged with LPS (170). Similarly, administration of anti-IL-12 mAb blocked the development of liver injury in B6 mice treated with *P. acnes* and challenged with LPS (14).

There are a number of important distinctions between the biological actions of IGIF and IL-12. Unlike IL-12, IGIF alone does not drive Th1 cell development (177). Furthermore, only IL-12 activates STAT4 phosphorylation. IGIF signals through the IL-1R associated kinase (IRAK), and induces translocation of p65/p50 NFκB in Th1 cells (177).

Role of IL-12 in Disease

Polarization of Th responses towards a Th1 or Th2 phenotype correlates with resistance or susceptibility of inbred mouse strains to bacterial and protozoal parasite infections (37). The powerful actions of IL-12 on the synthesis of IFN–γ, a strong inducer of macrophage microbicidal functions, and on Th subset selection, suggested that modulation of IL-12 activity could have pivotal effects on the outcome of infectious diseases. Moreover, the actions of IL-12 in enhancing NK cell lytic activity and CTL responses suggested potential applications for immunotherapy against tumors. A number of key studies addressing the role of IL-12 in disease, including blood-stage malaria, are discussed below.

Allergic Diseases

Broncoalveolar lavage (BAL) cells from allergic asthmatics show a predominance of cells that are positive for Th2 cytokine production compared to control non-asthmatics (178). This predominance of Th2 cells in BAL is further

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augmented in symptomatic versus non-symptomatic asthmatics. In a murine model of antigen-induced allergic asthma, rIL-12 treatment, given at the time of intratracheal antigen challenge, abolished airway hyper-responsiveness and BAL eosinophilia (178). In addition, whole lung tissue mRNA levels for IL-4 and IL-5 were decreased whereas IFN–γ mRNA levels were increased in IL-12 treated compared to untreated mice. Moreover, changes in protein levels of these cytokines in BAL, determined by ELISA, correlated with those for total whole lung mRNA expression. Therefore, treatment with IL-12 not only corrected the asthmatic symptoms, but also induced predominantly Th1 responses in the lung (178).

Cancer Immunotherapy

A recombinant vaccinia virus containing the genes for p40 and p35 subunits of IL-12 was effective against subcutaneously implanted murine fibrosarcoma tumors (179). Mice were injected s.c. with 10^5 MCA 105 sarcoma cells, followed by injection at an adjacent site with saline, recombinant vaccinia virus containing p40 and p35 genes for IL-12, or an irrelevant *E. coli* gene as control. Whereas 100% of mice treated with saline or control recombinant virus developed tumors, 60% of the recombinant virus-IL-12 treated mice remained tumor free. In another study, BALB/c mice bearing established RENCA or CT26 tumors that were treated daily with IL-12 showed complete tumor regression. In contrast, tumors grew progressively in untreated controls (180). Human rIL-12 has been approved for phase I clinical trials in patients with advanced malignancies (181).

The role of IL-12 in murine cutaneous infection with *Leishmania major* is particularly well documented (38). Resistant B6 mice exhibit self-healing lesions, marked by preferential expansion of IFN–γ producing Th1 cells. In contrast, susceptible BALB/c mice develop progressive and fatal infections that are preferentially associated with IL-4 producing Th2 cells (40, 41). Daily treatment with rIL-12 during the first week of infection cured 89% of normally susceptible BALB/c hosts. Moreover, IL-12-induced protection was associated with increased IFN–γ and decreased IL-4 mRNA expression and protein production by draining lymph node cells from treated vs. untreated mice (40, 41). Recombinant IL-12-induced protection against *L. major* was IFN–γ dependent, since mAb treatment against IFN–γ abrogated the therapeutic effects of IL-12 and led to the development of Th2 responses.

Treatment with rIFN–γ alone could not cure BALB/c mice from leishmaniasis, indicating that rIL-12-induced protection was not solely dependent on IFN–γ (41). Studies of *L. major* infection in mice genetically deficient in IL-12 p40 and p35 genes confirm a critical role for IL-12 in host resistance to leishmaniasis (156). Wild type 129/Sv/Ev mice were resistant to *L. major* infection and developed small, spontaneously resolving lesions. In contrast, IL-12 knockout mice developed large and progressive lesions. Lymph node cells from infected wild type mice produced predominantly IFN–γ in response to stimulation with *Leishmania* antigen in vitro. In comparison, similar to susceptible BALB/c mice, cells from IL-12 deficient mice preferentially secreted IL-4 in vitro.

Toxoplasmosis

A role for IL-12 has also been implicated in host resistance against murine toxoplasmosis. Acute *Toxoplasma gondii* infection in mice resulted in increased expression of IL-12 p40 mRNA levels in both spleen and peritoneal cells (182). Treatment with mAb against IL-12 resulted in increased susceptibility of B6 or BALB/c mice to acute *Toxoplasma* infection (183). Moreover, this treatment resulted in decreased IFN-γ and enhanced IL-4 and IL-10 synthesis by splenocytes from acutely infected animals. In chronic *Toxoplasma* infections, anti-IL-12 mAb treatment had no effect (183, 184). Recombinant IL-12 treatment, initiated before day 3 post infection, significantly increased survival of SCID mice compared to untreated controls against *T. gondii* infection (183, 185). Furthermore, splenocytes from the IL-12 treated mice produced significantly higher levels of IL-2 and IFN-γ in vitro compared to cells from untreated controls.

Malaria

IL-12 has also been found to play a role in host resistance to malaria. Treatment with rIL-12 protected 100% of susceptible BALB/c mice against challenge with *P. yoelii* sporozoites, whereas control animals receiving normal mouse serum in PBS were not protected (186). Protection was defined as the absence of detectable parasites for up to two weeks following *P. yoelii* inoculation. In order to be protective, treatment (30 ng/day) had to be initiated 1 or 2 days prior to *P. yoelii* sporozoite infection and continued for five days. A single dose of 150 ng of rIL-12, given 2 days prior to *P. yoelii* sporozoite challenge, was also effective. Furthermore, simultaneous administration of IL-12 and mAb against IFN-γ completely eliminated the protective effects of IL-12 against *P. yoelii* sporozoite infection (186). Depletion

of CD4+ or CD8+ T cells by mAb treatment had no effect on IL-12-induced protection. In addition, rIL-12-induced protection against *P. yoelii* sporozoite challenge was reduced by 50% when N^G-monomethyl-L-arginine, an inhibitor of NO synthesis, was also given to the mice.

Similar to the results of IL-12 treatment on *P. yoelii* sporozoite infection in mice, human rIL-12 (10 µg/kg), given 2 days prior to *P. cynomolgi* sporozoite challenge, protected 100% of rhesus monkeys from developing malaria (187). In contrast, control animals receiving 1% normal monkey serum in PBS developed patent parasitemia on days 10-12 after sporozoite challenge. Compared to controls, monkeys receiving IL-12 treatment developed marked increases in plasma IFN-γ and mRNA levels for IFN-γ, TNF-α, and IL-6 in peripheral blood mononuclear cells (PBMC). Taken together, these studies demonstrated that prophylaxis with IL-12 could protect susceptible hosts against liver-stage malaria.

Previous studies from our laboratory have shown that, in blood-stage *P. chabaudi* AS malaria, susceptible A/J mice could also be protected by exogenous IL-12 treatment (16). Daily treatment of A/J mice with rIL-12 (0.1 µg/day), beginning on the day of infection and continuing for six days resulted in significant decreases in peak parasitemia and increased survival. Whereas 75% of IL-12 treated A/J mice recovered from blood-stage *P. chabaudi* AS infection, all untreated controls died by 10-12 days post infection. In addition, spleen cells from mice receiving IL-12 produced significantly greater quantities of malaria parasite-induced IFN-γ in vitro compared to cells from control animals. Serum levels of IFN-γ, TNF-α, and NO₃- were also significantly higher in IL-12 treated mice compared with their untreated counterparts. The studies presented in **Chapter 4** of this thesis focus on the immunotherapeutic potential of exogenous IL-12 combined with the anti-malarial drug chloroquine for curing established blood-stage malaria.

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To address the role of IFN-γ, TNF-α and NO in IL-12-induced protection against blood-stage malaria, *P. chabaudi* AS infected A/J mice receiving IL-12 were also given mAb against IFN-γ or TNF-α, or aminoguanidine, a selective inhibitor of iNOS (100). IL-12 induced protection against *P. chabaudi* AS infection could be completely abrogated by simultaneous depletion by mAb treatment of IFN-γ and TNF-α . Moreover, there was a significant increase in mortality, but not parasitemia, in mice given IL-12 and aminoguanidine. In contrast, treatment of resistant B6 mice with anti-IL-12 mAbs resulted in significantly higher peak parasitemia and a 3 day delay in the time required to clear *P. chabaudi* AS infection, but there was no effect on survival. The mechanism(s) of action of the protective network of immunomodulatory molecules involving IFN-γ, TNF-α and NO in IL-12-induced protection against blood-stage malaria is the main subject of **Chapter 5**. Specifically, we asked whether or not TNF activity is required for IL-12 synthesis and induction of Th1 responses against blood-stage malaria.

Unlike in liver-stage *P. yoelii* sporozoite challenge in BALB/c mice (186), IL-12 induced protection against blood-stage *P. chabaudi* AS infection in A/J mice was CD4+ T cell dependent. Additional studies from our laboratory have recently demonstrated that IL-12 treatment results in marked amplification of bone marrow and splenic erythropoiesis in *P. chabaudi* AS infected A/J mice, characterized by significant increases in erythroid progenitor cells in these organ sites in IL-12 treated compared with untreated controls (188). Taken together, these results suggested that the combined effects of enhanced erythropoiesis and IFN-γ, TNF-α, and NO-dependent Th1 responses mediate IL-12-induced protection of A/J mice against blood-stage malaria (16).

IL-12 is a potent proinflammatory cytokine. Thus, excessive production or overactivity of this molecule could result in an uncontrolled inflammatory response

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that could potentially damage host tissues. For example, treatment of lymphocytic choriomeningitis virus (LCMV)-infected B6 mice with IL-12 doses in excess of 100 ng/day resulted in significant toxicity manifested by marked increases in serum TNF–α and glucocorticoids levels, severe cachexia and thymic atrophy (189). To be effective, yet avoid adverse effects, the dose of IL-12 that could rescue susceptible A/J mice from blood-stage *P. chabaudi* AS malaria had to be carefully titrated (16). Doses of IL-12 greater than 0.1 μg/day resulted in significantly lower parasitemias but increased mortality compared to mice given the standard therapeutic dose of 0.1 μg/day. Interestingly, treatment of normally resistant B6 mice with 0.1 μg/day resulted in a high number of deaths (16). Resistant B6 mice normally mount predominantly Th1 responses during early *P. chabaudi* AS infection and clear the infection with no mortalities. Therefore, these results suggested that endogenous IL-12 levels in these mice during early infection might already be optimal. Thus, further addition of exogenous IL-12 could result in net toxic levels of this potent cytokine. Exogenous IL-12 treatment was shown to induce in vivo gene expression of both IFN–γ and IL-10 (35). It is possible that IL-10 plays an important antagonistic function in modulating excessive IL-12 activity when no longer needed (111). Alternatively, IL-12 p40 homodimers may function as a natural IL-12 antagonist (114).

Moderate vs. excessive IL-12 production has been correlated with protective vs. pathological responses, respectively, in blood-stage *P. berghei* malaria (190). Protection against the irradiation-induced attenuated *P. berghei* XAT strain occurs by IL-12-induced IFN–γ -dependent mechanism(s). In contrast, infection with lethal *P. berghei* NK65 strain results in liver injury associated with elevation of liver enzymes. Anti-IL-12 mAb treatment of mice infected with the lethal strain resulted in partial

protection against liver damage and prolonged survival. However, all the mice eventually died whether treated or untreated.

Taken together, these studies suggest that IL-12-induced Th1 responses can dramatically influence host resistance or susceptibility to malaria. The mechanism of IL-12-induced protection against malaria appears to be partially dependent on the induction of cytokines, particularly, IFN-γ and TNF-α, as well as NO production. In addition, enhanced extramedullary erythropoietic effects of IL-12 may also contribute significantly to recovery of murine and other hosts from malaria.

Chapter Two

In Vivo IL-12 Production and IL-12R β1 and β2 mRNA Expression in the Spleen are

Differentially Up-Regulated in Resistant B6 and Susceptible A/J Mice During Early

Blood-Stage *P. chabaudi* AS Malaria

Hakeem Sam and Mary M. Stevenson

McGill University Centre for the Study of Host Resistance,

and the Montreal General Hospital Research Institute

Running title: In Vivo IL-12 Production in Blood-Stage Malaria

ABSTRACT

As previously reported, blood-stage *P. chabaudi* AS malaria is lethal by days 10-12 post infection in susceptible A/J mice, which mount an early, predominantly Th2 response. In contrast, resistant B6 mice clear the infection by 4 weeks with an early Th1 response. In this study, we analyzed in vivo production of IL-12, a potent Th1-inducing cytokine, during the first five days after *P. chabaudi* AS infection in these mice. By day 2, serum IL-12 p70 levels were significantly increased in B6 mice over basal levels and were also significantly higher compared to A/J mice that showed no significant changes in serum p70 levels following infection. Splenectomy of resistant B6 mice prior to infection demonstrated that the spleen is the major source of systemic IL-12 in these hosts. Splenic mRNA levels of both p40 and p35 were significantly higher in A/J mice, however, the ratios of p40/p35 mRNA levels were similarly up-regulated in both strains. Furthermore, B6, but not A/J, mice showed significant up-regulation of splenic IL-12R β2 mRNA over basal levels by days 3 and 4, coincident with sustained up-regulation of splenic IFN-γ mRNA levels on days 3-5. IL-12R β1 mRNA levels in the spleen were, however, similarly up-regulated in both mouse strains by day 3. Taken together, these data suggest that high systemic IL-12 production, accompanied by an early and sustained up-regulation of both IL-12R β1 and β2 mRNA levels in the spleen, as occurs in resistant B6 mice, appears to preferentially induce protective Th1 responses against blood-stage malaria.

INTRODUCTION

Interleukin-12 (IL-12) was originally described as natural killer cell stimulatory factor (NKSF) (103), and as a cytotoxic lymphocyte maturation factor (CLMF). Subsequent cloning of both mouse (104, 108) and human (105) IL-12 genes, and characterization of the protein revealed that IL-12 is a disulphide-linked heterodimeric cytokine, composed of 40 kDa (p40) and 35 kDa (p35) subunits. The two subunits together form the 70-75 kDa (p70) protein, which accounts for the biologic actions of IL-12. The p40 and p35 genes are located on separate chromosomes. However, coexpression of both genes is required for the synthesis of p70 (105, 106). Secretion of free p35 by cells that synthesize IL-12 does not occur, but rather, p35 must be complexed with p40 to be released. In contrast, p40 can be freely secreted on its own by IL-12 synthesizing cells and, in fact, p40 is often produced in large excess of p70 (104, 107, 108). Under these conditions, free p40 can form disulphide-linked homodimers that have been demonstrated to be capable of antagonizing the physiologic actions of p70 both in vitro (113) and in vivo (114).

IL-12 induces IFN-γ synthesis by both NK and T cells, augments NK cell cytotoxicity, induces T cell proliferation, and is also a potent stimulus for Th1 cell development both in vitro (152) and in vivo (41). Th1 cells produce IL-2, IFN-γ and TNF-α, provide activation signals for macrophages, mediate delayed type hypersensitivity (DTH) reactions, and provide B cell help for the production of IgG2a antibodies. Importantly, Th1 responses have been implicated in host protective immunity against parasitic diseases, including *Leishmania major* (41), *Toxoplasma gondii* (184), and blood-stage malaria (11, 43, 44). Th2 cells, on the other hand, secrete IL-4, IL-5, IL-6, IL-10, and IL-13, and provide B cell help for antibody synthesis of other isotypes, especially IgE and IgG1 (32-34, 165).

Malaria poses a major global health threat today, a worrisome prospect mainly attributable to the resurgence of drug-resistant forms of the parasite and the lack as yet of an effective vaccine (4). To address the host immune mechanism(s) against blood-stage malaria, our laboratory has utilized the model of *P. chabaudi* AS infection in resistant B6 and susceptible A/J mice (191). Briefly, resistant B6 mice mount an early Th1 response, show moderate levels of acute primary parasitemia and anemia, and clear the infection by 4 weeks. In contrast, susceptible A/J mice mount an early Th2 response, show very high levels of acute primary parasitemia and anemia, and infection is fatal after 10-12 days. We have recently reported that systemic administration of murine rIL-12 for 6 days beginning on the day of infection could protect susceptible A/J mice against i.p. challenge with 10^6 *P. chabaudi* AS parasitized erythrocytes. Moreover, this IL-12-induced protection occurred by IFN-γ, TNF-α, and nitric oxide (NO)-dependent mechanisms.

Despite the pivotal role of IL-12 in mediating Th1 cell development, it is presently unknown whether or not endogenous IL-12 synthesis, or lack thereof, plays any crucial role in the induction of host immunity against blood-stage malaria. To further understand the role of IL-12 in host defense against malaria, we have analyzed in vivo IL-12 production in resistant B6 and susceptible A/J mice during early blood-stage *P. chabaudi* AS malaria. We have also analyzed splenic mRNA expression of the two recently cloned IL-12R subunits (137, 138) in these hosts following malaria infection. Our results show an important correlation between resistance and an early presence of high levels of systemic IL-12 p70, concomitant with significant up-regulation of mRNA levels of both IL-12R β1 and β2, and IFN-γ in the spleen.

MATERIALS AND METHODS

Mice, Parasite and Experimental Infections. Mice, 6-8 week old, were age- and sex-matched in all experiments. A/J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and B6 mice were from Charles River Laboratories (St. Constant, Quebec, Canada). *P. chabaudi* AS was maintained as previously described (31). Infection was initiated by i.p. injection of 10^6 *P. chabaudi* AS PRBC and the course of infection was monitored by previously described procedures (31).

Reagents and Sera. Murine rIL-12 was a generous gift of Dr. S. Wolf (Genetics Institute, Cambridge MA). Rat anti-murine IL-12 mAbs, C15.1 and C15.6 (IgG1 isotype), and C17.8 (IgG2a isotype) were generated as previously described (182). Hybridomas producing these mAbs were a kind gift of Drs. M. Wysocka and G. Trinchieri (Wistar Institute, Philadelphia, PA). All three monoclonal antibodies detect the p40 subunit of IL-12. Red-T/G297-289, a mixture of mAbs against the p35/p70 subunit of IL-12, was purchased from PharMingen (Mississauga, Ontario, Canada). At indicated times, blood was obtained from A/J and B6 mice by cardiac puncture, allowed to clot, and sera were separated by centrifugation at $13,800 \times g$ for 3 min. Sera were kept at 4°C and immediately analyzed for IL-12 levels by ELISA.

Splenectomy and spleen cell preparation. Splenectomy and sham-splenectomy procedures were performed using standard techniques previously published (192). Mice were rested for 3 weeks following surgery before initiating *P. chabaudi* AS infection. At the indicated times, single cell suspensions of spleen cells in RPMI 1640 (Flow) supplemented with 5% heat-inactivated FCS (Hyclone, Logan UT), 2% HEPES buffer (Flow), and 0.12% gentamicin (Schering Canada, Inc., Montreal, Quebec) were prepared under aseptic conditions as previously described (16). To obtain non-adherent spleen cells, splenic adherent cells were depleted by 2 h adherence to plastic at 37°C in a humidified, CO_2 incubator. Non-adherent spleen

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cells, pooled from 3 uninfected A/J or B6 mice, were adjusted to 4×10^6/ml and aliquots of 2-3 ml were placed in 6-well culture dishes (Nunc, Roskilde, Denmark). Cells were stimulated for 24 h in the presence of 5 μg/ml Con A (Calbiochem, La Jolla, CA), 1.8 ng/ml rIL-12, or medium as control.

ELISAs. Two-site sandwich ELISAs were used to measure serum levels of IL-12; p40- and p70-specific ELISAs were used. The former detects all species of IL-12 including p40 monomers, homodimers and p40-p35 heterodimers, whereas the latter detects only levels of the heterodimer. For the p40-specific ELISA, the capturing antibody was C15.1 and the detecting antibody was biotinylated C15.6. For the p70-specific ELISA, the capturing antibody was Red-T/G297-289 and the detecting antibody was biotinylated C17.8. Streptavidin-HRPO conjugate (GIBCO BRL, Grand Island, NY) was added for final detection. Washing, blocking, incubation and final detection of products were performed using methods previously established in our laboratory for the IFN-γ ELISA (63) with minor modifications. The coating buffer for Red-T/G297-289 was 0.1 M $NaHCO_3$ (pH 8.2). Incubation conditions for serum samples and standard rIL-12 were overnight at 4°C, and following addition of biotinylated antibody, plates were incubated for 4-5 h at room temperature. Plates were read in a microplate reader (Fischer Scientific, Nepean, Ontario, Canada) at 405 ηm, with a reference wavelength of 492 ηm.

Cytokine mRNA determination by RT-PCR. Spleens were aseptically removed from uninfected or infected mice at indicated times, immediately frozen in liquid nitrogen and stored at -70°C. The procedure for RNA isolation was based on a modification of the single-step method described by Chomzynski and Sacchi (193). Briefly, frozen tissue samples were homogenized in TRIzol reagent (GIBCO) using a polytron homogenizer (Brinkman, Kimematica, Switzerland). Cells were directly

lysed by adding TRIzol reagent. Total RNA was subsequently isolated following the manufacturer's instructions.

RT-PCR was performed as previously described by Kichian et al. (194). Cycling conditions and the number of PCR cycles were empirically determined for each primer pair. Typically, 30 cycles were performed and the cycling conditions used were: 94°C for 1 min, 54°C for 20 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. To increase the specificity of PCR amplification, the "hot start" method, involving an initial denaturation of the reaction mixture containing all reagents except the enzyme for 3 min at 94°C was utilized. Taq polymerase (GIBCO), in 1X PCR buffer, was then added to the reaction mixture at 85°C before cycling was initiated. Both positive and negative controls were included in each assay to ensure efficacy of the reaction and to rule out possible cDNA contamination of reagents. The housekeeping gene G6PDH was simultaneously amplified in each assay to verify that equal amounts of cDNA were added in each PCR reaction. Nucleotide sequences for primers and probes for IL-12 p40 and IL-12 p35 (183), IFN-γ (195), and G6PDH (194) were used as previously published. Primers and probe sequences were designed in our laboratory for IL-12R β1 and β2, based on the recently cloned cDNAs for these genes (Genebank accession no. U23922 and U64199 respectively). The sequences were: IL-12R β1 sense, 5'-TGA-AGA-CGG-CGC-GTG-GGA-GTC-A-3', antisense, 5'- TCG-CGG-GTA-CAA-CAC-CTC-CGG-G-3', probe, 5'-GCG-AGC-GGA-CAC-TGC-GAG-GC-3' (product size, 412 bp); IL-12R β2 sense, 5'-GGT-TGC-TGG-CTC-CTC-ACC-AGG-3', antisense, 5'-ATG-CAG-CCC-CTT-TGC-TCC-GGG-3', probe, 5'-TCC-CCC-ACA-CTG-GCT-GCG-GA-3' (product size, 424 bp).

Fifteen microlitres of final PCR products were analyzed by electrophoresis in a 1.2% agarose gel, denatured, neutralized, and transferred onto a Hybond-N membrane (Amersham, Arlington Heights, IL) by Southern blotting. After UV cross-linking (UV

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Stratalinker 1800, Stratagen, La Jolla, CA) and baking in a vacuum oven (2 h at 80°C), membranes were hybridized with cytokine-specific γ-^{32} P-ATP (Amersham) end-labelled oligonucleotide probes that hybridize to a portion of the amplified segment between the primers. Probes were radioactively labelled with 10 U T4 polynucleotide kinase (GIBCO) in a 0.5X One-Phor-All buffer supplied with the enzyme. After hybridization and washing, cytokine or cytokine receptor mRNA was detected by autoradiography with Biomax MR Kodak film (Rochester, NY). The intensity of bands corresponding to specific cytokines was analyzed by high-resolution optical densitometry (SciScan 500, United States Biochemical) and normalized to those of G6PDH. As shown in Figure 1, titration of input cDNA was performed for each cytokine or cytokine receptor, followed by PCR and Southern detection in order to ensure linearity between input cDNA and final PCR products.

Statistical Analysis. Statistical significance of differences in mean between groups of mice was determined by one-way ANOVA, followed by Bonferonni post-test. The Student-Newman-Keuls' post-test was used in the analysis of serum IL-12 levels. $p < 0.05$ was considered significant.

RESULTS

Resistant B6 mice produce higher levels of biologically active IL-12 p70

To establish possible differences in the in vivo level of systemic IL-12 production between resistant B6 and susceptible A/J mice, sera were collected from both strains during the first five days following *P. chabaudi* AS infection. Sera were also obtained from uninfected mice of either strain. All serum samples were analyzed for IL-12 levels by the p70-specific ELISA, which only detects levels of the biologically active IL-12 p40-p35 heterodimer, and by the p40-specific ELISA, which

detects all forms of the IL-12 p40 subunit including p40 monomers and dimers, as well as p40-p35 heterodimers. As shown in Figure 2A, serum levels of p70 on day 2 were significantly higher in B6 compared to A/J mice ($p < 0.01$), or compared to basal levels on day 0 ($p < 0.001$). Serum p70 levels remained substantially elevated in B6 mice on day 3 and decreased rapidly thereafter, returning to basal levels by day 5. In contrast, A/J mice showed no significant changes in serum p70 levels following *P. chabaudi* AS infection on any of the days examined.

Unlike p70, the kinetics of serum levels of total p40 was surprisingly quite similar in B6 and A/J mice (Fig. 2B). In both strains, p40 levels were significantly increased on day 3 compared to basal levels on day 0 ($p < 0.05$). By day 5, serum levels of total p40 had returned to basal levels in both strains. Interestingly, there were no significant differences in total levels of serum p40 between B6 and A/J on any of the days examined. It is of interest to point out that levels of total p40 in the serum of either strain were at least 10-fold in excess of p70 levels, in agreement with previous observations (196) of excessive release of free p40 by IL-12 producing cells. Taken together, these data suggest the existence of distinct and complex mechanisms for regulating p70 vs. p40 synthesis and secretion in vivo.

The spleen is the major source of endogenous IL-12 p70

To determine the source of systemic IL-12 production, we examined the spleen as well as the liver for up-regulation of mRNA levels of IL-12 p40 and p35 subunits. Resistant B6 and susceptible A/J mice were infected with *P. chabaudi* AS and during the first five days post infection, the spleen and liver were removed and analyzed for tissue IL-12 mRNA levels by RT-PCR. Spleens and livers from uninfected B6 and A/J mice were also analyzed. As shown in Figure 3A, mRNA levels of p40 in the

spleen were significantly increased in A/J mice on day 3 compared to basal levels on day 0 ($p < 0.001$). Moreover, p40 mRNA levels in the spleen on day 3 were significantly higher in A/J compared to B6 mice ($p < 0.001$). By days 4 and 5, p40 mRNA levels in the spleen of A/J mice had returned to basal levels. In B6 mice, p40 mRNA levels in the spleen were slightly increased on day 3, followed by a return to basal levels by days 4 and 5.

Unlike p40, the expression of p35 mRNA levels in the spleen of both strains was constitutive on all days examined (Fig. 3B). However, splenic p35 mRNA levels were significantly higher in A/J compared to their B6 counterparts on day 2 ($p < 0.001$), day 3 ($p < 0.01$), and day 4 ($p < 0.05$). Since A/J mice appeared to express higher mRNA levels for both p40 and p35, p40 mRNA levels were normalized against p35 mRNA levels for each mouse strain. The kinetics of the ratios of p40/p35 mRNA levels in the spleen are shown in Figure 3C. Interestingly, very similar patterns were seen in the kinetics of p40/p35 mRNA ratios in A/J and B6 mice. In both strains, there was a significant increase in p40/p35 mRNA ratios on day 3 compared to basal levels ($p < 0.001$ and $p < 0.01$, in A/J and B6 mice respectively). However, no significant differences between strains in p40/p35 mRNA ratios in the spleen were evident on any day post infection examined.

Compared to the spleen, p40 mRNA levels in the livers of B6 and A/J mice were very low (data not shown). Autoradiographic bands corresponding to p40 mRNA levels in the spleen were readily detectable after 2 h exposure. In contrast, under identical experimental conditions, bands corresponding to p40 mRNA levels in the liver required up to a week of exposure in order to be detectable. Moreover, mRNA levels of p35 in livers of both strains were virtually undetectable on all days examined (data not shown), in agreement with observations of Schoenhaut et al. (107).

Taken together, these data suggest the spleen, rather than the liver, to be the likely source of systemic IL-12 production during blood-stage malaria.

To confirm the spleen as the major source of in vivo IL-12 production during early blood-stage *P. chabaudi* AS infection, experiments were conducted using splenectomized and sham-splenectomized B6 mice. As shown in Table I, sham-splenectomized controls had significantly elevated levels of serum p70 on day 2, comparable to levels seen in intact B6 mice on this day. In contrast, splenectomized animals failed to show the significant increase in serum p70 levels seen in spleen-intact B6 mice on day 2 post infection.

Kinetics of IL-12R β 1 and β2, and IFN-γ mRNA levels in the spleen

Recent studies indicate that full biological responses to IL-12 require the expression of both IL-12R β1 and β2 at the surface of cells responding to this cytokine (162, 197). Therefore, we asked whether or not A/J and B6 mice differ in the levels of expression of IL-12R mRNA in the spleen during early *P. chabaudi* AS malaria. As shown in Figure 4*A*, the kinetics of IL-12R β1 mRNA levels in the spleen were similar in A/J and B6 mice, except on day 4 when IL-12R β1 mRNA levels were significantly higher in B6 compared to A/J mice ($p < 0.01$). In A/J mice, IL-12R β1 mRNA levels in the spleen were significantly increased on day 3 ($p < 0.001$) and day 5 ($p < 0.001$), compared to basal levels on day 0. In B6 mice, significant increases over basal levels of IL-12R β1 mRNA levels in the spleen occurred on day 3 ($p < 0.05$), day 4 ($p < 0.001$), and day 5 post infection ($p < 0.001$).

In contrast to similar kinetics of IL-12R β1 mRNA expression between infected A/J and B6 mice, significant differences between A/J and B6 mice in IL-12R β2 mRNA levels in the spleen were evident as early as day 3 post infection ($p < 0.01$)

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and were sustained on day 4 (Figure 4*B*; $p < 0.001$). However, by day 5, there was no significant difference between strains in IL-12R β2 mRNA levels in the spleen. In A/J mice, only on day 5 were IL-12R β2 mRNA levels in the spleen significantly higher ($p < 0.001$) than basal levels on day 0. In contrast, IL-12R β2 mRNA levels in the spleen were significantly higher than basal levels in B6 mice on day 3 ($p < 0.001$), day 4 ($p < 0.001$), and day 5 post infection ($p < 0.001$). Taken together, these data suggest that resistant B6 mice, compared to susceptible A/J mice, show an earlier and sustained up-regulation of IL-12R β2 mRNA levels in the spleen.

Earlier up-regulation of IL-12R β2 mRNA in the spleen of B6 mice was accompanied by higher up-regulation IFN-γ mRNA expression. As shown in Figure 4*C*, splenic IFN-γ mRNA levels were significantly higher in B6 compared to A/J mice on days 4 and 5 post infection ($p < 0.01$). This finding is consistent with previous observations from our laboratory (43) and others (44) of an important correlation between early predominantly Th1 responses and resistance to blood-stage *P. chabaudi* AS malaria.

A/J mice are not inherently deficient in up-regulation of IL-12R or IFN-γ mRNA expression

We next addressed the question of whether or not A/J mice, compared to their B6 counterparts, are as capable of up-regulating IL-12R and IFN-γ mRNA expression in the absence of *P. chabaudi* AS infection. Non-adherent spleen cells from uninfected A/J and B6 mice were cultured in vitro for 24 h in medium alone, or medium containing Con A or murine rIL-12. After the incubation period, total RNA was isolated and mRNA levels of IL-12R and IFN-γ were analyzed by RT-PCR. As shown in Figure 5, treatment with Con A or rIL-12 in vitro induced IL-12R β1, and

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β2 as well as IFN-γ mRNA expression to comparable levels in non-adherent spleen cells from either A/J or B6 mice.

We then asked if IL-12R and IFN-γ mRNA expression in the spleen could be modulated in vivo during infection by the administration of exogenous rIL-12 to A/J mice. *P. chabaudi* AS infected A/J mice were treated with either the standard protective dose of rIL-12 (0.1 µg/mouse/day for six days beginning on the day of infection) or PBS as control. Figure 6 shows that treatment of *P. chabaudi* AS infected A/J mice with rIL-12 resulted in significant increases in mRNA levels of IL-12R β2 and IFN-γ expressed by unfractionated spleen cells on day 7 post infection compared to PBS-treated controls. These data suggest that the apparent defect in up-regulation of IL-12R β2 mRNA seen in the spleen of A/J compared to B6 mice during early *P. chabaudi* AS malaria may be due in part to reduced production of p70 in A/J mice. Moreover, the deficiency in IL-12R β2 mRNA expression during blood-stage malaria in these hosts can be corrected by treatment with exogenous rIL-12.

DISCUSSION

The importance of an appropriate Th response in the development of protective immunity against blood-stage *P. chabaudi* AS malaria as well as other parasitic diseases is well established (11, 41, 43, 44). IL-12 has potent and pleiotropic biologic actions on NK and T cells (103). The discovery of IL-12 and recognition of its capacity to direct the differentiation of naive and undifferentiated CD4+ Th0 cells towards a Th1 phenotype suggested that innate immune responses play a critical role in the development of adaptive immunity (120). Cytokines like IL-12 produced during innate immunity appear to play an instructive role in influencing the type of acquired immunity and, hence, the phenotype of resistance vs. susceptibility that

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develops in a given host during infection. Exogenous rIL-12 treatment was shown to be curative in several animal models of tumors and parasitic diseases (174, 179). In malaria, exogenous rIL-12 treatment was effective against both liver and blood-stage malaria (16, 186), suggesting that IL-12 might be important in host defense against this disease.

In the present report, evidence is presented that demonstrates for the first time, significant differences between resistant B6 and susceptible A/J mice in the level of systemic production of IL-12 during early blood-stage *P. chabaudi* AS malaria. As early as day 2 post infection, serum levels of p70, the form of IL-12 that accounts for most of its known biological actions, were significantly higher in B6 compared to A/J mice. In B6 mice, the serum levels of IL-12 on day 2 represented a significant increase over basal levels seen in uninfected mice. In contrast, no significant increases in serum p70 over basal levels were observed in A/J mice during the first five days of infection. Previous studies in our laboratory have shown that during early *P. chabaudi* AS infection, spleen cells recovered from B6 compared to A/J mice, produced substantially higher levels of IFN-γ in vitro following stimulation with Con A or PRBC (43). In addition, IFN-γ mRNA levels in the spleen were found to be significantly higher in B6 compared to A/J mice, whereas IL-4 mRNA levels in the spleen were significantly higher in A/J mice during the first week of infection (81). The results reported here of significant differences between A/J and B6 mice in systemic production of IL-12 are consistent with these earlier observations and provide further understanding of the underlying mechanism(s) for the observed dichotomy of Th responses seen in the two inbred mouse strains during blood-stage malaria.

Differences between resistant and susceptible mice in early in vivo IL-12 production were also recently reported in a model of *Mycobacterium avium* infection

(198). BALB/c mice, which are genetically susceptible and mount predominantly Th2 responses to *M. avium* infection, exhibited reduced IL-12 p70 levels in aqueous spleen extracts on days 1 and 3 post infection and developed large and numerous granulomas. In contrast, genetically resistant DBA/2 mice, which mount predominantly Th1 responses to this infection, had reduced mycobacterial burdens and increased IL-12 p70 levels in aqueous spleen extracts on these days post infection. Furthermore, it was found that IL-12 p40 mRNA levels in the spleen were higher in resistant DBA/2 compared to susceptible BALB/c mice shortly (3-24 h) after the infection, whereas p35 mRNA levels were constitutively expressed. In our studies, detectable up-regulation in p40 mRNA levels was seen in the spleens of both A/J and B6 mice on day 3 post infection, whereas p35 mRNA was constitutively expressed. The fact that mRNA synthesis would have to precede protein synthesis and release from the spleen suggests that this increase in splenic p40 mRNA levels on day 3 is probably not directly related to the increase in serum p70 levels in B6 mice on day 2. This elevated serum p70 levels in day 2 infected B6 mice could be related to transient increases in splenic p40 mRNA levels occurring hours after initiating *P. chabaudi* AS infection that could have been missed at the days post infection chosen for our study.

Despite this lack of direct correlation with serum p70 levels, the fact that both p40 and p35 mRNA were readily detectable in the spleens of infected A/J and B6 mice, but not in the liver, was important in suggesting that the spleen might be an important source of IL-12 production during early *P. chabaudi* AS infection. It is presently unclear why both p40 and p35 mRNA levels were higher in spleens of A/J compared to B6 mice, although the ratios of p40/p35 mRNA levels were similar in both strains. Studies have shown that for several IL-12 producing cells, mRNA levels for p40 are about 10-fold more abundant than for p35, consistent with observations that p40 is often produced 10-100-fold in excess of secreted p70 levels (105, 117,

199). This wide range of excess in p40 over p70 levels might be involved in the fact that, for similar serum p40 levels, serum p70 levels varied significantly between *P. chabaudi* AS infected A/J and B6 mice.

The regulation of IL-12 mRNA expression and secretion of p40 vs. p70 protein secretion appears to be complex. Firstly, the secretion of p70 requires that both p40 and p35 genes be expressed in the same cell. In situ hybridization studies on sections of mouse spleen by Bette and co-workers (200) revealed that large populations of spleen cells appear to express either p40 or p35 mRNA, but not both. Secondly, even when both p40 and p35 mRNA are expressed in the same cell, recent evidence suggests that different signals can modulate secretion of p70 vs. p40. Following stimulation with *Salmonella dublin* LPS, murine macrophages released substantial amounts of p40 but very little p70 (127). However, LPS and IFN-γ co-stimulation was accompanied by secretion of high levels of both p40 and p70 (127). Similar results have been reported for IL-12 p70 vs. p40 secretion by microglial cells in the CNS (119).

The spleen has long been recognized as a key site for the induction of anti-plasmodia mechanism(s) (18). During early blood-stage *P. chabaudi* AS infection, resistant B6 mice develop massive splenomegaly (17), marked by significant changes in spleen cells expressing CD3, B220 and Mac-1 (55). Moreover, Yap and Stevenson (19) demonstrated that an architecturally intact spleen was necessary for resolving blood-stage *P. chabaudi* AS infection in resistant B6 mice. In this report, we show that the spleen is the major source of systemic IL-12 production in *P. chabaudi* AS infected B6 mice. The spleen was also the site of significant up-regulation of IL-12R mRNA levels in both A/J and B6 mice during early blood-stage malaria, suggesting an important mechanism by which the protective effects of IL-12 in the spleen are mediated.

Monocyte/macrophages are generally regarded as the major physiologic sources of IL-12 (196). However, recent studies indicate that other cell types, including neutrophils (116, 117) and dendritic cells (118), can produce IL-12. Our recent study demonstrated that splenic macrophages recovered from B6 compared to A/J mice acutely infected with *P. chabaudi* AS produced significantly greater quantities of IL-12 p70 in vitro (manuscript submitted). Studies are currently in progress to identify the specific phenotype of cells in the spleen that express IL-12R mRNA. Evidence from FACs analysis studies suggests that the major cell types that express IL-12R are NK and T cells (131). In addition, recent analysis of lymph node cells from *Leishmania major*-infected mice revealed significant up-regulation of IL-12R on CD4+, CD8+ and B220+ cells (201).

Significant differences between A/J and B6 mice were evident in the kinetics of up-regulation of splenic IL-12R mRNA levels following *P. chabaudi* AS infection. In B6 mice, splenic IL-12R β2 mRNA levels were significantly elevated over basal levels by day 3 and levels remained significantly elevated on days 4 and 5 post infection. In contrast, significant up-regulation of IL-12R β2 mRNA over basal levels in the spleen of A/J mice was not evident until day 5 post infection. On the other hand, IL-12R β1 mRNA levels in the spleen were significantly up-regulated in both strains by day 3 post infection.

The delayed kinetics of up-regulation of IL-12R β2 mRNA levels in the spleens of susceptible A/J mice could have important immunological implications. Recent evidence suggests that the expression of both IL-12R β1 and β2 is required in order for high affinity interaction between IL-12 p70 and the IL-12 receptor complex to occur (163, 197). Importantly, the earlier and sustained expression of mRNA levels of both IL-12R in the spleens of malaria-infected B6 mice correlated with higher mRNA levels of IFN-γ in this organ on days 3 to 5 post infection, compared to A/J

mice. These results are consistent with our earlier observations that, for protective efficacy against blood-stage malaria, rIL-12 treatment in A/J mice had to be initiated on or one day prior to *P. chabaudi* AS infection (16). Treatment with rIL-12 was not protective when initiated in A/J mice beginning on day 3 after infection, emphasizing the importance of the presence of IL-12 early in infection. In both A/J and B6 mice, up-regulation of IL-12R mRNA levels in the spleen was accompanied by the disappearance of both p40 and p70 species of IL-12 from sera, possibly reflecting the increasing predominance of membrane bound forms of IL-12 in the spleen.

Taken together, this study demonstrates significant differences between resistant B6 and susceptible A/J mice in the level of systemic IL-12 production that correlates with the developmental dichotomy of Th responses previously observed in these hosts during early *P. chabaudi* AS malaria. In addition, we show that the spleen is the major source of systemic IL-12 production in B6 mice, and that an early and sustained up-regulation of both IL-12R β1 and β2 appears to be required for IL-12-induced protective immunity against blood-stage malaria.

ACKNOWLEDGEMENTS

Figure 1. Optimization of PCR amplification conditions. Synthesis of cDNA was performed with 1 µg of total RNA isolated from the spleen of a B6 mouse at day 5 post infection with *P. chabaudi* AS. The input cDNA for PCR was serially diluted such that each dilution contained two-thirds of the cDNA in the previous dilution. PCR was performed with primers for cytokine, cytokine receptor or housekeeping gene, and products were detected by southern blotting. Optimal cycling conditions for each cytokine or cytokine receptor and housekeeping gene were determined empirically, as illustrated here for p35, to ensure linearity of amplification. The density of p35-specific autoradiographic bands (*A*) were determined by densitometry. A linear relationship between input cDNA and PCR amplification (regression coefficient $r^2=0.97$) is shown (*B*). Amount of input cDNA in undiluted sample is arbitrarily set at 100%.

A

B
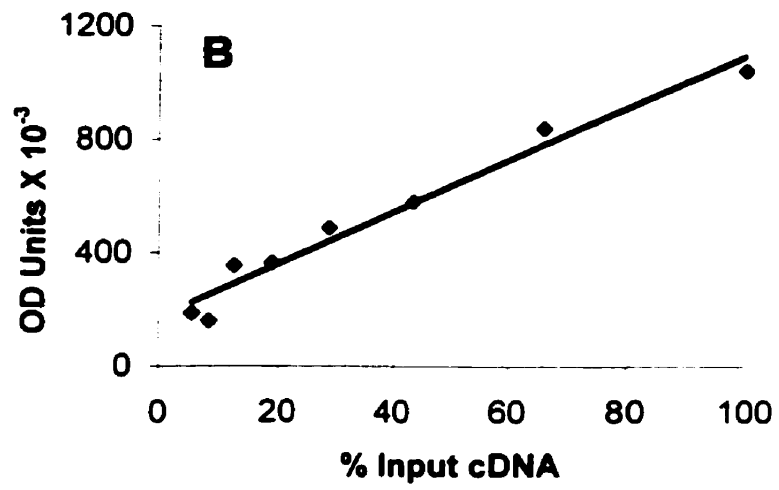
Figure 2. Kinetics of IL-12 p70 (*A*) and p40 (*B*) levels in serum of susceptible A/J and resistant B6 mice during the first five days of infection with *P. chabaudi* AS. Mice on day 0 were normal uninfected controls. Serum cytokine levels were determined by IL-12 p70- or p40-specific ELISA. Mice from two independent experiments were pooled for analysis. Data are presented as mean ± SEM of 5-12 mice per time point analyzed individually. Statistically significant differences shown are: *$p < 0.01$ vs. A/J mice on same day, #$p < 0.05$, ##$p < 0.001$ vs. uninfected controls of the same strain.

A

B

Days Post Infection
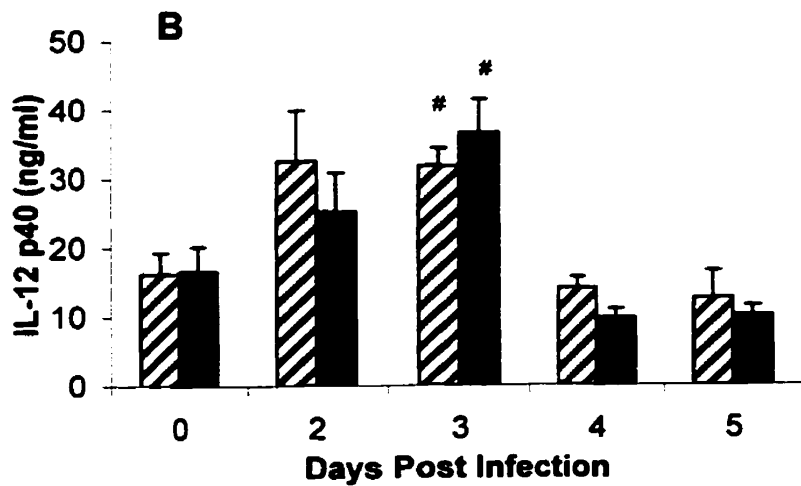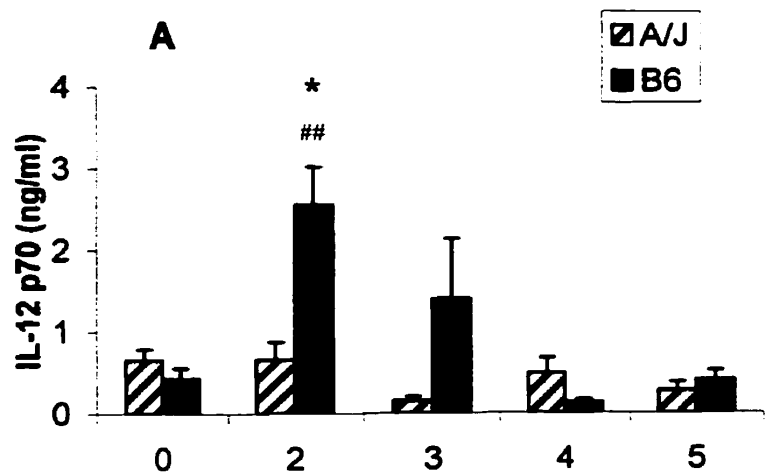
Figure 3. Kinetics of IL-12 p40 (*A*), p35 (*B*), and ratios of p40/p35 (*C*) mRNA levels in spleens of susceptible A/J and resistant B6 mice during the first five days of infection with *P. chabaudi* AS. Mice on day 0 were normal uninfected controls. After RT-PCR, electrophoresis, southern detection and autoradiography, the density of bands corresponding to cytokine mRNA was normalized to those of the housekeeping gene G6PDH (G6). Mice from two independent experiments were pooled for analysis. A representative result of three RT-PCR determinations is shown. Data are presented as mean ● SEM of 3-5 mice per time point analyzed individually. Statistically significant differences shown are: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. B6 mice on same day, $^{#}p < 0.01$, $^{##}p < 0.001$ vs. uninfected mice of the same strain on day 0.

A

p40/G6

***

A/J
B6

0   2   3   4   5

B

p35/G6

***   **   *

0   2   3   4   5

C

p40/p35

#

0   2   3   4   5

Days Post Infection
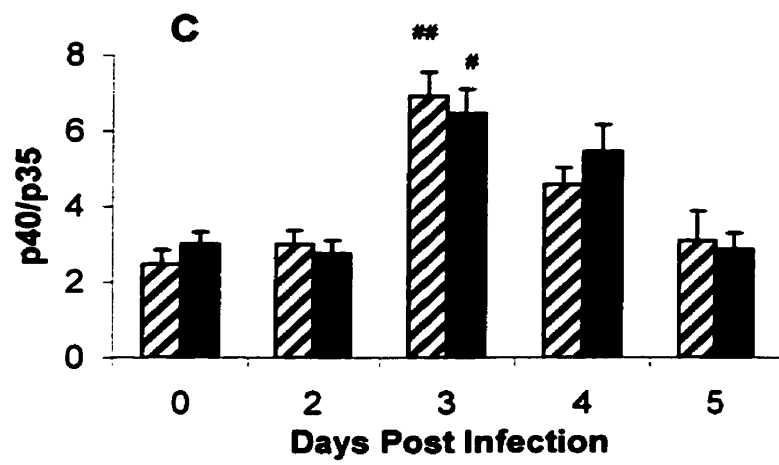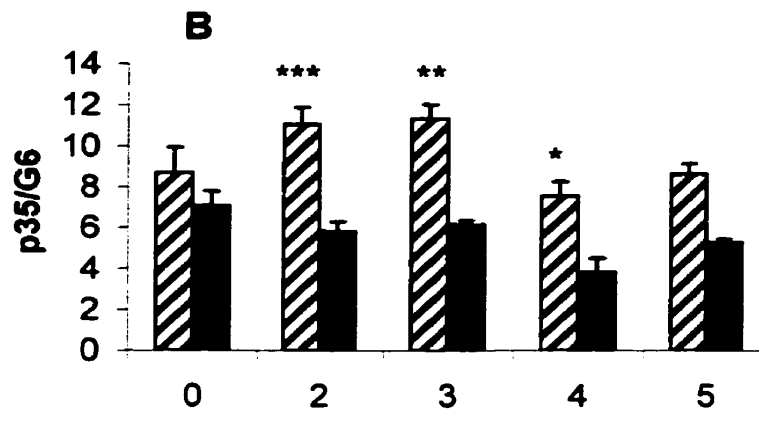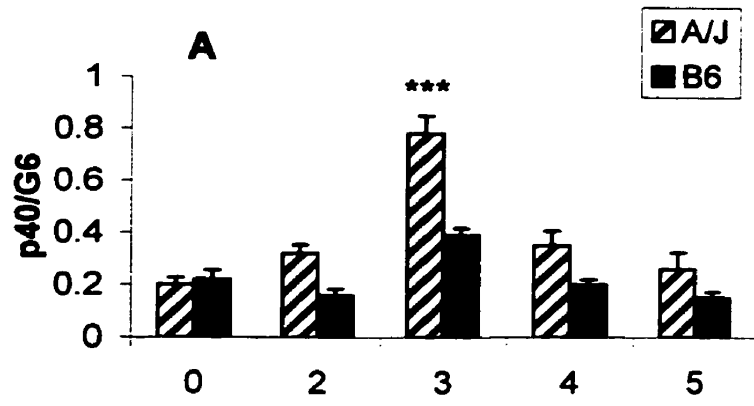
Figure 4. Kinetics of IL-12R β1 (*A*), IL-12R β2 (*B*), and IFN-γ (*C*) mRNA levels in spleens of susceptible A/J and resistant B6 mice during the first five days of infection with *P. chabaudi* AS. Mice on day 0 were normal uninfected controls. After RT-PCR, electrophoresis, southern detection and autoradiography, the density of bands corresponding to cytokine or cytokine receptor mRNA was normalized to those of the housekeeping gene G6PDH (G6). Mice from two independent experiments were pooled for analysis. A representative result of three RT-PCR determinations is shown. Data are presented as mean ± SEM of 3-5 mice per time point analyzed individually. Statistically significant differences shown are: *$p < 0.01$, **$p < 0.001$ vs. A/J mice on same day.

A

IL-12Rβ1/G6

A/J
B6

*

Days Post Infection: 0, 2, 3, 4, 5

B

IL-12Rβ2/G6

*

**

Days Post Infection: 0, 2, 3, 4, 5

C

IFN-γ/G6

*

*

Days Post Infection

0, 2, 3, 4, 5
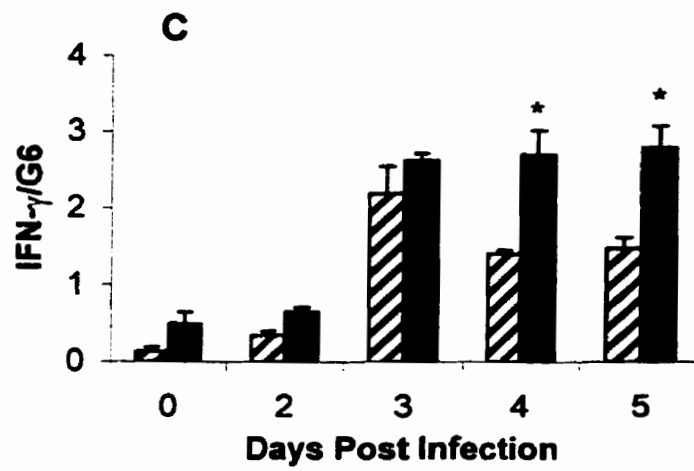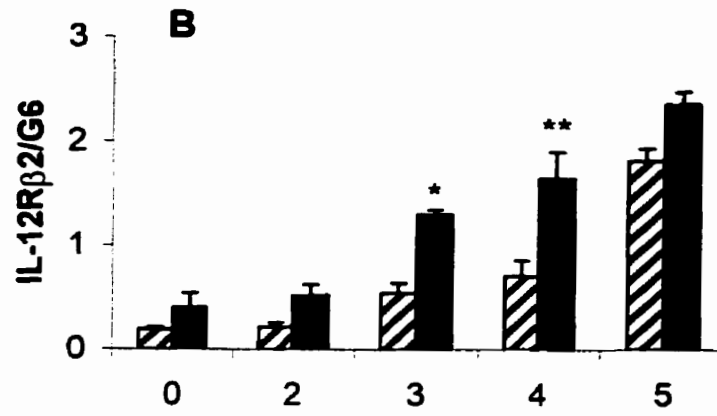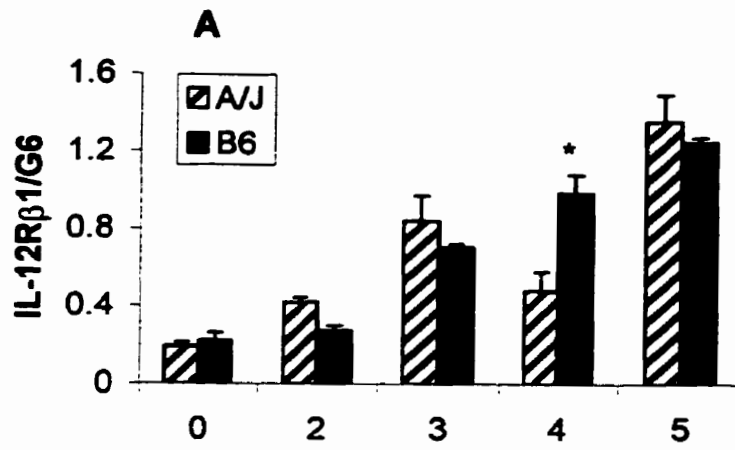
Figure 5. Effect of in vitro rIL-12 treatment on IL-12R β1 and β2, and IFN-γ mRNA expression. Autoradiograph (*A*), non-adherent spleen cells were pooled from 3 uninfected A/J or B6 mice. Cells were cultured for 24 h in the presence of medium alone (*lanes 1 and 4*), or medium containing either 5 µg/ml of Con A (*lanes 2 and 5*) or 1.8 ng/ml of rIL-12 (*lanes 3 and 6*). After RT-PCR, electrophoresis, southern detection and autoradiography, the density of bands corresponding to cytokine or cytokine receptor mRNA was normalized to those of the housekeeping gene G6PDH (G6). Results are shown for IL-12R β1 (*B*) and β2 (*C*), and IFN-γ (*D*). Similar results were obtained in a replicate experiment.

66

A

	A/J			B6		
	1	2	3	4	5	6
IL-12R β1						
IL-12R β2						
IFN-γ						
G6PDH						

B ⬚ A/J ■ B6

IL-12Rβ1/G6

5
4
3
2
1
0

C

IL-12Rβ2/G6

5
4
3
2
1
0

D

IFN-γ/G6

4
3
2
1
0

med   Con A   rIL-12
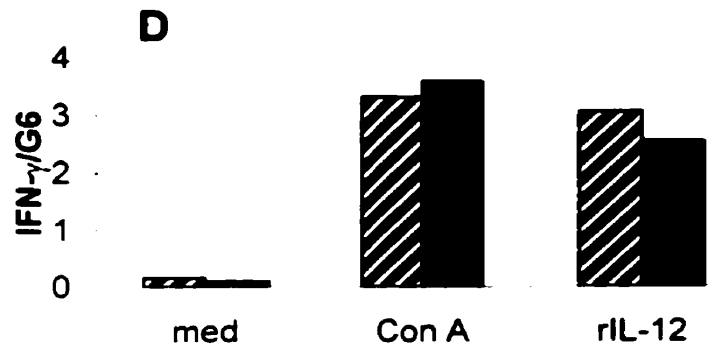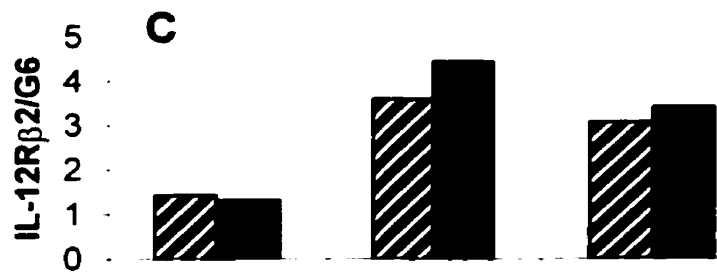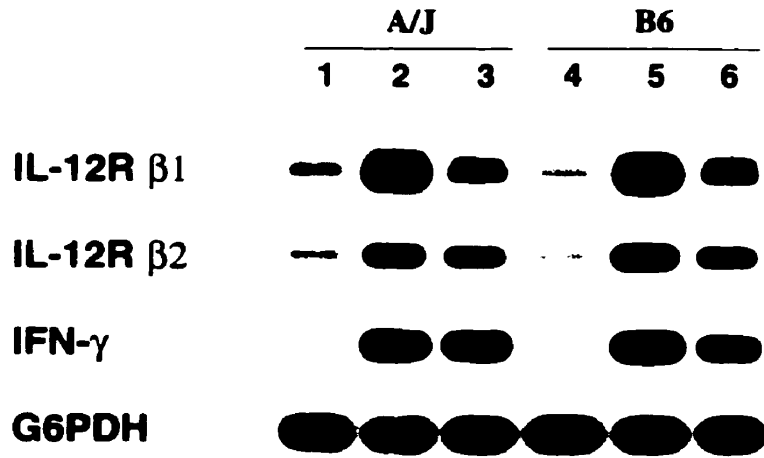
Figure 6. Effect of in vivo rIL-12 treatment on IL-12R β1 and β2, and IFN-γ mRNA expression. *P. chabaudi* AS infected A/J mice were treated daily, beginning on the day of infection until day 5 post infection, with either murine rIL-12 (0.1 μg/mouse/day) or sterile PBS as a control. Mice were sacrificed at day 7 and cytokine or cytokine receptor mRNA expression in unfractionated spleen cells was analyzed by RT-PCR. After electrophoresis, southern detection and autoradiography, the density of bands corresponding to cytokine or cytokine receptor mRNA was normalized to those of the housekeeping gene G6PDH (G6). A representative result of three RT-PCR determinations is shown. Data are presented as mean ● SEM of 3 mice individually analyzed. Statistically significant differences shown are: *$p < 0.001$ vs. vehicle-treated controls.
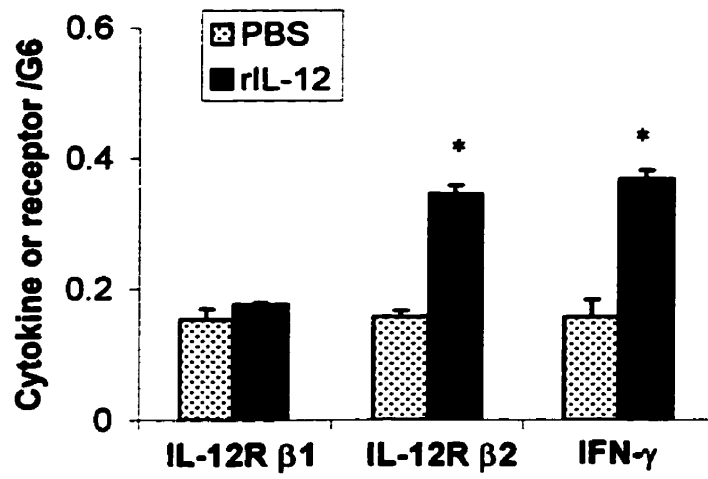
Table I

Effect of splenectomy on serum IL-12 p70 levels

Treatment[a]	Splenectomized[b]	IL-12 p70 (ng/ml)[c,d]
Uninfected	No	0.4 ± 0.1
Infected	No	$2.5 \bullet 0.5^*$
Infected	Yes	$0.8 \pm 0.2^\#$
Infected	Sham	$2.8 \pm 0.3^*$

[a]B6 mice were either uninfected or infected i.p. with 10^6 *P. chabaudi* AS PRBC and sera recovered on day 2 post infection.

[b]Splenectomized or sham splenectomized animals were rested for 3 weeks prior to initiating malaria infection.

[c]IL-12 p70 levels were determined by a p70-specific ELISA. Mice from two independent experiments were pooled for analysis. Values shown are mean $\bullet$ SEM of 7-12 mice analyzed individually.

[d]Statistically significant differences are shown: $^*p < 0.001$ vs. uninfected mice on day 0, $^\#p < 0.001$ vs. infected sham.

Chapter Three

Early IL-12 p70, But Not p40, Production by Splenic Macrophages Correlates With

Host Resistance to Blood-Stage *Plasmodium chabaudi* AS Malaria

Hakeem Sam and Mary M. Stevenson*

McGill University Centre for the Study of Host Resistance,

and the Montreal General Hospital Research Institute

Running title: Splenic macrophage derived IL-12 in blood-stage malaria

PREFACE

In chapter 2, significant differences were presented in systemic IL-12 p70 levels between resistant B6 and susceptible A/J mice during early blood-stage *P. chabaudi* AS malaria. It was unclear whether these differences between the strains in endogenous IL-12 levels were due to greater IL-12 synthesis on a per cell basis or the presence of greater numbers of IL-12 producing cells in the spleens of B6 mice. Moreover, the phenotype of the IL-12 producing cells was not addressed. Monocyte/macrophages are generally regarded as the major cellular sources of IL-12, although IL-12 synthesis has been described in a variety of other cell types. Hence, splenic macrophages were chosen for further analysis of IL-12 production in the two mouse strains at the cellular level. The results of these studies are the subject of the present chapter.

ABSTRACT

In this study, we compared synthesis of IL-12, a potent Th1-inducing cytokine, by splenic macrophages recovered from resistant C57BL/6 (B6) mice, which develop predominantly Th1 responses, and susceptible A/J mice that mount primarily Th2 responses during early *P. chabaudi* AS infection. Quantitative analysis of IL-12 p40 and p70 release by ELISA revealed significant differences between resistant B6 and susceptible A/J mice in the synthesis of biologically active IL-12 p70, but not p40, by splenic macrophages during early blood-stage *P. chabaudi* AS infection. Despite up-regulation in p40 and p35 mRNA levels, spontaneous release of p40 in vitro by splenic macrophages was not significantly increased following infection in either mouse strain. In contrast, spontaneous release of p70 by splenic macrophages was increased in both mouse strains but levels were significantly higher in cells from B6 compared to A/J mice. Furthermore, compared to infected A/J hosts, splenic macrophages recovered from infected B6 mice produced significantly greater quantities of IL-12 p70, but not p40, in vitro, following stimulation with LPS or malaria parasite antigen (PRBC). Moreover, we found earlier and significant increases in the percentage of macrophages in the spleens of infected B6 mice that could further contribute to differences in total p70 levels in vivo. Taken together, these data suggest that macrophage IL-12 synthesis may contribute significantly to the polarization of Th responses seen in resistant B6 and susceptible A/J mice during acute blood-stage malaria.

INTRODUCTION

Recent studies indicate that cytokines can profoundly influence the developmental maturation of CD4+ helper T cells towards either a Th1 or Th2 phenotype (143). Emerging evidence suggests that IL-12 plays a central role in the development of CD4+ T cells towards a Th1 phenotype, whereas IL-4 favors a Th2 phenotype differentiation (148, 152). IL-12 is unique among the cytokines in having a heterodimeric structure consisting of disulfide-linked 35 and 45 kDa subunits (p35 and p40, respectively). Together, these two subunits form a 70-75 kDa protein (p70) that accounts for the main biological actions of IL-12 (107). The genes for p35 and p40 are located on separate chromosomes and must be co-expressed in the same cell type in order for p70 to be produced (105, 106). IL-12 has potent and pleiotropic effects on NK and T cells, in particular the ability to induce the synthesis of IFN-γ (103). Importantly, protective effects of IFN-γ have been implicated in host resistance to protozoan parasite infections, including blood-stage malaria (63, 64, 182).

Resistance or susceptibility of inbred mouse strains to infections with *P. chabaudi AS* (11, 43, 44), *Leishmania major* (41), or *Toxoplasma gondii* (182) appears to be critically dependent on the timing and activation of an appropriate Th response. Evidence from our laboratory (43, 191) and others (44) have previously shown that, during *P. chabaudi* AS infection, resistant B6 mice mount early, predominantly Th1 responses, develop moderate levels of primary peak parasitemia and anemia, and clear the infection by 4 weeks post infection. In contrast, susceptible A/J mice mount early, predominantly Th2 responses, develop high levels of primary peak parasitemia and severe anemia, and mortality occurs in these hosts by days 10-12 following *P. chabaudi* AS infection.

Evidence for a crucial role of IL-12 in host defense against lethal blood-stage malaria has come from recent observations from our laboratory that daily treatment with murine rIL-12 for six days, beginning on the day of infection, could cure susceptible A/J mice against blood-stage *P. chabaudi AS* infection (16). Treatment with rIL-12 protected up to 75% of susceptible A/J hosts, compared to 100% mortality in vehicle-treated controls. Furthermore, this IL-12-induced protection occurred by IFN-γ, TNF-α and nitric oxide (NO) dependent mechanism(s). These data suggest that early IL-12 production might be impaired in *P. chabaudi AS* infected A/J mice. IL-12 synthesis has been described in a variety of cell types, including dendritic cells (118), neutrophils (116, 117) and microglial cells (119) in the CNS. Monocyte/macrophage(s), however, are generally regarded as the major physiological sources of IL-12 (120).

In the present study, we have compared IL-12 production by splenic macrophages recovered from resistant B6 and susceptible A/J hosts during acute *P. chabaudi AS* infection. Our results demonstrate that, during early blood-stage malaria, splenic macrophages obtained from resistant B6 mice produce significantly higher levels of biologically active IL-12 p70 in vitro compared to macrophages from susceptible A/J hosts. Moreover, higher p70 release by splenic macrophages from infected B6 compared to A/J mice correlated with synthesis of greater quantities of IFN-γ by unfractionated spleen cells obtained from these mice and stimulated in vitro with malaria antigen.

MATERIALS AND METHODS

Mice, Parasite and Experimental Infections. Mice, 6-8 week old, were age- and sex-matched in all experiments. A/J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and B6 mice were from Charles River Laboratories (St. Constant,

Quebec, Canada). *P. chabaudi* AS was maintained as previously described (31). Infection was initiated by i.p. injection of 10^6 *P. chabaudi AS* infected erythrocytes (PRBC) and the course of infection was monitored by previously described (31) procedures.

Cytokine Reagents and Antibodies. Murine rIL-12 was a generous gift of Dr. S. Wolf (Genetics Institute, Cambridge MA). Rat anti-murine IL-12 mAbs, C15.1 and C15.6 (IgG1 isotype), and C17.8 (IgG2a isotype) were generated as previously described (182). Hybridomas producing these mAbs were a kind gift of Drs. M. Wysocka and G. Trinchieri (Wistar Institute, Philadelphia, PA). All three monoclonal antibodies detect the p40 subunit of IL-12. Red-T/G297-289, a mixture of mAbs against the p35/p70 subunit of IL-12, was purchased from PharMingen (Mississauga, Ontario, Canada).

Preparation of splenic macrophage monolayers. At the indicated times, single cell suspensions of spleen cells were prepared under aseptic conditions in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 5% heat-inactivated FCS (Hyclone, Logan UT), 2% HEPES buffer (GIBCO-BRL) and 0.12% gentamicin (Schering Canada, Inc., Montreal, Quebec), as previously described (202). Total cell counts were performed and differential cell counts were determined on Cytocentrifuge (Shandon Corporation, Sewickley, PA, USA) preparations of spleen cells stained with Dif-Quik (American Scientific Products, McGraw Park, IL, USA). Spleen cell suspensions (pooled from 2-3 mice) were adjusted to a concentration of 2×10^6 macrophages/ml. Aliquots of 500 µl of spleen cell suspensions were added to 24-well flat-bottomed plates (Nunc, Roskilde, Denmark) and allowed to adhere for 2 h at 37°C in a humidified 5% CO_2 incubator. Following incubation, non-adherent cells were removed by washing adherent cell monolayers three times with warm HBSS (GIBCO-BRL). Adherent cells were cultured for 24 h in freshly added medium alone, with 100

ng/ml of *E. coli* O127:B8 LPS (Difco, Detroit, MI, USA) or 10^6/ml of malaria parasite antigen (PRBC). In some experiments, adherent cells were cultured in the presence of indicated concentrations of 1.2 µm diameter latex beads (Sigma Chemical Co. (St. Louis, MO, USA). Cell culture supernatants were removed and assayed for cytokine concentrations by ELISA. Adherent cells were >95% macrophages based on morphology, phagocytosis of inert latex beads, and nonspecific esterase staining. Where indicated, 500 µl aliquots of unfractionated spleen cells (4 × 10^6/ml), in medium or PRBC (10^6/ml), were cultured in 24-well plates and 48 h cell-free culture supernatants were assayed for IFN-γ levels by ELISA.

ELISAs. IFN-γ ELISA was performed as previously described (63) and had a detection limit of 100 pg/ml. Two-site sandwich ELISAs were used to measure levels of IL-12 in macrophage culture supernatants; both p40- and p70-specific ELISAs were used. The former detects all forms of IL-12 and does not distinguish between levels of single IL-12 species such as p40 monomers, homodimers or p40-p35 heterodimers, whereas the latter detects only levels of biologically active heterodimer. For the p40-specific ELISA, the capturing antibody was C15.1 and the detecting antibody was biotinylated C15.6. For the p70-specific ELISA, the capturing antibody was Red-T/G297-289 and the detecting antibody was biotinylated C17.8. Streptavidin-HRPO conjugate (GIBCO-BRL) was added for final detection. The coating buffer for Red-T/G297-289 was 0.1 M $NaHCO_3$ (pH 8.2). Incubation conditions for samples and standard rIL-12 were overnight at 4°C, and following addition of biotinylated antibody, plates were incubated for 4-5 h at room temperature. Plates were read in a ELISA reader at 405 ηm, with a reference wavelength of 492 ηm. The limit of detection for the p40-specific ELISA was 500 pg/ml, and for the p70-specific ELISA was 100 pg/ml.

Cytokine mRNA determination by RT-PCR To prepare splenic macrophages for RNA isolation, aliquots of 2-3 ml of spleen cell suspensions adjusted to 2×10^6 macrophages/ml were placed in 6-well culture dishes (Nunc) and allowed to adhere as described above. The procedure for RNA isolation was based on a modification of the single-step method described by Chomzynski and Sacchi (193). Briefly, splenic macrophages were directly lysed by adding TRIzol reagent (GIBCO-BRL) to macrophage monolayers immediately following the removal of non-adherent cells by washing with warm HBSS. Total RNA was subsequently isolated following the manufacturer's instructions.

RT-PCR was performed as previously described (194) to detect changes in mRNA levels of cytokine or the housekeeping gene, G6PDH. To determine optimal cycling conditions, titrations of input cDNA were performed followed by PCR amplification to ensure that for the selected number of cycles, a linear relationship exists between input cDNA and PCR product. Typically, 30 cycles were performed and the cycling conditions used were: 94°C for 1 min, 54°C for 20 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. To increase the specificity of PCR amplification, the "hot start" method was utilized. This method involves an initial denaturation for 3 min at 94°C of the reaction mixture containing all reagents except the enzyme. Taq polymerase (GIBCO-BRL), in 1X PCR buffer, was then added to the reaction mixture at 85°C before cycling was initiated. Both positive and negative controls were included in each assay to ensure efficacy of the reaction and to rule out possible cDNA contamination of reagents. The housekeeping gene G6PDH was simultaneously amplified in each assay to verify that equal amounts of cDNA were added in each PCR reaction. Nucleotide sequences for primers and probes for IL-12 p40 and IL-12 p35 (183), and G6PDH (194) were used as previously published.

Fifteen microlitres of final PCR products were analyzed by electrophoresis in a 1.2% agarose gel, denatured, neutralized, and transferred onto a Hybond-N membrane (Amersham, Arlington Heights, IL) by Southern blotting. After UV cross-linking (UV Stratalinker 1800, Stratagen, La Jollier, CA) and baking in a vacuum oven (2 h at 80°C), membranes were hybridized with cytokine-specific γ-^{32} P-ATP (Amersham) end-labelled oligonucleotide probes that hybridize to a portion of the amplified segment between the primers. Probes were radioactively labelled with 10 U T4 polynucleotide kinase (GIBCO-BRL) in a 0.5X One-Phor-All buffer supplied with the enzyme. After hybridization and washing, cytokine mRNA was detected by autoradiography with Kodak Biomax MR film (Rochester, NY). The intensity of bands corresponding to specific cytokines was analyzed by high-resolution optical densitometry (SciScan 500, United States Biochemical, Cleveland, OH) and normalized to those of G6PDH.

Statistical Analysis. Results are presented as mean ± SEM. Statistical significance of differences in means between two groups of mice was determined by Student's t-test. Where three or more groups were compared, one-way ANOVA, followed by Dunnett's post-test was used. Differences in IL-12 p70 levels were analyzed by the Mann-Whitney test. $p < 0.05$ was considered significant.

RESULTS

Ex-vivo p40 and p35 mRNA expression and IL-12 protein release by splenic macrophages

As an initial assessment of IL-12 production, we examined whether splenic macrophages from A/J and B6 mice differ in the kinetics of IL-12 p40 and p35 mRNA

expression during the first five days of *P. chabaudi* AS infection (Fig. 1). Besides their isolation from unfractionated spleen cells by 2 h adherence to plastic culture dishes, macrophages were not subjected to any further antigenic stimulation in vitro. Thus, it is expected that cytokine mRNA levels and protein secretion by these cells should closely reflect in vivo activation just prior to their isolation from *P. chabaudi* AS infected A/J or B6 mice. Splenic macrophages from uninfected controls of either mouse strain were used as controls. We found low and constitutive expression of p40 and p35 mRNA in splenic macrophages from control animals of either mouse strain (Fig. 1). Following *P. chabaudi* AS infection, the expression of both p40 and p35 mRNA by splenic macrophages was up-regulated in both mouse strains, suggesting an increased capacity for IL-12 protein synthesis by these cells.

Splenic macrophages from uninfected controls or malaria-infected resistant B6 or susceptible A/J mice at day 4 were selected for a quantitative analysis and comparison of IL-12 p40 and p70 protein secretion in vitro. Substantial increases in IL-12 protein levels in splenic macrophage culture supernatants from infected mice of either strain were not detectable at days earlier than day 4 post infection (data not shown). Splenic macrophages from uninfected controls of both mouse strains spontaneously secreted basal levels of p40 protein (Fig. 2A). Despite increases in mRNA expression by splenic macrophages for IL-12 p40 following *P. chabaudi* AS infection, there were no significant increases in the spontaneous secretion of p40 protein in supernatants of cells from infected compared with uninfected controls of either strain. In contrast, spontaneous p70 production was undetectable in cells from uninfected controls of both mouse strains (Fig. 2B). Importantly, p70 levels were significantly greater in supernatants of splenic macrophages from infected B6 compared to A/J mice ($p < 0.05$, Fig 2B). We also analyzed the levels of IFN-γ in 48 h culture supernatants of spleen cells obtained from uninfected or infected mice of both

strains. Consistent with previous studies (43, 81), and in correlation with higher IL-12 p70 release by splenic macrophages from infected B6 mice, malaria parasite antigen (PRBC)-induced IFN-γ synthesis was significantly greater ($p < 0.05$) in spleen cells recovered from day 4 *P. chabaudi* AS infected B6 compared to A/J mice (Fig. 2C).

IL-12 p40 and p70 release in response to stimulation with various microbial products

Next we analyzed the capacity of splenic macrophages to produce IL-12 p40 and p70 following stimulation in vitro with microbial antigens. As previously reported, LPS is a potent stimulus for monocyte/macrophage IL-12 p40 release in vitro (128) and systemic IL-12 p40 production in murine hosts (123). Differences in the potency of different microbial products to induce IL-12 p40 release by splenic macrophages were revealed by the comparison of stimulated vs. unstimulated release in medium alone (Table I). In cells from infected mice of either strain, LPS-induced p40 release was significantly higher than production in medium alone ($p < 0.01$). In cells from infected B6, but not A/J mice, PRBC-induced p40 release was also significantly greater than production in medium alone ($p < 0.01$). In contrast, small inert latex beads (1.2 μm diameter) were ineffective in inducing greater release of p40 over production in medium alone, consistent with studies by other investigators (128, 203).

In contrast with spontaneous production, LPS-induced p40 levels were significantly higher in splenic macrophage culture supernatants from infected versus uninfected mice of both strains ($p < 0.01$, Table I). However, LPS-induced p40 protein levels were not significantly different in culture supernatants of splenic macrophages obtained from infected B6 compared to A/J mice. Similar results were obtained when a range of doses of LPS, from 25 to 1000 ng/ml, were used (data not

shown). As seen with LPS, PRBC-induced p40 levels in culture supernatants of splenic macrophages were not significantly different between the two mouse strains (Table I).

In contrast to p40, LPS-induced p70 production by splenic macrophages in vitro was significantly greater in infected B6 compared to A/J mice ($p < 0.01$), whereas similar production was seen in cells from uninfected controls of either mouse strain (Fig. 3A). In addition, in B6, but not A/J mice, LPS-induced p70 production was significantly greater in splenic macrophages from infected versus uninfected controls ($p < 0.05$). Secretion of p70 in response to PRBC was also significantly higher in supernatants of splenic macrophages from infected versus uninfected B6 mice ($p < 0.01$) or compared to cells from infected A/J ($p < 0.01$) hosts (Fig 3B). Taken together, these data suggest that, following P. chabaudi AS infection, splenic macrophages from B6 compared with cells from A/J mice produce greater quantities of IL-12 p70, but not p40, both spontaneously and in response to stimulation with LPS or PRBC.

Kinetics of increases in spleen cellularity in vivo

In the results presented above, comparisons between resistant B6 and susceptible A/J hosts for IL-12 p40 and p70 synthesis by splenic macrophages were done on a per cell basis. However, following P. chabaudi AS infection, rapid and dramatic changes occurred in spleen cellularity of both mouse strains. These changes could impact significantly on total levels of IL-12 produced by splenic macrophages in vivo. In both mouse strains, spleen cell numbers were significantly higher on days 4 ($p < 0.05$ and $p < 0.01$ for A/J and B6, respectively) and 5 ($p < 0.01$ for both strains) post infection compared to uninfected controls on day 0 (Table II). However, spleen

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cell numbers were significantly higher in uninfected B6 compared to A/J mice ($p <$ 0.05), and during the first five days of $P.$ $chabaudi$ AS infection, with the exception of day 2. Unlike the total splenocyte numbers, the percentage of macrophages in the spleen was not significantly different between A/J and B6 mice before $P.$ $chabaudi$ AS infection. Following infection, the percentage of splenic macrophages was significantly higher in B6 mice on days 2-3 ($p < 0.001$) and 4-5 ($p < 0.01$). Thus, resistant B6 mice show earlier increases in the percentage of macrophages in the spleen. As a result of these differences between the strains, in terms of total spleen cell numbers and percentage of macrophages, the absolute number of splenic macrophages was significantly higher in B6 mice on all days examined.

DISCUSSION

The development of adaptive host responses to blood-stage malaria has been shown to be dependent on the timely activation of both cellular (31, 191, 202, 204) and humoral (19, 75) immune mechanism(s). It is thought that cell-mediated mechanism(s) can result in the activation of cells of the mononuclear phagocyte lineage that are capable of destroying $Plasmodia$-infected erythrocytes (205). In vivo depletion of monocyte/macrophages by treatment with silica or liposome-encapsulated muramyl dipeptide-glycerol dipalmitate demonstrated that these cells play a critical role in the elimination of $P.$ $chabaudi$ AS infection (205). The role of activated monocyte/macrophages in host resistance against blood-stage malaria may be mediated in part by the release of cytokines, most notably TNF-α, as well as reactive nitrogen and oxygen species. Our laboratory recently demonstrated that an early Th1-associated increase in TNF-α mRNA expression in the spleen correlates with resistance of B6 mice against $P.$ $chabaudi$ AS malaria (81). Moreover, IL-12-induced

protection of susceptible A/J mice was shown to occur by a mechanism involving IFN-γ, TNF-α and NO (16).

In the present study, we report significant differences between resistant B6 and susceptible A/J mice in splenic macrophage synthesis of biologically active IL-12 p70 during early blood-stage *P. chabaudi* AS infection. Compared to infected A/J hosts, splenic macrophages recovered from infected B6 mice produced significantly greater quantities of IL-12 p70 in vitro, both spontaneously and in response to LPS or malaria parasite stimulation. Importantly, greater release of p70 by splenic macrophages in infected B6 mice correlates with the development of early, predominantly Th1 responses and resistance of this host to this infection. In contrast, reduced p70 synthesis by splenic macrophages from infected A/J mice is consistent with the development of early, primarily Th2 responses and susceptibility to blood-stage malaria.

Since p70, but not p40, production by splenic macrophages was significantly different between the two mouse strains following *P. chabaudi* AS infection, our results suggest differences between the regulation of IL-12 p70 versus p40 secretion by splenic macrophages from the two mouse strains. This conclusion was suggested by the fact that while basal secretion of p40 could readily be detected in cells from uninfected mice of either strain, p70 production was undetectable. Moreover, following infection, substantial increases in p40 mRNA levels occurred in splenic macrophages from both mouse strains, yet these changes in p40 mRNA expression resulted in no significant increases in spontaneous release of p40 protein in vitro. The expression of p35 mRNA was constitutive in cells from uninfected mice of either strain. Following infection, p35 mRNA expression was up-regulated in cells from both strains. The suggestion that p70 secretion might be much more tightly regulated than p40 release is supported by earlier studies showing a tendency for p40 to be

produced in large excess over p70 levels (104, 107, 117). The essential difference in IL-12 release by splenic macrophages from susceptible A/J compared to resistant B6 mice appears to be reduced p70 synthesis in the presence of unimpaired p40 release. The capacity for similar production of p40 protein by splenic macrophages recovered from the two mouse strains acutely infected with *P. chabaudi* AS was demonstrated when cells were cultured in medium alone, or in the presence of LPS or PRBC. These results are in agreement with our recent findings on the differential production of IL-12 p70 versus p40 in vivo between the two mouse strains during early blood-stage malaria (Chapter 2). We found significantly greater levels of p70 in sera from infected B6 compared to A/J mice, whereas total serum p40 levels were comparable between the two mouse strains

In earlier studies addressing the role of IL-12 production during parasitic infections with *Leishmania major* (206), *Schistosoma mansoni* (207), *Toxoplasma gondii* (182), and *Listeria monocytogenes* (208), as well as in graft-versus host disease (194), p40 mRNA and protein production were used as markers of biologically active IL-12 p70 release. However, in recent years, the availability of reagents for specifically quantifying p70 levels has revealed important differences between p40 versus p70 release. For example, it was recently demonstrated that *Salmonella dublin* LPS stimulation induced substantial production of p40 by macrophages while p70 release was minimal (127). In the present study, we have analyzed production of both p40 and p70 by splenic macrophages, and to our knowledge, this is the first study to demonstrate significant differences between resistant and susceptible mouse strains in splenic macrophage IL-12 p70 release during early blood-stage malaria.

Comparisons between A/J and B6 mice for IL-12 production by splenic macrophages were done on a per cell basis. The percentage of macrophages in unfractionated spleen cells was not significantly different in uninfected controls of

either strain, even though B6 mice had higher numbers of splenocytes per spleen. Following *P. chabaudi* AS infection, the percentage of macrophages in the spleen was significantly higher in B6 compared to A/J mice on days 2-5, in agreement with our previous observation of a higher percentage of Mac-1+ cells in the spleens of B6 mice as detected by FACs analysis (55). A more severe depletion of marginal metallophilic macrophages in A/J versus B6 mice may be in part responsible for these differences (17). The recruitment of higher numbers of macrophages to the spleens of B6 mice could further augment the differences between the strains in splenic macrophage-derived IL-12 levels in vivo.

Taken together, the results of the present study suggest an important role of macrophage-derived IL-12 in host resistance to blood-stage *P. chabaudi* AS malaria. In addition to aiding in the destruction of *P. chabaudi* AS infected host erythrocytes, production of IL-12 by splenic macrophages may be crucial in shaping the development of an appropriate Th phenotype during early blood-stage malaria. In turn, which Th phenotype is activated has vital consequences for host resistance or susceptibility to this infection.

ACKNOWLEDGEMENTS

Figure 1. Ex vivo IL-12 p40 and p35 mRNA levels in splenic macrophages (pooled from 2-3 mice) recovered from uninfected or *P. chabaudi* AS infected A/J and B6 hosts during the first five days of infection. Cytokine mRNA levels were determined by RT-PCR followed by agarose gel electrophoresis, southern detection and autoradiography (*A*). The density of bands corresponding to p40 (*B*) or p35 (*C*) mRNA was normalized to those of the housekeeping gene G6PDH (G6). A representative result of three experiments is shown.

A

A/J

Days Post Infection
U 1 2 3 4 5

p40

p35

G6PDH

B6

Days Post Infection
U 1 2 3 4 5

p40

p35

G6PDH

B

p40/G6

0.5
0.4
0.3
0.2
0.1
0

A/J
B6

Days Post Infection
0 1 2 3 4 5

C

p35/G6

1.6
1.2
0.8
0.4
0

Days Post Infection
0 1 2 3 4 5
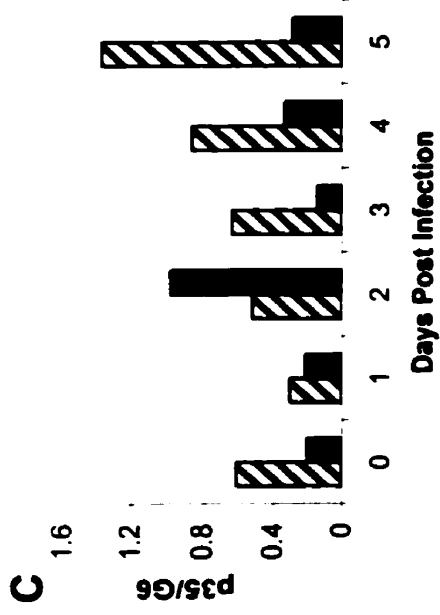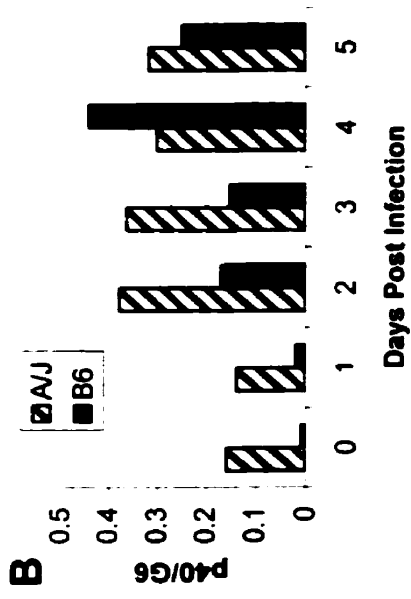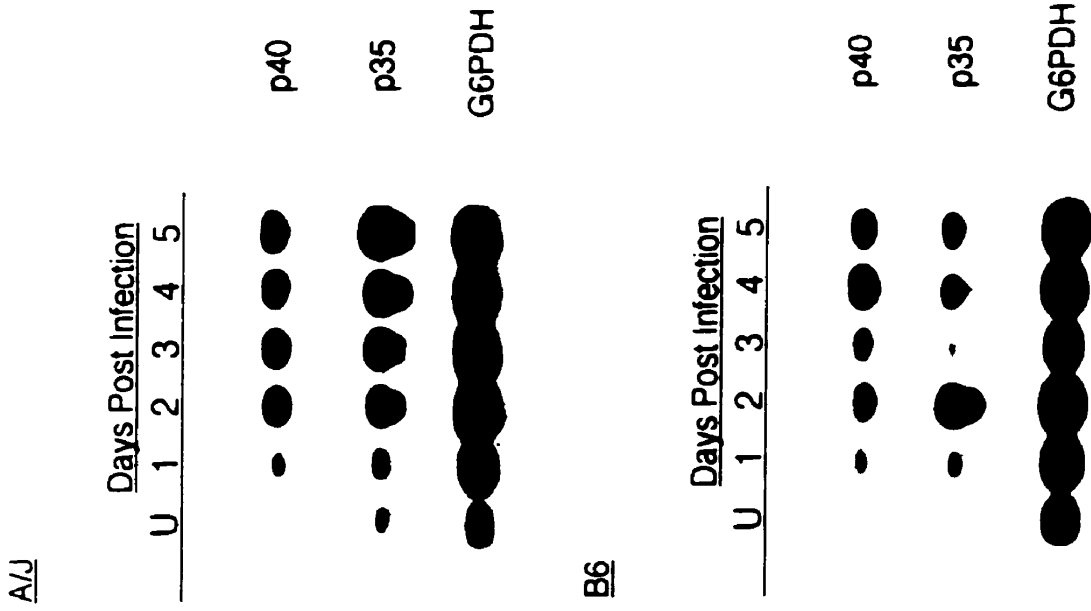
Figure 2. Spontaneous release of IL-12 p40 (*A*) and p70 (*B*) by splenic macrophages recovered from uninfected controls or day 4 *P. chabaudi* AS infected A/J and B6. Cells were cultured in the presence of medium alone for 24 h. IFN-γ production by unfractionated splenocytes following 48 h culture in the presence of medium alone or 10^6/ml of PRBC (*C*). IL-12 p40, p70 or IFN-γ levels culture supernatants were determined by ELISAs. Data are pooled from 2-3 replicate experiments and are presented as mean $\pm$ SEM (n = 4-15). Statistically significant differences shown are: *$p < 0.05$ vs. infected A/J mice. Abbreviation: N.D., not detectable.

A

IL-12 p40 (ng/ml)

A/J
B6

uninfected   Infected

B

IL-12 p70 (ng/ml)

ND   ND   *

uninfected   Infected

C

IFN-γ (ng/ml)

ND   ND   *

uninfected   Infected
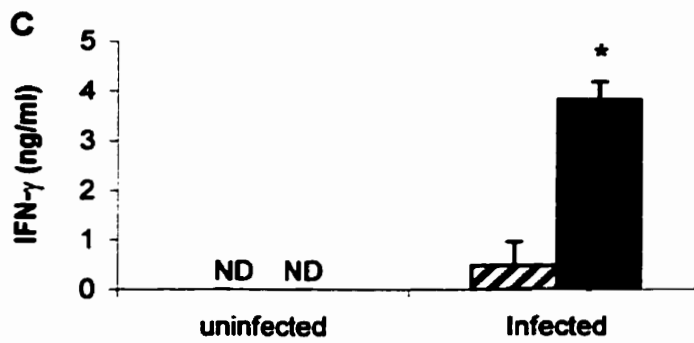
Figure 3. Stimulated release of IL-12 p70 in vitro by splenic macrophages recovered from uninfected controls or day 4 *P. chabaudi* AS infected A/J and B6 mice. Cells were cultured in the presence of 100 ng/ml of LPS (*A*) or 10^6/ml of PRBC (*B*). IL-12 p40 p70 levels in 24 h culture supernatants were determined ELISAs. Data are pooled from three replicate experiments and are presented as mean ± SEM (n = 6-8). Statistically significant differences shown are: *$p < 0.01$ vs. infected A/J mice, #$p < 0.05$ and ##$p < 0.01$ vs. uninfected B6 mice.

A

IL-12 p70 (ng/ml)

* #

☑ A/J
■ B6

uninfected   Infected

B

IL-12 p70 (ng/ml)

* ##

uninfected   Infected
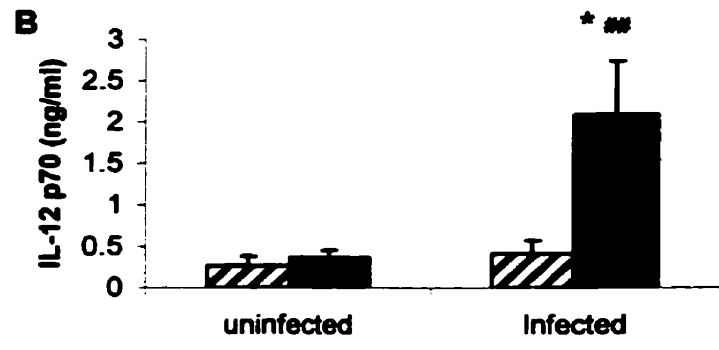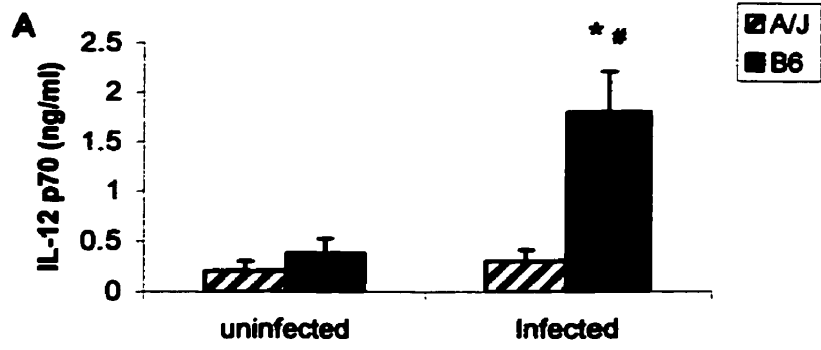
Table I

Effect of stimulation with microbial products on IL-12 p40

production by splenic macrophages[a]

Stimulus[b]	Concentration	IL-12 p40 (ng/ml)[c]			
		Normal A/J	Normal B6	Infected A/J	Infected B6
Medium	N/A.	1.6 ± 0.4	1.6 ± 0.2	2.2 ± 0.2	1.9 ± 0.3
Latex beads	0.01% aq. susp.	2.5 ± 0.7	1.7 ± 0.6	1.7 ± 0.4	2.0 ± 1.1
LPS	100 ng/ml	3.2 ± 0.3	2.2 ± 0.2	$6.6 \pm 1.3^{\#\dagger}$	$4.7 \pm 1.0^{\#\dagger}$
PRBC	10^6/ml	2.6 ± 0.9	1.7 ± 0.6	2.4 ± 0.8	$4.1 \pm 0.1^{\dagger}$

[a]Splenic macrophages were recovered from either normal or day 4 *P. chabaudi* AS infected mice. Splenic macrophages were prepared as described in 'Materials and Methods'.

[b]Splenic macrophages were cultured for 24 h in medium with the indicated concentrations of various microbial products. Abbreviations: aq. susp., aqueous suspension; N/A, not applicable.

[c]IL-12 p40 (ng/ml) levels in 24 h supernatants were analyzed by ELISA. Data are pooled from two replicate experiments and are presented as mean ± SEM, n=3-15.

[d]Statistically significant differences shown are: [†] vs. spontaneous release in medium alone, [#] vs. uninfected mice of the same strain.

Table II

Changes in percentage and number of macrophages in the spleen during *P. chabaudi* AS infection

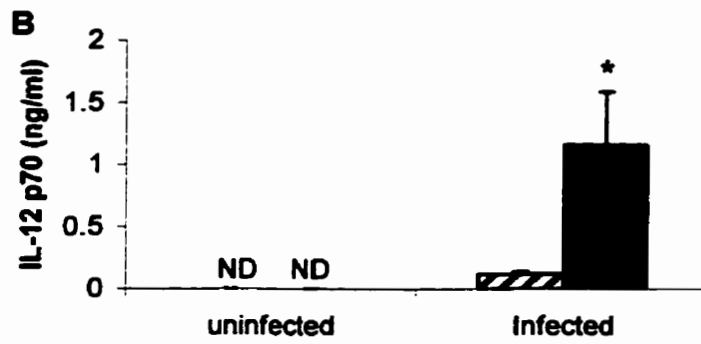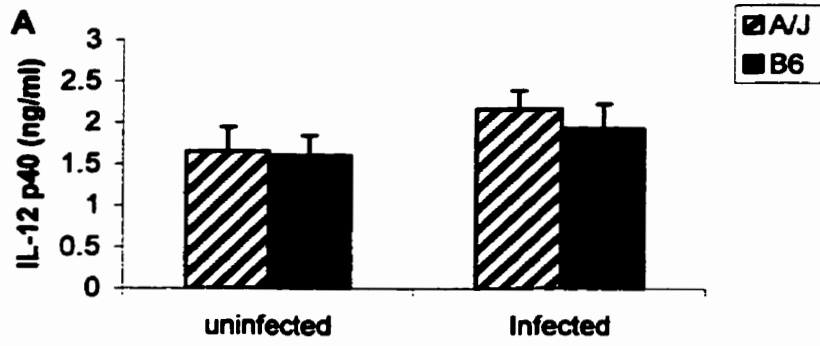| Days Post Infection	Total splenocytes[a]/spleen (× 10^7)		Number of macrophages[b] (× 10^6)	
	A/J	B6	A/J	B6
Uninfected	6.3 ± 0.4	$9.5 \pm 0.4^*$	6.0 ± 0.4	$10.5 \pm 0.8^*$
	(9.3 ± 0.4)	(10.5 ± 0.6)		
1	5.9 ± 0.7	$11.6 \pm 0.7^*$	5.1 ± 1.0	$16.3 \pm 0.9^{*\dagger}$
	(9.4 ± 0.9)	(11.4 ± 1.1)		
2	7.1 ± 0.8	9.6 ± 1.8	5.5 ± 0.9	$13.5 \pm 3.6^*$
	(9.2 ± 0.4)	$(14.7 \pm 1.0)^{*\dagger}$		
3	6.9 ± 0.9	$12.5 \pm 0.7^*$	7.6 ± 1.2	$20.0 \pm 2.8^{*\dagger}$
	(10.9 ± 0.6)	$(17.5 \pm 1.0)^{*\dagger}$		
4	$10.0 \pm 1.2^{\dagger}$	$18.1 \pm 2.8^{*\dagger}$	$13.6 \pm 1.7^{\dagger}$	$27.5 \pm 4.6^{*\dagger}$
	$(14.9 \pm 0.7)^{\dagger}$	$(18.3 \pm 0.7)^{*\dagger}$		
5	$15.7 \pm 1.7^{\dagger}$	$24.3 \pm 1.8^{*\dagger}$	$26.4 \pm 2.8^{\dagger}$	$53.2 \pm 2.6^{*\dagger}$
	$(17.1 \pm 1.2)^{\dagger}$	$(22.1 \pm 1.0)^{*\dagger}$		

[a]Single cell suspensions of spleen cells from normal or *P. chabaudi* AS infected mice were prepared and total splenocyte numbers and percentage (indicated in parentheses) of macrophages were determined as described in 'Material and Methods'. Values shown represent mean ± SEM of 3-15 mice analyzed individually.

[b]Number of splenic macrophages per spleen was determined on the basis of the total splenocytes per spleen and percentage of macrophages.

[c]Statistically significant differences shown are: * A/J vs. B6 mice; † vs. uninfected controls.

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Chapter Four

Low Dose Interleukin 12 and Chloroquine Combined Therapy Completely Cures
Blood-Stage Malaria, Prevents Severe Anemia and Induces Reinfection Immunity

Karkada Mohan, Hakeem Sam and Mary M. Stevenson

Centre for the Study of Host Resistance, McGill University and the Montreal General
Hospital Research Institute, Montreal, PQ, H3G 1A4, Canada

Running Title: IL-12 and chloroquine combined therapy for malaria

PREFACE

The studies presented in Chapters 2-3 are based on the report by our laboratory that exogenous IL-12 treatment, given daily during the first five days of infection, could protect susceptible A/J mice from the lethal effects of *P. chabaudi* AS malaria. The dose-dependent nature of IL-12-induced protection was evident in the efficacy as well as toxic effects of this molecule. A standard therapeutic dose of 0.1 μg/day of rIL-12 was found to be effective. To be effective, however, treatment had to be initiated on or a day prior to infection. In the studies presented in this chapter, a novel therapeutic strategy that combines 0.05 μg/day of rIL-12 with half the standard dose of the anti-malarial drug chloroquine is described. This strategy allows treatment to be initiated on day 3, by which time infection is established, and the total time for treatment is reduced from 6 to 4 days. The cytokine or cytokine receptor-mediated mechanism(s) of protection are addressed.

ABSTRACT

The immunoregulatory cytokine interleukin (IL)-12 induces host resistance against experimental malaria. In this study, we tested the feasibility of using IL-12 in combination with chloroquine (CQ) to rescue susceptible A/J mice from lethal blood-stage *Plasmodium chabaudi* AS infection. Combined treatment with low doses of CQ and IL-12 resulted in >15 fold reduction in the parasite load and 100% survival rate of A/J mice with established infections. Compared to control mice which succumbed to severe anemia, CQ+IL-12 treated mice had significantly higher early and late stage erythroid progenitors in the bone marrow and spleen, resulting in significantly higher hematocrits, RBC counts and percentage reticulocytes. Production of parasite-specific IFN-γ by splenocytes from these mice was up-regulated >20 fold relative to controls in parallel with enhanced IFN-γ mRNA expression. Further, enhanced responsiveness to IL-12 and increased downstream IFN-γ production in CQ+IL-12 treated mice was evident from increased mRNA expression for $\beta 1$ and $\beta 2$ subunits of IL-12R in the splenocytes. Moreover, this combined therapy induced higher levels of anti-malaria antibodies as well as sterile immunity against reinfection. Because IL-12 can be used at low doses, and is effective even in established infections, it may be feasible to use this immuno-chemotherapeutic approach in human malaria.

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INTRODUCTION

Malaria remains a major public health problem in most tropical countries, particularly sub-Saharan Africa. It has been estimated that between 300-500 million individuals are infected annually and between 1.5 and 2.7 million people die of this disease every year (1). Despite decades of frustrating research, an effective vaccine against this deadly disease is still not a reality (1, 209). In the meantime, however, we must rely on effective therapeutic strategies for treating acute infections in order to prevent malaria-associated complications and mortality, especially those due to *Plasmodium falciparum*. Chloroquine (CQ) has been both an affordable and well-tolerated drug for use in third world countries but this drug now faces severe limitations because of the wide-spread emergence of CQ-resistant *P. falciparum* strains, and, more recently, *P. vivax* strains (210, 211). To overcome this problem, different combinations of anti-malarial drugs have been utilized, but in most instances, multidrug resistant *P. falciparum* strains have emerged (212). Thus, efforts are intense to find an effective method to successfully treat acute malaria infections.

IL-12, a potent immunomodulatory cytokine, has been proven to be effective in conferring protection against bacterial, viral and intracellular parasitic infections (213, 214). This pleiotropic cytokine not only enhances cell mediated immune responses but also influences humoral immunity by inducing isotype switching through both IFN-γ-dependent and -independent mechanisms (215). IL-12 also appears to stimulate enhanced antibody production in switched B cells (215). In malaria, both mice and non-human primates can be protected against pre-erythrocytic infections following IL-12 treatment (186, 187). Our laboratory has demonstrated the effectiveness of IL-12 in inducing protective immunity against blood-stage infection in the murine model of *P. chabaudi* AS (16). In addition to its NK cell activating,

IFN-γ stimulatory, and Th1 polarizing effects early during *P. chabaudi* AS blood-stage infection, IL-12 also induces remarkable up-regulation of splenic erythropoiesis, thereby preventing fatal anemia associated with this infection (16, 55, 188). However, the dose of IL-12 appears to be critical given the potential toxic effects of this molecule (187, 189).

Although IL-12 can induce protective Th1 type immunity against experimental malaria infections, its therapeutic value is limited given the need to begin treatment prior to or on the day of establishing infection (16, 186, 187). The main goal of this study was to improve the efficacy of IL-12 treatment, especially in terms of its efficacy in established infections. This study examines the possibility of using IL-12 as a therapeutic agent, in combination with CQ, for treating established *P. chabaudi* AS infection in susceptible A/J mice. Our findings demonstrate that low dose CQ+IL-12 treatment of mice with established blood-stage infection, induced a protective Th1 immune response, and efficient up-regulation of erythropoiesis during primary infection and higher anti-malaria antibody production following reinfection.

MATERIALS AND METHODS

Mice, Parasites and Infection Protocol. Male A/J mice, 8-12 week old, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were infected i.p. with 10^6 *P. chabaudi* AS parasitized red blood cells (PRBC) in pyrogen-free saline, and parasitemia and survival rate were monitored as described previously (16). To assess reinfection immunity, mice were challenged with the same dose of parasites 4 weeks after recovery from primary infection and parasitemia monitored for 2 weeks.

IL-12 and CQ Treatment. Murine rIL-12 was a gift from Dr. S. Wolf, Genetics Institute (Cambridge, MA). Chloroquine diphosphate was purchased from Sigma (St.

Louis, MO). To establish an optimal subcurative dose of CQ, mice were treated orally with 25 mg/kg body wt CQ, the therapeutic dose, or 12.5 and 6.25 mg/kg, divided according to the WHO recommendation (216). For the therapeutic dose, an initial dose of 10 mg/kg was given on day 3 post infection (p.i.) followed by 5 mg/kg at 6, 24 and 48 h. For 12.5 and 6.25 mg/kg, the dose of CQ used was half and one quarter of the therapeutic dose, respectively, at each treatment time point. Since our intention was to use lower doses of IL-12 in combination with a subcurative dose of CQ, the dose of IL-12 was decreased from our previously reported (16) protective dose of 0.6 μg/mouse (0.1 μg per day for 6 days) administered i.p. starting on the day of infection, to 0.3 μg/mouse (0.05 μg for 6 days). Further, to test the protective efficacy of IL-12 in an established infection in combination with a subcurative dose of CQ, the dose of IL-12 was further decreased to 0.2 μg/mouse (0.05 μg per day for 4 days) and treatment started on day 3 p.i., once parasitemia was established at 0.5 to 1 percent. Infected mice receiving CQ alone, IL-12 alone or CQ plus IL-12 (CQ+IL-12) combinations were monitored daily for parasitemia and survival.

Hematology and Erythropoietic Progenitor Assays. Hematocrit, total red cell counts and percentage reticulocytes were determined on heparinized blood from individual mice using standard hematological procedures. Single cell suspensions were obtained from bone marrow and spleen and the number of erythrocyte burst (BFU-E) and colony forming units (CFU-E) were determined in colony forming assays using methylcellulose semisolid media in IMDM as previously described (188). The number of erythrocyte progenitor colonies were counted after 48 h for CFU-E and after 7 d for BFU-E and data are presented as mean ± SEM per organ. The number of BFU-E in the peripheral blood was determined using blood mononuclear cells separated by density gradient and are expressed as mean ± SEM per 5×10^6 total cells.

Quantitation of IFN-γ and Anti-malaria Antibodies. Single cell suspensions of unfractionated spleen cells were plated at 2×10^6 cells per well and incubated for 48 h with either medium alone, 5 μg/ml Con A or *P. chabaudi* AS Ag (mpAg) equivalent to 2×10^6 PRBC. A two-site sandwich ELISA was used to measure IFN-γ in the culture supernatants as described earlier (16). Malaria-specific, total IgG in sera obtained two wks after reinfection was determined by ELISA. Total anti-malaria IgG was captured using soluble mpAg coated plates and the level of malaria-specific IgG in the sera was estimated using goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) and 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] substrate (Boehringer Mannheim Canada, Laval, PQ). The levels of Ab are expressed as OD at 405 nm. Sera from uninfected, normal A/J mice served as negative controls.

RT-PCR for IFN-γ, IL-12Rβ1 and IL-12Rβ2 mRNA Expression. Reverse transcriptase PCR was performed essentially as described previously to determine relative quantities of mRNA for IFN-γ and the two IL-12 receptors, β1 and β2 (194). The primers and probe used for IFN-γ have been described (195). For IL-12R subunits, primers and probes were designed based on the recently cloned cDNA sequences, GeneBank accession numbers U23922 and U64199, for the β1 and β2 subunits, respectively (137, 138). The sequence of primers and probes used were: IL-12Rβ1, sense primer, 5'-TGA-AGA-CGG-CGC-GTG-GGA-GTC-A-3'; antisense primer, 5'-TCG-CGG-GTA-CAA-CAC-CTC-CGG-G-3'; probe, 5'-GCG-AGC-GGA-CAC-TGC-GAG-CG-3' (product size, 412 bp); IL-12Rβ2, sense primer, 5'-GGT-TGC-TGG-CTC-CTC-ACC-AGG-3'; antisense primer, 5'-ATG-CAG-CCC-CTT-TGC-TCC-GGG-3'; probe, 5'-TCC-CCC-ACA-CTG-GCT-GCG-GA-3' (product size, 424 bp). Both positive and negative controls were included in each assay to ensure efficacy of the reaction and to rule out possible genomic DNA contamination.

The primers and probe for G-6-PDH, the housekeeping gene used in this study, have been described previously (194). For RT-PCR, 1 µg total RNA, isolated from unfractionated spleen cells using TRIzol reagent (Gibco BRL, Grand Island, NY), was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco). The reaction mixture was diluted 1:8 and 10 µl were used for PCR amplification of IFN-γ (30 cycles), IL-12 receptor subunits β1/β2 mRNA (30 cycles) and G-6-PDH (26 cycles) using Taq DNA polymerase (Gibco). Following electrophoresis and Southern transfer onto nylon membranes (Hybond-N; Amersham, Arlington Heights, IL), PCR products were hybridized with internal cytokine-specific oligonucleotide probes labeled with {^{32}P}ATP and visualized by autoradiography. The intensity of bands corresponding to specific cytokine/cytokine receptors was analyzed by high-resolution optical densitometry (SciScan; United States Biochemical, Cleveland, OH) and normalized to those of G-6-PDH.

RESULTS AND DISCUSSION

Effect of combined CQ+IL-12 treatment on the course of infection and survival.

To determine a subcurative dose of CQ suitable for use in combination with IL-12 in later experiments, we first performed a CQ dose response study in *P. chabaudi* AS infected A/J mice. As demonstrated previously by our laboratory, mice of this strain are extremely susceptible to *P. chabaudi* AS infection and experience fulminant parasitemia and severe anemia with 100% mortality within a few days of peak parasitemia (16). Mice were treated beginning on day 3 p.i. with the curative dose of 25 mg/kg body wt CQ or with two subcurative doses of 12.5 and 6.25 mg/kg. Since the parasite strain used is CQ sensitive, as expected, the curative dose of CQ did not allow the parasitemia to exceed 0.5% and no parasites were detectable from day 7

through day 28 p.i. (Fig. 1A). Untreated mice showed high parasitemia of more than 50% and all the mice succumbed to infection by day 11 p.i. (Fig. 1C). Similarly, mice given 6.25 mg/kg body wt CQ developed mean peak parasitemia in excess of 40% and all the mice in this group succumbed to infection. On the other hand, mice receiving half the curative dose, that is, 12.5 mg/kg CQ showed a modest reduction in peak parasitemia to around 20% and the mortality rate was about 30%. We selected this latter dose of CQ to be used in combination with IL-12 in order to explore the possibility of reducing parasite burden and rescuing all of the infected animals.

In the preliminary experiments, we identified the optimum dose of IL-12 suitable for use with half the therapeutic dose of CQ. Initially, 0.05 µg/mouse IL-12 treatment was given for six days, starting on the day of infection (a total of 0.3 µg/mouse). Using this combination therapy, it was possible to reduce the peak parasitemia to about 3 percent and all the mice survived infection (data not shown), while the mice receiving 0.3 µg IL-12 alone did not survive infection, suggesting the need for combined treatment for survival. Since our aim was to test the efficacy of CQ+IL-12 treatment combination in established infection, experiments were conducted using 0.05 µg/mouse/day of IL-12, starting on day 3 p.i., and continued until day 6 p.i., so as to deliver a total IL-12 dose of 0.2 µg/mouse along with 12.5 mg/kg of CQ. Untreated mice, mice treated with CQ or IL-12 alone, and CQ+IL-12 treated mice had an initial mean parasitemia of 0.6 ± 0.08% (range 0.3-1.6%) on day 3 p.i., before treatment was initiated. As shown in Fig. 1B, following CQ+IL-12 treatment, parasite proliferation was markedly suppressed and the mean peak parasitemia was only 3.1% and was reached only on day 10 p.i. In contrast, untreated mice had a mean peak parasitemia of 63.5% by day 7 p.i. and all the mice were dead by day 11 p.i. (Fig. 1C). Untreated mice appeared severely anemic, hypothermic and were very lethargic for 3 to 4 days prior to death. Similarly, mice given a total dose of

0.2 µg IL-12 alone had a mean peak parasitemia of >60% by day 7 p.i. and all the mice succumbed to infection by day 9 p.i. (Fig. 1, B and C). Mice receiving 12.5 mg/kg body wt CQ alone had a mean parasitemia of 33.5% by day 9 p.i. (Fig. 1B), showed a survival rate of 70% (Fig. 1C) and were also lethargic, although these mice did not show signs of severe anemia or hypothermia. Parasites were detectable in these mice most of the time during the first four wks of infection, with a recrudescent parasitemia on day 21 p.i. (Fig 1B).

We also tested the curative efficacy of combining 6.25 mg/kg body wt CQ with or without a total IL-12 dose of 0.2 µg/mouse (day 3 through day 6 p.i.). These mice developed severe infection, anemia and succumbed to infection during the second week of infection (data not shown). In contrast to mice receiving lower doses of CQ and IL-12, either alone or in combination, mice on combined therapy with 12.5 mg/kg body wt CQ and 0.2 µg/mouse total dose of IL-12 remained healthy and active throughout the course of infection. Parasites were not detectable after day 12 p.i., and all the mice survived infection in repeated experiments (Fig.1, B and C). Thus, this dose of IL-12, in combination with half the curative dose of CQ, is as efficient as using 0.3 µg/mouse of IL-12, and is effective even in established infections. Moreover, this dose also represents the critical curative level required in combined therapy, since lowering the total dose of IL-12 further to 0.15 µg results in higher parasite burden (>30%), even when combined with half the curative dose of CQ (data not shown). Because a total IL-12 dose of 0.2 µg/mouse given along with 12.5 mg/kg CQ markedly reduces the parasite load and rescues 100% of infected mice, we consider this combination therapy to be optimal for successful treatment of established P. chabaudi AS infection.

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Effect of combination therapy on the development of malarial anemia.

Having established the protective efficacy of 12.5 mg/kg CQ in combination with 0.2 μg IL-12, we explored the possible mechanism(s) involved. *P. chabaudi* AS susceptible A/J mice suffer from severe anemia and shock prior to death following infection (217). We previously showed that severe anemia is a main factor contributing to mortality since blood transfusion given to these mice after the development of peak parasitemia rescued up to 90% of the mice (217). Our recent study examining the erythropoietic role of IL-12 in infected A/J mice showed that 0.1 μg IL-12 treatment for 6 days, starting on the day of infection, results in more than a seven-fold increase in splenic erythropoiesis (188). Here, we examined mice receiving CQ+IL-12 therapy, using a substantially lower dose of IL-12 in an established infection, for possible inhibition of infection-induced development of anemia. The peripheral blood picture of CQ+IL-12 treated animals was compared with that of untreated mice and mice receiving CQ or IL-12 alone. As shown in Table 1, mice receiving combined therapy had a significantly higher hematocrit and total RBC count at day 7 p.i. compared to untreated or mice treated with 12.5 mg/kg CQ or 0.2 μg IL-12 alone. Signs of severe anemia were apparent in untreated or IL-12 treated mice while mice treated with 12.5 mg/kg CQ retained hematocrit and RBC counts at significantly higher levels compared to untreated mice. However, mice in the combined treatment group alone had significantly higher levels of both hematocrit and RBC counts after 7 days of infection compared to controls and showed no signs of anemia.

It is possible that since the mice receiving optimum combined therapy had >15 fold less parasitemia, the absence of anemia could be due to decreased direct red cell destruction by the developing parasites. On the other hand, it is also possible that the extent of up-regulation of erythropoiesis following infection could have been of

several orders of magnitude higher in the mice receiving combined therapy. To address this question, we first examined the percentage of reticulocytes in the peripheral blood. On day 7 p.i., 12.5 mg/kg CQ treated mice, with or without IL-12 treatment, showed significantly higher percentages of reticulocytes than untreated mice (Table I). However, mice treated with IL-12 alone failed to show significantly higher percentage reticulocytes compared to untreated mice. Since reticulocytosis is apparent during second and third wk of infection in *P. chabaudi* AS resistant C57BL/6 mice (10), we also examined blood films of surviving mice at day 10 p.i. At this time, A/J mice receiving CQ+IL-12 treatment had more than a two fold increase in the percentage of reticulocytes as compared to mice receiving 12.5 mg/kg CQ alone.

Next, we examined the effect of CQ and IL-12 treatment on erythropoiesis by determining the number of erythroid progenitors in the bone marrow, spleen and blood. Treatment of infected A/J mice with 0.05 μg IL-12/mouse for 4 days, starting on day 3 p.i., along with half the curative dose of CQ significantly enhanced bone marrow and splenic erythropoiesis when compared to mice which were either untreated or treated with CQ or IL-12 alone (Table II). A marked increase was seen in the extent of splenic erythropoiesis in terms of both BFU-E and CFU-E. Mice receiving the combined treatment had a nearly two-fold increase in the numbers of splenic BFU-E and more than a three-fold increase in splenic CFU-E numbers compared to untreated animals or mice treated with CQ alone. Mice treated with 12.5 mg/kg CQ alone showed a small but significant increase only in the bone marrow CFU-E compartment. However, mice receiving IL-12 monotherapy had significantly enhanced CFU-E numbers in bone marrow and spleen, and higher BFU-E in the spleen compared to untreated controls.

Our earlier study showed that IL-12 treatment of normal, but not infected, A/J mice results in bone marrow suppression (188), possibly due to the ability of this

cytokine to enhance mobilization of bone marrow precursors to the spleen (218). These studies suggest a role for IL-12 in enhancing extramedullary erythropoiesis, especially in the spleen. To examine the mobilization of bone marrow erythroid precursors to the spleen following low dose CQ+IL-12 treatment, we investigated the frequency of BFU-E in the peripheral blood. We observed a nearly three fold increase in the blood BFU-E numbers in mice treated with CQ+IL-12 compared to untreated mice and nearly a two fold increase compared to mice receiving either CQ or IL-12 alone (Table II).

It is interesting that even though the dose of IL-12 used was similar in mice receiving either 12.5 or 6.25 mg/kg CQ, the extent of erythropoiesis was higher in the former group of mice which had a lower parasite load. This indirectly suggests that high parasitemia may in fact inhibit efficient erythropoietic up-regulation, possibly due to impaired levels of other hematopoietic co-factors such as IL-3, IL-6, IL-11 and steel factor in mice with high parasite burdens. Thus, the use of IL-12 treatment along with half the curative dose of CQ not only suppresses parasitemia and limits the extent of direct red cell destruction by the parasites, but also initiates a more efficient erythropoietic response. The erythropoietic stimulatory effect of IL-12 is not restricted to the combination of CQ+IL-12, since we observed similarly enhanced red cell genesis when IL-12 was used in combination with clindamycin to treat infected A/J mice (unpublished observations). This finding raises the possibility of using IL-12 in combination with other anti-malarial drugs in order to prevent fatal anemia. In human situations, this is particularly important in areas with widespread CQ resistance where one has to rely on other anti-malaria drugs for the treatment of acute infections. However, this strategy should be tested in infections due to drug-resistant parasite strains because such strains are usually resistant to higher than the therapeutic doses of the drugs.

Development of a novel therapeutic strategy to prevent malarial anemia is critical considering the importance of anemia in malaria morbidity and mortality. Studies in Gambian children with severe *P. falciparum* malaria and presenting with severe anemia showed marked dyserythropoietic changes, including erythroblast multinuclearity, karyorrhexis, incomplete and unequal mitosis, and cytoplasmic bridging (219). While blood transfusion has been proven to be beneficial in correcting malarial anemia (220), the risks involved are considerable, especially due to high levels of HIV infection in many malaria-endemic populations. This calls for a method of treatment, which can correct hyperparasitemia as well as infection-induced anemia, without the need for transfusion. The combination therapy used in this study appears to be promising in this direction. Endogenous up-regulation of erythropoiesis enhances reticulocyte numbers in the peripheral blood and prevents the risk of prolonged parasite patency observed following transfusion (217), since reticulocytes are refractory to infection by most normophilic malaria parasites.

Combination therapy significantly up-regulates parasite-specific IFN-γ production and IL-12R expression.

We next examined the level of IFN-γ production by splenocytes, both in terms of protein and gene expression, following combination therapy of infected A/J mice. We have previously demonstrated the essential role of IFN-γ in the protective host response against *P. chabaudi* AS in resistant C57BL/6 mice (43, 55, 81) as well as in IL-12 treated, susceptible A/J mice (16, 55). Anti-IFN-γ treatment of IL-12-treated A/J mice resulted in a significant increase in parasite load and high mortality (16). Our recent study also suggested that a defect in the production of IFN-γ by NK cells early during infection contributes to the extreme susceptibility of A/J mice to blood-

stage malaria (55). This finding is consistent with an earlier observation in resistant C57BL/6 mice that tissue specific expression of IFN-γ mRNA in the spleen is significantly higher by day 3 p.i. compared to A/J mice and higher expression of this cytokine mRNA persists for one wk post infection (81). Furthermore, both in vivo IL-12 treatment of A/J mice and in vitro IL-12 supplementation of enriched NK cell cultures from infected A/J mice significantly enhances the levels of spontaneous NK cell derived IFN-γ production (55). To achieve a protective response in A/J mice, our previous study utilized 0.1 μg IL-12/mouse/day for six days starting on the day of infection for a total dose of 0.6 μg IL-12 (16), while the present study used 0.05 μg IL-12 daily for only four days starting on day 3 p.i., a total dose of 0.2 μg IL-12, in combination with CQ, once the parasitemia was established between 0.5 to 1.0 percent. Hence, we asked whether inclusion of low, sub-protective doses of IL-12 in combined treatment influences the immune response in A/J mice by inducing a protective Th1 response. In response to non-specific stimulation with Con A, splenocytes from untreated mice, mice treated with CQ or IL-12 alone or those receiving CQ+IL-12 combined treatment secreted comparable amounts of IFN-γ on day 7 p.i. (Fig. 2A). In contrast, spleen cells from mice treated with both CQ and IL-12 secreted significantly higher levels of IFN-γ in response to specific stimulation with mpAg compared to untreated mice or mice treated with CQ or IL-12 alone. Following mpAg stimulation, splenocytes from mice receiving IL-12 alone produced more than four fold higher IFN-γ compared to untreated or CQ treated mice. The magnitude of up-regulation of IFN-γ in mice treated with CQ+IL-12 was more than 20 fold higher than in untreated or CQ treated groups of mice, and was nearly five fold higher than in mice given IL-12 alone. In parallel, on day 7 p.i., IFN-γ mRNA expression was also up-regulated in mice receiving combined treatment (Fig. 2B). This suggests the development of efficient anti-parasitic immunity in mice given combination therapy

during the early phase of infection by an enhanced IFN-γ production. Further, this finding has an important bearing on the development of the adaptive immune response, since it has been shown that during murine *Leishmania major* infection, IFN-γ, along with IL-12, acts as an essential co-factor in the development of parasite specific, Th1 type protective immunity (41).

Responsiveness to IL-12 and subsequent downstream signal transduction events, IFN-γ production and Th cell development has recently been shown to be dependent on the levels of IL-12Rβ1 and IL-12Rβ2 expression on the responder cells (197, 221). Co-expression of these two IL-12R subunits results in the expression of both high and low affinity IL-12 binding sites and confers IL-12 responsiveness in transfected cells (138). Hence, we questioned whether enhanced IFN-γ production and development of a protective Th1 immune response in mice receiving combined therapy correlate with enhanced IL-12R expression. For this, we analyzed the expression of β1 and β2 IL-12R subunit mRNA expression in splenocytes from day 7 infected mice given CQ+IL-12 combined treatment. Compared to untreated mice, CQ+IL-12 treated mice had a higher expression of both IL-12Rβ1 and β2 (Fig. 2*B*), indicating a role for IL-12 in up-regulating its own receptors on responder cells during *P. chabaudi* AS infection. Higher levels of IL-12R expression on responder cells may explain why spleen cells from mice receiving combined treatment produced significantly higher levels of IFN-γ than cells from control mice, following specific Ag stimulation. We have recently observed that on day 7 p.i. spleens from IL-12 treated, infected A/J mice contain more than four fold higher absolute numbers of asialo GM1$^+$ NK cells and more than two fold higher numbers of B cells compared to uninfected mice (55). While NK cells expressing increased IL-12R could contribute to higher levels of protective IFN-γ secreted during the early course of infection, IL-12 may also influence B cell production of parasite-

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specific Ab. To test this latter possibility, we quantitated anti-malaria Ab levels as well as the development of immunity against reinfection in the surviving A/J mice in CQ treated and CQ+IL-12 treated groups.

CQ+IL-12 treatment results in higher anti-malaria Ab and protects against reinfection.

Mice surviving primary infection were reinfected after 4 weeks of parasite clearance. The course of infection was monitored and sera were collected 14 days after reinfection. Anti-malaria Ab in terms of total serum IgG was found to be significantly higher in mice receiving combined therapy than those treated with CQ alone (Fig. 3A). High anti-malaria Ab levels correlated with protection since reinfection of mice treated with CQ+IL-12 during primary infection showed sterile immunity while low grade parasitemia was observed in mice given CQ alone (Fig. 3B).

Our present understanding of immunity against blood-stage *P. chabaudi* AS infection in resistant mice is that control of primary infection is dependent on an early Th1 response involving NK cell and macrophage activation, followed by a Th2 response involving protective Ab during the chronic phase (16, 43, 55, 81, 204), which eventually clears parasites from the circulation. The present study revealed that IL-12 used in the combined therapy of A/J mice, in addition to providing protection against acute infection, induces better Ab responses during reinfection. Although both cell- and antibody-mediated immune mechanisms are believed to be important in protection against malaria, the results of the present study do not distinguish between the relative importance of the two arms of the immune system, since we had to evaluate IFN-γ and antibody production at different phases of infection. In practice, unlike murine malaria, 'semi-immune' or non-immune humans are vulnerable to reinfection after

successful treatment of primary infection using anti-malaria drugs. By using CQ+IL-12 combination treatment, it may be possible not only to reduce primary parasite load and the associated complications, but also to induce a protective antibody response and reinfection immunity. Our findings raise the question regarding the role of IL-12 and IFN-γ in influencing antibody responses during human malaria infections where long lasting immunity in endemic populations is primarily associated with anti-malaria Ab levels (222).

In malaria endemic areas, the use of anti-malarial drugs has been observed to have an inhibitory effect on the acquisition of anti-malaria Ab, both in children and, more particularly, in adults on chemoprophylaxis (222). It is not clear yet whether this is due to direct immunosuppressive effects of drugs like CQ or due to decreased exposure to malaria parasite antigens. In the present study, we observed lower levels of expression of both IFN-γ and IL-12R mRNA in splenocytes of mice treated with CQ alone compared to untreated mice. These mice had lower anti-malaria antibodies than CQ+IL-12 treated mice, although it was not possible to compare the antibody levels of mice treated with CQ alone with those of untreated mice which did not survive till our reinfection studies. However, the issue of possible immunosuppression by CQ could be resolved by studying the effect of CQ treatment in *P. chabaudi* AS resistant C57BL/6 mice. In any event, as shown by the present study, any immunosuppressive effects of CQ can be effectively overcome by using CQ+IL-12 combined treatment, which resulted in marked up-regulation of IFN-γ and IL-12R mRNA levels as well as that of anti-malaria antibody levels.

IL-12, in combination with amphotericin B, has been used as immunotherapy in *Histoplasma capsulatum*-infected SCID mice (223) and more recently, in combination with antibiotics, for bacterial clearance in *Mycobacterium avium*-infected SCID mice (224). Furthermore, successful therapy of chronic,

nonhealing murine cutaneous leishmaniasis with the combination of sodium stibogluconate and IFN-γ was found to be dependent on continued production of IL-12 (225). Cure from established *Leishmania major* infection in mice following combined therapy with Pentostam and IL-12 involves a switch from a Th2 to a Th1 type immune response (226). However, in this study, IL-12 alone appeared unable to enhance Th1 cell expansion in vivo, which was thought to be due to high parasite loads in mice receiving only IL-12 compared to mice given combined treatment with the drug and IL-12. Similarly, A/J mice could be rescued from lethal malaria using IL-12 when the treatment is given on the day of infection (16), but not after the infection is established (unpublished observations). In contrast, as observed in the present study, IL-12-induced development of an early Th1 response appears possible even in established infections when CQ is given along with IL-12 to substantially reduce the parasite load. Moreover, in this treatment regime, doses of both CQ and IL-12 could be reduced to one half and one third, respectively, compared to the doses required to induce parasite suppression when either of these is used alone. In particular, cure of established infections with this combined treatment and the feasibility of using IL-12 at low, possibly non-toxic, doses suggest the usefulness of IL-12 in combination with anti-malarials in treating human malaria.

ACKNOWLEDGMENTS

Figure 1. Dose response to CQ (A) and the effect of CQ+IL-12 combined therapy (B) on the course of *P. chabaudi* AS infection (A,B) and survival rate (C) of A/J mice. Mice were infected i.p. with 10^6 PRBC and were treated with CQ alone (12.5 mg/kg) or IL-12 alone (0.05 µg/day for 4 days) or both, starting on day 3 p.i. after the parasitemia was established at 0.5-1.0%. Pooled parasitemia data represent mean ● SEM from 9 to 12 individual mice studied in two (A) or three (B) experiments. Mean percentage survival data shown are pooled from 10-12 mice in each group. † Indicates 100% mortality.
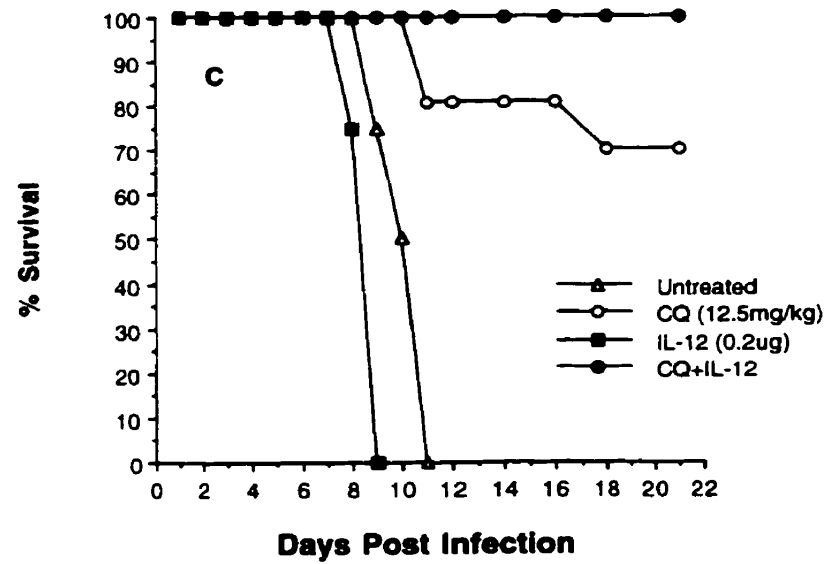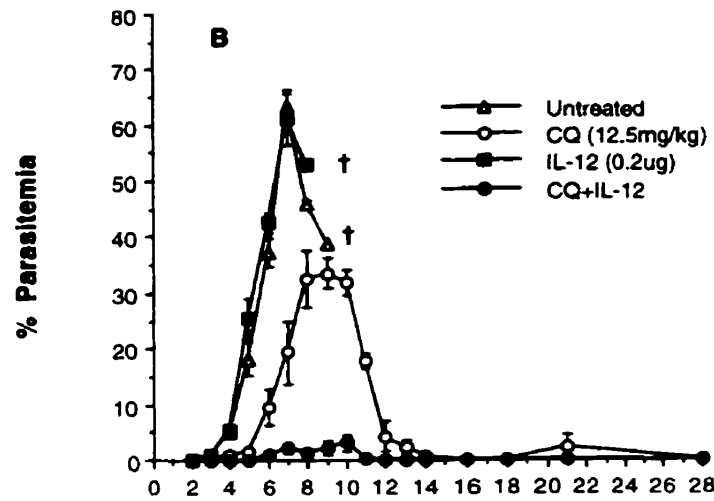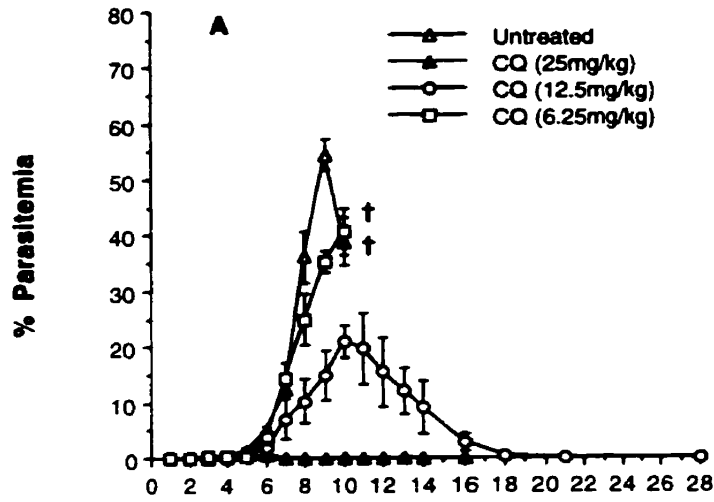
Figure 2. The effect of CQ+IL-12 combined therapy on the levels of IFN-γ secretion (A) and the expression of mRNA for IFN-γ, IL-12Rβ1 and IL-12Rβ2 by splenocytes obtained from *P. chabaudi* AS infected A/J mice on day 7 p.i. (B). IFN-γ secretion by unfractionated spleen cells was measured in the culture supernatants by ELISA after 48 h stimulation with Con A or mpAg as indicated. Data represent mean $\pm$ SEM from four individual mice from two experiments. Levels of IFN-γ and IL-12R mRNA expression was measured by RT-PCR and the results shown are from two representative mice in each group. [Lanes 1 and 2, untreated mice; lanes 3 and 4, mice given 12.5 mg/kg CQ alone; and lanes 5 and 6, mice given CQ+IL-12 combined therapy.] * $p<0.001$, by Student's unpaired-t-test.

A
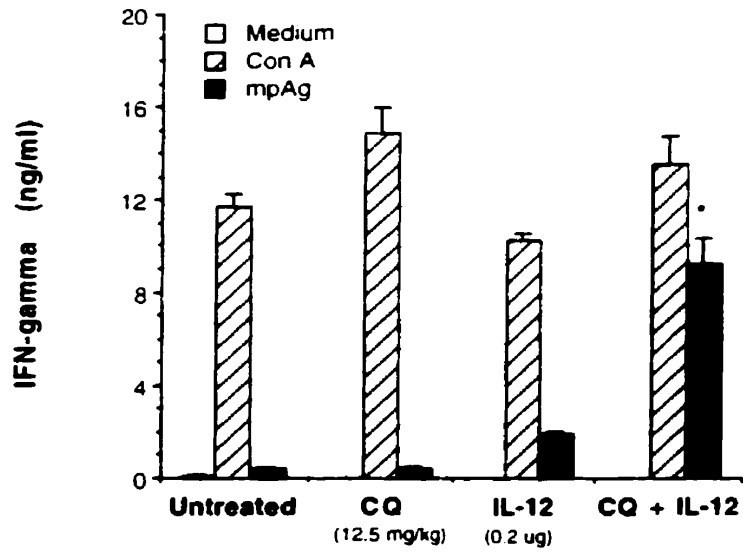
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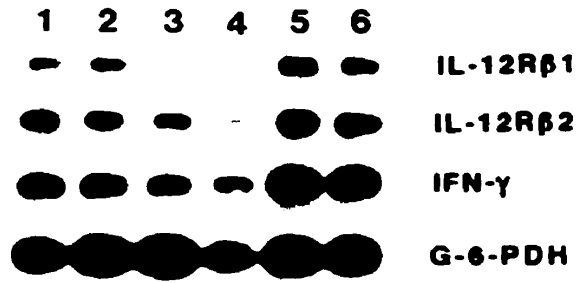
Figure 3. The effect of CQ+IL-12 combined treatment on the levels of anti-malaria Ab (total IgG) production (A) and the course of *P. chabaudi* AS reinfection (B) in A/J mice. Mice were reinfected using 10^6 PRBC four wks after recovery from primary infection and the total anti-malaria IgG was measured in 1:256 diluted sera by ELISA, 2 wks post reinfection. In (A), mean ± SEM data for optical densities representing Ab levels, and in (B) mean ● SEM of percent parasitemia shown are from 6 individual mice. * $p<0.001$, by Student's unpaired-t-test.

A

O.D. at 405 nm

A/J Normal   CQ   CQ + IL-12
                   (12.5 mg/kg)

B

% Parasitemia

○ CQ (12.5mg/kg)
● CQ (12.5mg/kg)+IL-12

Days Post Infection
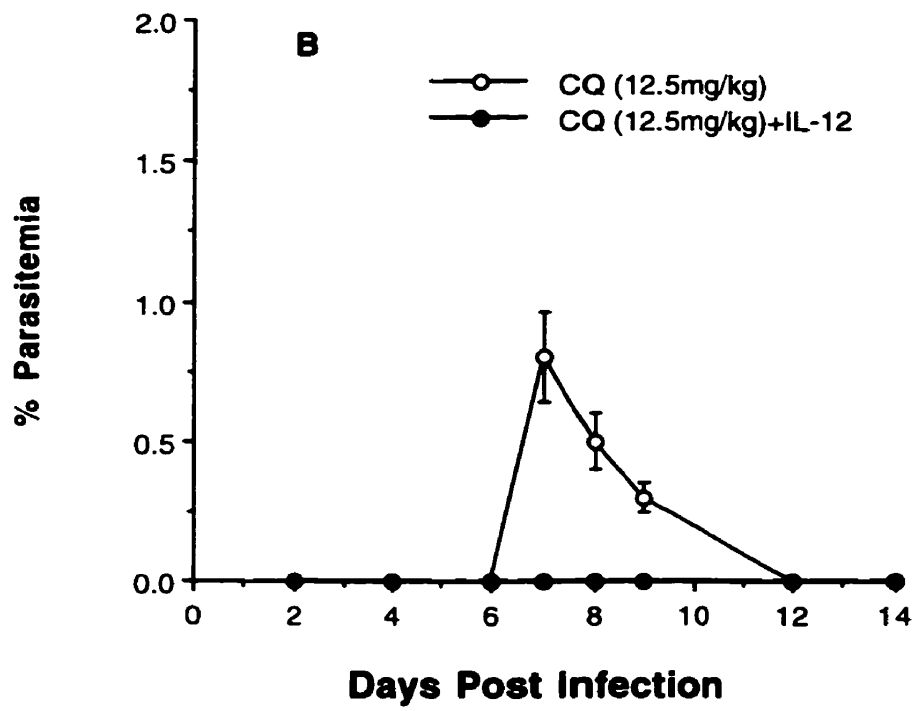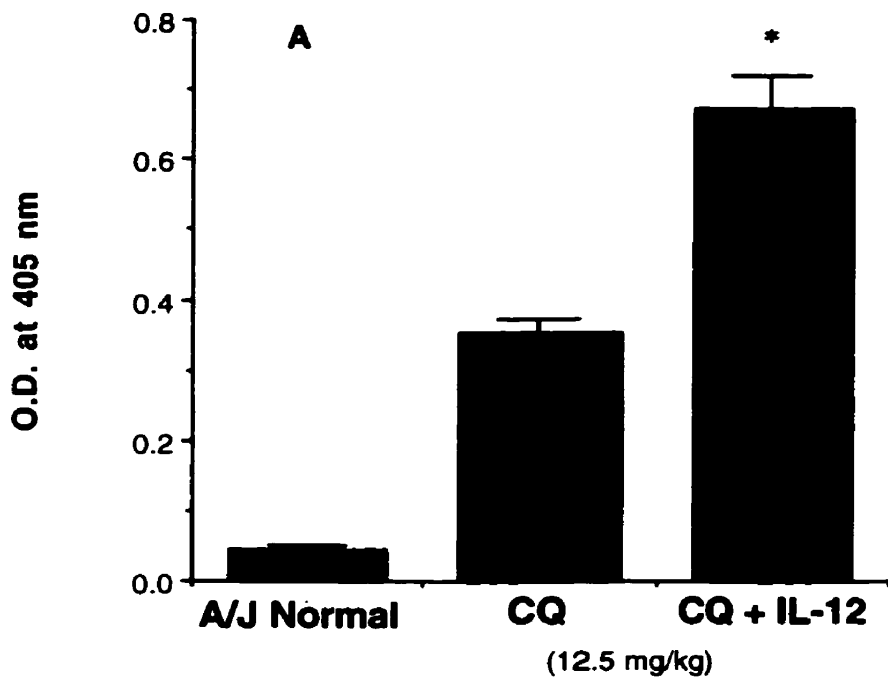
Table I

Effect of CQ and IL-12 Treatment on Hematological Parameters in A/J Mice With Established

P. chabaudi AS Infection.

Treatment	Hematocrit (%)	RBC Count ($\times 10^6$/mm^3)	Reticulocytes (%)	
			day 7 p.i.	day 10 p.i.
Untreated	25.0 ± 2.2	5.5 ± 0.3	5.0 ± 1.0	-
CQ (12.5 mg/kg)	44.0 ± 1.6[a]	7.3 ± 0.3[a]	9.0 ± 0.4[a]	11.3 ± 1.7
IL-12 (0.2 µg)	29.0 ± 1.1	5.6 ± 0.3	6.0 ± 0.5	-
CQ + IL-12	52.4 ± 0.9[a,b,c]	8.6 ± 0.2[a,b,c]	11.0 ± 1.0[a,c]	25.3 ± 2.5[b]

Mice were infected 7 d previously and were either untreated, CQ treated (12.5 mg/kg), IL-12 treated (0.05 µg/day/mouse for 4 days) or treated with both CQ and IL-12, starting on day 3 p.i. Heparinized peripheral blood was analyzed using standard hematological procedures. Data shown are mean ± SEM from 6-8 individual mice. Significant differences shown by unpaired-*t*-test are: [a] untreated vs. treated mice; [b] CQ treated vs. CQ+IL-12 treated mice; [c] IL-12 treated vs. CQ+IL-12 treated mice.

Table II

Chloroquine and IL-12 combination therapy markedly up-regulates erythropoiesis in A/J mice with established *P. chabaudi* AS infection.

Treatment	Bone Marrow		Spleen		Peripheral Blood
	BFU-E ($\times 10^3$)	CFU-E ($\times 10^3$)	BFU-E ($\times 10^3$)	CFU-E ($\times 10^4$)	BFU-E / 5×10^6 WBC
Untreated	1.1 ± 0.2	3.2 ± 0.2	9.8 ± 0.6	5.6 ± 0.4	39 ± 6
CQ (12.5 mg/kg)	1.3 ± 0.1	5.3 ± 0.4^a	8.3 ± 0.6	5.8 ± 0.2	57 ± 6
IL-12 (0.2 µg)	1.2 ± 0.1	5.0 ± 0.6^a	11.4 ± 0.7^a	10.1 ± 1.1^a	60 ± 4^a
CQ + IL-12	$1.8 \pm 0.1^{a,b,c}$	$7.9 \pm 0.2^{a,b,c}$	$16.3 \pm 0.8^{a,b,c}$	$19.0 \pm 1.3^{a,b,c}$	$111 \pm 7^{a,b,c}$

Mice were infected 7 d previously and were either untreated, CQ treated (12.5 mg/kg), IL-12 treated (0.05 µg/day/mouse for 4 days) or treated with both CQ and IL-12, starting on day 3 p.i. Single cell suspensions from femur, spleen and peripheral blood were cultured in methycellulose semisolid medium for either 7 d (BFU-E) or 48 h (CFU-E). Progenitor cell numbers are expressed as number of burst or colony forming units per organ. Data shown are mean ± SEM from 6-8 individual mice. Significant differences shown by unpaired-*t*-test are: [a] untreated vs. treated mice; [b] CQ treated vs. CQ+IL-12 treated mice; [c] IL-12 treated vs. CQ+IL-12 treated mice.

Chapter Five

Unimpaired IL-12 Production and Th1 Responses in TNFR Deficient
Mice During Early Blood-Stage *Plasmodium chabaudi* AS Malaria

Hakeem Sam and Mary M. Stevenson

McGill University Centre for the Study of Host Resistance,

and the Montreal General Hospital Research Institute

Running title: IL-12 in TNFR deficient mice during blood-stage malaria

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PREFACE

In Chapters 2 and 3, IL-12 production was assessed at the systemic and cellular levels in malaria-infected B6 and A/J mice. However, the question of what factors regulate IL-12 production in vivo during malaria infection was not addressed. Previous studies suggest that TNF–α may be critical for IL-12 and IFN-γ production. Moreover, TNF–α appears to be an important modulator of Th1 cytokine responses. In *P. chabaudi* AS malaria, our laboratory has shown a correlation between early protective Th1 responses in the spleen and up-regulation in splenic TNF-α mRNA levels. In this chapter, the effects of the absence of TNF activity on IL-12 production, IL-12R mRNA expression and Th1 responses during early *P. chabaudi* AS infection were investigated using the model of mice genetically deficient in both the p55 and p75 receptors for TNF.

ABSTRACT

Previous studies suggest that both TNF-α and IL-12 contribute to early protective Th1 responses against blood-stage malaria. Yet, the exact nature of the interactions between the two cytokines during acute malaria infection is presently unknown. In the present study, the course of *P. chabaudi* AS infection, early IL-12 production, and Th1 responses were examined in the absence of TNF activity using the model of mice genetically deficient in both the p55 and p75 receptors for TNF (TNFRp55p75-/-). All TNFRp55p75-/- mice recovered from *P. chabaudi* AS infection, as did wild type controls by 4 weeks post infection. However, female, but not male, TNFRp55p75-/- mice had significantly higher parasitemias compared to their wild type controls during the first few days immediately following the peak parasitemia. Similar to wild type controls, serum IL-12 p70 levels in TNFRp55p75-/- mice were significantly increased on day 2 compared to levels in uninfected animals. On day 7, IFN-γ production by spleen cells in vitro was similar, but LPS-induced NO synthesis by splenic macrophages was significantly reduced in TNRp55p75-/- compared with wild type mice. Taken together, these data suggest that deficiency of TNF activity alone does not impair IL-12 production or the development of protective Th1 responses against blood-stage malaria.

INTRODUCTION

TNF-α has been implicated in protective as well as pathological roles in resistance of inbred mouse strains to bacterial and protozoan parasite infections, including blood-stage malaria (227-231). The balance between protective versus pathological actions of TNF-α depends on several factors including the quantity, timing and duration of TNF-α production, as well as the organ-specific site of its synthesis (81, 227). The biological activities of TNF-α, and also TNF-β, which shares many functions with TNF-α, are mediated by two structurally related but functionally distinct receptors known as TNFRp55 or TNFR1, and TNFRp75 or TNFR2 (232, 233). Most of the common biological actions of TNF are attributed to signaling via TNFRp55 (234, 235). The TNFRp75 is thought to function both as a TNF antagonist, and as an agonist by facilitating the cell surface interaction between TNF and TNFRp55 (236).

In recent years, mice with gene-targetted deletion of both the TNF p55 and p75 receptors have been used to study the role of TNF-α in host defense against parasitic infections. *Toxoplasma gondii* infection resulted in higher parasite burdens and 100% mortality within 20 to 26 days in TNFRp55p75-/- mice, compared with wild type controls that survived for at least 60 days (237). In contrast, parasite burdens and susceptibility to infection were comparable in TNFRp55p75-/- and wild type controls infected with *Mycobacterium avium* (238).

Previous studies from our laboratory have demonstrated that, an early, Th1-associated increase in TNF-α is involved in resistance of C57BL/6 (B6) mice against blood-stage *P. chabaudi* AS malaria (81). Furthermore, we demonstrated that the mechanism of rIL-12-induced protection of susceptible A/J mice against *P. chabaudi* AS infection is dependent on TNF-α, acting in concert with nitric oxide (NO) and

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IFN-γ (16). In the present study, mice genetically deficient in both TNFR were used to further define the role of TNF in resistance of inbred mouse strains to blood-stage malaria. Our results reveal that, similar to wild type controls, doubly deficient TNFRp55p75-/- mice produce IL-12 in vivo, mount unimpaired Th1 responses, and clear *P. chabaudi* AS malaria by four weeks post infection. In contrast to wild type controls, however, splenic macrophages from TNFRp55p75-/- mice exhibited significantly reduced LPS, but not PRBC, -induced NO synthesis in vitro.

MATERIALS AND METHODS

Mice, Parasite and Experimental Infections. Mice, 9-10 week old, were age- and sex-matched in all experiments. TNFRp55p75-/- mice were bred in the animal facilities of the Montreal General Hospital Research Institute from breeders provided by Genentech, Inc., San Francisco, CA. Wild type (B6 × 129) F1 from Jackson Laboratories (Bar Harbor, ME) were used as wild type and genetic background controls. *P. chabaudi* AS was maintained as previously described (31). Infection was initiated by i.p. injection of 10^6 *P. chabaudi* AS infected erythrocytes (PRBC) and the course of infection was monitored by previously described procedures (31).

Reagents and Sera. Murine rIL-12 was a generous gift of Dr. S. Wolf (Genetics Institute, Cambridge MA). Rat anti-murine IL-12 mAbs, C15.1 and C15.6 (IgG1 isotype), and C17.8 (IgG2a isotype) were generated as previously described (182). Hybridomas producing these mAbs were a kind gift of Drs. M. Wysocka and G. Trinchieri (Wistar Institute, Philadelphia, PA). All three monoclonal antibodies detect the p40 subunit of IL-12. Red-T/G297-289, a mixture of mAbs against the p35/p70 subunit of IL-12, was purchased from PharMingen (Mississauga, Ontario, Canada). At the indicated times, blood was obtained from wild type or TNFRp55p75-/- by

cardiac puncture, allowed to clot, and sera were separated by centrifugation at 13,800 × g for 3 min. Sera were kept at 4°C and immediately analyzed for IL-12 levels by ELISA.

Preparation of unfractionated spleen cells and macrophage monolayers. At the indicated times, single cell suspensions of spleen cells were prepared under aseptic conditions in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 5% heat-inactivated FCS (Hyclone, Logan UT), 2% HEPES buffer (GIBCO-BRL) and 0.12% gentamicin (Schering Canada, Inc., Montreal, Quebec), as previously described (192). Total cell counts were performed and differential cell counts were determined on Cytocentrifuge (Shandon Corporation, Sewickley, PA, USA) preparations of spleen cells stained with Dif-Quik (American Scientific Products, McGraw Park, IL, USA). Spleen cell suspensions were adjusted to a concentration of 2×10^6 macrophages/ml. Aliquots of 500 µl of spleen cell suspensions were added to 24-well flat-bottomed plates (Nunc, Roskilde, Denmark) and allowed to adhere for 2 h at 37°C in a humidified 5% CO_2 incubator. Following incubation, non-adherent cells were removed by washing adherent cell monolayers three times with warm HBSS (GIBCO-BRL). Adherent cells prepared by this method were >95% macrophages based on morphology, phagocytosis of inert latex beads, and nonspecific esterase staining.

Splenic macrophages were cultured for 24 h in freshly added medium as control, with PRBC (2×10^6/ml) or 1 µg/ml of *E. coli* O127:B8 LPS (Difco, Detroit, MI, USA). Cell culture supernatants were removed and assayed for nitrite levels by the Griess reaction (239) or cytokine concentrations by ELISA. Where indicated, 500 µl aliquots of unfractionated spleen cells (4×10^6/ml), in medium alone or stimulated with Con A (5 µg/ml) or PRBC (2×10^6/ml) were cultured in 24-well plates and 48 h cell-free culture supernatants were assayed for IFN-γ levels by ELISA.

ELISAs. IFN-γ ELISA was performed as previously described (63) and had a detection limit of 100 pg/ml. Two-site sandwich ELISAs were used to measure serum IL-12 levels; both p40- and p70-specific ELISAs were used. The former detects all forms of IL-12 and does not distinguish between levels of single IL-12 species such as p40 monomers, homodimers or p40-p35 heterodimers, whereas the latter detects only levels of biologically active heterodimer. For the p40-specific ELISA, the capturing antibody was C15.1 and the detecting antibody was biotinylated C15.6. For the p70-specific ELISA, the capturing antibody was Red-T/G297-289 and the detecting antibody was biotinylated C17.8. Streptavidin-HRPO conjugate (GIBCO-BRL) was added for final detection. The coating buffer for Red-T/G297-289 was 0.1 M $NaHCO_3$ (pH 8.2). Incubation conditions for samples and standard rIL-12 were overnight at 4°C, and following addition of biotinylated antibody, plates were incubated for 4-5 h at room temperature. Plates were read in a ELISA reader at 405 ηm, with a reference wavelength of 492 ηm. The limit of detection for the p40-specific ELISA was 500 pg/ml, and for the p70-specific ELISA was 100 pg/ml.

Cytokine mRNA determination by RT-PCR. Spleens were aseptically removed from uninfected or infected mice, immediately frozen in liquid nitrogen and stored at -70°C. The procedure for RNA isolation was based on a modification of the single-step method described by Chomzynski and Sacchi (193). Briefly, frozen tissue samples were homogenized in TRIzol reagent (GIBCO) using a polytron homogenizer (Brinkman, Kimematica, Switzerland). Unfractionated spleen cells were directly lysed by adding TRIzol reagent. Total RNA was subsequently isolated following the manufacturer's instructions.

RT-PCR was performed as previously described (194) to detect changes in cytokine or cytokine receptor mRNA levels. To determine optimal cycling conditions, titrations of input cDNA were performed followed by PCR amplification to ensure

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that for the selected number of cycles, a linear relationship exists between input cDNA and PCR product. Typically, 30 cycles were performed and the cycling conditions used were: 94°C for 1 min, 54°C for 20 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. To increase the specificity of PCR amplification, the "hot start" method, involving an initial denaturation of the reaction mixture containing all reagents except the enzyme for 3 min at 94°C was utilized. Taq polymerase (GIBCO), in 1X PCR buffer, was then added to the reaction mixture at 85°C before cycling was initiated. Both positive and negative controls were included in each assay to ensure efficacy of the reaction and to rule out possible cDNA contamination of reagents. The housekeeping gene G6PDH was simultaneously amplified in each assay to verify that equal amounts of cDNA were added in each PCR reaction. Nucleotide sequences for primers and probes for IFN-γ (195) and G6PDH (194) were used as previously published. Primers and probe sequences were designed in our laboratory for IL-12R β1 and β2, based on the recently cloned cDNAs for these genes (Genebank accession no. U23922 and U64199 respectively). The sequences were: IL-12R β1 sense, 5'-TGA-AGA-CGG-CGC-GTG-GGA-GTC-A-3', antisense, 5'- TCG-CGG-GTA-CAA-CAC-CTC-CGG-G-3', probe, 5'-GCG-AGC-GGA-CAC-TGC-GAG-GC-3' (product size, 412 bp); IL-12R β2 sense, 5'-GGT-TGC-TGG-CTC-CTC-ACC-AGG-3', antisense, 5'-ATG-CAG-CCC-CTT-TGC-TCC-GGG-3', probe, 5'-TCC-CCC-ACA-CTG-GCT-GCG-GA-3' (product size, 424 bp).

Fifteen microlitres of final PCR products were analyzed by electrophoresis in a 1.2% agarose gel, denatured, neutralized, and transferred onto a Hybond-N membrane (Amersham, Arlington Heights, IL) by Southern blotting. After UV cross-linking (UV Stratalinker 1800, Stratagen, La Jollier, CA) and baking in a vacuum oven (2 h at 80°C), membranes were hybridized with cytokine-specific γ-^{32} P-ATP (Amersham) end-labelled oligonucleotide probes that hybridize to a portion of the amplified

segment between the primers. Probes were radioactively labelled with 10 U T4 polynucleotide kinase (GIBCO) in a 0.5X One-Phor-All buffer supplied with the enzyme. After hybridization and washing, cytokine or cytokine receptor mRNA was detected by autoradiography with Kodak Biomax MR film (Rochester, NY). The intensity of bands corresponding to specific cytokines was analyzed by high-resolution optical densitometry (SciScan 500, United States Biochemical) and normalized to those of G6PDH.

Measurement of NO synthesis. NO production by splenic macrophages was measured as nitrite concentration in cell culture supernatants using the Griess reaction, as previously established in our laboratory (82, 239). Briefly, 50 µl of supernatant were incubated with an equal volume of Griess reagent for 5 min at room temperature. Plates were read at A_{550} using an ELISA reader. The concentration of nitrite (µM) in supernatants was determined using $NaNO_2$ as a standard.

Statistical Analysis. Results are presented as mean ± SEM. Statistical significance of differences in means between two groups of mice was determined by Student's t-test. Where three or more groups were compared, one-way ANOVA, followed by Student-Newman-Keul post-test was used.

RESULTS AND DISCUSSION

Course of P. chabaudi AS infection in wild type and TNFR deficient mice

It was previously demonstrated that intravenous treatment of susceptible A/J mice with human rTNF-α during *P. chabaudi* AS infection results in a significant decrease in the level of primary peak parasitemia and 80% survival, compared with 100% mortality in untreated controls (240). Therefore, it was of interest to investigate the effect of TNFR deficiency on survival and the course of parasitemia following *P.*

chabaudi AS infection. As shown in Figure 1, TNFRp55p75-/- male and female mice recovered from blood-stage *P. chabaudi* AS malaria, as did wild type controls by the end of four weeks post infection. In contrast, blood-stage *P. chabaudi chabaudi* AS infection resulted in 56% mortality within 20 days in female, but not male, mice with gene-targetted deficiency in IL-10, whereas 100% of heterozygous controls survived (68).

The level of primary peak parasitemia was similar in TNFR deficient animals compared with wild type controls. In female, but not male, mice however, parasitemias were significantly higher in TNFR deficient mice compared to wild type controls ($p < 0.05$) on days 9-14, immediately following the peak parasitemia (Figure 1). These results suggested that TNF might be important, but is not a critical requirement, for resolving blood-stage malaria.

P. chabaudi AS infection results in significant increases in splenic index

The development of massive splenomegaly, likely to be due in part to the dramatic amplification of splenic erythropoiesis that helps combat malaria-induced anemia, was previously correlated with resistance to *P. chabaudi* AS infection in resistant B6, but not susceptible A/J, mice (10, 17). Treatment of *P. chabaudi* AS infected B6 mice with mAbs against TNF-α alone, or TNF-α and IFN-γ, resulted in significant reductions in spleen weight compared to control animals (83). In addition, treatment with mAbs against TNF-α also prevented the development of massive splenomegaly in mice infected with *Brucella abortus* (241). Therefore, it was of interest to determine if the development of splenomegaly might be affected in TNFR deficient compared to wild type mice infected with *P. chabaudi* AS.

As shown in Figure 2, spleen weights were expressed as splenic index (spleen mass/body mass) since male mice, wild type or TNFRp55p75-/-, had significantly higher body weights compared to their female counterparts (data not shown). Based on earlier studies, marked increases in spleen weight can be demonstrated in *P. chabaudi* AS infected mice by day 7 post infection (17). In TNFR deficient or wild type controls, *P. chabaudi* AS infection resulted in significant and comparable increases in splenic index on day 7 post infection compared to uninfected controls (Fig. 2A, $p < 0.05$ and $p < 0.01$, for male wild type and TNFRp55p75-/- mice, respectively; Fig. 2B, $p < 0.001$ for female wild type and TNFRp55p75-/- mice).

P. chabaudi AS infected female wild type or TNFR deficient animals had significantly higher splenic indices compared to their respective male counterparts ($p < 0.001$ and $p < 0.01$, for wild type and TNFRp55p75-/- mice, respectively). In contrast, there were no significant differences in splenic indices between uninfected mice, male or female, wild type or TNFR deficient. Thus, TNFR deficiency did not affect the development of splenomegaly following *P. chabaudi* AS infection. The differences between these results and those of earlier studies using mAb treatment against TNF-α could be related to unknown compensatory mechanisms that develop in TNFRp55p75-/- mice.

Unimpaired IL-12 p70 synthesis in TNFR deficient mice during early P. chabaudi AS malaria

TNF may play a role in IL-12 production during murine mycobacterial infections. Studies of *M. bovis* strain BCG infection in mice deficient in TNFRp55 demonstrated a significant impairment in IL-12 synthesis in vivo and by bone-marrow derived monocyte/macrophages in vitro (242). Possibly reflecting the consequences

of deficient IL-12 production on host resistance to mycobacterial infections, TNFRp55-/-, but not wild type controls, succumbed to *Mycobacterium tuberculosis* (243). In the present study, systemic IL-12 p70 production was analyzed in TNFRp55p75-/- and wild type hosts during early blood-stage *P. chabaudi* AS infection. Earlier studies demonstrated significant increases in systemic IL-12 p70 levels in blood-stage malaria-infected B6 mice by day 2 post infection compared with uninfected controls (Chapter 2). In TNFR deficient as well as wild type mice of either sex (Table I), serum levels of biologically active IL-12 p70 were significantly increased ($p < 0.001$) on day 2 post infection compared to uninfected controls. There were no significant differences in serum p70 levels between TNFR deficient compared with their wild type controls, either uninfected or after malaria infection. Similar to the changes in splenic indices, serum p70 levels were significantly higher ($p < 0.01$) in infected female compared to male mice, TNRp55p75-/- or wild type (Table I).

Daily treatment with murine rIL-12 during the first five days of *P. chabaudi* AS malaria was found to rescue susceptible A/J mice from a lethal course of infection (16). Simultaneous treatment with rIL-12 and mAbs against TNF-α and IFN-γ completely abrogated IL-12-induced resistance in A/J mice to blood stage malaria. Furthermore, a close association was reported between significant up-regulation of TNF-α mRNA levels in the spleen and the induction of early protective Th1 responses in resistant B6 mice during the first week of *P. chabaudi* AS infection (81). Whereas these studies suggested that both IL-12 and TNF-α play an important role in early Th1-dependent immune responses against blood-stage malaria, it was unclear whether IL-12 synthesis and Th1 responses were events downstream of TNF activity. The results of the present investigation demonstrate that TNF activity is not required for systemic IL-12 production during early blood-stage malaria.

Normal Th1 responses but defective NO production in TNFR deficient mice

Early Th1 responses, dominated by expression of high levels IFN-γ mRNA and protein production in vitro by spleen cells, correlates with resistance to blood-stage *P. chabaudi* AS malaria (43, 81). It has been shown that TNF may play a role in the generation of Th1 responses (149). In addition, TNF-α was found to be an important co-factor for IL-12-induced production of IFN-γ by NK cells from mice with severe combined immunodeficiency (SCID) (145, 146).

Therefore, we next examined TNFR deficient and wild type hosts for Th1 responses downstream of IL-12 p70 production, namely, up-regulation of splenic mRNA levels for IL-12R β1 and β2 and IFN-γ, as well as IFN-γ protein production in vitro by spleen cells recovered from *P. chabaudi* AS infected animals. Female wild type and TNFRp55p75-/- mice were selected for these additional studies. Developing Th2 cells appear to lose mRNA expression for IL-12R β2 while maintaining mRNA expression for IL-12R β1 (163). Evidence suggests that both IL-12R β1 and β2 are required for high affinity interaction between IL-12 and its receptor complex (163, 197). Our earlier studies demonstrated significant increases in IL-12R β1 and β2 mRNA levels in the spleens of blood-stage malaria-infected B6 mice by day 5 post infection compared with uninfected controls (Chapter 2).

As shown in Table II, fold increases in splenic IL-12R β1 mRNA levels in day 5 infected vs. uninfected controls were 2-3 for TNFR deficient mice, compared with 2-5 for wild type controls. Splenic IL-12R β2 mRNA levels increased by 1.5-3-fold in day 5 infected vs. uninfected controls for both TNFR deficient and wild type controls. Based on our earlier studies in B6 mice, splenic IFN-γ mRNA levels were determined at day 7 when peak IFN-γ mRNA levels were expected. Fold increases in infected vs. uninfected controls were approximately 3 in wild type and 3-6 in TNFRp55p75-/- hosts (Table II). Correlating with these splenic IFN-γ mRNA levels, PRBC or Con A-

induced IFN-γ secretion in vitro by unfractionated spleen cells from day 7 infected wild type or TNFRp55p75-/- mice was significantly higher than spontaneous production in medium alone ($p < 0.05$ and $p < 0.01$, for PRBC and Con A, respectively, Figure 3A). However, there were no significant differences between TNFR deficient and wild type mice in IFN-γ production by unfractionated spleen cells.

TNF-α is an important activator of macrophages for NO synthesis (244). It was previously found that splenic macrophages recovered from resistant B6, compared with susceptible A/J, mice displayed significantly greater LPS-induced release of NO in vitro (82). Treatment of *P. chabaudi* AS infected resistant B6 mice with mAbs against TNF-α resulted in significant decreases in serum nitrate levels compared to untreated controls (83). Furthermore, significant reductions in splenic iNOS mRNA levels were observed in *P. chabaudi* AS infected resistant B6 mice treated with a combination of mAbs against TNF-α and IFN-γ (83). Hence, in this study, NO production by splenic macrophages recovered from malaria-infected TNFR deficient or wild type controls was assessed. As shown in Figure 3B, LPS, but not PRBC, - induced NO synthesis was significantly higher in splenic macrophages from infected wild type compared to TNFRp55p75-/- mice ($p < 0.01$). Interestingly, mice deficient in both TNFR or in TNFRp55 alone, but not wild type controls, were protected against lethal endotoxin challenge with the combination of LPS and D-gal, suggesting the possible relevance of differences between TNFR deficient animals and wild type hosts in LPS responses (236).

The mice used in the present study lacked both the TNF p55 and p75 receptors, in other words, TNF-α activity was completely absent. However, in mice with selective gene-targetted deficiency of either the p55 or p75 TNFR, but not both, TNF-α signaling could still occur via the remaining TNF receptor. Use of these mice has

been particularly useful in dissecting the differential roles of TNF-α signaling through the TNF p55 or p75 receptors in host defense against parasitic infections. For example, TNFRp75-/- mice were significantly protected from cerebral malaria, whereas TNFRp55-/- hosts were as susceptible as wild type controls (245). In contrast, TNFRp55 deficiency resulted in increased susceptibility to infection with *Listeria monocytogenes* (234, 235) and *Mycobacterium tuberculosis* (243), compared with wild type controls.

Taken together, our results suggest that TNF-α activity is not a critical requirement for resolving blood-stage infections with *P. chabaudi* AS malaria. Furthermore, neither IL-12 production nor protective Th1 responses appear to be impaired in the absence of TNF-α activity during early blood-stage malaria.

ACKNOWLEDGEMENTS

Figure 1. Course of *P. chabaudi* AS infection in male (*A*) and female (*B*) mice. Wild type or TNFRp55p75-/-animals were infected and the course of parasitemia was monitored as described in "Materials and Methods". Data are pooled from 2-3 replicate experiments and are presented as mean ± SEM of 10-15 mice per time point analyzed individually. Statistically significant differences shown are: $*p < 0.05$ vs. wild type mice on same day.

A

Parasitemia

—○— Wild Type
—■— TNFRp55p75-/-

B

Parasitemia

Days Post Infection
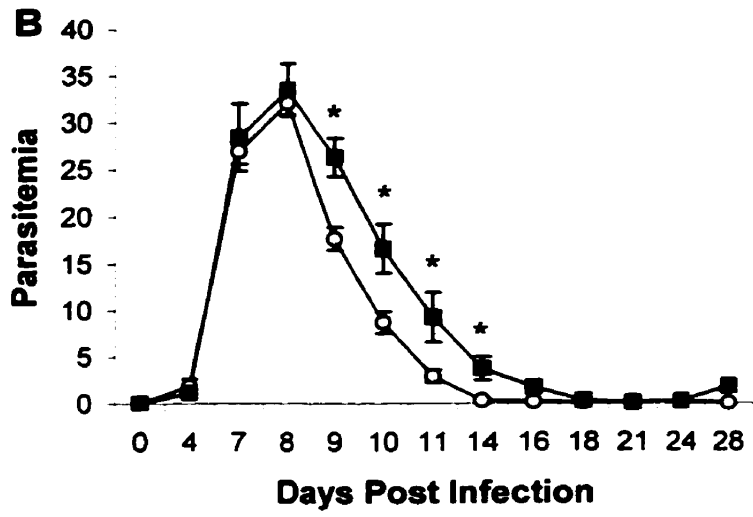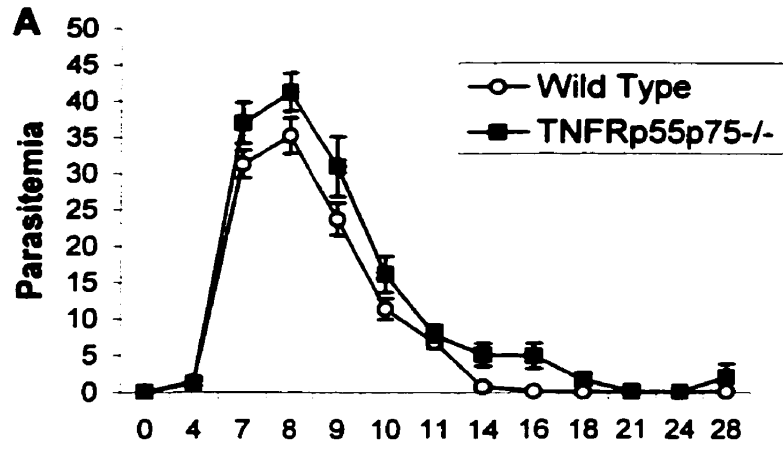
Figure 2. Increases in splenic index (spleen weight/body weight) during *P. chabaudi* AS infection in male (*A*) and female (*B*) mice. Body and spleen weights were determined in uninfected or day 7 infected wild type or TNFRp55p75-/- animals. Data are pooled from two replicate experiments and are presented as mean ± SEM of 4-8 mice per time point analyzed individually. Statistically significant differences shown are: *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ vs. uninfected controls, #$p < 0.01$, ##$p < 0.001$ vs. infected male mice.

A

Splenic Index (X 10⁻³)

□ Wild Type
■ TNFRp55p75-/-

Uninfected   Infected

B

Splenic Index (X 10⁻³)

Uninfected   Infected
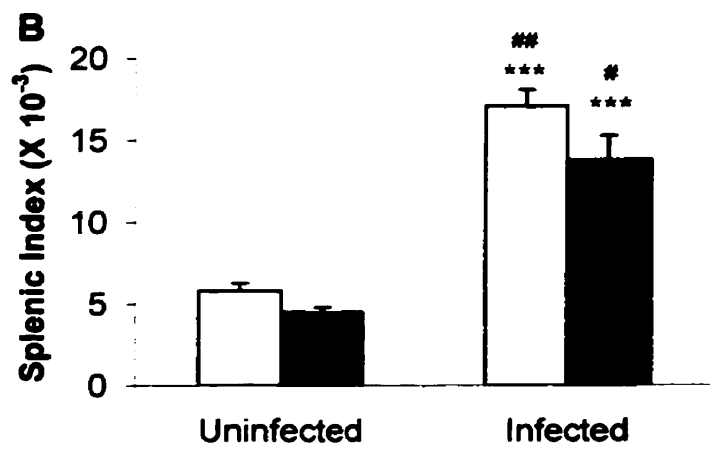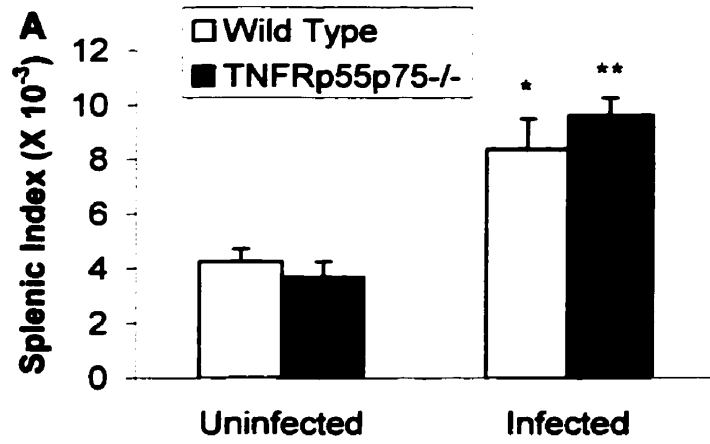
Figure 3. IFN-γ production by unfractionated spleen cells (*A*) and NO synthesis by splenic macrophages (*B*) in vitro from day 7 *P. chabaudi* AS infected wild type or TNFRp55p75-/- mice. Unfractionated spleen cells were cultured in the presence of medium alone, Con A (5 μg/ml) or PRBC (2 × 10^6/ml) and IFN-γ levels in 48-h cell culture supernatants were analyzed by ELISA. Splenic macrophages were cultured for 24 h with medium alone, LPS (1 μg/ml) or PRBC (2 × 10^6/ml) and NO levels in 24 h culture supernatants were measured as nitrite using the Greiss reaction. Data are pooled from two replicate experiments and are presented as mean ± SEM of 4-8 mice analyzed individually. Statistically significant differences shown are: *$p < 0.05$, **$p < 0.01$ vs. medium controls of the same strain, #$p < 0.01$ vs. TNFRp55/p75-/- mice.

A

IFN-γ (ng/ml)

Medium
Con A
PRBC

** **

*

Wild Type   TNFRp55p75-/-

B

Nitrite (Micromolar)

Medium
LPS
PRBC

#
**

*

Wild Type   TNFRp55p75-/-
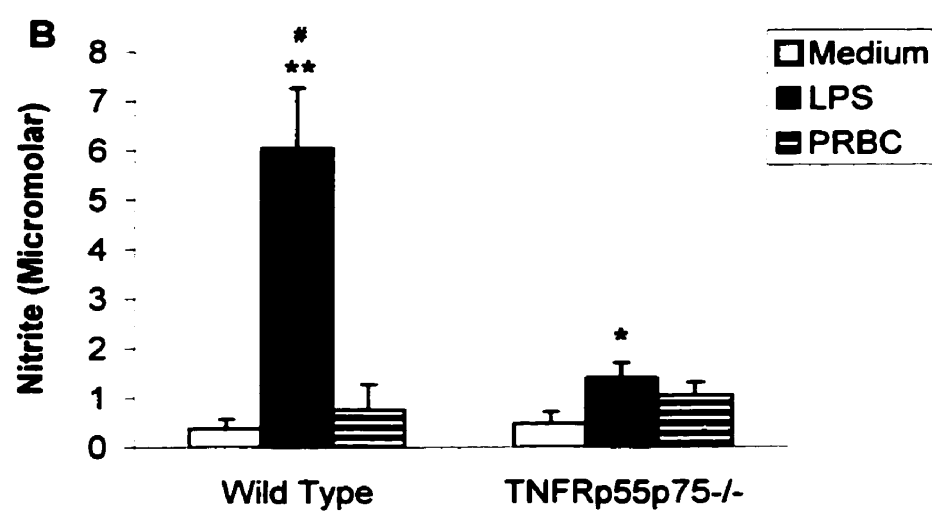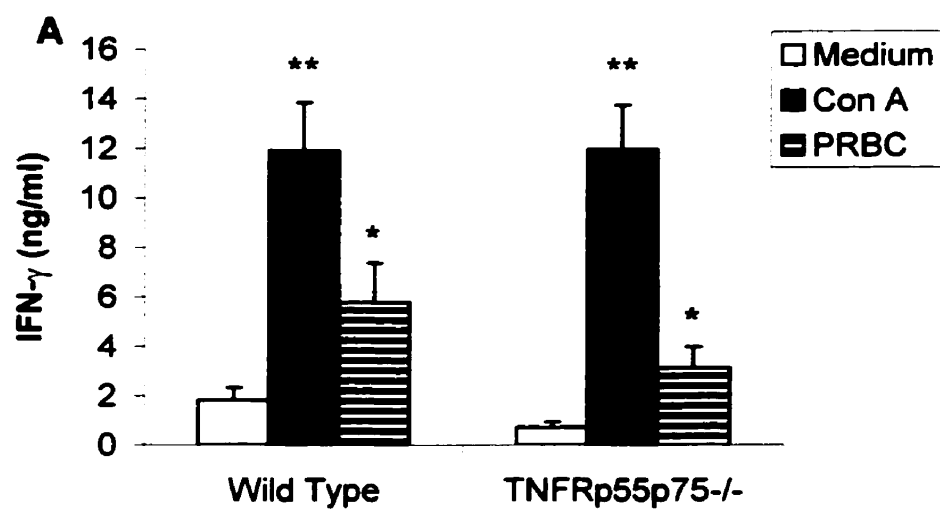
Table I

Effect of TNFR deficiency on serum IL-12 p70 levels following

P. chabaudi AS infection[a]

	IL-12 p70 (ng/ml)[b]	
Strain	Uninfected	Infected
Male		
Wild Type	0.3 ± 0.1	4.0 ± 1.0[*]
TNFRp55p75-/-	0.1 ± 0.03	3.2 ± 0.7[*]
Female		
Wild Type	0.3 ± 0.1	8.8 ± 0.3[*†]
TNFRp55p75-/-	0.1 ± 0.0	7.0 ± 0.6[*†]

[a]Mice were either normal or *P. chabaudi* AS infected on day 2.

[b]Serum IL-12 p70 levels were analyzed by ELISA. Data are pooled from three replicate experiments and are presented as mean ± SEM of 4-8 mice analyzed individually.

[c]Statistically significant differences shown are: $*p < 0.001$ vs. uninfected controls, $^{†}p < 0.01$ vs. male mice of the same strain on day 2 post infection.

Table II

Effect of TNFR deficiency on IL-12R β1 and β2, and IFN-γ

mRNA levels in the spleen following *P. chabaudi* AS infection[a]

| | Fold Increases[b] | | |
Strain	IL-12R β1	IL-12R β2	IFN-γ
Experiment 1			
Wild Type	4.7	1.5	3.1
TNFRp55p75-/-	3	1.5	6.1
Experiment 2			
Wild Type	2.3	2.6	3.3
TNFRp55p75-/-	1.8	2.7	2.5

[a]RT-PCR was used to detect changes in cytokine or cytokine receptor mRNA levels in whole spleen tissue (Experiment 1) or unfractionated spleen cells (Experiment 2) from normal or *P. chabaudi* AS infected female wild type or TNFRp55p75-/- mice. Infected mice analyzed were on day 5 (for IL-12R β1 and β2) or day 7 (for IFN-γ).

[b]Fold increases indicate the ratios of cytokine or cytokine receptor mRNA levels (normalized to those of housekeeping gene G6PDH) in infected vs. uninfected controls. Data for fold increases are pooled from 2-3 mice in each experiment.

Chapter Six

GENERAL DISCUSSION AND CONCLUSION(S)

It is estimated that well over 300 million people suffer a bout of malaria infection every year (1). The mortality rate associated with these infections is in the order of 2 million deaths annually, predominantly in children living in malaria-endemic regions in Africa and Southeast Asia. Despite this huge burden on public health, an effective vaccine is yet to be found. The situation has further been worsened by the development of drug-resistant forms of *Plasmodia*. Studies in humans have shown that host resistance to this infection varies between those living in endemic versus non-endemic regions (2). Resistance also varies among inbred mouse strains (15). This heterogeneity in host resistance is determined by a variety of factors, including prior exposure to malaria, sex and genetic background of the host (15, 21, 22, 24).

As reviewed in the general introduction to this thesis, a role for almost every immune cell type has been implicated in protective or pathological host responses to malaria. The *P. chabaudi* AS model of blood-stage malaria is well established in our laboratory. Using this model, our laboratory's primary research interest has been to address the role of cytokines in host defense against blood-stage malaria. Previous studies from our laboratory (63, 64, 81, 240) and others (65, 87) demonstrated that both IFN-γ and TNF-α contribute to host resistance to blood-stage malaria. These two cytokines have also been implicated in protective, as well as pathological roles, in host resistance to infections with *M. tuberculosis* (243, 246), *L. major* (39, 244, 247), *L. monocytogenes* (248-250), and blood-stage malaria (16, 55, 63, 66, 67, 88, 89). Furthermore, it has been shown that CD4+ T helper cells are required for the successful resolution of blood-stage malaria (11, 31, 44, 45, 204, 248-250). In particular, the timing and activation of particular subsets of CD4+ T helper (Th) cells is important (43, 44). Resistant B6 mice mount predominantly Th1 responses during the first week of *P. chabaudi* AS malaria, whereas susceptible A/J mice exhibit

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predominantly Th2 responses during this stage of the infection (43). Whereas 100% of B6 mice completely recover from *P. chabaudi* AS infection by the end of 4 weeks, there is a 100% mortality in A/J mice by days 10-12 post infection (191).

IL-12 is a heterodimeric cytokine with potent and pleiotropic effects, primarily on NK and T cells (103, 104, 107). IL-12 induces the activation of both cell types and stimulates their production of cytokines, in particular IFN–γ. Moreover, IL-12 directly induces the differentiation of CD4+ T cells towards a Th1 phenotype, in contrast to IL-4, which promotes Th2 cell differentiation (36). These effects of IL-12 on Th subset selection suggested that it might be involved in the polarization of Th responses seen in *P. chabaudi* AS infected B6 and A/J mice during early *P. chabaudi* AS infection (43). Daily treatment of susceptible A/J mice with rIL-12 (0.1 μg/day) during the first five days of *P. chabaudi* AS infection was able to rescue 75% of these hosts from a normally lethal infection (16). Confirming the efficacy of IL-12 administration against malaria, one s.c. injection of 10 μg/kg recombinant human IL-12 (rhIL-12) 2 days before challenge with *P. cynomolgi* sporozoites was found to protect seven of seven rhesus monkeys (187).

Treatment of *P. chabaudi* AS infected resistant B6 mice, however, with 0.1 μg/day of IL-12 resulted in mortalities in treated compared to untreated controls (16). The protective efficacy of exogenous IL-12 treatment against *P. chabaudi* AS infection in A/J mice was significantly reduced when higher doses of IL-12 were given. Consistent with these results, high dose IL-12 therapy was shown to cause remarkable toxicity in lymphocytic choriomeningitis virus (LCMV) infected B6 mice, characterized by severe thymic atrophy, marked cachexia, and elevated serum TNF–α and glucocorticoid levels (189). Taken together, these results suggested that optimal levels of IL-12 might be synthesized in *P. chabaudi* AS resistant B6 mice, whereas IL-

12 production might be significantly reduced or impaired in susceptible A/J mice during early blood-stage malaria.

Despite the effects of exogenous IL-12 treatment in protecting susceptible A/J mice against *P. chabaudi* AS malaria, no previous studies had addressed endogenous synthesis of biologically active IL-12 p70 in resistant or susceptible hosts during early blood-stage malaria. Therefore, the differences between *P. chabaudi* AS infected B6 and A/J mice in IFN-γ mRNA levels and protein synthesis by spleen cells, during the first week of infection, could be due entirely to strain variations in the production of other well known inducers of IFN-γ synthesis. These include members of the IL-1 family of cytokines, IL-2, and possibly the recently identified interferon-gamma inducing factor or IL-18 (147, 170, 251). The primary goal in this thesis was to determine whether or not endogenous IL-12 synthesis occurs in resistant B6 and susceptible A/J mice during early blood-stage *P. chabaudi* AS malaria. It was important to establish whether any differences between the two mouse strains in the kinetics of endogenous IL-12 synthesis correlated with the polarization of Th responses seen in these hosts during early blood-stage malaria. Furthermore, we sought to analyze the requirements for IL-12 production, the possible role of TNF, as well as the effects of exogenous IL-12 administration on the induction of cytokine or cytokine receptor gene expression in malaria-infected mice.

The main emphasis in Chapter 2 was to analyze systemic IL-12 production in *P. chabaudi* AS infected B6 and A/J mice during the first five days of infection. The key findings in this study were that, firstly, serum levels of biologically active IL-12 p70 were significantly higher on day 2 in *P. chabaudi* AS infected B6 compared to A/J mice. In B6, but not A/J, mice serum IL-12 p70 levels on day 2 also represented a significant increase over basal levels in uninfected controls. Secondly, IL-12 p40 and p35 mRNA levels were readily detectable in the spleens of *P. chabaudi* AS infected

A/J and B6 mice. However, under similar conditions of analysis, only a weak expression of p40 mRNA and a virtually undetectable p35 mRNA were observed in the livers of these mice, suggesting the spleen, rather than the liver, as the main source of IL-12 p70 synthesis in vivo. Splenectomy abolished the significant increase in serum IL-12 p70 levels seen in resistant B6 mice, confirming the spleen as the major source of the systemic IL-12 production. Thirdly, compared with uninfected controls, splenic mRNA levels of both IL-12R β1 and β2 were significantly increased in B6 mice by day 3. In contrast, in A/J mice, splenic IL-12R β1 mRNA levels were significantly up-regulated by day 3, but IL-12R β2 mRNA levels were not significantly increased until day 5. Correlating with the earlier up-regulation of both IL-12R, splenic IFN–γ mRNA levels were significantly higher in the spleens of malaria-infected B6 compared to their A/J counterparts on days 4 and 5, consistent with our earlier findings (43, 81).

These results confirm and extend earlier studies demonstrating the polarization of early Th responses in *P. chabaudi* AS infected resistant B6 and susceptible A/J mice (43, 81), and the pivotal role of the spleen as a site for the development of anti-plasmodial mechanisms (18, 19). Importantly, our results support, in an infectious disease model, recent observations showing the critical requirement of both IL-12R β1 and β2 expression for full functional interaction between IL-12 p70 and its receptor complex (163, 197). There were, nonetheless, a number of unexpected findings that deserve further comment. As presented in Chapter 2, whole tissue IL-12 p40 mRNA levels in the spleen were slightly increased on day 3 in B6 mice compared to uninfected controls, whereas in A/J hosts, this increase on day 3 was statistically significant. Compared to basal levels in uninfected controls, IL-12 p35 mRNA levels were not significantly up-regulated following *P. chabaudi* AS infection in either mouse strain, although levels tended to be higher in A/J mice. The exact nature of the

relationship between whole spleen IL-12 mRNA levels and serum p70 levels is unclear. Since gene transcription would have to precede translation and protein secretion, it appears that the changes in splenic IL-12 p40 mRNA levels on day 3 would not be directly responsible for the early increases in serum p70 in infected B6 mice, which had already peaked by day 3.

Although serum p70 levels were significantly different between the two strains, the total p40 levels, referring to the combined levels of p40 monomers, p40-p40 homodimers, and p40-p35 heterodimers, were not significantly different. It is well established that p40 tends to be secreted in large excess (10-100 fold) of p70 levels (104, 107). It follows that, at a given level of p40 concentration, corresponding p70 levels could vary widely and thus measurement of p40 levels alone can lead to an over-estimation of biologically active p70 levels. This point appears to be relevant to our results since, although serum p40 levels were comparable, serum p70 levels varied significantly between *P. chabaudi* AS infected A/J and B6 mice.

Exactly what regulates how much excess p40 will be released over p70 is presently unknown, but the nature of this regulation seems to be complex. For example, recent analysis of *Salmonella dublin* LPS-induced p40 release by murine peritoneal macrophages indicated that, LPS, when used alone, was a potent stimulus for IL-12 p40, but not p70, release (127). However, the combination of *S. dublin* LPS and IFN-γ was a potent stimulus for the secretion of high levels of both p40 and p70, suggesting that other cytokines may be involved in the regulation of p40 versus p70 secretion.

IL-12 p40 monomers and dimers have been shown to be capable of antagonizing the actions of biologically active p70, both in vitro and in vivo (113, 114). Since the excess in serum p40 over p70 levels is greater in *P. chabaudi* AS infected A/J compared to B6 mice, it is tempting to speculate whether or not this

excess of p40 could contribute to greater antagonistic activity against p70 in A/J compared to B6 hosts. Since p40 is normally secreted in large excess of p70 in virtually all IL-12 producing cells, the question of just how much p40 would be required to significantly inhibit p70 actions in vivo is important.

To evaluate the antagonistic activity of serum p40 in vivo, IL-12 p40 transgenic mice were generated by Yoshimoto and co-workers (252). Treatment of p40 transgenic mice and control littermates with 30 ng of rIL-12 given i.p. for 2 consecutive days resulted in greatly enhanced NK cell lytic activity in spleens of control, but not p40 transgenic, mice. Furthermore, keyhole limpet hemocyanin (KLH)-induced IFN-γ production in vitro was markedly reduced in lymph node cells from p40 transgenic compared to normal controls previously immunized with KLH. In addition, daily administration of 0.1 µg/day of rIL-12, from day -1 to day 4, to blood-stage *P. berghei* XAT malaria-infected p40 transgenic and control mice resulted in significant protection of control, but not transgenic, hosts from the disease.

The concentration of p40 in the sera of uninfected transgenic mice was as high as 100 µg/ml, whereas p70 levels were around 30 pg/ml. In comparison, serum IL-12 p40 levels in normal or *P. chabaudi* AS infected A/J or B6 were over 1000-fold less, ranging from 15 to 40 ng/ml, but p70 levels were higher, ranging from 0.4-0.5 ng/ml in uninfected A/J or B6 mice, to about 3 ng/ml in day 2 *P. chabaudi* AS infected B6 mice. Based on the relative levels of p40 compared to p70 in transgenic compared to normal or malaria-infected A/J or B6 mice, it would appear that serum p40 levels in our model would only account for minimal antagonistic effects against p70. Support for such a conclusion comes from the observation that, following treatment with 0.1 µg/day of rIL-12 for five days (16), A/J mice develop readily detectable Th1 responses and are protected against lethal challenge with malaria, unlike transgenic mice infected with *P. berghei* and treated with IL-12 (252).

Monocyte/macrophages are generally regarded as the major physiological sources of IL-12, although IL-12 synthesis has also been described in a variety of cell types, including neutrophils and dendritic cells (116, 118, 196). The main focus of the studies presented in Chapter 3 was to determine whether differences in IL-12 production in vivo between A/J and B6 mice during early blood-stage *P. chabaudi* AS malaria were also reflected at the level of macrophage IL-12 synthesis in vitro. As seen earlier in Chapter 2, there were significant differences between the two mouse strains in systemic p70, but not p40, levels. Similarly, in Chapter 3, we found that splenic macrophages recovered from day 4 infected B6 compared to A/J mice produced significantly greater quantities of IL-12 p70, but not p40, both spontaneously and following stimulation with LPS or PRBC. Moreover, these differences in splenic macrophage p70 synthesis correlated with a significantly greater level of IFN–γ synthesis by spleen cells from day 4 infected B6 compared to A/J mice. In addition, during early blood-stage *P. chabaudi* AS malaria, we found significant increases in the percentage of macrophages earlier in the spleens from resistant B6 compared to susceptible A/J mice. These differences between the two mouse strains in the population of splenic macrophages could further contribute to in vivo differences in total p70 levels produced by these cells during early infection.

Although these results are consistent with a major role for splenic macrophages in IL-12 synthesis during blood-stage malaria, we cannot rule out the possible contribution of production by other cell types. Support for the role of spleen cell types other than macrophages in IL-12 production was suggested by preliminary studies that showed up-regulation of IL-12 p40 and p35 mRNA expression in non-adherent spleen cell populations from *P. chabaudi* AS infected A/J and B6 mice (data not shown). In previous studies from our laboratory, in vivo depletion of macrophages by treatment with silica or liposome-encapsulated macrophage poisons demonstrated a critical role

for macrophages in host resistance to blood-stage malaria (205). Therefore, macrophage IL-12 production and IL-12-induced protective effects might contribute significantly to the dependence of host resistance on macrophage activity. In chapter 3, splenic macrophages were found to be maximally primed for IL-12 p40 and p70 release in vitro on day 4 post infection, whereas serum IL-12 p70 levels had already reached a peak in B6 mice on day 2. These results raised the possibility that other cell types in the spleen might be responsible for the early increases in serum p70 observed in B6 hosts. Alternatively, splenic macrophages might be primarily responsible for the early increases in serum p70, suggested by the fact that up-regulation of p40 mRNA levels was detectable in splenic macrophages by day 2. However, progressive activation of splenic macrophages for further Il-12 p70 release between days 2-4 might not be reflected in further increases in serum p70 after day 2, owing to increased up-take from the serum by enhanced IL-12R expression in the spleen on days 3-5.

In Chapter 4, the therapeutic potential of exogenous IL-12 treatment in blood-stage malaria was further explored. Previous studies using rIL-12 treatment to cure malaria were limited by the fact that treatment had to be initiated on or one day prior to the infection (16). During the usual course of malaria infections in humans, patients are usually diagnosed by positive identification of the malaria parasites on blood films. By the time of such diagnoses, patients would often have been exposed to the parasite for over two weeks due to the long incubation period of the liver stage of the disease. Another important consideration in the therapeutic use of IL-12 is the issue of dose-dependent toxicity (189).

Using half the usual therapeutic dose of chloroquine (CQ) (12.5 instead of 25/kg body wt) and half of the standard protective dose of IL-12 (0.05 instead of 0.1 μg/day), daily treatment of *P. chabaudi* AS infected A/J mice could be delayed until day 3 when infection is well established. Moreover, the time course of treatment was

reduced from 6 to 4 days. This translates to delivering a combined dose of 0.2 μg of IL-12 per mouse, compared with 0.6 μg when the standard protective dose of 0.1 μg/day for six days is used. Combined treatment of *P. chabaudi* AS infected A/J mice with low doses of IL-12 and CQ resulted in a greater than 15-fold reduction in peak parasitemia compared with untreated controls. Moreover, 100% of mice treated with IL-12 and CQ recovered from the infection. It should be recalled that a cumulative survival rate of 75% was obtained when mice were treated with the standard therapeutic IL-12 dose of 0.1 μg/day for six days. Compared to untreated controls, combined IL-12 and CQ treatment resulted in significantly enhanced erythropoiesis in the bone marrow and spleen of infected A/J mice.

To further understand the mechanism(s) of IL-12-induced protection against blood-stage malaria, one of the objectives in this thesis was to determine the downstream effects of IL-12 treatment on the induction of cytokine or cytokine receptor gene expression in *P. chabaudi* AS infected A/J mice. In infected A/J mice receiving combined IL-12 and CQ, compared to untreated controls or those receiving CQ alone, malaria parasite -induced production of IFN-γ by splenocytes in vitro was significantly increased. Consistent with the increased IFN-γ production, the IL-12 and CQ treated animals also displayed increased splenocyte mRNA levels for IL-12R β1 and β2, as well as for IFN-γ. Moreover, combined IL-12 and chloroquine therapy induced higher levels of anti-malaria antibodies as well as sterile immunity against reinfection.

A close association between splenic TNF-α mRNA expression and early Th1 responses in *P. chabaudi* AS infected B6 mice was previously demonstrated (81). Moreover, IL-12-induced protection of susceptible A/J mice from *P. chabaudi* AS infection was shown to be mediated by IFN-γ, TNF-α, and NO-dependent mechanisms (16). Additionally, evidence from our laboratory and others have shown

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that TNF–α is a potent modulator of NO synthesis (83, 244). However, the exact relationship between IL-12 and TNF–α is unclear. The studies presented in Chapter 5 were designed to determine whether or not TNF is required for IL-12 synthesis and the induction of protective Th1 responses against blood-stage malaria. TNF activity was previously shown to be required for IL-12 synthesis in vivo and in vitro, using the mouse model of *Mycobacterium bovis* strain BCG (242). Mice deficient in both TNFR are accepted as a model for studying the effects of the total absence of TNF–α activity (247). These mice were chosen for the studies in Chapter 5.

As presented in this chapter, TNFRp55p75-/- mice recovered from *P. chabaudi* AS infection by 4 weeks post infection as did wild type controls. Female, but not male, TNFRp55p75-/- mice were found to have significantly higher parasitemias compared to their wild type littermates, however, during the second week of infection, immediately following the peak parasitemia. Following *P. chabaudi* AS infection, systemic IL-12 p70 production was similar in TNFR deficient compared to wild type controls. Serum levels of p70 were significantly higher in *P. chabaudi* AS infected female mice compared to their male counterparts, wild type or TNFR deficient. The significance of these differences is presently unknown. As reviewed in Chapter 1 of this thesis, studies have shown that the sex of the host can influence resistance to malaria infection, and females tend to be generally more resistant than males.

In addition to unimpaired IL-12 production, TNFRp55p75-/- mice displayed normal Th1 responses compared to their wild type controls. IL-12R β1 and β2 mRNA levels were similarly up-regulated in the spleens of both TNFR deficient and wild type controls on day 5 post infection. Moreover, splenic IFN–γ mRNA levels and protein synthesis by unfractionated spleen cells in vitro were similar in day 7 *P. chabaudi* AS infected wild type or TNFRp55p75-/- mice. LPS, but not PRBC, -induced NO production by adherent splenic macrophages was significantly reduced in cells from

day 7 infected TNFR deficient compared to wild type controls. Interestingly, TNFR deficient, but not wild type, mice were protected against lethal endotoxic challenge with the combination of LPS and D-gal (236). The underlying mechanisms for these differences in LPS response between the two types of mice may also be related to the differential effects of LPS versus PRBC-induced macrophage NO production seen in our studies.

The emerging picture of the mechanism(s) of cytokine-mediated host resistance to blood-stage malaria is one of cooperative and coordinated interactions within a cytokine network involving IL-12, IFN-γ and TNF-α, in particular, as well as the important immunomodulatory molecule, NO. Other mediators are likely to be involved, but await discovery. Resistance depends on the timely and organ-specific syntheses and activities of these cytokines, whereas their uncoordinated production or actions can result in severe pathology and host susceptibility. The pleiotropic nature of cytokine activities suggests a remarkable plasticity in the function of the cytokine network in determining host resistance to blood-stage malaria.

In summary, the studies presented in this thesis demonstrate significant differences between resistant B6 and susceptible A/J mice in endogenous synthesis of IL-12 p70, but not p40, that correlate with the polarization of early Th responses seen in the two mouse strains during early blood-stage malaria. On a per cell basis, significant differences were also found between the two mouse strains in IL-12 p70, but not p40, release by splenic macrophages during early *P. chabaudi* AS infection. In addition, the requirements for systemic IL-12 production, splenic IL-12R β1 and β2 mRNA expression and Th1 responses in *P. chabaudi AS* infected mice appear to be independent of TNF activity. Finally, combined therapy with low dose IL-12 and CQ, compared with untreated controls or mice receiving CQ alone, resulted in increased splenocyte expression of IL-12R β1 and β2, as well as IFN-γ mRNA, together with

enhanced parasite-induced IFN–γ synthesis in vitro by spleen cells. This novel combined IL-12 and CQ therapeutic strategy advances earlier treatment protocols by offering significantly reduced parasitemia, enhanced survival and induction of re-infection immunity, as well as effectiveness against established blood-stage malaria. In conclusion, IL-12 appears to be a key member of a network of early-acting cytokines whose actions have important consequences for the development of host adaptive immunity to blood-stage malaria.

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