MECHANISMS OF INNATE IMMUNITY TO BLOOD-STAGE MALARIA INFECTION: ROLE OF DENDRITIC CELLS IN HOST-PARASITE INTERACTIONS AND INDUCTION OF PROTECTIVE IMMUNITY

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Your file Votre référence
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Our file Notre référence
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For my parents and brother

ABSTRACT

A favorable outcome of blood-stage malaria infection requires immune responses that effectively eliminate the infection with minimum pathology to the host. There is strong evidence that the innate immune system is critical for initiating and shaping the subsequent adaptive immune response to blood-stage *Plasmodium* parasites. As sentinels of infection and potent activators of immunity, dendritic cells (DCs) are pivotally positioned to control the immune response to malaria and thereby influence the outcome of infection. Experiments performed in this thesis work aimed to determine the role of DCs in host-parasite interactions and subsequent induction of protective immunity to blood-stage malaria. Moreover, the studies described herein emphasize the critical contributions of key cytokines to DC responses and interactions with other cells of the innate and adaptive immune system during blood-stage malaria. Based on previous linkage analyses that identified interleukin (IL)-15 as a candidate in genetic regulation of host resistance to blood-stage malaria, we demonstrated that IL-15 is important for timely resolution of a primary infection with *Plasmodium chabaudi* AS and maximal production of Th1-type cytokines and antibodies. Notably, IL-15 was revealed to be a key early cytokine for optimal DC and natural killer (NK) cell function as well as IL-12-dependent IFN-γ production following *P. chabaudi* infection. Next, we demonstrated the ability of DCs to recognize and phagocytose parasitized red blood cells (pRBCs) in a highly selective and actin-dependent manner. Following interaction with pRBCs in vitro or in vivo, DCs from malaria-resistant mouse strains were shown to express upregulated costimulatory molecules and secrete abundant Th1-polarizing cytokines, particularly IL-12 and IFN-γ. These signals enable DCs to prime other immune cells such as NK cells and $CD4^+$ T cells for maximal IFN-γ production. Reciprocally, malaria-activated NK cells were shown to induce DC maturation and production of Th1-polarizing cytokines, thus propagating an innate feedback loop leading to induction of Th1 immunity. DCs from malaria-susceptible A/J mice, however, showed impaired Th1 priming in favor of selective induction of IL-10-secreting $CD4^+$ T cells, suggesting DC-mediated activation of tolerance, not immunity, in hosts unable to control and survive blood-stage malaria. These findings provide novel and important insights into the DC-mediated immune mechanisms that initiate and regulate host resistance to blood-stage malaria infection.

RESUMÉ

Pour combattre efficacement une infection causée par la phase érythrocytaire du parasite du paludisme (malaria), une réponse immune adéquate est requise, capable d'éliminer le parasite sans provoquer de dommages pathologiques chez l'hôte. De nombreuses études mettent en évidence l'importance du système immunitaire inné dans l'initiation et la modulation de la réponse immune acquise nécessaire pour éradiquer le parasite. En tant que sentinelles et activateurs par excellence de l'immunité contre les infections, les cellules dendritiques (CDs) jouent un rôle primordial dans le contrôle de la réponse immune contre la malaria et influencent ainsi la résolution de la maladie. Les expériences réalisées dans cette thèse avaient pour but de déterminer le rôle de CDs dans les interactions entre l'hôte et le parasite ainsi que dans l'induction subséquente d'une réponse immune protectrice contre la phase érythrocytaire de la malaria. De plus, les études décrites ici ont permis de mettre en évidence le rôle critique de certaines cytokines dans les réponses générées par les CDs et dans les interactions de celles-ci avec d'autres cellules du système immunitaire inné et acquis lors d'une infection causée par le parasite de la malaria. Des études génétiques préalables ont identifié inteleukine-15 (IL-15) comme étant un candidat responsable de la régulation génétique de la résistance de l'hôte contre la phase érythrocytaire du parasite. En accord avec cette observation, nous avons démontré que IL-15 est importante pour une résolution plus rapide de l'infection primaire causée par le parasite *Plasmodium chabaudi* AS ainsi que pour une production optimale de cytokines et d'anticorps de type Th1. Notamment, IL-15 s'est révélé être une cytokine clé pour permettre un fonctionnement optimal des CDs et des cellules « natural killer » (NK) ainsi que pour optimiser la production d'IFN-γ dépendante d'IL-12 induite par une infection à *P. chabaudi*. Nous avons ensuite démontré la capacité des CDs à reconnaître et à phagocyter les globules rouges parasités (GRp) de façon hautement spécifique et dépendante de la polymérisation d'actine. Suite à l'interaction avec les GRp in vitro ou in vivo, les CDs provenant de souris résistantes à la malaria ont présenté une augmentation de l'expression de molécules co-stimulatrices et de la production de cytokines du type Th1, en particulier IL-12 et IFN-γ. Ces signaux permettent aux CDs de stimuler d'autres cellules du système immunitaire comme les NK et les cellules T $CD4^+$ pour une production maximale d'IFN-γ. De façon réciproque,

les cellules NK activées par la malaria induisent la maturation des CDs et la production de cytokines de type Th1 par celles-ci, contribuant ainsi à l'amplification de la réponse innée nécessaire à la génération d'une immunité de type 1. Cependant, les CDs provenant de souris A/J, susceptibles à la malaria, ont présenté une réponse de type 1 diminuée, en favorisant par le fait même une production sélective de cellules T $CD4^+$ sécrétrices d'IL-10. Ce type de réponse mènerait à une tolérisation du système immunitaire dans des hôtes incapables de contrôler et de survivre à la phase érythrocytaire du parasite. Les résultats de cette thèse fournissent de nouvelles et importantes connaissances sur les mécanismes immunitaires reliés aux CDs dans l'initiation et la régulation de la résistance de l'hôte à la malaria.

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
B6	C57BL/6
CFSE	carboxyfluorescein succinimidyl ester
Char	chabaudi resistance locus
CM	cerebral malaria
CpG	CpG oligonucelotides
DC	dendritic cell
DDC-IDC	dermal dendritic cell and interstitial dendritic cell
ELISA	enzyme-linked immunoabsorbent assay
FcR	Fc receptor
GM-CSF	granulocyte/monocyte colony-stimulating factor
GPI	glycosyl-phosphatidylinositol
ICAM	intracellular adhesion molecule
IFN	interferon
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
moDC	monocyte-derived dendritic cell
MSP	merozoite surface protein
NK cell	natural killer cell
NKT cell	natural killer T cell
NO	nitric oxide
nRBC	normal or noninfected red blood cell

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
p.i.	post-infection
pRBC	parasitized red blood cell
PRR	pattern recognition receptor
RBC	red blood cell
SEM	standard error of the mean
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TLR	Toll like receptors
TNF	tumor necrosis factor
Treg	regulatory T
Tr1	regulatory T type 1
VCAM	vascular adhesion molecule

STATEMENT FROM THE THESIS OFFICE

According to the "Guidelines for Thesis Preparation" of the Faculty of Graduate and Postdoctoral Studies, manuscripts of papers which have been published, have been submitted for publication, or are to be submitted, have been incorporated in this thesis.

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 (not the reprints) of one or more published papers.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

The thesis must include the following: 1) a table of contents; 2) a brief abstract in both English and French; 3) an introduction which clearly states that rational and objectives of the research; 4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); 5) a final conclusion and summary; 6) a thorough bibliography; and 7) Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and /or radioactive material.

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

In general, when coauthored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors as a preface to the thesis. The supervisor must attest to the accuracy of this statement 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 clearly specify the responsibilities of all the authors of the co-authored papers.

CONTRIBUTIONS OF THE AUTHORS

The work described in this thesis was performed by the author under the supervision and guidance of Dr. Mary M. Stevenson at the Centre for the Study of Host Resistance, Research Institute of the McGill University Health Centre. The author and Dr. Stevenson are coauthors of all manuscripts presented. For all experimental work, the author designed and performed the experiments, compiled and analyzed the data, and wrote the manuscripts. Dr. Stevenson provided scientific expertise and edited the manuscripts. The author maintained breeding of the IL-$15^{-/-}$ mice. Mifong Tam maintained the parasite and breeding of the IL-$12p40^{-/-}$ and IFN-$\gamma^{-/-}$ mice, provided technical guidance, and assisted with the flow cytometric analyses.

In Chapter 2[b], Dr. Philippe Gros co-supervised the project and provided expertise on genotyping of IL-$15^{-/-}$ mice. In Chapter 3[c], Dr. Mariela Segura devised the BM-derived DC culture system and assisted with all BM-derived DC experiments. Neeta Thawani also contributed to the BM-derived DC experiments. Mifong Tam provided technical guidance and performed the flow cytometric analyses jointly with the author. In Chapter 5[e], Jessica St. Pierre performed the flow cytometry analyses of CD25 and Foxp3 expression and Dr. Ciarico Piccirillo will provide expertise on regulatory T cells.

Parts of Chapter 1[a], including a version of Figure 1B, have been published previously as a book chapter. The work in Chapters 2[b] and 3[c] has been published previously. The work in Chapter 4[d] has been submitted for publication and a manuscript based on the work in Chapter 5[e] is currently in preparation.

a. Urban, B., R. Ing, and M. M. Stevenson. 2005. Early Interactions between blood-stage *Plasmodium* parasites and the immune system. *Curr. Top. Microbiol. Immunol.* 297:25-70.

b. Ing, R., P. Gros, and M. M. Stevenson. 2005. Interleukin 15 enhances innate and adaptive immune responses to blood-stage malaria infection in mice. *Infect. Immun.* 73:3172-3177. Copyright 2005 American Society for Microbiology Journals Dept.

c. Ing, R., M. Segura, N. Thawani, M. Tam, and M. M. Stevenson. 2006. Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation and antigen presentation. *J. Immunol.* 177:441-450. Copyright 2006 The American Association of Immunologists, Inc.

d. Ing, R. and M. M. Stevenson. Dendritic cell and NK cell crosstalk promotes early IFN-γ production and Th1 immune responses to blood-stage malaria infection. Manuscript in revision.

e. Ing, R., J. St. Pierre, C. A. Piccirillo, and M. M. Stevenson. Dendritic cells regulate induction of effector or regulatory T cell responses to blood-stage malaria infection Manuscript in preparation.

CHAPTER 1

INTRODUCTION

1.1 Overview of Malaria Infection

Malaria, a term derived from Italian words meaning 'bad air,' is a parasitic disease caused by protozoans of the genus *Plasmodium*. It is a disease that has afflicted mankind since antiquity and yet today, despite concerted efforts in research and prevention, the global prevalence of malaria continues to rise at an alarming rate and is a major cause of high morbidity and mortality in populations of the developing world. Thus far, there is no effective vaccine, and many insecticides and anti-malarial drugs are increasingly ineffective due to widespread drug resistance. Eradication efforts in developing countries are frequently hampered by a lack of adequate health care due to poverty, social unrest, and political conflict. In areas where malaria is endemic, individuals acquire partial immunity against malaria only after repeated exposure to the parasite. It may take many years of frequent infection episodes to establish protective immunity capable of preventing clinical disease. The recent completion of the *Plasmodium* and anopheline genome projects has renewed hope that better drugs can be developed against the parasite or mosquito vector. However, given that the population at risk from malaria is estimated at over 500 million, the costs of implementing drug-based control programs on a regular basis would be substantial. Alternatively, an effective vaccine against malaria infection that may provide immunity in humans or at least reduce clinical disease would be of great benefit. Therefore, a better understanding of the mechanisms that induce protective immunity to malaria infection may help identify strategic targets for development of effective malaria vaccines and improved immunotherapies.

1.1.1 Epidemiology of Malaria Infection

There are four *Plasmodium* species that infect humans: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*. Together, these parasites are responsible each year for 1-2 million deaths, of which 90% occur mainly in sub-Saharan Africa (1). It is estimated that 200-300 million people in Africa alone are infected with malaria. A recent study suggests that this figure may not reflect the true extent of the malaria burden worldwide and indicate that in 2002 there were over 500 million clinical episodes of *P. falciparium* malaria (2). Indeed, malaria is an increasing health problem in many parts of Asia and South America due in part to resistance to

anti-malarial drugs and insecticides, population growth, agricultural initiatives such as dam building and irrigation schemes, and climate changes such as global warming (2, 3). These factors, along with inadequate health care, poverty and civil disturbances, also have been linked to the increasing malaria burden in Africa.

Young children and pregnant women are disproportionately affected by the morbidity and mortality due to malaria. Each year, young children in Africa experience several clinical attacks of malaria, but only 1-2% of these attacks result in severe or life-threatening disease (3). It is not clear why some children infected with *P. falciparum* experience only fever and other minor symptoms, whereas others develop serious complications such as severe anemia and cerebral malaria. The parasite species, transmission patterns, host genetics and socioeconomic factors all likely play a role. It is clear, however, that malaria mortality rates are increasing as a result of drug resistance and reduced use of blood transfusions in areas where the prevalence of infection with the human immunodeficiency virus (HIV) is high (4). While the overall mortality in African children under the age of 5 years has decreased, mortality due to malaria has increased in the same period and now accounts for 23-37% of mortality from all causes (5, 6). Malaria is also very harmful to pregnant women who experience diminished immune function associated with pregnancy. In low endemic areas, malaria in pregnancy may lead to maternal death, abortion, or stillbirth (4). In high endemic areas, mothers may survive the malaria infection but heavy parasite loads in the placenta may impair placental function, resulting in low birth weight, premature delivery, hypoglycemia, intrauterine growth retardation, acute pulmonary edema, and increased risk of infant mortality (4, 7).

1.1.2 Parasite Life Cycle

The *Plasmodium* parasite has a complex life cycle involving an insect and vertebrate host, three distinct phases of infection, and multiple host tissue compartments (Fig. 1.1; reviewed in reference 8). The *Anopheles gambiae* mosquito is the insect host for the sexual stage of development as well as the transmission vector of the parasite. During a blood meal, a female *Anopheles* mosquito draws blood containing male and female gametocytes from an infected individual. Inside the midgut of the mosquito, the

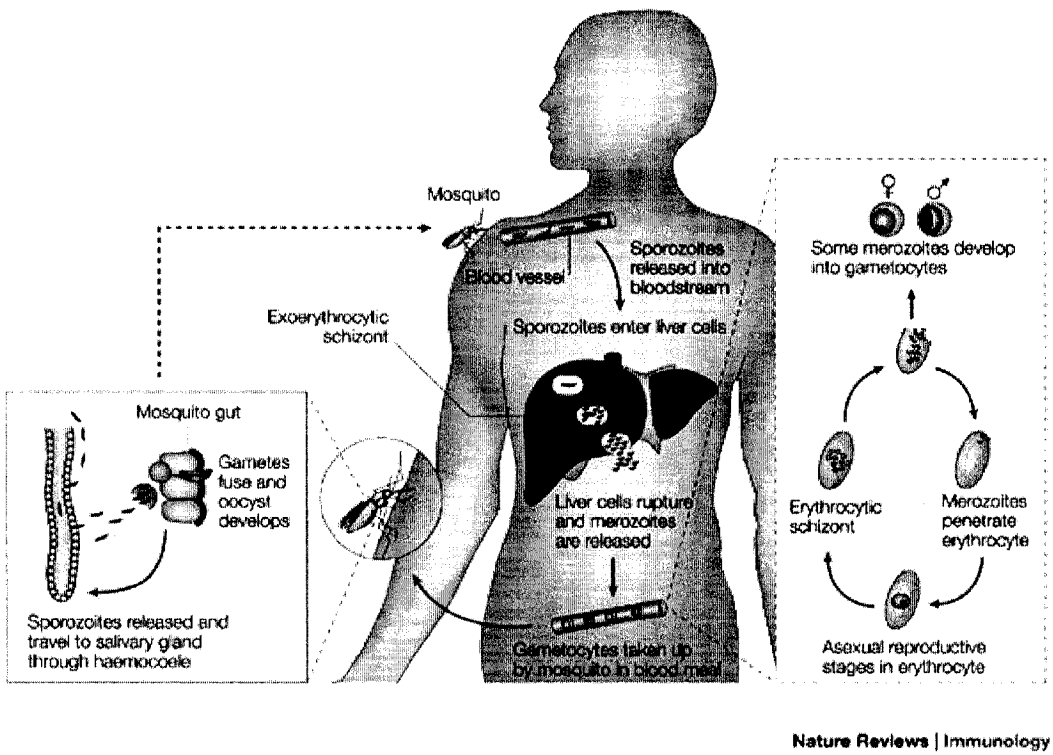

Figure 1.1: *Plasmodium* Life Cycle (Adapted from Ref. 8)

gametocytes mature and fuse to form a zygote, called an oökinete, which then penetrates through the midgut wall to the outer surface and develops into an oöcyst in which a large number of thread-like sporozoites, also called liver-stage parasites, develop. The oöcyst then bursts, liberating the sporozoites which migrate to the mosquito's salivary glands, from where they can be inoculated into another host during the next blood meal. The entire sexual stage varies from 8 to 35 days, depending on the parasite species.

After inoculation by the mosquito into the subcutaneous tissue or, less frequently, the bloodstream of the mammalian host, the sporozoites reach the liver and begin the pre-erythrocytic or liver stage of development, which lasts for a period of about two weeks. Sporozoites must traverse several hepatocytes by disrupting host cell membranes before they enter a final hepatocyte and start asexual replication. It is thought that the initial migration process triggers intracellular signaling pathways within the sporozoite that are needed to induce apical exocytosis of certain molecules required for final internalization (9). Circumsporozoite protein (CSP) is a major protein found on the surface of sporozoites and plays an important role in both sporozoite motility and hepatocyte invasion (9, 10). The specific binding of CSP to surface proteins expressed by hepatocytes, such as heparin sulfate proteoglycans (HSPGs), allows the sporozoite to recognize and attach to hepatocytes (11, 12). Final hepatocyte invasion involves invagination of the hepatocyte plasma membrane to form a vacuole, wherein the sporozoite develops and replicates (13). Asexual replication of parasites in hepatocytes results in the formation of liver-stage schizonts, which rupture via parasite-induced proteolysis to release thousands of merozoites into the bloodstream (14). Infected hepatocytes do not induce inflammatory responses and the host typically does not experience any clinical symptoms at this stage of infection. For *P. vivax* and *P. ovale*, some of the sporozoites may enter the hypozoite stage before undergoing asexual multiplication, and reactivation of the hypozoite may cause the relapses in infections with these plasmodial species (15).

During the blood stage of infection, the merozoites invade host erythrocytes and undergo a second stage of asexual development. Invasion of erythrocytes is a complicated process that involves many parasite proteins and host receptors. The initial attachment of the merozoite to the red blood cell (RBC) is mediated through binding of

highly polymorphic merozoite surface proteins (MSPs) with surface RBC receptors (16). After the initial binding, the merozoite undergoes reorientation to bring its apical end in contact with the RBC membrane (17). A tight junction, called an apical complex, is then formed between the merozoite surface and specific ligands on the RBC membrane. The apical complex contains microneme and rhoptry proteins, which are thought to be important for the invasion process (18) and bind to two major families of host receptors. First, a microneme protein called Duffy binding protein is used by *P. vivax* to recognize the Duffy blood group antigen at the surface of human reticulocytes (19). In West Africa, where the Duffy negative blood group is widely prevalent, *P. vivax* infections have largely disappeared (20, 21). The second family includes receptors not related to the Duffy blood group on the RBC or reticulocyte surface that recognize reticulocyte binding proteins (21). In contrast to *P. vivax*, *P. falciparium* uses many redundant pathways to invade erythrocytes, which may explain its high virulence and unique ability to invade erythrocytes of different stages of maturation (22). In one pathway used by *P. falciparium*, a family of microneme proteins, including the 175 kDa erythrocyte binding antigen (EBA-175) and its paralogues such as BAEBL, bind a group of RBC membrane sialoglycoproteins called glycophorins (22, 23). Two other pathways involve either sialic acid components but not glycophorins or trypsin-resistant reticulocyte binding proteins (18, 24).

Apical attachment of the merozoite induces invagination of the RBC membrane. The tight junction formed between parasite and RBC membrane moves from the anterior to the posterior end of the parasite, creating a parasitophorous vacuole inside the host cell (25). Inside the vacuole, the merozoite first develops into the trophozoite, which appears as a ring form inside the infected RBC. The trophozoite matures and undergoes asexual replication, called erythrocytic schizogeny, to form a blood-stage schizont. About 20 merozoites are produced in a schizont (16). After many replicative cycles, the infected RBC ruptures and releases parasite-derived toxins and merozoites into the host bloodstream to invade other erythrocytes, thus propagating blood-stage infection. Within the RBC, some of the merozoites undergo sexual differentiation to form gametocytes, which are ingested in the blood meal of the next biting female *Anopheles* mosquito. The erythrocytic cycle occurs repeatedly to produce gradual increases in parasitemia, and the

host experiences nonspecific symptoms associated with malaria, including shivering, headache, chills and fever. Each erythrocytic cycle takes 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, and 72 hours for *P. malariae* (26).

1.1.3 Malaria Pathology

A wide spectrum of clinical manifestations is observed in human malaria, ranging from asymptomatic infections to fulminant disease. The clinical symptoms and severity of disease may vary depending on the *Plasmodium* species, the age and immune status of the host, and other genetic and environmental determinants. Of the four species that infect humans, *P. falciparum* and, to a much lesser extent, *P. vivax* are responsible for the majority of infections (21). *P. falciparum* also causes the most severe symptoms (21). *P. ovale* and *P. malariae* cause relatively benign infections, although an immune complex disease called glomerulonephropathy can develop after chronic *P. malariae* infection (27). Clinical symptoms are linked to the replication cycles of the parasite. Rupture of infected RBCs and release of malarial toxins into the bloodstream at each replicative cycle causes repeated episodes of chills, headaches, and fevers. Malaria infections are naturally chronic, leading to the development of splenomegaly, hepatomegaly, renal failure, pulmonary edema, and anemia. Both *P. falciparum* and *P. vivax* can cause severe anemia, but only *P. falciparum* causes the serious complications of cerebral malaria, hypoglycemia, metabolic acidosis and respiratory distress (21). In addition to host genetics and immune status, transmission patterns and host age are important determinants of disease. In high endemic areas, infants and young children are most susceptible to severe anemia, cerebral malaria and metabolic acidosis; while in areas of lower transmission and in travelers, primary infections occur mainly in adults and may cause renal failure, pulmonary edema, shock, and jaundice (27).

Anemia is the most common serious complication of malaria infection and accounts for the majority of deaths from malaria, causing 5-16% morality in children (28, 29) and 6% in pregnant women in Africa (30). Severe anemia is associated with tissue hypoxia and respiratory distress, which can lead to cardiac arrest. In humans, severe anemia is not correlated with high parasite densities and does not arise simply from destruction of infected RBCs (31, 32). Rather, severe anemia is thought to be a result of two processes:

increased destruction of uninfected RBCs and decreased production of new RBCs (also called erythropoietic suppression) to replenish the depleted RBC pool. Phagocytosis of uninfected, bystander RBCs for a period beyond the initial control of parasitemia has been demonstrated during human infection (27, 33). Indeed, long-term studies indicate that the duration of infection is a better predictor of risk of severe anemia than is the level of parasitemia (32). Erythropoietic suppression resulting in insufficient numbers of reticulocytes, or young functional RBCs, to be released into the circulation to alleviate the anemia has been observed during acute infection with *P. falciparum* and with rodent *Plasmodium* parasites (34, 35, 36). The acute phase of infection is also associated with suppressed proliferation, differentiation and maturation of erythroid precursors into reticulocytes and mature RBCs (35, 36). Similarly, DNA microarray analysis in a murine model of malaria has revealed reduced transcription of erythroid-specific genes in the spleen and bone marrow during early infection (37). It is notable that increased transcriptional activity of erythroid-specific genes, marked splenomegaly and constitutive reticulocytosis are features of recombinant congenic strains of mice that are highly resistant to a mouse model of infection with *Plasmodium chabaudi* AS (38).

Cerebral malaria (CM), while less common, is more deadly and causes about 20% mortality, even in children living in areas where there is access to good health care facilities (39). About 10% of children who survive cerebral malaria continue to suffer neurological defects that may impede their education and limit their socioeconomic prospects (4). Accumulation of infected RBCs in cerebral microvessels is thought to be the main mechanism of CM pathogenesis. Cerebral sequestration of infected RBCs may disturb blood flow in brain capillaries and venules, leading to obstruction and hypoxia of the surrounding brain parenchyma and to hemorrhages (27). Although sequestration of infected RBCs occurs in nearly all patients infected with *P. falciparum*, only 1% of infected individuals develop CM, suggesting that cerebral cytoadherence of infected RBCs is not sufficient for the progression to CM (27). Research in humans with CM, as well as in mouse models of CM, show that infiltration and sequestration of host cells, such as leukocytes or platelets, may also be involved in CM pathogenesis (40, 41). In patients with CM and experimental models of CM, there is increased expression of chemokines and proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and

interferon (IFN)-γ, which act to recruit leukocytes including monocytes, macrophages and neutrophils (42, 43, 44). Importantly, the recruited macrophages and neutrophils are also major sources of cytokines and chemokines that may participate directly in the development of CM (43, 45). Depletion of neutrophils prevents the development of CM in mice by inhibiting the production of proinflammatory cytokines and decreasing sequestration of monocytes in the brain (43). T cells are also thought to contribute to CM pathogenesis through the secretion of cytokines, chemokines and cytotoxic mediators such as perforin (27).

1.1.4 Pathogenesis of Malarial Disease

Malarial disease is complex and involves multiple processes, including destruction of both infected and uninfected RBCs, microvascular obstruction, and inflammatory responses that singly or in combination lead to severe complications. Given the diverse range of disease syndromes in severe malaria, no single mechanism can fully explain the complex etiologies. Nonetheless, research suggests that a few basic processes appear to be involved in several major malarial diseases. These processes include the adherence of parasitized erythrocytes to vascular endothelium of target organs, the effects of bioactive parasite products on host cells, and the production of proinflammatory mediators and recruitment of inflammatory cells by the immune system in response to parasite products. Thus, overlapping inflammatory cascades in target tissues may lead to diverse systemic or organ-specific disease syndromes. Appropriate regulation of immune responses might therefore be important for both infection control and healthy outcomes.

Parasite adhesion and sequestration

A key feature of the biology of *P. falciparum* is its ability to cause infected RBCs to adhere to the linings of small blood vessels. By contrast, *P. vivax* does not show cytoadherence and sequestration, which explain why this strain does not cause cerebral malaria and is rarely fatal. This adhesion by *P. falciparum* allows the parasite to sequester in numerous tissues throughout the body and greatly reduces blood flow through the affected tissues, possibly leading to some of the serious complications associated with falciparum malaria. In addition, the asexual parasites and gametocytes

also can adhere to the placenta and contribute to increased morbidity and mortality of both mother and fetus. Adherence to the endothelium greatly benefits parasite survival; it protects the parasite from clearance as nonadherent infected RBCs are rapidly removed from the circulation by the spleen (46) Adhesion is mediated by the highly polymorphic *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed on the surface of infected RBCs and binds to multiple host receptors, such as intercellular adhesion molecule 1 (ICAM-1), vascular cell-adhesion molecule 1 (VCAM-1), CD31, CD36, thrombospondin, and E-selectin expressed the surface of vascular endothelial cells, and chondroitin sulphate A (CSA) and hyaluronic acid expressed on syncytiotrophoblasts (27). PfEMP1is encoded by a large and diverse *var* gene family that is involved in the parasite's antigenic variation and plays an important role in *P. falciparum* pathogenesis as well as immune evasion. Despite its considerable diversity, specific domains of PfEMP1 have been potential targets for vaccines (47).

The extent of adhesion and type of tissue affected vary according to properties of the parasite isolates and specific combinations of host receptors. CSA and the macrophage scavenger receptor CD36 are involved in sequestration of infected RBCs in many organs, including heart, lung, liver, kidney, and subcutaneous tissues (27). In the placenta, parasites bind to CSA but not to CD36; while CD36 is the crucial host receptor for sequestration in the microvasculature (48, 49). Sequestration of parasites in the brain leading to cerebral malaria is thought to involve ICAM-1 (50). There is evidence for a role of PfEMP1 and host receptors in malarial disease. A nonsense mutation in the CD36 gene is associated with protection from severe malaria (51). Moreover, the production of anti-PfEMP1 antibodies in pregnant women decreases the risk of complications from placental malaria (52).

Malaria toxins and products

In addition to cytoadherence, malaria parasites produce toxins and metabolic products such as hemozoin that may contribute to the pathogenesis of malarial disease. One putative toxin, glycosyl-phosphatidylinositol (GPI) that anchors MSP antigens on the surface of infected RBCs, is thought to bind to pathogen recognition receptors expressed by host cells of the innate immune system, leading to activation of Src family

kinases and protein kinase C (53, 54). These kinases mediate signaling pathways that induce gene transcription of various inflammatory mediators, such as TNF-α, interleukin (IL)-1, inducible nitric oxide synthase (iNOS), ICAM-1 and other adhesion molecules (54, 55). Some of these mediators act as endogenous pyrogens to cause fever and also contribute to parasite sequestration as well as to immunopathology (discussed below). In a sepsis shock model, GPI alone is sufficient to cause symptoms of acute malaria, such as transient pyrexia, hypoglycemia, and TNF-associated shock syndrome (53). GPI is thought to be a ligand for natural killer T (NK-T) cells which recognize glycolipids such as GPI presented on CD1d molecules expressed by dendritic cells (DCs). GPI-activated NK-T cells produce proinflammatory cytokines, particularly IL-1 and TNF-α, which may contribute to the pathogenesis of cerebral malaria (56). Hemozoin, also called malaria pigment, is a long-lived product of parasite-mediated hemoglobin digestion and has seemingly contradictory bioactivities. It stimulates macrophages to produce nitric oxide (NO) and TNF-α (57) and DCs to release IL-12, TNF-α and chemokines (58, 59). Conversely, ingestion of hemozoin by macrophages impairs their phagocytic function and expression of major histocompatibility complex (MHC) class II molecule (60-62). Hemozoin has also been shown to inhibit the differentiation and maturation of human monocyte-derived DCs, possibly by increasing expression of peroxisome proliferator-activated receptor (PPAR)-γ, an antognist of NF-κB and inhibitor of monocyte differentiation into DCs (63). In contrast, other studies show that hemozoin can favor DC maturation, as demonstrated by upregulated costimulatory molecule expression and production of chemokines and cytokines (58). Overall, hemozoin is thought to contribute to the immunosuppression observed in malaria-infected humans or experimental mice, although this remains controversial. Furthermore hemozoin converts arachidonic acid to hydroxyarachidonic acid (HETE), which can cause vasoconstriction, edema and parasite adhesion to the vascular endothelium (64).

Inflammatory mediators

Inflammatory mediators produced by the host immune system during the acute phase of malaria infection play key roles in both protective immunity against infection and development of malarial disease. Excessive production of proinflammatory

cytokines is thought to favor disease onset over immune protection. Children with CM or severe malaria show higher circulating levels of inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IFN-γ, than those with mild malaria (65, 66, 67). An earlier study showed that mice deficient in TNF receptor 2 (TNFR2) are resistant to experimentally induced CM (68). The role of TNF-α, however, has been challenged by recent studies indicating that lymphotoxin (LT)-α is more important than TNF-α in CM pathogenesis. Mice deficient in TNF-α are as susceptible as controls to CM, whereas LT-α-deficient mice are resistant (69). Notably, LT-α also binds to TNFR1 and TNRF2 (70), which may explain the resistance of TNFR2-deficient but not TNF-α-deficient mice to the development of CM.

IFN-γ, on the other hand, has been clearly linked to the onset of malarial pathology in mice as well as humans. In vivo neutralization of IFN-γ in a mouse model of CM reduces TNF-α production and prevents CM development (65). Moreover, strong evidence for the central role of IFN-γ in CM pathogenesis is provided by findings that mice deficient in IFN-γ or IFN-γ receptor are resistant to the development of CM (71). In humans, potent and timely IFN-γ production is indicative of an effective cell-mediated immune response to infection. However, high systemic levels of IFN-γ correlate with severe pathology in humans (72, 73), suggesting that control of clinical symptoms depends on the ability to regulate the inflammatory response. IFN-γ may induce onset of severe pathology by upregulating the production of TNF-α and quinolinic acid metabolites which also contribute to cerebral symptoms in humans (74). Despite these findings, an IFN-γ-dependent mechanism of malarial pathology may be overly simplistic because: 1) mice deficient in IFN-γ receptor remain susceptible to severe malaria and mortality; and 2) in vivo neutralization of IFN-γ does not ameliorate pathology in mice susceptible to *P. chabaudi* infection (75).

It is likely that the balance and timing of proinflammatory and anti-inflammatory cytokines critically determine the final outcome of infection. Indeed, the production of the T helper 2 (Th2) cytokine IL-4 by NK-T cells protects against severe disease (76). Mice deficient in the anti-inflammatory cytokine IL-10 suffer more severe disease and mortality from *P. chabaudi* infection than do wild-type mice or other resistant strains

(77, 78). Similarly, *P. chabaudi*-infected mice treated with antibodies neutralizing transforming growth factor (TGF)-β produce higher levels of TNF-α and IFN-γ and exhibit a higher rate of mortality (79). Importantly, the timing of TGF-β production determines whether protection or pathology develops. An early TGF-β response is associated with upregulated TNF-α and IFN-γ and rapid resolution of murine malaria, whereas TGF-β alone or in concert with IL-10 acts during the chronic stage to suppress inflammatory responses that might trigger onset of clinical disease (80-82).

1.1.5 *Plasmodium chabaudi* AS: murine model of blood-stage malaria

Infections of inbred mice with rodent strains of *Plasmodium* species are useful and convenient experimental tools for the elucidation of the complex interactions between *Plasmodium* parasites and the host immune system. Murine malaria models have been particularly informative for the study of immune responses against asexual blood stages of *Plasmodium* parasites. Four species, namely, *P. chabaudi, Plasmodium vinckei, Plasmodium yoelii*, and *Plasmodium berghei* cause either self-resolving or lethal infections in mice (83). *P. berghei, P. vinckei*, and *P. chabaudi* have also been used to study malaria pathology (84), while *P. berghei* and *P. yoelii* have been used in animal models of CM (85). The availability of inbred, congenic, transgenic, and gene knockout mice with naturally occurring or experimentally induced genetic mutations leading to defects in specific components of the immune system has allowed researchers to finely dissect mechanisms involved in protective immunity or pathology during blood-stage malaria. Although various *Plasmodium* species can differ in virulence and elicit disparate immune responses, many principles of immune recognition, activation, and effector pathways that were defined in mouse models are likely to be relevant to human malaria. Indeed, many findings obtained in mice infected with various rodent parasites have been confirmed in human studies and have established a framework for studying mechanisms of anti-malarial immunity in humans (83, 84).

The mouse model of *P. chabaudi* AS infection has been used extensively to investigate mechanisms of immunity and pathology during blood-stage malaria. Many studies have been performed in inbred mice with differential susceptibilities to a primary *P. chabaudi* infection, which is characterized by an acute phase parasitemia followed by

a chronic phase of low and recrudescent parasitemia. Resistant C57BL/6 mice show moderate levels of acute parasitemia, resolve the infection by week 4 post-infection (pi), and nearly 100% survive. In contrast, susceptible A/J mice develop fulminating parasitemia and severe anemia, and succumb to infection by day 10-12 p.i. (8, 86). These interstrain differences in susceptibility to blood-stage malaria have permitted mapping of quantitative trait loci using large crosses between resistant and susceptible progenitor strains. In early linkage studies, the control of blood-stage *P. chabaudi* replication was mapped to a 10-15 Cm locus—called the *chabaudi resistance locus* 2 or *char2*—on the mouse chromosome (Chr) 8, which accounts for 10% of variance in peak parasitemia between susceptible A/J and resistant B6 mice (87, 88). This locus contains several candidate genes, including the gene for IL-15. A number of other loci have also been identified for the control of *P. chabaudi* malaria in mice: 1) *char1* on Chr 9 controls peak parasitemia and mortality (87); 2) *char3* on Chr 17 controls parasite clearance during late infection (89); 3) *char4* on Chr 3, with an additive effect contributed by a locus on Chr 10, controls peak parasitemia (90); 4) *char5* and *char6*, found on Chr 5 which contains the gene encoding erythropoietin, control peak parasitemia (91); 5) *char7* on Chr 17 controls resistance to challenge infection and maps to the previously reported *char3* but outside the H-2 region (91); 6) *char8* on Chr 11 controls parasite clearance and contains genes for several Th2 cytokines including IL-4, IL-5 and IL-13 (92); and 7) *char9* on Chr 10 controls peak parasitemia and contains the candidate gene encoding vanin 3 (P. Gros personal communication). Together these findings indicate that susceptibility to malaria is a complex trait and likely influenced by both multiple genetic and environmental factors.

1.2 Overview of Innate and Adaptive Immunity

The immune system of vertebrate organisms is divided broadly into innate and adaptive immunity. Adaptive immunity is mediated by T and B lymphocytes, which express a highly diverse repertoire of antigen receptors, the T and B cell receptors, and undergo clonal selection and expansion into effector and memory cells capable of mounting strong primary responses with high specificity and long-lived immunological memory, respectively. However, adaptive immunity is limited by its randomly generated antigen receptors that are unable to distinguish the type or origin of antigens encountered and by the need for specific T or B cell clones to expand and differentiate into effector cells before they can mediate host defense. As a result, adaptive immune responses are slow to develop, typically delayed up to 4-7 days after infection, and thus are unable to respond promptly to contain rapidly replicating microbes. To overcome these limitations, vertebrates rely on innate immunity, an evolutionarily more ancient and conserved host defense system, to detect both the presence and nature of danger, provide the first line of host defense, and control the initiation and pattern of adaptive immune responses. DCs, found in peripheral sites where many pathogens invade host tissues, are highly efficient at capturing and presenting antigens to T cells as well as interacting with other cells of the innate immune system. Thus, DCs are pivotally positioned at the host-pathogen interface to initiate immune responses and provide an essential link between innate and adaptive immunity.

1.2.1 Innate Immunity

In recent years, there has been increasing recognition of the paramount importance of innate immunity in the host response to infection, injury and inflammation, and autoimmune disease. The central tenet of innate immunity is that the immune system is able to discriminate self from nonself through the interaction of pathogen-associated molecular patterns (PAMPs) expressed by invading microbes with pattern recognition receptors (PRRs) expressed on the surface of antigen presenting cells (APCs). This interaction provides pathogen-driven signals needed to activate antigen-specific immune responses capable of protecting the host from infectious agents. As sentinels of infection and efficient stimulators of T and B lymphocytes, DCs are key players in the innate

immune system that functions to recognize and respond quickly to pathogens and tumor cells. In addition, several other cell types and molecules interact to coordinate an innate immune response that leads to the activation of primary adaptive immune responses and boosting of immunological memory. The following sections review the various cells and cytokines in the innate immune system relevant to malaria immunology, with an emphasis on the central role of DCs in the immune response.

Dendritic cells

DCs are highly specialized APCs that initiate, control and regulate the immune response to self and nonself antigens. Long before Steinman and Cohn identified DCs in mouse lymphoid organs and named them based on their unique morphology (93), Paul Langerhans had described DCs in human skin but thought they were cutaneous nerve cells (94). In addition, it was known that antibody production in culture required the presence of accessory cells (95). Numerous studies since these early reports have revealed the immense complexity and diversity of the DC system. When activated, DCs display many fine dendrites that are mobile and extend in many directions from the cell body. The shape and motility of DCs enable these cells to sample the local microenvironment and carry antigen-driven signals to lymphoid tissues for priming of naive lymphocytes. The antigens taken up at the site of infection or injury are processed into peptides that are presented on major histocompatibility complex (MHC) molecules along with costimulatory molecules at the DC surface. As antigens are processed for presentation, the DCs mature and migrate to lymph nodes or the spleen where they interact with T cells as well as other cell types, including natural killer (NK) cells and B cells, to initiate an immune response of an appropriate magnitude and specificity, leading to the elimination of invading pathogens.

In the periphery, low numbers of DCs are present in an immature state in which they are highly capable of recognizing and capturing antigens using several uptake mechanisms. DCs take up particles, microbes and infected or damaged cells by phagocytosis. They also form large pinocytic vesicles to sample extracellular fluid and solutes, a process called macropinocytosis. Third, DCs express antigen uptake receptors that mediate absorptive endocytosis. These receptors include the family of C-type lectin

receptors, such as DC-SIGN (CD209) and DEC-205 (CD205), as well as Fc, complement and scavenger receptors (96). DCs also receive information about the nature of the infection or insult in peripheral tissues through PRRs (97). The most studied PRRs comprise the family of Toll-like receptors (TLRs) that recognize conserved microbial structures, which include bacterial products such as peptidoglycan (via TLR2) and lipopolysaccharide (TLR4), viral double-stranded (ds) RNA (TLR3), and unmethylated CpG motifs in bacterial DNA (TLR9). Signaling through most TLRs, except for dsRNA-specific TLR3, involves the sequential recruitment of the adaptor molecule MyD88 (98). In addition, DCs are able to distinguish between antigens from tissue cells that die by the normal process of apoptosis and those that die by externally activated necrosis (99). Immature DCs are weakly immunogenic; they are efficient at capturing and processing antigens, but they express few MHC class II or costimulatory molecules and therefore are unable to prime naïve T cells. Following uptake of antigens, the internalized proteins are processed in the endocytic compartment and loaded onto MHC class II molecules and the resultant peptide-MHC complexes are transported to the cell surface for presentation to T cell receptors (TCRs). In the course of antigen capture, DCs undergo several phenotypic and functional changes, including the loss of uptake receptors, upregulated expression of costimulatory molecules, such as CD40, CD80 and CD86, a shift in lysosomal compartments with upregulated expression of lysosomal-associated membrane protein 3 (LAMP3), enhanced efficiency of assembly of peptide-MHC complexes, production of inflammatory mediators, and increased expression of chemokine receptors which mediate their trafficking from peripheral tissues to draining lymph nodes (100). All these changes are associated with DC maturation, also called DC activation, and enable DCs to migrate to lymphoid organs where they present to resident lymphocytes the array of pathogen-derived epitopes and signals that fully reflects the antigenic situation encountered at the site of infection.

The maturation of DCs is thus crucial for the initiation of immune responses. It was once thought that all DCs have the capacity to induce immunity or tolerance, depending on the developmental stage of the DC (100, 101). In the original concept, DCs that sample antigens from apoptotic cells and the normal gut flora do not become activated and as a result these DCs present self-antigens to T cells without costimulation, resulting

in tolerance during the steady state (102). Immature DCs induce tolerance by eliciting deletion or anergy of self-reactive T cells or by promoting the generation of regulatory T cells that suppress the function of other T effector cells (103-105). Recent research, however, suggests that tolerance can be induced by DCs that are sufficiently mature to express moderate levels of MHC class II and costimulatory molecules but are not fully activated to induce immunity (106, 107). It has also been proposed that a subtype of specialized regulatory or tolerogenic DCs participates in the maintenance of peripheral tolerance (108). Immunogenic DCs, on the other hand, provide additional signals derived from pathogens and inflammatory stimuli required for T cell activation and subsequent induction of adaptive immunity. In this revised paradigm, at least three signals are essential for DCs to prime T cells and control the type of initiated response (109). As illustrated in Fig. 1.2, DCs present MHC-bound antigens to TCR in the presence of TLR ligation (signal 1), express costimulatory molecules (signal 2), and secrete Th-cell polarizing cytokines that induce inflammation and regulate the ensuing immune response (signal 3). More recent studies have further refined this model to show that inflammatory mediators alone are insufficient for full DC activation, that is, they can expand but not initiate adaptive immune responses (110). DCs matured indirectly by inflammatory signals stimulate T cell proliferation but these DCs do not produce cytokines and fail to direct $CD4^+$ T cell differentiation. Rather, TLR engagement is required for functional activation of DCs and TLR-mediated signaling then enables DCs to produce immunoregulatory cytokines, mainly IL-12, and to drive differentiation of $CD4^+$ T helper (Th) cells into specialized Th effector cells in vivo (110). Thus, pathogen-mediated DC activation through direct PAMP recognition provides the instructive signals for initiation of adaptive immunity, while cytokines and other inflammatory mediators act to amplify rather than direct the immune response.

Given that DCs induce both immune activation and tolerance, the different and often opposing immune responses initiated by DCs are thought to be performed by distinct DC subsets with defined phenotypic and functional properties. The diversity of DC subsets is related to their developmental stage, specific tissue location and interactions with different antigens and cells of the immune system (the functional

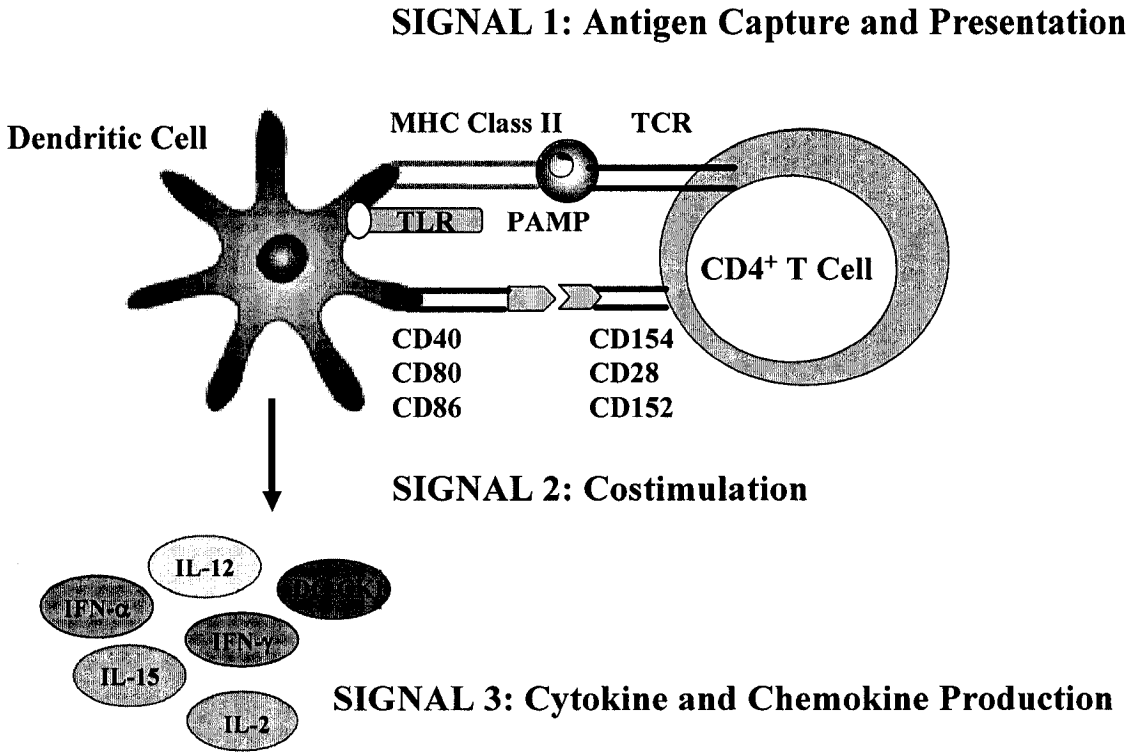

Figure 1.2: Dendritic Cell Signals for Priming of $CD4^+$ T cells

plasticity model). Alternatively, DC subsets may be products of distinct hematopoietic lineages that segregate according to signals received during early DC development (the specialized lineage model). While some DC subsets are derived from distinct precursors (e.g., lymphoid versus myeloid DCs), most DCs demonstrate a considerable degree of functional plasticity depending on the type and dose of antigen, cytokine milieu, and crosstalk with other immune cells (111). DC function is therefore not fixed but adaptable to signals provided by the local environment and the pathogen, thus permitting flexibility in the evolution of the immune response during the course of an infection.

In humans, there are four DC subsets that are all derived from $CD34^+$ hematopoietic precursors in the bone marrow or umbilicial cord blood and are defined under cytokine-driven conditions in vitro (112, 113). These include: 1) blood monocyte-derived DCs (moDCs), derived from $CD14^+$ blood monocytes and also called conventional or myeloid DCs; 2) dermal DCs and interstitial DCs (DDC-IDCs), derived from $CD14^+$ precursors; 3) Langerhans cells, derived from $CD14^-$ precursors; and 4) plasmacytoid DCs (pDCs), named for their morphological resemblance to plasma cells and derived possibly from lymphoid precursors under the control of Flt 3 ligand. There is also a small population of DCs circulating in human blood that is lineage marker negative, $CD11c^+$, $CD86^+$, and $HLA\text{-}DR^+$, and expresses CD83 after in vitro activation (107, 113).

Human DCs differ in their tissue location but share many phenotypic and immunostimulatory features, resulting in overlapping functions and biologic flexibility in the immune response. moDCs access lymphoid tissues via afferent lymphatics and also migrate via the blood to the spleen, where they concentrate in the marginal and perifollicular zones, hence, in regions where the blood circulation enters the spleen. moDCs are major producers of IL-12 and therefore selectively drive a T helper 1 (Th1) rather than a Th2 response (114, 115). DDC-IDCs and Langerhans cells are abundant in cutaneous and mucosal tissues. pDCs are found mainly in blood and many lymphoid tissues. Virus-activated pDCs produce high amounts of type 1 interferons (IFNs) and are potent stimulators of NK and $CD8^+$ T cell cytotoxicity and IFN-γ secretion during viral infection (116). Myeloid and plasmacytoid DCs differ also in their expression of TLRs and Fc receptors, thus accounting for their differential responses to distinct but overlapping repertoire of pathogens. For instance, pDCs preferentially express TLR7

and TLR9 which recognize viral structures and bacterial CpG sequences, respectively, whereas myeloid DCs express TLR1, TLR2, TLR4, TLR5 and TLR8, and therefore react to a different set of microbial ligands (107). Given their different tissue locations and varied expression of PRRs, it is thought that myeloid DCs are the first responders to pathogens, allergens and other causes of inflammation in peripheral tissues and, upon encounter with foreign antigens, migrate to lymphoid organs to initiate immune responses, whereas pDCs circulate in the blood and respond to systemic viral infection and blood-borne antigens (112). Compared to myeloid DCs, pDCs are poor stimulators of T cell proliferation and do not capture, process and present antigens as efficiently nor express upregulated costimulatory molecules following activation. Therefore, pDCs might not be essential for initiating immune responses but may specialize in controlling the strength, duration and type of NK, T and B cell responses by secreting immunomodulating chemokines and cytokines (112). Although these DC subsets may have distinct roles during an immune response, there is considerable flexibility in their course of development under inflammatory conditions. After virus infection, bone marrow-derived pDCs can differentiate into myeloid DCs (117) and conventional DCs can act as IFN-α-producing plasmacytoid cells (118). This reciprocal switch in lineage commitment requires TLR ligation by viral dsRNA as well as type I IFNs and provides compelling evidence in support of the functional plasticity model of DC development.

In mice, DC subsets have been identified based on their tissue location as well as differential expression of the T cell markers CD4 and CD8. While most mouse DCs express CD11c (the integrin-α^x chain), the expression of CD8α, in the αα homodimeric form rather than the αβ heterodimer typical of T cells, was proposed to be a marker of lymphoid DCs. However, myeloid progenitors from both the thymus and spleen can become $CD8\alpha^+$ DCs in vitro upon stimulation (119, 120) and in vivo following adoptive transfer into CD8-deficient mice (121). Thus, CD8α expression is not indicative of a lymphoid origin and is now commonly used to ascribe functional differences among murine DC subsets. The mouse spleen contains four distinct subsets: the $CD4^-CD8\alpha^+$, the $CD4^+CD8\alpha^-$, the $CD4^-CD8\alpha^-$, and pDCs (122). $CD8\alpha^+$ DCs are concentrated in the spleen T cell areas and $CD8\alpha^-$ DCs in the marginal zones but can migrate into the T cell zones following stimulation by microbial products (122). Notably, $CD8\alpha^+$ DCs are high

producers of IL-12 and were initially called DC1 based on their ability to direct Th1 cell differentiation; whereas $CD8\alpha^-$ DCs (or DC2) do not produce IL-12 and were shown to preferentially promote Th2 cells (123). However, displaying the functional plasticity shared by most DCs, $CD8\alpha^-$ DCs can be induced by different combinations of cytokines and microbial stimuli to produce IL-12 and IFNs (124), thereby questioning the validity of the DC1 and DC2 designations. Murine pDCs express B220, Gr1 and TLR9 and, like their human counterparts, secrete high levels of IFN-α/β following activation by viral or microbial stimuli (125, 126). The four DC subtypes are also found in the liver; however, liver DCs are less mature and less efficient than splenic DCs in activating naïve T cells, suggesting that liver DCs may contribute to tolerance induction in the steady state (127, 128). However, liver DCs can produce large amounts of proinflammatory cytokines after CpG activation and thus may act to augment T cell responses initiated by other DCs during infection (128). Mouse lymph nodes contain two additional DC subsets not normally found in the spleen and these DCs show many characteristics of either tissue interstitial DCs or epidermal Langerhans DCs (122). The defining features of the different mouse DC subsets, as well as those of the immature and mature stages, are summarized in Table 1.

Natural killer cells

NK cells are populations of lymphocytes that provide protection against infections and cancer through their cytotoxic activities and production of cytokines and chemokines. Rapid induction of NK cell responses (on the order of 2-6 days) limits an infection until adaptive immunity develops (129, 130). NK cells do not express T-cell antigen receptors (TCR) and preferentially recognize and kill cells that express altered levels of MHC class I molecule, without the need for pre-activation or antibodies. To kill aberrant cells and avoid targeting of normal cells, NK cells use a complex repertoire of surface activation and inhibitory receptors. NK cell activating receptors (e.g., human NKp30, NKp44 and NKp46; mouse Ly49D and Ly49H) bind a variety of ligands, including human MHC-I-related chain, pathogen-specific epitopes, and stress signals and inducible molecules expressed by infected or damaged cells (131). Alternatively,

Table 1: Mouse Dendritic Cell Subsets: Lineages, Surface Markers, and Function

DC Subset	Surface Markers	Characteristics
Immature	$CCR7^{lo}$ Low or absent MHC class II Low costimulation	• Found in nonlymphoid tissues • Act as sentinels of infection and injury • Antigen uptake and processing • Poor T cell stimulators due to lack of costimulatory and MHC molecules
Mature	$CCR7^{hi}$ ↑ MHC class II ↑ CD40 (CD154)[a] ↑ CD80 (CD28/CTLA-4)[a] ↑ CD86 (CD28/CTLA-4)[a]	• Found in secondary lymphoid tissues • CCR7 expression is essential for migration to lymphoid tissues • Expression of costimulatory and MHC class II molecules enable antigen-specific activation of $CD4^+$ T cells
DC1[b]	$CD11c^+$ $CD4^-CD8\alpha^+$ $CD11b^-CD205^+$	• Localized in spleen T cell zones • High producers of IL-12 and preferentially induce Th1 cells • Protect against $CD4^+$ T cell apoptosis • Stimulate strong $CD8^+$ T cell responses
DC2[b]	$CD11c^+$ $CD4^+CD8\alpha^-$ $CD11b^+CD205^-$	• Localized in spleen marginal zones • Low producers of IL-12 and preferentially induce Th2 cells • Produce large amounts of IFN-α and stimulate strong $CD8^+$ T cell responses
Plasmacytoid	$CD11c^+B220^+$ $CD8\alpha^-$	• Can be derived from bone marrow progenitors stimulated with Flt 3 ligand • High producers of type 1 IFNs and preferentially induce Th1 cells • Express high levels of TLR9
Lymph Node	$CD4^-CD8\alpha^-CD11b^+CD205^+$	• Two subsets in lymph nodes are not found in high numbers in the spleen • One subset expresses CD11b and CD205 and resembles interstitital DCs • A second subset expresses high levels of langerin and resemble Langerhans DCs

[a]Ligands for the DC costimulatory molecules are given in the parentheses
[b]The concept of DC1 and DC2 subsets is controversial. DCs demonstrate functional plasticity depending on the state of maturation, cytokine stimulus, and type and dose of antigen. Accordingly, $CD8\alpha^-$ DCs can be induced to produce IL-12 and IFN-γ.

normal host cells present sufficient MHC class I molecules to MHC-I-sensing inhibitory receptors (e.g., human KIR and CD94/NKG2; mouse Ly49), resulting in downregulation of NK cell function (131). Several NK cell receptors perform both activation and inhibition, depending on which isoforms are expressed; the activating isoforms lack inhibitory motifs in their short cytoplasmic domains and are able to associate with additional subunits that transduce activating signals to the cell nucleus. Therefore, the commitment to NK cell effector function is regulated by a fine balance in the expression of activating versus inhibiting receptors, the sequence of which can be influenced by the density and array of ligands, cytokine milieu, and class I and other surface molecules expressed by APCs, such as DCs (discussed below).

NK cells were discovered for their unique ability to spontaneously lyse tumor cell lines. This cytolysis is mediated by several pathways and involves phenotypically distinct NK cell subsets. The main pathway is mediated by perforin and granzymes released by mature activated NK cells, although both immature and mature human NK cells employ Fas ligand (CD178) and/or TNF-related apoptosis-inducing ligand (TRAIL) to induce target cell death (132). In addition, NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) through an Fc-receptor complex (CD16) that recognizes the Fc portion of antibodies bound to target cell-associated antigens (133).

The production of immunoregulatory cytokines is an important mechanism by which NK cells direct hematopoietic cell differentiation and modulate other immune cells. In humans, subsets of $CD56^{hi}$ NK cells with weak natural cytolytic activity produce high levels of IFN-γ, TNF-α, LT-α, granulocyte/monocyte colony-stimulating factor (GM-CSF), IL-10 and IL-13 following selective stimulation (134). Based on their cytokine profiles, human NK cells, like DCs, have been categorized into NK1 and NK2 subsets (135). Recent studies (136) showed that the maturation of human NK cells progresses sequentially from an early stage of proliferation and IL-13 production (type 2) to a late stage of terminal differentiation and IFN-γ production (type 1), suggesting that NK cell cytokine production may be developmentally regulated. However, mature $CD56^{+}$ NK cells cultured in vitro and some $CD56^{+}$ NK-cell clones have mixed type 0 profiles (IFN-γ, IL-4, IL-13), whereas $CD56^{dim}$ NK cells produce low amounts of these cytokines but are highly cytolytic (131, 137). Given the need for efficient production of

cytokines by NK cells early in the immune response to infection, the physiological relevance of cytokine production by clonal NK cells or after prolonged stimulation in vitro is presently unclear.

Murine NK cells are similar to their human counterparts in their ability to lyse target cells, produce cytokines, and express activating or inhibiting receptors. However, murine NK cells do not express a homolog of CD56 and, as a result, there are no comparative studies demonstrating functionally distinct subsets of NK cells in mice. Nonetheless, murine NK cells are potent producers of cytokines, particularly IFN-γ, following intracellular infection or stimulation with various combinations of cytokines. Specifically, NK cells derived from murine splenocytes stimulated by a combination of IL-12 and IL-18 secrete large amounts of IFN-γ compared to NK cells derived in IL-2 or IL-15 (138). As the main producers of IFN-γ early after viral (129) or malaria infections (139-141), NK cells play a critical role in the innate immune response to stimulate Th1 cell development and induction of Th1-type adaptive immune responses.

Dendritic cell and natural killer cell interactions

DCs and NK cells are specialized cells of the innate immune system that perform distinct but mutually dependent functions in the innate immune response to infection. Both cell types are known to rapidly accumulate at the site of pathogen entry and in the marginal zone of the spleen, acting as sentinels against infection. DCs are the principal cells responsible for presenting antigens and activating immune responses, whereas NK cells are innate effector cells that through their cytolytic activity and secretion of pro-inflammatory cytokines, particularly IFN-γ, help control infection and augment downstream antigen-specific responses. Recent studies show that DCs and NK cells participate in bi-directional crosstalk which results in potent, reciprocal activation (142, 143). Although much of the evidence for this cross talk has been obtained from in vitro studies, NK cells have recently been shown to co-localize with DCs in lymph nodes during infection or antigen challenge (142, 144). Thus, DC-NK cell interactions in the periphery as well as in secondary lymphoid organs may shape both the type and magnitude of innate as well as adaptive immune responses.

Upon contact with invading pathogens, DCs capture antigens for presentation and secrete chemokines, including CCL2 and IL-8, which recruit circulating NK cells to inflamed tissues. DCs then prime resting NK cells through cell-cell contact and release of soluble mediators, such as IFN-α, IL-2, IL-12, IL-15, and IL-18 (145-149). It was initially difficult to reconcile the need for both cell-cell contact and cytokines for DC-mediated NK cell activation. Recent studies, however, showed that secretion of IL-12 and IL-18 is restricted to the DC–NK cell immunological synapse (150, 151) and IL-15 is presented in trans to NK cells via DC-bound IL-15Rα (152, 153). Some of these cytokines, mainly IL-2 and IL-15, act as potent growth factors for NK cell development and proliferation. IL-2 is secreted by DCs and T cells that co-localize with NK cells in secondary lymphoid organs and acts directly on NK cells to promote optimal IFN-γ production and cytolytic activity (145, 147, 154). DC-derived IL-15 and IFN-α induce autocrine expression of MHC class I-related chain A and B (MICA/B), ligands for the activating receptor, NKG2D, expressed on human resting NK cells, resulting in increased NK cytotoxicity (148). Moreover, membrane-bound IL-15 is required for DC-mediated activation of NK cell IFN-γ production (153). Upon contact with NK cells, DCs also release IL-18 (151), which in turn activates a subset of effector NK cells that enhance the ability of DCs to produce IL-12 and to promote Th1 cell differentiation (149). Indeed, DC-derived IL-12 and IL-18 are required for NK cell IFN-γ production and cytotoxicity against murine cytomegalovirus infection (155).

The maturation status of DCs is an important determinant of the ability of DCs to activate resting NK cells. Immature DCs are recognized via the NKp30 natural cytotoxicity receptor and TRAIL and consequently are targeted for killing. However, DCs matured by microbial stimuli, proinflammatory cytokines or CD40 ligation are resistant to NK cell lysis (145, 156, 157). Upregulated expression of MHC class I molecules, such as HLA-E on human DCs, following antigen uptake by DCs, also confers protection against NK cell lysis (145, 156), thereby enabling mature DCs to survive and to recruit and activate additional NK cells as well as T cells. A recent study showed that TGF-β significantly down-regulates NKp30 expression, rendering NK cells incapable of killing DCs (158). This finding suggests a potential role for DC-derived cytokines in mediating DC-NK interactions that lead to either immune tolerance or

activation, although TGF-β production by DCs in response to maturation signals has yet to be documented.

Reciprocally, NK cells have been to shown to induce DC maturation and cytokine production (159, 160). Stimulation of DC responses by activated NK cells is critically dependent on cell-cell contact and, to a lesser extent, on cytokines secreted by NK cells (160, 161). Following interaction with DCs, NKp30 is engaged and this induces NK cells to secrete TNF-α and IFN-γ, which directly promote DC maturation (162). While it is unclear exactly how NK cell-derived TNF-α acts to stimulate DCs, IFN-γ secreted by NK cells has been shown to upregulate expression of the co-stimulatory molecule inducible T cell antigen (4-IBB), which promotes autocrine and paracrine DC expansion, maturation, and migration (161). As discussed above, NK cells lyse immature but not mature DCs using an array of cell surface receptors such as NKp30 and TRAIL. By recognizing and killing immature DCs, NK cells may play an immunoregulatory role in selecting an immunogenic DC population that could direct subsequent adaptive immune responses. In addition to their selective editing of DCs, NK cells may be the primary source of signals for type 1 polarization of DCs and T cells. DCs act as carriers of NK cell-derived signals that induce early polarization of $CD4^+$ Th cell responses (163). Following stimulation by NK cells, DCs carry NK cell-derived signals, including TNF-α and IFN-γ, into draining lymph nodes where DCs induce strong Th1 responses in naïve $CD4^+$ T cells. Further research is required to determine in vivo the ability of NK cells to lyse DCs and whether NK cells alter the numbers and functions of specific DC subsets.

Natural killer T cells

Natural killer T (NK-T) cells are a heterogeneous population of lymphocytes with phenotypic and functional characteristics of both NK cells and classical T cells. NK-T cells are especially abundant in the liver and are also found in the bone marrow, thymus, blood, and spleen, but their presence is rare in lymph nodes. NK-T cells express NK1.1 (CD161) and an invariant αβ-TCR (76). Murine NK-T cells express a TCR α-chain (Vα14-Jα281, known now as Jα18) in association with Vβ2, Vβ7, or Vβ8 (164). Human NK-T cells express an invariant Vα24-JαQ TCR α-chain in association with the Vβ11-TCR β-chain, the homologues of the mouse Vα14-Jα18 and Vβ8.2 chains, respectively.

Most NK-T cells are $CD4^+$ or $CD4^-CD8^-$, but a small population, abundant in the liver but scarce in the thymus and other lymphoid organs, expresses the CD8αα homodimer (164). Classical NK-T cells recognize glycolipid antigens via a repertoire of invariant VαVβ-TCR in the context of CD1d, a MHC class-I-like, β2-microglobulin-associated molecule. The expression of CD1d is closely linked to the development of classical NK-T cells, although recent studies suggest that IL-15 is more important than CD1d for survival and expansion of NK-T cells in the liver (165, 166). Mouse CD1d-restricted NK-T cells can recognize human CD1d and vice versa, indicating a high level of conserved specificity between the two species (167). The nonclassical $CD8^+$ NK-T cells are CD1d independent and may express either TCRαβ or TCRγδ.

CD1d-restricted NK-T cells have been shown to play a role in host resistance to viral, bacterial, yeast, and parasitic infections, including malaria (discussed below). There are at least two possible mechanisms by which NK-T cells contribute to immunity to infection. NK-T cells possess some of the machinery, including perforin, Fas ligand, and receptors, such as NKG2D, required for cytotoxicity and have been found to be directly cytotoxic against virus-infected cells (76, 167). A more likely role for NK-T cells, however, is as immunoregulatory cells that activate effector cells through a cytokine-dependent pathway (76, 167). Classical NK-T cells are known for their rapid ability to secrete immunomodulatory cytokines, especially IFN-γ, IL-4 and TNF-α, following NK1.1 engagement or antigenic stimulation, and therefore these cells may influence the polarization of $CD4^+$ T cells as well as activate bystander macrophages, NK cells and conventional $CD8^+$ T cells (164, 168, 169). $CD8^+$ NK-T cells, in contrast to classical NK-T cells, produce IFN-γ but not IL-4 after stimulation, suggesting that this population is involved in the induction of Th1-type immune responses (170). NK-T cell responses and their capacity to direct differentiation of Th cells are also modulated by cytokines: IL-7 enhances IL-4 production by thymic and splenic NK-T cells, whereas IL-12 enhances IFN-γ production (164). These results suggest that NK-T cells secrete distinct cytokines depending on their lineage and the microenvironment and therefore have divergent effects on the regulation of the immune response.

γδ T cells

γδ T cells comprise a small fraction of circulating T cells (2-5%) and are distinct from the αβ T cells based on their ability to recognize antigens in the absence of classical MHC presentation (171). Recent studies, however, show that activated human γδ T cells can efficiently process and display peptide antigens to αβ T cells, thus displaying the antigen-presenting function of DCs and B cells and forming a unique link between innate and adaptive immunity (172). γδ T cells also can recognize antigens through nonconventional MHC proteins such as the human class IB MHC molecules, which do not present pathogen-derived peptide antigens (173). This is consistent with findings that γδ T cells recognize and respond to unusual nucleotide derivatives and autologous proteins, such as the thymus leukaemia antigen, that are derived from infected, damaged or transformed host cells (173, 174). Although γδ T cells are a small subset of peripheral T cells, they constitute the majority of intraepithelial lymphocytes (IELs) in the gut and other mucosal tissues (175-177). Given their prevalence in peripheral blood and mucosal tissues, γδ T cells participate in innate immunity against blood-borne pathogens and also maintain the integrity of epithelia tissues both in the steady state and during infection (178). γδ T cells also perform key immunoregulatory functions by secreting chemokines and cytokines that regulate the homing and maturation of other lymphoid cells (178, 179). While clonal γδ T cell responses can be of either a Th1 or Th2 type (180), most γδ T cells subsets, particularly IELs, are biased toward Th1-type responses (181). Similarly, γδ T cells play a role in the adaptive immune response to a variety of intracellular pathogens, including mycobacteria, salmonella, toxoplasma, plasmodia, and HIV (182, 183).

Macrophages

Monocytes circulate in the blood and then migrate into tissues where they receive instructive signals to differentiate into either macrophages or DCs (called monocyte-derived DCs as discussed above). During the steady state, macrophages perform an important scavenging function by removing cellular debris, apoptotic cells, and proteinaceious particles. Following infection, macrophages are activated by

proinflammatory cytokines, such as IFN-γ, TNF-α, IL-1 and IL-6, or by ligation of PRRs, particularly the macrophage scavenger receptor CD36 and the macrophage mannose receptor (184, 185). When activated, macrophages provide a first line of nonspecific defense through phagocytosis and release of NO and other reactive oxygen and nitrogen species (186, 187). These mediators contribute to the destruction of the ingested pathogen or infected cell (188) and, as the case of NO, also exert modulatory effects on the immune and hematopoietic systems (189, 190). Macrophages, compared to DCs, are less efficient at priming naïve T cells because they possess fewer MHC class II molecules and most of the internalized antigens are directed to lysosomes where the proteins are fully digested into amino acids (95). For this reason, macrophages are regarded as professional phagocytes that benefit the control and clearance of systemic infection rather than as classical APCs in the initiation of immune responses (95). However, activated macrophages secrete B cell growth factors and proinflammatory cytokines and express costimulatory molecules, which allow them to support the survival and differentiation of B cells as well as to activate and expand effector or memory T cells (95).

In the mouse spleen, two subtypes of macrophages are found abundantly in the marginal zone, an area that surrounds the white pulp and is rich in cells that are leaving the bloodstream and entering the white pulp. Marginal-zone macrophages lie in the outer ring of the marginal zone and express the C-type lectin SIGNR1, a mouse homologue of human DC-SIGN, and the type I scavenger receptor MARCO (191-193). These receptors allow marginal-zone macrophages to recognize polysaccharide antigens found at the surface of mycobacteria and streptococci (192, 194) and binding of these antigens to macrophage PRRs leads to internalization of bacteria, facilitating their clearance. SIGNR1, like DC-SIGN, may also be involved in the uptake of viruses (193, 195). Marginal-zone metallophilic macrophages form the inner ring of macrophages, are the main producers of type I IFNs after virus infection, and express the adhesion molecule SIGLEC1 which recognizes sialic-acid residues present at the cell surface of pathogens and therefore is involved in the phagocytosis of blood-borne pathogens (196). In humans, splenic macrophages are found in the perifollicular zone surrounding the marginal zone and also express high levels of SIGLEC molecules (197). When activated

by pathogens or their products, macrophages can interact with DCs, T cells and B cells in the marginal zone, although only marginal-zone DCs and B cells are known to migrate further into the white pulp to activate naïve T cells (197). The majority of macrophages are found in the red pulp in the splenic cords and function to remove aged or damaged erythrocytes, thereby contributing to the turnover of erythrocytes and the recycling of iron (197, 198).

Interleukin 15

IL-15 is a pleiotropic cytokine expressed early in the inflammatory cascade in response to infection or tissue injury. At the time of its discovery in 1994, IL-15 was thought to share many functional and biochemical characteristics with IL-2 although the two cytokines share no sequence homology at the DNA or protein level. The functional similarities between IL-15 and IL-2 are due primarily to their sharing of the IL-2 receptor (IL-2R) subunits, specifically the β chain (IL-2Rβ) and the common γ chain (IL-2Rγ or γc). IL-15 also has its own high affinity binding receptor (IL-15Rα), which mediates IL-15-specific signaling (199, 200). Despite their similarities, much evidence indicates that IL-15 and IL-2 are not redundant cytokines (201). IL-15 and IL-15Rα are widely expressed in lymphoid and nonlymphoid cells, suggesting that IL-15 has a broader range of function in multiple cell types than does IL-2 (201-203). That IL-15 is produced by APCs, whereas IL-2 is produced mainly by activated T cells, strongly implicates a role for IL-15 as an early host factor that directs the generation of an effective adaptive immune response against intracellular pathogens (202, 203). Of the two cytokines, only IL-15 supports the differentiation and maturation of NK cells, NK-T cells, memory phenotype $CD8^+$ T cells, and subsets of γδ IELs (203). Mice deficient in IL-15 have a severe reduction or absence of NK cells and memory $CD8^+$ T cells (204, 205). IL-15 induces the lymphocyte anti-apoptotic factor Bcl-2 (206) and is thought to be absolutely critical for the survival and proliferation of NK cells and memory $CD8^+$ T cells during both homeostasis and viral infections (207-210). IL-15 costimulates NK cell IFN-γ production in synergy with IL-12 (211) and IL-21 (212). Although several studies indicate that IL-15 increases NK cell cytotoxicity and IFN-γ production, others have

reported that other cytokines, namely IL-12 and IL-18, are more effective than IL-15 alone (138, 213).

While IL-15 is not required for the development of $CD4^+$ T cells, it may promote differentiation of $CD4^+$ T cells into subsets with distinct cytokine profiles. To this end, IL-15 synergizes with IL-12 to induce development and proliferation of Th1 cells and to increase production of IFN-γ and TNF-α, leading to Th1 cell-mediated immunity against intracellular pathogens (211, 213, 214). IL-15 stimulates the production of IFN-γ and IL-12 by macrophages and DCs, augments their responsiveness to IL-12 by up-regulating the expression of IL-12Rβ1, and induces functional maturation of DCs (215). IL-15, combined with IL-2, upregulates CD40 ligand (CD154) expression on activated $CD4^+$ T cells, leading to effective generation of $CD4^+$ T cell responses (216). These data are compelling evidence implicating IL-15 as a pivotal cytokine for the activation of innate immune responses in a manner that provides a "jump-start" or an initial push toward Th1 cell development. IL-15 has also been shown to play a role in B cell proliferation and immunoglobulin synthesis by activated B cells and promoting Ig class switching, thereby augmenting or maintaining antibody defenses initiated by other cytokines (217, 218). Thus, IL-15 appears to be critical for innate immune responses as well as for the induction of protective Th1 cell-mediated immunity against intracellular pathogens.

1.2.2 Adaptive Immunity

The adaptive immune system has evolved to recognize and respond to an extensive range of antigens. Antigen recognition is mediated by large repertoires of T cell antigen receptors (TCRs) and immunoglobulin B cell antigen receptors (BCRs) that can detect and recall any possible antigen encountered throughout life. The diversity of the TCR and BCR repertoires is generated through somatic DNA recombination, and each receptor of a particular specificity is expressed in a clone of lymphocytes. Given that there is a limited number of T or B cell clones in a person at any one time, each repertoire has considerable capacity to recognize different antigens expressed by various pathogens. This cross-reactivity also allows the system to promptly reactivate memory lymphocytes with distinct specificities to microbes encountered at an earlier time. Engagement of the antigen receptors along with additional information provided by

APCs then dictates the class of lymphocyte effector response, which results in different functional outcomes. $CD8^+$ T cells perform cytolysis to eliminate the pathogens, regulatory T cells suppress immune response to induce tolerance and prevent immunopathology, and $CD4^+$ T cells help B cells develop into plasma cells that secrete antibodies. The secreted antibodies bind to antigens with high affinity and trigger a wide range of effector responses that efficiently neutralize or destroy the pathogen. Activated T and B cells also can develop into long-lived memory cells, which carry out rapid, highly specialized responses that protect against a second infection.

$CD4^+$ Th cells

In 1986, Mosmann et al. (219) described the presence of two types of $CD4^+$ Th cells that have distinct profiles of cytokine production. Th1 cells produce IL-2, IFN-γ, and GM-CSF for control of cell-mediated immunity or delayed-type hypersensitivity responses. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 for control of B cell and antibody responses. This Th1/Th2 paradigm is regarded as a conceptual revolution in cellular immunology and its functional relevance to many areas of the field has been validated in diverse models of infection, cancer, transplantation, tolerance, and autoimmunity. In infection models, cell-mediated (type 1) responses are required for protection against intracellular pathogens such as viruses, intracellular bacteria and protozoa, while humoral (type 2) responses eliminate extracellular bacteria and helminth parasites. Although recent studies have challenged the universality of the Th1/Th2 paradigm (220), particularly in human disease which does not show as stringent a delineation in Th cell responses, the Th1/Th2 terminology remains very useful for classifying observable phenotypes with a particular set of cytokines and immune responses. It is important, nonetheless, to note that: 1) in many infectious diseases, both type 1 and 2 responses are required at different stages of the immune response for clearance of the infection and avoidance of immunopathology, respectively; 2) individual cytokines may exert opposing effects, depending on the amount and timing of their synthesis as well as the context, such as the presence of other mediators, in which they are produced; 3) many cytokines have overlapping functions, allowing redundant pathways to induce and maintain a Th1 or Th2 response; 4) cytokines modulate the

function of many non-adaptive cells, such as DCs, macrophages, NK cells and γδ T cells, which may contribute to host defense independently of $CD4^+$ T cells; and 5) Th1 cells also support humoral responses by promoting B cell production of IgG2a in mice and IgG1 in humans (discussed below). Recently, both Th1 and Th2 cells are considered as effector T cells, as opposed to regulatory T cells (see below), that perform specific, often opposing stimulatory functions in the immune response.

The differentiation of $CD4^+$ T cells into Th1 or Th2 cells is tightly regulated by a complex series of molecular interactions that result in transcription of cytokine genes. These signaling pathways involve many transcriptional factors that are activated after instructive signals, such as the dose of antigen, cytokines and costimulation, are received through the TCR and cytokine receptors during interaction of $CD4^+$ T cells with APCs. Distinct transcriptional factors, including the group of signal transducer and activator of transcription (STAT) proteins, control the expression of specific cytokine genes, leading to differential cytokine production between Th1 and Th2 cells. For example, IL-12 and IFN-γ signal through STAT4, IL-2 and IL-15 through STAT3 and STAT5, and IL-4 through STAT6 (221, 222). The activation of specific STAT proteins and other transcriptional factors can induce the naïve $CD4^+$ T cell to follow a Th1 or Th2 differentiation pathway. STAT6 activation may lead to the expression of GATA3, which in turn activates expression of IL-4, IL-5 and IL-13, resulting in Th2 cell differentiation (223, 224). In contrast, the expression of T-bet, a member of the T-box family of transcription factors, induces IFN-γ production in naïve $CD4^+$ T cells to initiate Th1 differentiation and also in committed Th2 cells to convert them into IFN-γ-secreting Th1 cells (225). However, latest research indicates that T-bet does not positively regulate IFN-γ expression directly; rather, it suppresses GATA-3 expression, leading to increased STAT4 and IL-12Rβ2 expression and subsequent induction of IFN-γ production and Th1 cell development (226). These observations have led to the proposal that a balance among different STAT proteins, as well as between Th1-specific T-bet and Th2-specific GATA3, determines whether a naïve $CD4^+$ T cell becomes committed toward the Th1 or Th2 lineage (222).

Regulatory T cells

Suppressor T cells with inhibitory activity were first identified in 1960s, but major problems in obtaining sufficient numbers of purified cells impeded research to confirm their existence and functional relevance. This concept was revived in 1995 when Sakaguichi et al. showed that adoptive transfer of T cells depleted of cells constitutively expressing the IL-2 receptor α-chain (CD25) induce multi-organ autoimmunity in recipient mice (227). There is now compelling evidence for at least three types of regulatory T (Treg) cells that regulate or suppress the function of other effector $CD4^+$ and $CD8^+$ T cells to induce peripheral tolerance (228, 229): 1) $CD4^+CD25^+$ T cells are produced in the thymus, inhibit T cell IL-2 production, and promote cell cycle arrest in $CD4^+$ and $CD8^+$ T cells by a mechanism that requires cell-cell contact and possibly TGF-β but not IL-10; 2) Tr1 cells are derived from peripheral $CD4^+CD25^-$ precursors, produce high levels of IL-10 and low to moderate levels of TGF-β, and require IL-10 for their differentiation and function; and 3) Th3 cells are also derived from $CD4^+CD25^-$ cells, produce preferentially TGF-β and low amounts of IL-10 and IL-4, and suppress via a TGF-β-dependent mechanism. $CD4^+CD25^+$ T cells, also called naturally occurring Treg because they are produced during neonatal development and are present in the periphery of normal mice, are involved in protection against autoimmune disorders, such as gastritis, thryoiditis, diabetes, and oöpheritis (108). Tr1 cells, also called inducible Treg cells because they are induced mainly in the presence of IL-10 and TGF-β, have been shown to suppress alloreactivity associated with graft versus host disease as well as immune function in certain infections (108).

Although the expression of CD25 has been useful in defining Treg cells, other nonregulatory T cells may upregulate CD25 levels during immune activation and other markers, such as CD28, CD62L (L-selectin) and CD103, have also been associated with Treg cells. These data question the validity of CD25 as an exclusive marker of Treg cells. Recently, the forkhead family transcription factor Foxp3 was shown to be specifically expressed in $CD4^+CD25^+$ T cells. Foxp3, a transcriptional repressor, controls both the development and function of $CD4^+CD25^+$ T cells, and $CD4^+CD25^-$ T cells can acquire suppressive activity upon induced expression of Foxp3 (230, 231). However, not all Foxp3-expressing cells show suppressive activity and low expression

of Foxp3 has been detected in $CD4^+CD25^-$ and $CD8^+$ T cells (232). Other Treg cells induced under particular regimens of antigenic stimulation have also been described. For instance, IL-10-secreting Treg (IL-10-Treg) cells do not express Foxp3 and produce little IL-2, but are as potent as $CD4^+CD25^+$ T cells in suppressing T cell proliferation in a mechanism that is independent of IL-10 (233, 234).

Although Treg cells play a critical role in preventing allergic and autoimmune diseases, there is increasing evidence that these cells control immunity to infection. The suppressive function of Treg cells can favorably affect the outcome of infection or result in damage to the host, depending on the timing of effector versus regulatory T cell responses (235, 236). In the early phase of the immune response, excess inhibition of effector T cell responses by Treg cells may lead to uncontrolled growth of the pathogen, resulting in disease and death of the host. Dampening Treg cell responses at the time of immune activation may help boost host defenses to control the infection. On the other hand, an effective host response to infectious agents may eliminate the infection but often results in tissue damage as well. Enhanced Treg cell responses at the chronic stage of infection may limit harmful immunopathology and, paradoxically, may also benefit the persistence of pathogens. Indeed, many pathogens have evolved strategies to favor activation of Treg cells as a way to delay or prevent host death, thus facilitating pathogen survival, replication and eventual transmission (236). Induction and expansion of Treg cells have been observed following diverse infections, including HIV, leishmania, schistosoma, and malaria (235, 237, 238).

B cells and antibody

B cells can act as APCs to recognize and respond to antigens, leading to $CD4^+$ T cell expansion and differentiation into effector cells (239, 240). However, in vivo, B cells are unable to prime resting T cells in naïve animals (241) and can induce tolerance in both $CD4^+$ and $CD8^+$ T cells (242, 243). As APCs, B cells are not as efficient as DCs in presenting antigens and priming T cells because B cells express lower levels of MHC class II and costimulatory molecules, and produce less immunoregulatory cytokines, such as IL-12, needed for Th cell differentiation (95). A key regulator of whether B cells induce T cell tolerance or effector function is the interaction between the receptor CD40

on B cells and CD40L (CD154) expressed by T cells. During cognate interaction with T cells, B cells capture antigens via their antigen-specific immunoglobulin (Ig) receptors and present the MHC-peptide complexes to T cells that bear TCRs specific for the BCR-bound MHC-peptide complex. Following high affinity contact, CD40/CD154 interaction results in T cell proliferation and induction of other costimulatory molecules, such as CD80 and CD86, for further T cell stimulation (244). In turn, the engagement of CD40 and secretion of cytokines by primed T cells provide potent signals for B cell activation, growth and differentiation into Ig-secreting or memory cells. Thus, the major roles of B cells in the immune response are to support T cell expansion and to produce specific Ig.

Secreted antibodies display an extraordinary range of antigenic specificity. Each Ig has a variable antigen-binding site, a heterodimer with heavy and light Ig chains, that mediates antigen recognition, and a constant region composed of a heavy chain tail that triggers effector responses from the rest of the immune system. Immature B cells secrete IgM and IgD, while activated B cells may undergo class switch recombination to secrete IgG, IgE or IgA isotypes. The different Ig isotypes and IgG subclasses are related to distinct classes of cytokine and Th cell effector responses. IL-12, IFN-γ and Th1 cells promote IgG2a in mice and IgG1 in humans, whereas IL-4, IL-13 and Th2 cells induce the production of IgG1 and IgE in mice and IgG4 and IgE in humans (245). Although B cells require help from antigen-primed $CD4^+$ T cells to undergo Ig class switching, DCs were recently shown to directly trigger B cell production of IgG and IgA. DCs express two newly discovered ligands, the B lymphocyte stimulatory protein (BLys) and a proliferation-inducing ligand (APRIL), that are recognized by receptors expressed on the surface of B cells, thereby directly triggering class switch recombination even in the absence of T cells and CD40-CD40L interactions (218). Cytokines may modulate the ability of DCs to induce Ig class switching. In response to viral infection, DCs produce IFN-α and IFN-γ that can upregulate expression of BLyS and APRIL, leading to enhanced B cell differentiation and induction of class switching (218). IL-15 induces B cell proliferation (217) and differentiation, as well as Ig class switching through direct effects on B cells or via stimulation of crosstalk between DCs and B cells (218, 246).

Recent studies suggest that B cells cooperate with DCs to stimulate T cell responses, an interaction called a "ménage a trois" of DC-T-B lymphocytes (247). Early in the

immune response, B cells are an important source of cytokines and chemokines, which influence the migration of DCs and T cells. Naïve B cells constitutively release IL-16, a cytokine that induces chemotaxis of $CD4^+$ T cells, monocytes, and blood-borne moDCs in the steady state (248). Activated B cells secrete macrophage inflammatory proteins, MIP-1α and MIP-1β, and other chemokines to promote homing of DCs to the spleen (249-251), as well as a B-cell-derived lymphotoxin to direct DC migration into B cell follicles (252). Once the three types of lymphocytes co-localize in lymphoid tissues, their interaction determines which effector class of $CD4^+$ T cell response is initiated (discussed below) and also provides a negative feedback loop to regulate Th1 cell development. In the latter scenario, IL-12 produced by Th1-promoting DCs stimulates B cells to release IL-6 and IL-10, which in turn induce IL-4 synthesis in $CD4^+$ T cells (253). B-cell-derived IL-10 also acts to inhibit IL-12 production by DCs, thus decreasing their Th1-polarizing capacity (254, 255).

Cytokine regulation of dendritic cell-mediated adaptive immunity

Antigen-specific Th cells control the effector mechanisms of adaptive immunity through the differential secretion of cytokines. As discussed above, committed Th1 cells are high producers of IFN-γ whereas Th2 cells secrete large amounts of IL-4, IL-13, and IL-5, and these distinct Th cell subsets induce the production of different antibody isotypes/subclasses. In the classical paradigm of $CD4^+$ Th cell commitment toward either the Th1 or Th2 phenotype, the priming of naïve T cells is thought to be influenced to a large extent by the levels of IL-12p70 produced by APCs early in the innate immune response. IL-12 is the pivotal cytokine directing Th1 cell differentiation and the absence or inhibition of IL-12 production leads to the development of Th2 cells.

However, this paradigm has been recently challenged by studies showing that IL-12p40-deficient mice infected with *Toxoplasma gondii* develop comparable frequencies of $CD4^+$ T cells secreting IFN-γ, suggesting that other Th1 switch factors besides IL-12 control the initial stage of $CD4^+$ T cell activation (256). Rather than focusing on IL-12 or any single cytokine as the sole inducer of Th cell differentiation, multiple signals released during DC-pathogen interactions could influence Th1/Th2 effector choice and activation of downstream immune responses (257). Cytokines released in the innate

immune response control the capacity of DCs to produce IL-12 and syngerize with IL-12 to efficiently drive Th1 responses (258, 259). IFN-γ enhances the ability of myeloid DCs activated by CD40L or TLR ligands to produce IL-12 and induce Th1 cells (259, 260). IL-15 augments IL-12, IFN-γ and NO production by macrophages and DCs and upregulates IL-12R expression, hence, the responsiveness of APCs to IL-12 (215). IL-18, produced constitutively by immature DCs, activates NK cells to secrete IFN-γ (151) and increases IL-12-dependent IFN-γ production by both DCs and T cells (261-263). This early production of IL-12, IL-15, IL-18 and IFN-γ by APCs results in their autocrine activation and cytokine production during antigen presentation. In turn, these cytokines also stimulate development of IFN-γ-secreting Th1 cells and IFN-γ production from NK cells, all of which further activate APCs in a paracrine positive feedback loop (264). These findings indicate that immune regulation by cytokine networks is a key process in DC-mediated differentiation and commitment of Th1 cell responses.

Conversely, a different set of factors released during the early phase of infection or tissue injury can initiate Th2 cell differentiation. IL-10, phosphoinositide-3-kinase, prostaglandin E_2, and glucocorticoids inhibit IL-12 production by maturing DCs (265-268). There are conflicting data on the effect of TGF-β on DC function: TGF-β has been reported to inhibit LPS-induced IL-12p40 production (269), but other studies showed that either TGF-β has no effect on IL-12-mediated signaling or IL-12 production (270, 271) or it upregulates CD40-stimulated IL-12 production by mouse Langerhans cells (272). Unlike TGF-β, IL-10 is more conclusively involved in Th2 deviation due to its ability to stimulate DCs to prime $CD4^+$ T cells for IL-4 production (273). IL-4 is a potent cytokine secreted by $CD4^+$ T cells and many non-T-cell types that induce Th2 cell differentiation. However, IL-4 has been shown to play complex and paradoxical roles in cytokine-mediated regulation of Th cell priming. IL-4, alone or in combination with IFN-γ, instructs DCs to secrete high levels of IL-12p70 and enhances the capacity of DCs to induce Th1 cell differentiation (274). While IL-4 increases bioactive IL-12p70, it reduces the production of inactive IL-12p40 (274, 275), a finding which may explain the previous notion of IL-4 as an IL-12 inhibitor. Furthermore, IL-4 synergizes with IL-18 to enhance IL-12-dependent production of IFN-γ (276), which in

turn further promotes IL-12 production by DCs (260) as well as induces Th1 cell differentiation (277, 278). IL-4 upregulates IL-12 production by decreasing histone acteylation of the IL-10 promotor in a STAT6-dependent manner, resulting in decreased IL-10 mRNA transcription (279). That IL-4 does not inhibit Th2-inducing cytokine production by DCs nor does it inhibit LPS-stimulated IL-10 production by B cells has led to the suggestion that the ability of IL-4 to influence the shift from Th1 to Th2 responses is based on the type of APC presenting antigens to T cells. In this scenario, IL-4 enhances the Th1-promoting capacity of DCs in the absence of B cells and the resultant Th cell polarization therefore is determined in part by a balance between DCs and B cells in the innate immune response. Indeed, DCs produce more IL-12 in the absence of B cells to result in Th1 cell development and B cells upregulate the capacity of DCs to promote IL-4 secretion for induction of a Th2 response (254).

While differential cytokine secretion contributes to the ability of DCs to direct divergent classes of Th cell development, cytokines also play a vital role in feedback mechanisms that antagonize the excessive development of one type of immune response. IL-10 downregulates IL-12 production by DCs and thus inhibits the capacity of DCs to induce Th1 responses (280, 281). While it remains controversial whether TGF-β modulates DC homeostasis or IL-12 production, several studies have reported that TGF-β suppresses Th1 cell development by downregulating IL-12-dependent IFN-γ production by NK cells (271, 282, 283). Alternatively, IL-4 induces pDCs to produce IFN-γ in a mechanism that does not require IL-12, type I IFNs, induction of T-bet, or changes in the maturation state of the pDC (284). Rather, IL-4 signaling via STAT6 induces the expression of STAT4, the transcription factor required for IFN-γ production, by pDCs (284). IL-4 also inhibits IL-10 production while enhancing IL-12 production by bone marrow-derived DCs (279). Given that IL-4 is produced by NK-T cells and/or basophils in the early phase of immune responses (285, 286), the IL-4-induced production of IL-12 and IFN-γ by diverse DC subsets provides a broad negative feedback regulation against a Th2-type deviation in favor of activating a Th1 response, thereby dampening excessive allergic inflammation (287). Similarly, there are intrinsic mechanisms to control Th1-dependent cell-mediated immune responses. IFN-γ is an important Th1 cytokine; it elicits Th1 polarization of $CD4^+$ T cells independently of IL-

12 (288) and induces expression of T-bet in Th2 cells, causing reversal of the Th2 phenotype by suppressing IL-4 production and inducing IFN-γ secretion (289). However, studies have shown that IFN-γ may synergize with IL-4 to prime $CD4^+$ T cells for IL-4 production and thus Th2 differentiation (290). Clearly, cytokine regulation of Th cell polarization is more complex than the earlier models showing IL-12/IFN-γ and IL-4 as the principal cytokines mediating Th1 and Th2 effector choice, respectively. The precise timing and levels of cytokine production are crucial to their differential effects on APCs in skewing Th cells toward the Th1 or Th2 phenotype.

In addition to immune activation, cytokines play an important role in tolerogenic DC and T cell responses. IL-21, a cytokine that shares the common γ chain for signaling and other structural similarities with IL-2, IL-4 and IL-15, was shown to inhibit LPS-induced activation and maturation of DCs (291). In contrast to IL-15 which is a potent stimulator of DCs (215), IL-21 maintains DCs in a immature state characterized by low expression of MHC class II and costimulatory molecules, impaired production of the proinflammatory cytokines IL-1β, IL-12, IL-6 and TNF-α in response to LPS, and inhibition of antigen-specific T cell proliferation (291). Cytokines also regulate the homeostasis of peripheral Treg cells. While $CD4^+CD25^+$ T cells in the thymus develop spontaneously to maintain central tolerance, cytokines expand and activate the pool of peripheral Treg cells during the course of an immune response. TGF-β signaling increases Foxp3 expression and suppressive function of $CD4^+CD25^+$ T cells (293, 294). Although IL-2 is a potent growth factor for T cells, recent studies have shown that IL-2 is essential for induction of tolerance, not immunity (294). IL-2 is not required for the development and differentiation of Treg cells in the thymus (295), but this cytokine, together with STAT5 signaling, is indispensable for the maintenance and suppressive function of $CD4^+CD25^+$ T cells (296-299). Both IL-2 and TGF-β can convert antigen-stimulated $CD4^+CD25^-$ cells to become $CD4^+CD25^+$ T cells that express Foxp3 and produce IL-10 and TGF-β, which in turn convert other $CD4^+CD25^-$ cells to acquire regulatory phenotype and function (299, 300). Intriguingly, although IL-15 supports IL-12 and IFN-γ production for Th1 cell development (211, 215), it can induce tolerance by promoting the proliferation and survival of IL-10-producing Tr1 cells (301-303)

Role of dendritic cells in T cell differentiation

DCs are the major APCs that produce IL-12 following antigenic stimulation and engagement of CD40 on the DC with CD40 ligand (CD40L or CD154) on the Th cell. Ligation of TLRs by intact bacteria, LPS, bacterial DNA, or dsRNA at the time of DC-T cell interaction can also induce or enhance IL-12 production by DCs (304-307). DC-derived IL-12 then primes the activated Th cell for differentiation and commitment toward IFN-γ production, resulting in strong Th1 cell responses. Early studies showed that murine $CD8\alpha^+$ DCs from the thymus, lymph nodes and especially the spleen are high producers of IL-12 and thus are the main DC subtype to induce a Th1 response in vivo (123, 308, 309). In contrast, murine $CD8\alpha^-$ DCs produce low amounts of IL-12 and preferentially induce a Th2 response in vivo (123, 308, 309).

However, such a system involving separate DC lineages for promoting Th1 versus Th2 responses would greatly limit the robustness of the immune system to respond to changing conditions in pathogen-invaded tissues and also would require different surface receptors among DC subsets to discriminate between Th1- and Th2-inducing pathogens. More recent studies have challenged this specialized lineage model by demonstrating that DCs display an exceptional capacity for functional plasticity depending on signals from the pathogen and local environment. $CD8\alpha^-$ DCs can be induced by IFN-γ to increase their Th1-promoting capacity (310). Moreover, $CD8\alpha^-$ DCs pulsed with Th1-polarizing antigens from the bacterium *Propionebacteriuam acnes* induce Th1 responses while the same DCs pulsed with Th2-polarizing antigen from the helminth *Schistosoma mansoni* stimulate Th2 responses (311). Despite conflicting data regarding the relative levels of IL-12 produced among different DC subsets, both $CD8\alpha^+$ and $CD8\alpha^-$ DCs have been shown to produce IFN-γ after stimulation by microbial products or CD40 ligation (309, 312). Ligation of the Fas (CD95) receptor also induces DCs to mature, release IL-1β, and stimulate T cells to produce IFN-γ even in the absence of IL-12 (313). Taken together, these studies suggest the presence of alternate IL-12-independent pathways in DC-mediated differentiation of naïve $CD4^+$ T cells into Th1 cells. Furthermore, high doses of OVA peptide stimulate both mouse myeloid and plasmacytoid DCs to induce Th1 cell development, while low doses lead to Th2 cell development (314). pDCs, which express high levels of TLR9, are more responsive to CpG than to LPS in

promoting Th1 cells, and myeloid DCs, which express high levels of TLR4, also induce Th1 responses when stimulated with LPS (314). Thus, the net effect of antigen dose, stimulation of DCs by specific TLR ligands and cytokine milieu, regardless of the DC subset, determines whether a Th1 or Th2 response develops.

The state of maturation of the DC can also influence the initiation of immune responses. Immature DCs are thought to induce T cell anergy or deletion or, alternatively, the development of regulatory T cells (101, 103). Early after stimulation by microbes or CD40 ligation alone or in combination with IFN-γ, DCs produce high amounts of inflammatory mediators including IL-12p70, TNF-α, IL-1β and IL-6 (304-307, 315). DCs that are matured solely by inflammatory stimuli, such as TNF-α, and in the absence of TLR engagement express high levels of MHC class II and costimulatory molecules but do not produce Th cell-skewing cytokines (110, 316). These DCs, called semi-mature DCs, are able to stimulate T cell proliferation, but importantly fail to induce Th1 effector cells and instead promote suppressive IL-10-secreting $CD4^+$ T cells, leading to tolerance induction (106, 110, 316). The kinetics of DC activation can also influence the type of effector T cells generated. As DCs mature, they lose their capacity to produce IL-12, become refractory to further stimulation by both microbial and inflammatory stimuli, and preferentially prime Th2 and nonpolarized T cells (315, 317, 318). The specific mechanisms responsible for the reduced responsiveness of fully mature DCs are presently unclear; both IL-10 and prostaglandin E_2 inhibit IL-12 production by maturing DCs (265, 266) but have no effect on DCs that have completed final maturation (266). Mature DCs, however, continue to secrete IL-6, a cytokine that costimulates the proliferation of naïve Th cells (319) and promotes differentiation of B cells (320, 321).

Myeloid DCs are not the only DC population that can promote divergent Th cell responses. pDCs also show a similar degree of flexibility in their cytokine profiles and capacity to direct T cell polarization. pDCs activated by viral infection, CD40 ligation or TLR9 engagement by bacterial CpG produce large amounts of type I IFNs and can elicit potent cytotoxicity and IFN-γ production by NK cells and $CD8^+$ T cells (322, 323). Virus-activated pDCs also secrete Th1-promoting IL-12, chemokines to attract activated T cells to the site of inflammation, and IL-6 to direct differentiation of plasma cells (320). On the other hand, pDCs can be activated by IL-3 and CD40 ligation to drive

Th2-type responses by allogeneic T cells (306). This priming of Th2 cells is associated with upregulated expression of OX40L and decreased type I IFN production by pDC, but does not depend on IL-4 (324). However, viral activation of pDCs reverses the Th2-polarizing effects of OX40L expression and induces the pDCs to produce a strong type I IFN response, resulting in Th1 cell development (324).

The activation state of DCs may influence differentiation of naïve T cells into effector or regulatory T cells. Under steady-state conditions, immature pDCs can induce the development of $CD4^+CD25^+$ Treg cells and IL-10-secreting $CD8^+$ T cells (325-327). Activation of DCs is a key process in DC-mediated differentiation of naïve T cells into effector Th1 or Th2 cells (discussed above). Moreover, TLR-activated DCs abrogate the suppression mediated by Treg cells, a process that is partially dependent on DC-derived IL-6 (328, 329). However, many recent studies suggest that mature DCs do not block the activity of Treg cells but can induce tolerance by promoting their proliferation and suppressive function. After stimulation by TLR ligands, both myeloid and plasmacytoid DCs can promote the development of $CD4^+CD25^+$ Treg cells (330, 331). DCs may induce Treg cells by providing antigenic and cytokine signals, such as autoantigenic MHC-peptide complexes (332, 333) as well as IL-2, IL-10 and TGF-β (154, 300, 330, 334, 335). Indeed the cytokine profile produced by the DC appears be instrumental in influencing the type of Th cell response: DCs that produce little to no IL-12 but high IL-10 are critical for the induction of Tr1 cells that suppress $CD4^+$ T cell responsiveness to nonself antigens (327, 334, 336, 337). Generation of $CD4^+CD25^+$ T cells after exposure to Th1-promoting antigens is thought to exert a negative feedback mechanism on immune responses by suppressing the development of Th1 cells induced by mature DCs (338). Moreover, a recent study showed that tumor cells stimulate myeloid DCs to secrete TGF-β, which induces proliferation and activation of $CD4^+CD25^+$ T cells (339). Reciprocally, Treg cells suppress the maturation and antigen-presenting function of DCs. IL-10 released by Treg cells reduces the expression of costimulatory molecules by DCs, thus impairing the priming of naïve T cells (340, 341). Treg cells isolated from allograft-tolerant mice were able to induce the generation of tolerogenic DCs from bone marrow progenitors (342). Conversely, CD40 and TLR ligation renders DCs resistant to suppression by Treg cells, restoring the capacity of DCs to stimulate naïve T cells (343).

1.2.3 Summary: Role of Dendritic Cells in Innate and Adaptive Immunity

DCs, the most potent APC, play a key role in the initiation of innate and adaptive immunity. DCs orchestrate many elements of the immune response by presenting antigens to T cells, interacting with other immune cells that carry out distinct effector functions, and secreting immunoregulatory cytokines. Although research over the past 30 years has undoubtedly revealed the complexity of DC development and function, some general mechanisms and responses have emerged. In Fig. 1.3, a composite model is presented to summarize the different stages of DC maturation and the role of DCs in the induction of innate and adaptive immune responses.

As shown in Fig. 1.3, circulating precursor DCs enter tissues as immature DCs and continually sample the microenvironment. After capture of antigens derived from pathogens (e.g., viruses) or altered host cells (e.g., malaria-infected RBCs), DCs migrate to secondary lymphoid tissues and undergo maturation. In the absence of inflammatory stimuli, DCs may remain in an immature state and present antigens to T cells without costimulation, leading to deletion of T cells or generation of inducible regulatory T cells. Conversely, antigen-stimulated DCs may interact with NK cells to elicit potent cytotoxicity and high IFN-γ production. Activated NK cells, reciprocally, promote further DC maturation and enhance the capacity of DCs to prime naïve $CD4^+$ T cells for Th1 cell polarization. Cytokines released during the innate immune response may activate macrophages to phagocytose pathogens and infected or damaged cells and to secrete proinflammatory mediators. Mature DCs present MHC class II-peptide complexes, along with appropriate costimulatory molecules and cytokines, to naïve $CD4^+$ T cells, thereby activating T cell expansion and differentiation into effector Th1, including cytotoxic T lymphocytes (CTL), Th2, or Treg cells. Mature DCs also trigger B cell activation, proliferation and Ig class switching for production of antigen-specific antibodies that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and other host defenses against infection. Consequently, antigen-activated DCs possess much of the machinery needed to stimulate a diverse number of innate and adaptive effector mechanisms for control and resolution of an infection.

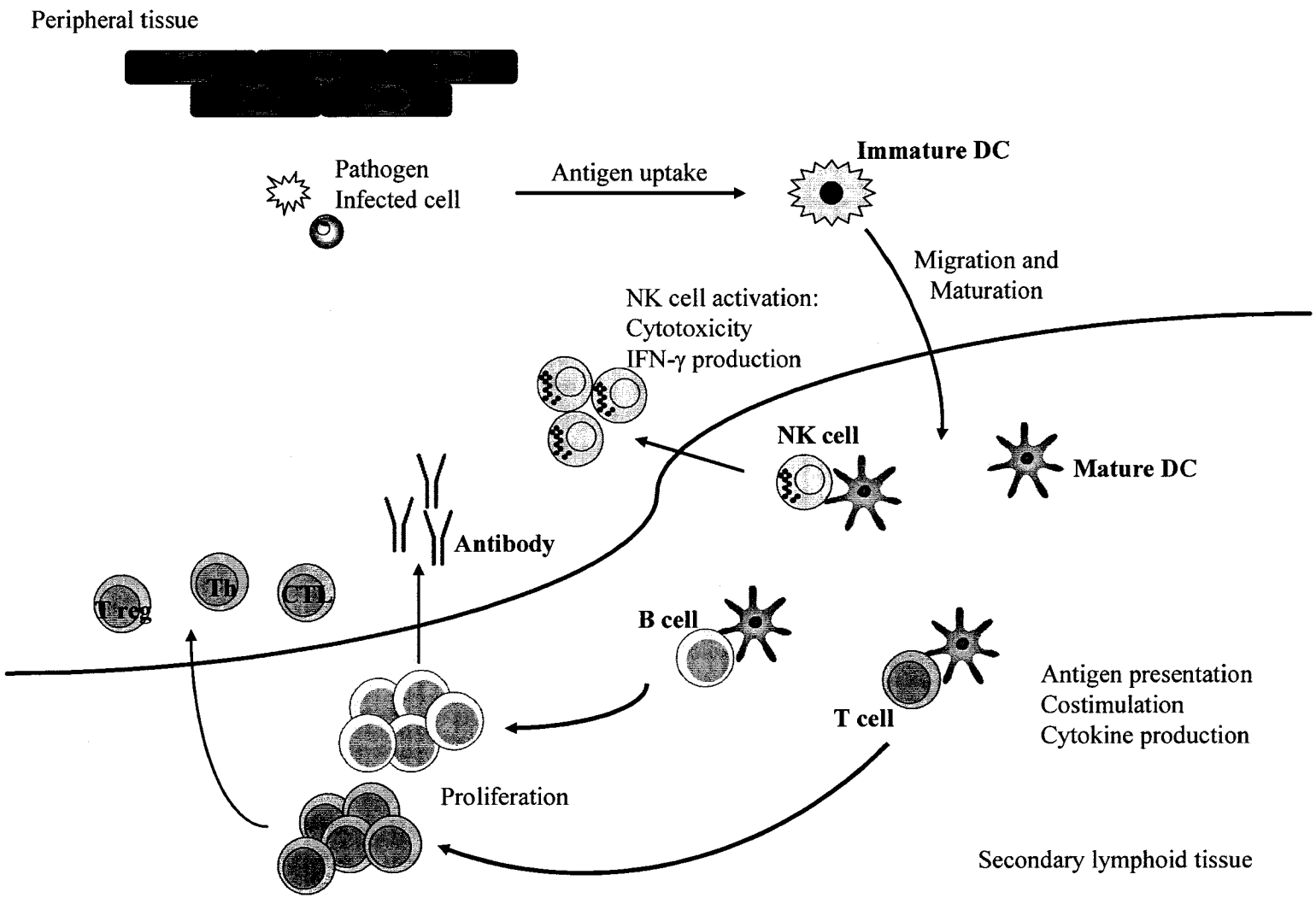

Figure 3: Model of Dendritic Cell Responses and Crosstalk in the Immune Response to Pathogens

1.3 Immunity to Blood-Stage Malaria Infection

Although the blood stage is responsible for all clinical symptoms and pathology associated with malaria infection, immunity to this stage is yet to be fully understood. The blood stage is an important target for vaccine development because a malaria vaccine against this stage would offer immense benefits in terms of reduction of parasite growth and prevention of malarial pathogenesis. Thus, it is crucial to understand the nature of both innate and adaptive immune mechanisms targeted against blood-stage malaria. In human and murine malaria, effective control and resolution of infection require DCs, macrophages, NK cells, $CD4^+$ T cells and production of proinflammatory cytokines during the acute phase of infection as well as Th1-dependent Ab responses during the curative phase. The following sections review our current knowledge of immunity to blood-stage malaria in both humans and experimental mouse models.

1.3.1 Genetic Factors

Humans infected with *Plasmodium* parasites experience a wide range of disease severity, from very mild symptoms to severe, life-threatening complications. The genetics of the host is a major factor that affects susceptibility to malaria. Different genetic factors may determine an individual's initial susceptibility to infection, the likelihood of developing severe complications once infection has occurred, and the ability to acquire effective long-term immunity. Studies in both humans and mouse models have shown that the genetic component is complex, multigenic and controlled by three-way interactions among host genes, the environment, and the malaria parasite. There are several inherited genetic polymorphisms that protect humans living in malaria endemic areas. These include erythrocyte variants (e.g., Duffy blood group antigen), glucose-6-phosphate dehydrogenase deficiency, and hemoglobin disorders (e.g., sickle-cell anemia, α and β thalassaemias). Studies on these genetic determinants of the susceptibility to malaria have also provided important information on host receptors and parasite proteins involved in parasite invasion and malaria pathogenesis.

Genetic factors also regulate the immune response to *P. falciparum*. A study of several West African ethnic groups show clear inter-ethnic differences in infection rates, malaria febrile episodes, and antibody responses to a major *P. falciparum* surface protein

that were not related to other environmental, social or genetic factors (344). Moreover, a study in twins showed that regulation of the anti-malarial antibody responses to a variety of *P. falciparum* antigens is controlled by MHC and non-MHC genes (345). Among populations residing in Cameroon and Burkina Faso, levels of parasitemia are linked to 5q31-5q33, a region that contains many genes for cytokines and other immune factors (346, 347) A number of studies have identified polymorphisms in genes for IFN-γ, TNF, IL-1, IL-4, IL-10, IL-12, HLA antigens, ICAM-1, iNOS, PECAM, complement receptor 1 and CD36 that control resistance to human malaria (348). In general, the roles of these genes in susceptibility to human malaria are complicated. For example, polymorphisms of iNOS and CD36 have been associated with both protection and susceptibility to severe malaria (51, 349-351). Complex genetic traits, such as host resistance to malaria, can be finely dissected using genetically well-defined strains of mice in which single gene effects may have naturally segregated, have been experimentally isolated by breeding, or have been experimentally induced by targeted gene mutation or deletion (352). Studies in nude mice (353) or SCID mice (354) as well as $CD4^+$ T-cell-depleted mice have established a key role for $CD4^+$ T cells in resistance to blood-stage infection (355, 356). Genetic analyses of different inbred, recombinant inbred and recombinant congenic strains have identified several loci that are associated with control of parasitemia (91, 92, 352). Several candidate genes found in these loci may have possible functions in the host immune response and RBC physiology. As discussed above, the genetic interval for *Char2* on mouse chromosome 8 contains genes encoding the cytokine IL-15, the class A scavenger receptor (SR-AI and SR-AII), and several erythrocyte structural proteins (87, 88). The role of IL-15 in innate and adaptive immune responses to blood-stage malaria was the focus of experiments performed in Chapter 2.

1.3.2 Innate Immunity to Blood-Stage Malaria

The early interaction of the parasite with the host activates nonspecific innate immune responses that result in antigen-specific adaptive responses, which might contribute to both parasite clearance and malarial disease. Despite increasing awareness of the vital role of the innate immune system in induction of adaptive immunity, much of the immunological research on malaria has been focused on adaptive immune responses

to facilitate vaccine development. However, a recent review of the literature concluded that the control of the early peak of parasitemia in murine malaria infections is critically dependent on innate as opposed to adaptive cell-mediated immune responses (357). Studies in humans also provide strong evidence that innate immunity is critical for control of blood-stage malaria (358).

Dendritic cells

Investigation of DC function in blood-stage malaria has yielded conflicting results. Early studies showed that CD36-adherent *P. falciparum* strains inhibit DC maturation and stimulation of allogeneic T cell proliferation (359, 360). These early findings were taken as evidence that *Plasmodium* parasites modulate host innate defenses to escape immune clearance and thereby establish persistent infection. These data were supported by a subsequent ex vivo study that showed a reduction in HLA-DR expression on myeloid DCs in Kenyan children suffering from acute malaria (361). It is important to note, however, that the same study reported comparable percentages of peripheral $CD83^+$ DCs, a mature population, between healthy and malaria-infected children (361). More extensive studies, especially in murine models, indicate that DCs respond differently depending on the *Plasmodium* species. While it is possible that cytoadherent *P. falciparum* modulate DC function through adherence via CD36 (359, 360), such a mechanism of immune evasion has not been observed with rodent *Plasmodium* species and may represent an important difference between human and rodent malaria species.

In an in vitro study, *P. chabaudi* schizonts were shown to induce bone marrow-derived DCs to express the co-stimulatory molecules CD40, CD86, and MHC class II, and to produce the proinflammatory cytokines IL-12, TNF-α, and IL-6 (362). Bone marrow-derived DCs from SCID mice and from gene-deficient Rag or CD40 knockout mice are able to produce these cytokines in vitro, demonstrating that DC cytokine response to *P. chabaudi* schizonts is not dependent on the presence of T cells, NK cells, or CD40 expression. Importantly, *P. chabaudi* schizonts did not inhibit LPS-induced upregulation of MHC class II and costimulatory molecule expression. While these data were obtained using bone marrow-derived DCs, it is the splenic DCs that are of major

interest given the central role of the spleen in filtering parasitized RBCs from the blood and in generating protective immunity to blood-stage malaria.

Enlargement of the spleen or splenomegaly is a hallmark of malaria endemicity in humans and often serves as a phenotypic marker of host resistance in malaria-infected mice (198, 363). APCs isolated from spleens of *P. yoelii* 17X-infected mice have been observed to have increased expression of CD80 and MHC class II molecules and to stimulate high levels of IFN-γ production by naïve T cells (364). Of the APC subsets, there is growing evidence that DCs are the cells primarily responsible for stimulating production of proinflammatory cytokines and activation of naïve $CD4^+$ T cells, leading to induction of protective type 1 responses to blood-stage malaria. Within 5 days of infection with *P. chabaudi*, $CD11c^+$ DCs migrate from the marginal zone of the spleen to the $CD4^+$ T-cell-rich periarteriolar lymphoid sheath (365). In contrast, macrophage and B-cell populations expand but remain confined to the red pulp area. Concurrent with their migration within the spleen, $CD11c^+$ DCs have upregulated expression of CD40, CD54, and CD86 as well as increased production of IFN-γ (365). Similarly, splenic $CD11c^+$ DCs, but not $CD11b^+$ macrophages or B cells, from *P. yoelii* 17X-infected mice were shown to stimulate high levels of IL-2, IFN-γ and TNF-α production by naïve $CD4^+$ T-cells (366). These responses were found to be antigen-specific as demonstrated by the ability of purified $CD11c^+$ DCs from infected mice to stimulate a *P. yoelii* 17X-specific T-cell hybridoma to secrete high levels of IL-2. Furthermore, both myeloid and lymphoid $CD11c^+$ DCs from infected mice were found to produce IL-2, IFN-γ, TNF-α and IL-12p40 and, importantly, to induce IFN-γ secretion by $CD4^+$ T-cells through an IL-12-dependent mechanism (366, 367). In addition to providing the requisite co-stimulation and cytokines to stimulate T cells, there is evidence that DCs from mice infected with *P. yoelii* 17XNL secrete DC-derived CC chemokine 1 (DC-CK1) that acts preferentially on naïve T and B cells (368). DC-CK1 treatment in vitro stimulates chemotaxis of T and B cells; in vivo, this chemokine induces a strong IFN-γ-producing $CD8^+$ T cell response to liver-stage *P. yoelii* 17XNL and also acts as a vaccine adjuvant by increasing protection to liver-stage malaria mediated by immunization with irradiated *P. yoelii* 17XNL sporozoites. The role of DC-CK1 as a chemotactic or immunostimulatory factor in cell-mediated immunity to blood-stage malaria remains to

be determined. In addition to infected RBCs, parasite products may activate DCs. Innate immune cells recognize hemozoin via TLR9, leading to MyD88-dependent production of cytokines and chemokines as well as upregulation of costimulatory molecules (57, 58). Taken together, DCs activated in vitro and in vivo in mouse models of malaria are capable of presenting *Plasmodium* antigens (signal 1), expressing co-stimulatory molecules (signal 2), and producing proinflammatory cytokines and chemokines (signal 3) to stimulate antigen-specific T-cell-dependent immune responses (as shown above in Fig. 1.2). The experiments conducted in Chapter 3 offer novel data concerning these important DC functions in blood-stage *P. chabaudi*-infected mice.

These findings provide increasing evidence that, in contrast to some isolates of *P. falciparum*, the rodent parasites *P. yoelii* and *P. chabaudi* do not inhibit DC function. Activation of DCs is not limited, however, to evidence from murine models of malaria. Importantly, *P. falciparum* schizonts stimulate human plasmacytoid DCs and mouse splenic $B220^+$ DCs to upregulate CD86 expression, produce IFN-α, and promote $\gamma\delta$ T cell proliferation and IFN-γ production through a TLR9-dependent pathway (369). Mice immunized with bone marrow-derived DCs pulsed with the respective intact parasitized RBCs survive challenge infections with lethal blood-stage *P. yoelii* YM or *P. chabaudi* (370). Transfer of antigen-pulsed DCs also can induce cross-strain protection and is associated with malaria-specific production of IFN-γ and IL-4 as well as malaria-specific antibody after challenge infection. Moreover, no protection was observed in IL-12p40$^{-/-}$ mice after lethal *P. yoelii* YM challenge, demonstrating that DC-induced immunity is dependent on host expression of IL-12. It is likely that T cells play an important role in mediating immunity conferred by transferred DCs given that splenic T cells isolated from mice immunized with antigen-pulsed DCs protected mice from *P. yoelii* YM challenge, although to a lesser degree compared to immunization by transfer of DCs.

Contrary to reports showing that DCs are important mediators of protective immune responses to blood-stage malaria, *P. yoelii* 17XNL-infected RBCs have been shown to inhibit LPS-stimulated DC maturation and IL-12 secretion (371). Intriguingly, the parasite-induced modulation of DC functions was associated with suppressed protective $CD8^+$ T cell responses against liver-stage malaria. The ability of *P. yoelii* 17XNL-exposed DCs to inhibit immune responses to sporozoites was interpreted as a novel

mechanism by which the *Plasmodium* parasite evades host immunity, leaving the host unprotected against reinfection. In support of this concept, a recent study showed that *P. berghei*-infected RBCs selectively impair the ability of DCs to prime $CD8^+$ but not $CD4^+$ T cells for proliferation and IFN-γ production (367). Suppressed activation of $CD8^+$ T cells by pRBC-exposed DCs was attributed to IL-10-dependent cell cycle arrest. These results may help explain why naturally acquired immunity to malaria in humans develops very slowly, despite persistent exposure to *Plasmodium* parasites and intermittent episodes of infection in malaria endemic areas.

Natural killer cells

Enhanced NK cell activity in spleens of mice infected with irradiated *P. berghei* sporozoites was demonstrated many years ago (372). Subsequent studies confirmed the important role of NK cells, along with parasite-specific $CD8^+$ T cells and production of IL-12 and IFN-γ, in protective immunity to liver-stage malaria (373, 374). In blood-stage malaria, mouse strains resistant to *P. chabaudi* infection show high NK activity, including marked increases in NK cell numbers in the spleen and increased cytotoxic activity and IFN-γ secretion (139, 375). Depletion of NK cells in mice infected with *P. chabaudi* or *P. yoelii* increases parasitemia and mortality (376, 377). Similarly, depletion of NK cells in *P. chabaudi*-resistant C57BL/6 mice results in a more severe course of infection, and IL-12-treated but NK cell-depleted susceptible A/J mice are unable to fully control the infection (139). Importantly, NK cell IFN-γ production, not cytotoxicity, was demonstrated to play the major role in mediating protective immunity to blood-stage *P. chabaudi* AS infection (139). Together these results suggest that the primary role of NK cells in innate immunity to blood-stage malaria is to produce IFN-γ and therefore activate IFN-γ-dependent effector mechanisms. Indeed, early production of IFN-γ by NK cells and γδT cells is associated with resolution of nonlethal *P. yoelii* and *P. chabaudi* infections, but is absent in lethal *P. yoelii* and *P. berghei* infections (378). Other studies have reported opposite findings: depletion of $NK1.1^+$ cells in mice infected with nonlethal *P. berghei* XAT does not affect parasitemia or IFN-γ production by splenocytes, although IFN-γ production, possibly by $CD4^+$ T cells, is critical for resistance (379). Interpretation of these data may be complicated by the inadvertent

depletion of T cell subsets expressing the NK1.1 marker. Observations that NK cell-depleted SCID mice succumb to nonlethal *P. yoelii* infection at an earlier time than SCID mice or intact mice treated with anti-Thy 1.1 antibody (377) confirm a role for NK cells in anti-malarial immunity that is independent of NK-T or T cells.

In agreement with data from mouse models, *P. falciparum*-infected RBCs elicit rapid production of IFN-γ from human $CD3^{-}CD56^{+}$ NK cells, indicating that NK cells are among the first cells in peripheral blood to produce IFN-γ production during early infection (140). In contrast, γδ T cells and αβ T cells initiate IFN-γ production 2 to 4 days later, respectively. *P. falciparum*-infected RBCs also upregulate expression of NK cell activation receptors, CD25 and CD69, on human peripheral blood mononuclear cells (PBMCs) (380). The NK cell response is highly dependent on IL-12 and, to a much lesser extent, IL-18 released by accessory cells (140). There is now compelling evidence from studies on DC–NK cell interactions (discussed above) that DCs are the accessory cells that secrete IL-12, IL-18 and other NK cell-activating cytokines (146, 151, 152, 381). The studies presented in Chapter 4 support the hypothesis that IL-12 is a major cytokine whereby DCs activate NK cell IFN-γ production during blood-stage malaria. Alternatively, a recent study showed that IL-18-secreting monocytes/macrophages are responsible for maximal NK cell activation and IFN-γ production induced by *P. falciparum*-infected RBCs (380). However, this study did not investigate whether monocyte-derived DCs could also contribute to *P. falciparum*-stimulated NK cell activation. In addition to cytokines released by DCs and other APCs, human NK cells require direct contact with *P. falciparum*-infected RBCs for optimal induction of IFN-γ production (141). The IFN-γ-producing NK cells were found to express high levels of CD94/NKG2A, suggesting that NK cell receptors are able to directly recognize infected RBCs. In support of this idea, human NK cells have been shown to be more efficient at killing *P. falciparum*-infected RBCs than uninfected RBCs (382).

Macrophages

The concept that macrophages suppress immune responses to blood-stage malaria has been documented extensively (60, 61, 383, 384). Macrophages from mice infected with *P. chabaudi* (61, 384) or *P. yoelii* 17X (366) inhibit T cell proliferation and

production of the T cell growth factor, IL-2. Phagocytosis of the malarial pigment, hemozoin, is associated with suppression of various monocyte and T-cell functions (59-61, 385), leading to the notion that phagocytic cells produce a soluble suppressive factor, such as NO, prostaglandin E_2 (PGE_2), or TGF-β. Hemozoin has been shown to increase NO synthesis by macrophages through an ERK/NFκB-dependent pathway (56). Inhibition of both NO and PGE_2 reverses the suppressive effect of peritoneal macrophages from *P. chabaudi*-infected mice on mitogen-induced proliferation of normal splenocytes *in vitro* (384), whereas inhibition of NO alone did not restore IL-2 production by antigen-activated T cell hybridomas cultured with macrophages from infected mice (61). Malaria-infected RBCs and hemozoin generate large amounts of hydroxyl fatty acids that inhibit monocyte functions and can stimulate RBC cytoadherence, thereby contributing to hemozoin toxicity and malarial disease (64). Recent studies provide further evidence that a soluble factor(s) produced by splenic $CD11b^+$ macrophages from *P. yoelii* 17X-infected mice inhibits T cell proliferation and IL-2 production, but this factor does not appear to be NO, PGE_2, or TGF-β (364, 366). Interestingly, $CD11b^+$ macrophages from infected mice are unable to inhibit IFN-γ or TNF-α production by naïve T cells (364), suggesting that macrophages play an important immunoregulatory function in limiting T cell expansion to prevent malarial pathology while promoting high levels of IFN-γ production and, possibly as a result, Th1 cell differentiation during the acute stage of blood-stage malaria infection.

The suppressive effects of macrophages during malaria infection are not limited to T cell responses. Following infection with *P. berghei* ANKA, peritoneal macrophages exhibit suppressed IL-12p40, but not IL-12p35, gene expression. Macrophages that have engulfed parasitized RBCs in vitro are capable of inhibiting IL-12p40 mRNA expression by uninfected macrophages, suggesting the presence of a soluble suppressive factor (386). Indeed, this inhibition was found to be meditated by macrophage-produced IL-10, while PGE_2 and TGF-β do not seem to be involved. High levels of IL-12p70 synthesis by splenic macrophages correlate with host resistance to *P. chabaudi* AS infection (387, 388), although the expression of both p40 and p35 mRNA is upregulated in resistant C57BL/6 as well as susceptible A/J mice during infection (388). Together, these data raise the possibility that inhibition of IL-12, a key cytokine in the induction of protective

Th1 immunity to blood-stage malaria, at the level of p40 gene expression or IL-12p70 secretion, might be an important evasion strategy by the *Plasmodium* parasite. In addition, ingestion of *P. chabaudi*-infected RBCs or hemozoin by peritoneal macrophages stimulates the release of macrophage migration inhibitory factor (MIF), a proinflammatory mediator that stimulates T cell activation, antibody production, and delayed-type hypersensitivity reactions (389). MIF also can inhibit erythropoietin-dependent erythropoiesis in vitro, suggesting a potential role for macrophage-derived MIF in the pathogenesis of malarial anemia (389).

Despite the immunosuppressive effects of macrophages on selective monocyte and T cell functions during blood-stage malaria, phagocytosis of parasitized RBCs is an important nonspecific immune defense mechanism (390-392). High levels of opsonin-independent phagocytosis of pRBCs and free merozoites are observed in mice that successfully control and resolve a primary *P. chabaudi* infection (392). Levels of opsonin-independent phagocytosis correlate with the ability of infected mice to produce IFN-γ and treatment of macrophages with IFN-γ enhances phagocytic activity while IL-10 treatment inhibits this activity (392). Thus, opsonin-independent phagocytosis by macrophages contributes to IFN-γ-dependent control of blood-stage malaria during the early stage of infection, prior to the development of antigen-specific adaptive immunity.

Cytokine regulation of innate immunity to blood-stage malaria

A coordinated network of cytokine responses tightly regulates the innate immune response to invading pathogens. DC-derived IL-15 stimulates DCs and macrophages to secrete IL-12 and IFN-γ (215), which in turn enable these cells to become further activated via a positive autocrine feedback loop. The Th1-type cytokines produced by APCs also activate NK cells, NK-T cells, $CD8^+$ T cells, and γδ T cells to produce large amounts of IFN-γ, which is crucial for priming $CD4^+$ T-cells toward Th1 cell differentiation and subsequent development of type 1 adaptive immune responses required for control of blood-stage parasitaemia (139, 378, 393, 394). Moreover, IL-2 and IL-15 have been shown to enhance CD154 (CD40 ligand) expression by activated $CD4^+$ T cells, thus prolonging T cell stimulation even in the absence of cognate interaction with CD40-expressing APCs (216). Although IL-18 alone primes Th2 cell

development (395), it synergizes with IL-12 to induce IFN-γ-secreting DCs and Th1 cells (262, 396, 397). Indeed, IL-18 has been shown to increase IFN-γ production by NK cells in response to *P. falciparum*-infected RBCs and to mediate Th1-dependent protective immunity to blood-stage malaria in mice (381, 398). IFN-γ produced by activated APCs, NK cells, and $CD8^+$ T cells during the innate response enhances priming of Th1 cells via T-bet induction, inhibition of IL-4, and upregulation of IL-12Rβ2 expression on T cells (277, 278, 289). In the later phase of blood-stage malaria, activated Th1 cells result in additional IFN-γ production, further APC and NK cell activation, and B cell differentiation into plasma cells secreting Th1-dependent antibody isotypes. Therefore, multiple signals released during parasite-APC interactions can influence $CD4^+$ Th cell differentiation and activation of downstream immune responses.

In addition to their coordinated interactions, some cytokines produced during the innate immune response also perform distinct, non-redundant functions. IL-12 is crucial for the development of type 1 immune responses, mainly due to its potent ability to induce IFN-γ production and Th1 cell differentiation. Moreover, high systemic IL-12 production and up-regulated expression of IL-12Rβ2, the receptor subunit mediating IL-12 signaling, correlate with resistance to *P. chabaudi* malaria (388). IL-15, on the other hand, is important for NK cell development and function, and IL-15 has been shown to synergize with IL-12 to promote IFN-γ production and Th1 cell development. IL-15 functions mainly as a survival and proliferation factor for developing NK cells during both homeostasis and the immune response to infection (208, 210, 399). The production of IFN-γ by APCs might effectively jump-start Th1 cell development by inducing autocrine APC activation as well as by priming commitment of other innate immune cells and $CD4^+$ T cells toward type 1 responses. Although multiple cytokines may result in Th1 cell development, IFN-γ is considered to be the central effector cytokine mediating protective immunity to blood-stage malaria infection (400, 401).

1.3.3 Adaptive Immunity to Blood-Stage Malaria Infection

Adaptive immunity to blood stages of infection relies primarily on $CD4^+$ T and B cell responses that induce protection against not only natural infection but also clinical

disease. Although T cells and antibodies have been shown to control parasite growth in mouse models, natural immunity to human malaria takes several years to develop, is relatively short-lived in the absence of continuous or regular exposure to the parasite, and does not fully protect against reinfection. The inability to generate effective adaptive and memory responses is related partly to the variation and polymorphism of surface antigens that allow parasites to escape immune recognition. In humans and mouse models, some long-term immunity is observed and is highly dependent on Th1 cell-mediated immunity and parasite-specific antibodies. In addition, immunity without pathology requires rapid parasite clearance, appropriate resolution of the inflammatory response, and development of protective antibodies against multiple strains.

T cell immunity

Early studies showed that T cell responses are effective in controlling parasitemia in the absence of B cells and antibody (402, 403), although B cells are required in the chronic phase for complete elimination of parasites (404). Three types of T cells are involved in immunity to blood-stage malaria. $CD8^+$ T cells, via IFN-γ secretion and cytolytic activity, are critical effector cells of immunity against sporozoites (374, 405). A role for $CD8^+$ T cells in immunity to blood-stage malaria is less clear. Adoptive transfer of $CD8^+$ T cells from mice immune to *P. chabaudi adami* or *P. yoelii* accelerated the recovery of recipient mice (406, 407l), but other studies were unable to demonstrate a protective effect of $CD8^+$ T cells (355, 408). In vivo depletion experiments confirmed that $CD8^+$ T cells contribute to but are not necessary for resolution of primary *P. chabaudi* infection (356). Human γδ T cells stimulated by *P. falciparum* produce proinflammatory cytokines such as IFN-γ, IL-1 and TNF-α (409), and can inhibit the growth of blood-stage parasites in vitro (410, 411). Experiments in mice deficient in γδ T cells demonstrate that these T cells are not essential for clearance of blood-stage parasites, but help control parasite growth in *P. chabaudi*-infected mice that also lack B cells (393, 412). On the other hand, many studies have established a definitive role for αβ $CD4^+$ Th cells, particularly the IFN-γ-producing Th1 cell subset, in protective immunity to blood-stage malaria (355, 356, 413). Adoptive transfer of $CD4^+$ T cells from immune mice protected recipient naïve mice from *P. yoelii* 17X infection (414),

and mice depleted of $CD4^+$ T cells developed high levels of parasitemia throughout an eight-week primary *P. chabaudi* infection (356).

$CD4^+$ T cells have two major effector functions in immunity to blood-stage malaria: they help B cells produce high affinity, malaria-specific IgG antibodies and differentiate into long-lived memory B cells (357), and they secrete Th1-type cytokines that activate macrophages, NK cells and other cells to mediate cytolytic defenses and to secrete IFN-γ, TNF-α, NO and reactive oxygen species that help control parasite growth (84). Differentiation and activation of Th1 cells is critically regulated by IL-12 alone or in cooperation with IL-15, IL-18, IFN-γ and TNF-α (discussed above). In vivo depletion of $CD4^+$ T cells abrogates IL-12-dependent resistance to blood-stage malaria (415) and impairs vaccine-induced protection against challenge infection with *P. chabaudi* in mice immunized with crude malaria antigen (416). Although Th1 cells are thought to control acute infection, the role of Th2 cells during chronic infection is controversial. Following peak parasitemia, there is a switch from a Th1 to Th2 pattern of cytokine responses, which suggests that Th2 cells/cytokines regulate B cell expansion and antibody production (417). However, mice lacking IL-4 or IL-10 are able to resolve a primary infection with *P. chabaudi* or *P. yoelii*, although IL-4-deficient mice are susceptible to reinfection with *P. chabaudi* and IL-10-deficient mice show more severe malarial disease and higher mortality from a primary *P. chabaudi* infection (78, 418, 419). Importantly, following *P. chabaudi* infection, IL-12-deficient mice develop high levels of primary parasitemia and produce lower levels of IFN-γ as well as Th1-dependent IgG2a and IgG3 than do IL-12-sufficient mice (420). Together these data indicate that IL-12-dependent Th1 immune responses are crucial for protection during both acute and chronic phases of blood-stage malaria, while Th2 cells/cytokines act to augment Th1-associated antibody responses and to dampen excessive inflammation and prevent immunopathology.

Despite the compelling evidence from mouse models, it has been more difficult to establish a role of $CD4^+$ T cells during human malaria. Natural immunity to human malaria is slow to develop even in individuals living in high endemic areas. One possible reason for the host immunosuppression associated with malaria infection is that parasite-specific, but not nonspecific, $CD4^+$ T cells are deleted during blood-stage infection. An

in vitro study showed that *P. falciparum* induces apoptosis of human mononuclear cells obtained from patients with acute malaria, but it is not known whether these cells undergoing apoptosis are specific for malaria (421). Similarly, effector Th cells specific for MSP-1 are selectively targeted for apoptotic deletion (422). Rates of spontaneous and activation-induced apoptosis in T cells are higher in individuals with acute malaria than in healthy controls (423, 424). These data have been corroborated in mouse models: *P. berghei*-specific $CD4^+$ T cells that had been adoptively transferred into nude mice are depleted after challenge with homologous parasites (425). Moreover, protective parasite-specific $CD4^+$ T cells are depleted in mice after infection with both lethal and nonlethal *Plasmodium* species (426). This study and others show that IFN-γ is a key factor mediating the apoptotic deletion of activated $CD4^+$ T cells during malaria and other intracellular infections, suggesting a possible mechanism of IFN-γ-induced self-tolerance (426-428). Indeed, IFN-γ induces apoptosis of mammalian cells independently of Fas and TNF receptors (429l). Deletion of malaria-specific $CD4^+$ T cells would impair the ability of the host to control parasite growth and may explain in part why natural immunity to human infection takes many years of endemic parasite exposure to develop (430). Alternatively, substantial numbers of malaria-specific T cells can be recovered from the liver and bone marrow following their removal from the bloodstream (425), which suggests that exposure to infection induces migration of peripheral T cells to tissues where they differentiate into memory T cells. Thus, deletion of parasite-specific T cells from the circulation may be a normal homoeostatic mechanism rather than constitute an immunosuppressive pathway (431). In support of this idea, high levels of spleen cell apoptosis were observed to coincide with a decrease and normalization of spleen cell numbers after peak parasitemia in *P. chabaudi*-infected mice (432, 433). In these mice, spleen cell apoptosis did not interfere with their ability to fully resolve primary infection or to resist challenge infection. Similarly, acute malaria patients that have high rates of T cell apoptosis also have high numbers of activated circulating T cells compared to healthy controls (423). Taken together, these data suggest that T cell apoptosis might be a beneficial mechanism to restore immunological homeostasis and avoid pathology during resolution of infection

Regulatory T cells

Despite growing awareness of the importance of Treg cells in the regulation of immunity and immunopathlogy, the induction and function of these cells in immunity to blood-stage malaria are poorly understood. Despite the relative lack of information regarding the role of Treg cells, many studies have previously reported the effects of regulatory T cell cytokines, such as IL-10 and TGF-β, on malaria immunity and immunopathology (discussed below). In many infection models, Treg cells have been shown to determine the balance between immune activation and pathology following microbial infection (235). In vivo depletion of $CD4^+CD25^+$ Treg cells enables mice to control and resolve an otherwise lethal infection with *P. yoelii* PyL, although it has no effect on the course of infection with the nonlethal *P. yoelii* PyNL strain (434). Protection against lethal *P. yoelii* malaria was accompanied by an increased proliferative response of splenocytes to infected RBCs in vitro. Moreover, the lethal strain *P. yoelii* PyL elicits higher levels of IL-10 and TGF-β than nonlethal *P. yoelii* PyNL.

A more recent study has provided the first evidence that Treg cells influence parasite growth in humans infected with *P. falciparum* (238). In this study, 26 individuals received a candidate liver-stage vaccine that, unfortunately, failed to protect them from an experimental infection with *P. falciparum* sporozoites. Although the vaccine did not work, the investigators were able to follow the immune status of these subjects during blood-stage infection and to correlate their immune responses to levels of parasitemia. Subjects with early peak in plasma TGF-β levels also showed significantly higher rates of parasite growth, and the TGF-β response was associated with increased numbers of $CD25^+Foxp3^+$ Treg cells. In vitro depletion of $CD25^+$ cells resulted in enhanced proliferative and IFN-γ responses of PBMCs to *P. falciparum*-infected RBCs in vitro, which is consistent with the notion that Treg cells modulate T cell IFN-γ production. This study raises many questions relevant to malaria immunity and pathology. It is unknown why some individuals generate a high TGF-β response that predisposes them to higher parasitemias and whether such individuals would also experience malarial disease had the infection been allowed to proceed. It will also be important to identify the parasite molecule(s) that stimulate TGF- β production and the main cellular source(s) of TGF-β during malaria infection. Data from this study suggest

that monocytes and possibly non-blood cells such as hepatocytes are likely sources of TGF-β following the sporozoite inoculation (238). In addition, a direct link between TGF-β and development of Treg cells during malaria is not clear; that is, whether TGF-β, produced by non T cells, induces conventional $CD4^+$ T cells to become inducible Treg cells or whether naturally occurring Treg cells are activated by *Plasmodium* parasites to produce TGF-β. The function of APCs, such as DCs, in the induction of Treg cells and TGF-β production during blood-stage malaria is also not known. The role of DCs and DC-derived cytokines in the induction of T cell immunity versus tolerance in response to blood-stage *P. chabaudi* infection was the focus of experiments performed in Chapter 5.

B cells and antibody-mediated immunity

B cells are necessary for elimination of blood-stage malaria infection in most experimental models. Mice lacking B cells are unable to clear infections with *P. chabaudi* or *P. yoelii* (403, 435). The major role of B cells in protective immunity to blood-stage malaria is to produce high affinity antibodies. Antibodies were first shown to be important in the resolution of malaria infection by passive transfer experiments, whereby infection could be reduced or prevented via transfer of immune sera to naïve recipients (436, 437). In humans, humoral immune responses develop partly through acquisition of a diverse repertoire of protective antibodies that are specific for polymorphic antigens expressed by antigenically distinct parasite variants. Variant-specific antibodies against antigens expressed on the surface of infected RBCs, such as PfEMP-1, have been shown to be important in naturally acquired immunity in children (438). In adults, antibodies to nonvariable antigens are involved in mediating protection against different parasite strains (439).

The mechanisms whereby antibodies mediate elimination of parasites are not well understood. Mice lacking the classical or nonclassical pathway of complement are able to resolve primary or challenge infections with *P. chabaudi*, indicating that complement fixation by antibody is not required for protective immunity (440). Some studies indicate that the main protective role of antibodies is to interfere with merozoite invasion of RBCs or with processing of MSP-1 that is thought to be required for erythrocyte entry (441, 442). Serum IgG from mice that had recovered from *P. yoelii* infection can inhibit

intraerythrocytic growth of *P. falciparum* in vitro (443). However, the protective activity of human IgG does not correlate with inhibition of invasion or development of *P. falciparum* parasites but with antibody-mediated cellular inhibition involving mononuclear cells (437). This suggests that protective IgG isotypes function through interaction with Fc receptors to trigger phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and the release of inflammatory mediators after cross-linking by immune complexes. However, mice lacking Fc receptor common γ (Fcγ) chain are able to resolve a lethal *P. yoelii* 17XL infection (444). Moreover, IgG3 antibodies specific for *P. yoelii* MSP-1 are able to immunize mice lacking FcγRI, the receptor specific for IgG3, against challenge *P. yoelii* infection (445). Together, these data demonstrate that humoral immune responses are not dependent on Fc receptor-mediated effector mechanisms.

While cell-mediated immune responses during acute infection are necessary to control parasite growth, antibody-mediated responses are crucial for rapid clearance of chronic infection. During the chronic stage of primary blood-stage *P. chabaudi* infection, the Th cell response is thought to switch from a Th1 to a Th2 immune phenotype (417, 446). This is characterized by decreased production of Th1 cytokines and increased production of Th2 cytokines, and by the onset of antibody responses. It was originally believed that Th2 cytokines determine the nature of the antibody response in the chronic stage (417). However, many studies have shown that the protective IgG subclasses are those associated with the Th1 phenotype. In murine malaria, only parasite-specific IgG2a, which binds to Fcγ receptor with high affinity, has been shown to confer protection upon passive transfer (447, 448). In humans, IgG1 and IgG3, which are functionally similar to mouse IgG2a, are the main antibodies found in sera of immune adults (437, 449, 450). More recent evidence confirmed that IL-12, not IL-4, produced during the early phase of infection selectively promotes Th1-dependent IgG2a and IgG3, and these IgG subclasses are required for rapid resolution of *P. chabaudi* infection (420). Adoptive transfer of immune sera depleted of IgG2a, but not of IgG1, was unable to protect recipient mice from challenge *P. chabaudi* infection. Although IgG1 has been shown to enhance parasite killing in vitro (449), it is thought that the

presence of IgG1, which is not protective, during the curative phase of infection might be a homeostatic response to limit Th1-type inflammation (446).

Although antibody is important during blood stage malaria in humans, protective immunity develops only after repeated infection and is lost without continued exposure to the parasite. Antibody responses are often short-lived even in individuals living in high endemic areas—the level of serum antibodies to MSP-1, apical membrane antigen 1 (AMA1), rhoptry-associated protein, PfEMP1, and other proteins on the surface of infected RBCs is highest during infection but decreases rapidly once infection subsides or at the end of the malaria transmission season (451-454). This loss of antibody responses is also observed in people who have left the transmission area (455). These data contrast with long-lived antibody responses observed in mouse models of malaria, and help explain in part the delay in acquisition of immunity to human malaria. It is important, however, to distinguish between sterile and clinical immunity. Sterile immunity that fully prevents establishment of infection is very rare; in contrast, clinically immune individuals remain susceptible to malaria infection but suffer from mild to no morbidity. Although previously immune adults are at risk of reinfection when they revisit endemic areas, the mortality rate is significantly lower in these travelers than in non-immune travelers who contract malaria for the first time (456, 457). Maintenance of high levels of circulating antibodies appears to depend on the persistence of parasite antigen, and antibody responses rapidly reappear upon secondary infection or at the onset of a new period of malaria transmission (458, 459). Repeated exposures to *P. falciparum* fail to result in antigen-specific memory B cells in children (460), but, in adults who have been exposed for a longer period, memory B cells and antibody-secreting plasma cells are long-lived and can persist in the absence of antigen (431). It has been proposed that non-sterilizing immunity is a result of host and parasite co-evolution, which ensures host survival and thus parasite transmission. The costs of mounting a sterilizing immune response to blood-stage infection may be too high for the host in terms of resources and/or immunopathology.

Given the absence of sterile immunity in the vast majority of exposed individuals, a vaccine that limits but does not completely prevent blood-stage infection may be a more reasonable target. In this manner, the ideal malaria vaccine would avoid perturbing the

balance between immunity and immunopathlogy. Clinical trials of malaria vaccine candidates often rely on titers of antigen-specific antibodies to indicate vaccine efficacy and some of these studies have provided clues regarding antibody-mediated effector mechanisms of immunity to blood-stage malaria. Mice immunized with *P. falciparum* GPI produce antibodies that are able to block GPI-induced TNF-α production by macrophages and this inhibitory effect was associated with protection against cerebral malaria (461). Other effector mechanisms that might contribute to vaccine-induced immunity may involve: 1) antibodies that prevent binding of infected RBCs to endothelium, thereby preventing sequestration and possibly protecting against CM and placental malaria (66, 462, 463); 2) antibodies that mediate complement-dependent lysis of gametes may reduce parasite transmission (464-466); 3) antibodies raised against MSP-based vaccines are able to block invasion of merozoites into erythrocytes, thus reducing parasite burden (467, 468); and 4) vaccine-induced antibodies can completely block the ability of *P. vivax* to infect mosquitoes (469).

Cytokine regulation of adaptive immunity to blood-stage malaria

The balance between Th1 and Th2 immune responses, or between proinflammatory and anti-inflammatory responses, is important in determining the level of malaria parasitemia, onset and severity of disease, and degree of recovery. Proinflammatory cytokines are important during acute infection to activate immune mechanisms to control parasite growth and to initiate long-lasting or memory adaptive immune responses. Anti-inflammatory or regulatory cytokines are needed during chronic infection to complete parasite clearance and restore homeostasis, thereby preventing immune-mediated disease. The overproduction of both proinflamamtory and anti-inflammatory cytokines can lead to increased disease severity and mortality (470, 471). Murine models suggest that control of blood-stage infection is mediated by an early IL-12-dependent IFN-γ response (400, 420), followed by IL-10 and TGF-β responses to minimize pathology and mortality (78, 82, 472). Human studies also show that strong IL-12 and IFN-γ responses correlate with host resistance to infection with *P. falciparum* in young African children (473, 474), although high levels of proinflammatory cytokines, including IL-12, IL-18, IFN-γ, TNF-α, IL-1 and IL-6, are associated with development of CM, febrile disease,

and other manifestations of severe malaria in both humans and mice (471, 475, 476). Taken together, the timing and magnitude of different cytokine responses determine the ability of the host to effectively resolve blood-stage infection with minimum pathology.

As discussed above, resistance to blood-stage malaria is absolutely dependent on IFN-γ (400, 477), and failure to maintain early Th1 responses can result in rapid increases in parasite load (378). IL-12 is a key cytokine in inducing abundant IFN-γ synthesis, Th1 cell development and production of Th1-dependent antibodies, all of which are critically required for protection against blood-stage malaria (415, 420). IL-15 acts as a costimulator of IL-12-mediated IFN-γ production by $CD4^+$ T cells (138, 211, 212) and enhances Ig class switching by activated B cells, thus supporting strong Th1-type cellular and antibody-mediated responses to infection and tumor cells (217, 218, 246). Splenic TNF-α production correlates with resistance to blood-stage malaria in mice (478), although studies in TNF-α receptor-deficient mice show that TNF-α activity is not required for the ability to control a primary blood-stage infection but may promote recall responses to secondary infection (479, 480). Although plasma levels of IL-2 are correlated with resistance to *P. falciparum* reinfection and severe malaria (481), other studies show that IL-2 is not essential for cell-mediated immunity to blood-stage malaria in mice (394, 482). Rather, IL-2 may contribute more to pathology than to immunity by recruiting γδ T cells to the brain and rendering otherwise resistant mice susceptible to experimentally induced CM (483). In other systems, IL-2 has been shown to be essential for tolerance rather than immunity by inducing regulatory T cells (294).

The presence of IL-10 during T cell priming may inhibit the generation of a Th1 response, leading to impaired protective immunity to blood-stage malaria. Indeed, a recent study showed that the presence of IL-10 and absence of IFN-γ mediate downregulation of antigen-specific Th1 responses in neonates whose mothers had *P. falciparum*-induced placental malaria (484). During the chronic phase of blood-stage malaria, IL-10 functions primarily to downregulate inflammatory responses to prevent the onset of immune-mediated pathology. A deficiency of IL-10 leads to enhanced malarial disease and mortality from *P. chabaudi* infection in mice (78, 480). Likewise, TGF-β has two distinct roles in blood-stage malaria depending on the time of infection. Early after infection, low levels of TGF-β recruit monocytes, T cells and neutrophils to

the site of infection and have been shown to induce protective immune responses leading to slower parasite growth (80, 81). However, at high concentrations, TGF-β suppresses production of TNF-α and NO from macrophages, inhibits production of IFN-γ and TNF-α from NK cells, and inhibits IFN-γ-stimulated upregulation of MHC class II antigens (282, 485, 486). Similar immune suppression by TGF-β results in failure of IFN-γ- and NO-dependent resistance to blood-stage *P. chabaudi* infection (487), and the inhibition of early TGF-β responses promotes IFN-γ and TNF-α responses as well as rapid resolution of *P. yoelii* infection (82). TGF-β production increases as blood-stage infection progresses (488) and, in the later stages, TGF-β downregulates Th1-type immune responses to limit inflammation-associated pathologies in both humans and mice (79, 82, 475).

1.4 Study Objectives

The work performed in this thesis aimed to delineate the role of DCs in activating innate and adaptive immune response to blood-stage malaria infection. Some of the key questions addressed in this work to unravel DC-mediated mechanisms of host immunity to blood-stage malaria were: What are the key cytokines involved in supporting innate immune responses to blood-stage malaria? How do DCs recognize and respond to parasitized erythrocytes to initiate adaptive immunity? What are the critical immunomodulatory cytokines involved in DC interactions with other immune cells to influence the development of protective immunity to blood-stage malaria? Which DC functions are important for regulating the nature and quality of the immune response against blood-stage malaria?

Genetic analyses of inter-strain differences in susceptibility to *P. chabaudi* malaria identified IL-15 as a candidate immunoregulatory cytokine in host resistance to blood-stage malaria. Previous studies reported that IL-15 is a key cytokine regulating the function of innate immune cells, development of Th1 responses, and antibody production. The objective of the work described in Chapter 2 was to determine the role of IL-15 in innate and adaptive immune responses to blood-stage *P. chabaudi* infection. IL-15 was found to be important for effective control and resolution of a primary infection by supporting optimal Th1-type cytokine synthesis by DCs and NK cells as well as production of Th1-dependent parasite-specific antibodies.

DCs provide a critical link between innate and adaptive immune responses. As effective APCs, DCs are specialized for the uptake, processing and presentation of pathogen-derived antigens to $CD4^+$ T cells. The work described in Chapter 3 aimed to determine whether DCs discriminate between parasitized and uninfected RBCs through preferential uptake, maturation, and antigen presentation to $CD4^+$ T cells. The results of this study indicate that DCs recognize and respond to parasitized RBCs in a highly selective manner, leading to initiation of type 1 innate and adaptive immune responses.

Recent research shows that DCs and NK cells interact during early innate immune responses to result in activation of Th1 immunity (155, 381). Although IL-15 is important for NK cell function and can enhance DC cytokine production, IL-12 is critically required for DC-mediated development of Th1-type protective immunity to

intracellular pathogens. The objective of the study described in Chapter 4 was to investigate if reciprocal interactions occur between DCs and NK cells during blood-stage malaria and the key cytokine mediators involved in this crosstalk. Interactions between DCs and NK cells were observed to be a critical event in the early innate immune response to blood-stage malaria that promotes early IFN-γ production and the induction of protective type 1 adaptive immune responses.

DCs initiate and regulate $CD4^+$ T cell responses through antigen uptake and presentation, costimulatory signals, and production of proinflammatory cytokines. The objective of the work described in Chapter 5 was, first, to characterize and compare DCs responses among inbred strains of mice with differential susceptibilities to *P. chabaudi* malaria, and, second, to determine which DC functions are required for effective induction of protective Th1 immunity. The results from this study showed that DCs from susceptible A/J mice were able to capture pRBCs and express high levels of costimulatory molecules, but these DCs were unable to produce Th1-polarizing cytokines and to induce IFN-γ-secreting NK cells and $CD4^+$ T cells compared to those from resistant B6 or H-2 compatible B10.A mice. Importantly, DCs from A/J mice stimulated the development of IL-10-secreting $CD4^+$ T cells, which may indicate selective induction of Treg cells in malaria-susceptible mice.

CHAPTER 2

INTERLEUKIN 15 ENHANCES INNATE AND ADAPTIVE IMMUNE RESPONSES TO BLOOD-STAGE MALARIA INFECTION IN MICE

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Preface

The inter-stain differences in susceptibility to blood-stage *P. chabaudi* malaria enabled genetic mapping of the control of blood-stage malaria to a locus (*Char*2) on mouse chromosome 8. IL-15 is one of several interesting candidates found in the genetic interval for *Char2*. To determine a functional basis for this cytokine in host resistance to blood-stage malaria, the study described in Chapter 2 investigated the role of endogenous IL-15 in innate and adaptive immunity to a primary infection with *P. chabaudi* in mice.

Abstract

This study investigated the role of IL-15 in innate and adaptive immunity to blood-stage *P. chabaudi* AS infection. Compared to C57BL/6 wild-type (WT) mice, IL-15 gene knockout (KO) mice showed significantly delayed parasite clearance. Infected IL-15 KO mice had lower serum levels of IL-12 p70 and IFN-γ and lower spleen cell production of IFN-γ and TNF-α in vitro compared to WT mice, suggesting dysregulated Th1 cell-mediated responses. IL-15 KO mice had lower numbers of DCs, NK cells, and $CD8^+$ T cells, as well as higher percentages of apoptotic NK cells and $CD8^+$ T cells, indicating the importance of IL-15 for innate immune cell expansion and survival during blood-stage malaria. In response to pRBCs, splenic $CD11c^+$ DCs from infected mice produced modest levels of IL-12 and IFN-γ in vitro, which were significantly increased by IL-15 treatment but to a greater extent in DCs from WT compared to IL-15 KO mice. NK cell cytotoxicity and IFN-γ production were also severely impaired in IL-15 KO compared to WT mice. Importantly, defects in cytokine synthesis by DCs and NK cells from IL-15 KO mice were not restored fully by addition of IL-15 in vitro. IL-15 KO mice showed profoundly lower titers of malaria-specific antibodies, suggesting a Th1-associated antibody-mediated mechanism by which IL-15 promotes rapid parasite clearance. Therefore, IL-15 supports early control and timely resolution of blood-stage malaria through regulation of innate immune mechanisms and subsequent Th1-dependent adaptive responses.

Introduction

In the experimental model of *P. chabaudi* AS infection, resistant C57BL/6 (B6) mice show moderate levels of acute primary parasitemia and resolve the infection by 4-5 weeks post-infection (p.i.), whereas susceptible A/J mice develop fulminating parasitemia and succumb to infection by day 10-12 p.i. (388, 415). Expression of resistance, including recovery from infection, requires $CD4^+$ T cells, macrophages, NK cells, and IFN-γ production during the acute phase of infection, as well as Th1-dependent antibody responses during the curative phase (139, 356, 415, 420). Our laboratory has previously shown the important role of IL-12 in the induction of IFN-γ-dependent protective immunity to blood-stage malaria infection (401, 415). Other factors released during the early immune response may also influence Th1/Th2 effector choice and activation of downstream immune responses. A possible candidate is IL-15, a cytokine that promotes the expansion and activation of type 1 immune responses. In this study, we investigated the role of endogenous IL-15 in innate and adaptive immune responses to blood-stage *P. chabaudi* infection.

Previous studies have implicated a role for IL-15 in host resistance to intracellular pathogens, including *Salmonella* (489), *Listeria* (490), *Toxoplasma* (491), and *Mycobacterium* (492). The mechanisms by which IL-15 enhances survival and host immunity to these pathogens involve the promotion of IFN-γ production, NK cell expansion and activation, and increased survival and cytolytic activity of γδT cells or $CD8^+$ T cells (489-492). The role of IL-15 in the development of protective immunity to blood-stage malaria infection is not well understood. Serum IL-15 is undetectable in patients with complicated malaria involving multiple organ dysfunctions, although higher parasitemia correlates with elevated IL-10 and IL-12 levels (493). However, IL-15 increases the subset, survival and parasiticidal activity of γδ T cells in human peripheral blood mononuclear cells cultured with *P. falciparum* (411). It remains unclear whether IL-15 is involved in anti-malarial immunity mediated by dendritic cells (DCs), NK cells, or Th1-dependent antibody. The results presented here show that IL-15 is required for type 1 cytokine production in vivo, NK cell responses, optimal IL-12 and IFN-γ synthesis by DCs, and malaria-specific antibody responses, all of which contribute to the early control and timely resolution of blood-stage malaria infection.

Mice, parasite, and experimental infections

Breeding pairs of IL-15$^{-/-}$ mice on the B6 background were kindly provided by Dr. Jacques Peschon (Amgen). IL-15$^{-/-}$ mice were generated by targeted disruption of the IL-15 gene in B6-derived embryonic stem cells and identified by PCR analysis (494). Age-matched littermates (IL-15$^{+/+}$) or B6 mice (Charles River Laboratories) were used as WT controls. Female mice were used in all experiments and maintained in the animal facility of the Montreal General Hospital Research Institute (QC, Canada). Infections were initiated by intraperitoneal injection of 10^6 *P. chabaudi* parasitized red blood cells (pRBC). Parasitemia of individual mice was monitored by counting the percentage of infected cells per 400 RBC on blood smears stained with Diff-Quik (American Scientific Products). Mice were sacrificed at the indicated times and blood was obtained by cardiac puncture. Sera were collected and stored at –20 °C until assayed for cytokine levels. To measure antibody titers, blood samples were collected from experimental mice by bleeding via the tail vein.

Spleen cell cultures

Spleens from experimental mice teased gently apart and pressed through a sterile fine wire mesh. Single cell suspensions were prepared in RPMI 1640 medium (Gibco-Invitrogen) supplemented with 5% heat-inactivated FCS (HyClone Laboratories), 10 mM HEPES (Gibco-Invitrogen), 20 μg/ml gentamicin (Schering-Plough), 2 mM glutamine (Gibco-Invitrogen), and 2 μM β-mercaptoethanol (Sigma-Aldrich). RBCs were lysed with NH_4Cl lysing buffer and membrane debris was removed by filtering the cell suspension through a 70 μm cell strainer (BD Labware). Viability of the cells was determined by trypan blue exclusion and was always >95%. To determine cytokine production, spleen cells were adjusted to 5 x 10^6 cells/ml, and aliquots of 1 ml were plated in triplicate in 48-well tissue culture plates in medium only (unstimulated controls) or in the presence of 10^6 pRBC and incubated for 48 h at 37 °C in a humidified CO_2 incubator. Supernatants were collected and stored at –20°C until assayed for cytokine levels by ELISA.

Flow cytometry

Unfractionated spleen cells were FcR blocked using anti-CD16/CD32 (clone 2.4G2) monoclonal antibody (mAb) (BD Biosciences), and then stained with mAbs to mouse CD11c-FITC (clone HL3), Pan-NK/CD49b-PE (clone DX5), and isotype-matched control mAbs (BD Biosciences) in sorting buffer. NK cell apoptosis was determined by staining FcR-blocked spleen cells with mAbs to Annexin V and DX5 (BD Biosciences). Cells were analyzed by FACSCalibur equipped with CellQuest software (BD Biosciences).

Spleen cell subset enrichment and culture

Spleen cells were enriched for NK cells by positive selection using anti-DX5 microbeads (Miltenyi Biotec). The resulting NK cells were >83% positive for DX5, a pan-NK marker, as determined by flow cytometry. To determine NK cell cytokine production, $DX5^+$ NK cells (5 x 10^5 cells/well) were cultured with rhIL-15 (100 ng/ml; kindly provided by Amgen) for 72 h and supernatants were assayed for IFN-γ by ELISA. The choice of 100 ng/ml concentration of rhIL-15 was the minimum concentration found in our preliminary experiments to induce increases in NK cell proliferation and IFN-γ production, whereas no significant increases were observed at lower concentrations (data not shown).

To obtain DCs, spleens were digested with collagenase D (Roche) and low-density cells were collected using Nycoprep (Axis-Shield). Cells were further purified using anti-CD11c microbeads (Miltenyi Biotec), and the resulting cells were 80 to 88% positive for CD11c as determined by flow cytometry. To determine DC cytokine production, 1 x 10^6 $CD11c^+$ cells were plated in complete medium in 96-well tissue culture plates for 48 h at 37°C. Some cultures were stimulated with rhIL-15 (100 ng/ml), with pRBC (10^6/ml), or with a combination of rhIL-15 and pRBC.

NK cell cytotoxicity assay

NK cell cytotoxicity was measured by a standard ^{51}Cr release assay performed as described previously (139). Briefly, $DX5^+$ NK cells were plated with murine YAC-1

cells at an optimum effector cell/target cell ratio of 10:1 and presented as percentage of specific lysis calculated according to the formula: percentage of specific lysis = 100 x (cpm experimental release – cpm spontaneous release) / (cpm maximum release – cpm spontaneous release).

Intracellular IFN-γ staining and flow cytometry

Enriched $DX5^+$ NK cells were stimulated in vitro with Golgi Stop (BD Biosciences), phorbol myristate acetate (Sigma) and ionomycin (Sigma) for 2 h, washed, FcR-blocked, and then stained on the cell surface with PE-conjugated anti-DX5 mAb (BD Biosciences) in sorting buffer. Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), and then were stained intracellularly with mAb (BD Biosciences) for isotype control (rat IgG1) or IFN-γ (clone XMG1.2) in permeabilization buffer. On each day of analysis, $DX5^+$ NK cells were gated on SSC versus FL2 using cells stained with isotype control to set the gating for the $DX5^+$ NK cell population. IFN-γ signals on FL1 were then generated from the gated cells and the percentages of $DX5^+$ NK cells expressing IFN-γ are presented in the histograms.

Cytokine ELISAs

Levels of IL-12p70, IFN-γ, TNF-α, and IL-10 in sera or cell culture supernatants were measured by ELISA using paired capture and detection antibodies as described previously (388, 400, 415). Standard curves for each cytokine ELISA were generated using recombinant cytokines (BD Biosciences). Reactivity was revealed using ABTS [2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid)] substrate (Roche) and optical density values were read at 405 nm with a reference wavelength of 492 nm.

Malaria-specific antibody titers

Serum levels of *P. chabaudi*-specific antibody isotypes and subclasses were determined by ELISA as described previously (420). Briefly, serum samples were serially diluted twofold, and 50 µl of each dilution was added to 96-well plates coated with parasite antigen (5 µg/ml in PBS) prepared as described previously (420). After extensive washing, horseradish peroxidase-conjugated goat anti-mouse isotype and

subclass mAbs (Southern Biotechnology Associates) were added, and reactivity was visualized using ABTS substrate (Roche). Antibody levels were expressed as endpoint titers, the reciprocal of the highest dilution that yielded the background optical density.

Statistical analyses

Data on serum cytokine levels, spleen cell cytokine production, parasitemia and antibody titers are presented as means ± SEM. All other data are presented as median values ± 95% confidence intervals. Statistical differences between the groups in serum cytokine levels, spleen cell cytokine production, and antibody titers were analyzed by ANOVA. Differences in parasitemia were analyzed by repeated measures ANOVA and in survival by the chi-square test. All other statistical comparisons were analyzed using the nonparametric Mann-Whitney U test. All statistical analyses were performed using SAS/STAT software (SAS Institute). A $P < 0.05$ was considered significant.

Results and Discussion

To determine the role of IL-15 in protective immunity to blood-stage malaria, the course of a primary *P. chabaudi* infection was monitored in wild-type (WT) and IL-15$^{-/-}$ mice. IL-15$^{-/-}$ mice had an earlier peak in parasitemia than WT mice, developed a small recrudescent parasitemia of 4-6% at 24 to 32 days p.i., and failed to resolve their infection by day 32 p.i. (Fig. 1*A*). IL-15$^{-/-}$ mice continued to demonstrate low parasitemias of 1-2% as late as day 48 p.i., whereas 100% of WT mice cleared the infection by day 32 p.i. (Fig. 1*A*, inset). Although 10% of IL-15$^{-/-}$ mice died by day 13 p.i., in contrast to the WT mice, 100% of which survived, this difference was not statistically significant (Fig. 1*B*).

The delayed parasite clearance observed in IL-15$^{-/-}$ mice in this study suggested impaired production of type 1 cytokines. IL-15 promotes CD40-dependent IL-12 production by monocytes (495) and co-stimulates IFN-γ production by NK and T cells in synergy with IL-12 (208, 211, 213) and IL-21 (212). We have previously reported that protection against blood-stage P. chabaudi infection is induced by IL-12 and mediated critically by IFN-γ (388, 400, 415). During the first week p.i., IL-15$^{-/-}$ mice had peak levels of IL-12p70, IFN-γ and TNF-α in the serum that were significantly lower than the levels found in WT mice (Table 1). Peak serum IL-10 levels were comparable between WT and IL-15$^{-/-}$ mice. Splenocytes from infected IL-15$^{-/-}$ mice appeared to be as proficient as those from WT mice at producing IL-12 and IL-10 in response to pRBC in vitro. However, despite their comparable IL-12 and IL-10 production, IL-15$^{-/-}$ splenocytes produced significantly less IFN-γ and TNF-α in vitro than WT splenocytes.

IL-15 activates NK cells, macrophages, and DCs, the last of which are influential in detecting infection and regulating innate immune responses. IL-15 has been shown to stimulate IL-12 production by DCs as well as monocytes in response to lipopolysaccharide or CD40 ligation (214, 215). Following *P. chabaudi* infection, IL-15$^{-/-}$ mice had significantly lower numbers of CD11c^{+} DCs in the spleen than WT mice (at day 7 p.i.: WT 12.03 x 10^6 vs. IL-15$^{-/-}$ 7.82 x 10^6; $P < 0.05$; Appendix I). To address the role of IL-15 in inducing cytokine production by DCs during blood-stage malaria, splenic CD11c^{+} cells from infected WT and IL-15$^{-/-}$ mice were cultured with no stimulation (medium), with pRBC, with recombinant human IL-15 (rhIL-15), or with a combination of pRBC and rhIL-15. WT DCs stimulated with pRBC in vitro produced significantly less IL-12p70 than

did unstimulated cultures (Fig. 2*A*), a result consistent with the ability of *Plasmodium*-infected erythrocytes to suppress IL-12p40 gene induction in macrophages (386). The addition of rhIL-15, in the absence of pRBC, significantly enhanced DC secretion of IL-12p70. Importantly, the elevated levels of IL-12p70 in IL-15-stimulated cultures were observed only with WT DCs, not with IL-15$^{-/-}$ DCs.

Recent studies which challenge the classical paradigm in which only lymphoid cells produce IFN-γ show that activated APCs secrete IFN-γ in an IL-15-dependent manner (215, 496). Based on this evidence, it was proposed that IL-15 is an early host factor that initiates a positive autocrine feedback loop for further Th1-type cytokine synthesis by DCs (215, 496). Therefore, we wanted to determine whether IL-15 modulates IFN-γ production by DCs during blood-stage malaria. DCs from infected WT mice produced significantly higher levels of IFN-γ in response to pRBC or rhIL-15 in vitro than did DCs from IL-15$^{-/-}$ mice (Fig. 2*B*). Stimulation with both rhIL-15 and pRBC further increased IFN-γ secretion, but this secretion occurred to a significantly greater extent in WT DCs than in IL-15$^{-/-}$ DCs. Taken together, these results suggest that IL-15 supports optimal synthesis of IL-12p70 and IFN-γ by splenic CD11c^{+} DCs during the innate immune response to *P. chabaudi* infection. The ability of IL-15 to increase both IL-12 and IFN-γ production by DCs as shown here and in other studies (215, 496), as well as the IL-12 responsiveness of APCs (215), places IL-15 in a critical position to induce Th1-type innate immune responses. Recent work in our laboratory suggests that IL-15 enhances the ability of DCs from malaria-infected mice to stimulate IFN-γ production by DX5^{+} NK cells and CD4^{+} T cells (R. Ing and M. M. Stevenson, unpublished data).

IL-15 maintains NK cell survival through activation of the lymphocyte anti-apoptotic factor Bcl-2 (208). Following *P. chabaudi* infection, IL-15$^{-/-}$ mice had significantly lower numbers of NK cells in the spleen (at day 7 p.i., 1.59 x 10^{6} [WT mice] versus 0.86 x 10^{6} [IL-15$^{-/-}$ mice]; $P < 0.05$; Appendix I) and higher percentages of NK cells staining positive for Annexin V, a surface marker of apoptosis, than did WT mice (Fig. 3*A*). IL-15 deficiency also impairs CD8^{+} T cell expansion and survival (Appendix I). These data are consistent with previous studies showing that IL-15 is required for survival and proliferation of NK cells and CD8^{+} T cells during homeostasis and infection (204, 208). In addition, several studies indicate that IL-15 increases NK cell cytotoxicity and IFN-γ

production (138, 204, 211), whereas others show that IL-12 and IL-18 are more effective than IL-15 alone (138, 399). Since IL-15$^{-/-}$ splenocytes were proficient at producing IL-12p70 in vitro (Table 1), we questioned whether splenic NK cells from IL-15$^{-/-}$ mice were capable of cytotoxicity and IFN-γ production. As shown in Fig. 3*B*, WT NK cells showed increasingly higher levels of cytolytic activity against NK cell-sensitive murine YAC-1 targets during the first week of *P. chabaudi* infection. Importantly, IL-15$^{-/-}$ NK cells exhibited undetectable or significantly lower cytolytic activity at 0 to 4 days p.i., suggesting that IL-15 deficiency impairs not only the expansion but also the function of splenic NK cells during blood-stage malaria infection. Interestingly, IL-15$^{-/-}$ NK cells showed increased cytolytic activity after day 4 p.i., likely reflecting the availability of other factors, such as IL-12 and IL-18, in IL-15$^{-/-}$ mice to induce NK cell cytotoxicity, albeit in a sub-optimal and severely delayed manner.

Previous work in our laboratory has shown that NK cell IFN-γ production, not cytotoxicity, plays a major role in mediating protective immunity to *P. chabaudi* infection (139). Accordingly, we examined IL-15-stimulated IFN-γ production by splenic DX5^{+} NK cells purified from WT and IL-15$^{-/-}$ mice. At 0 to 4 days p.i., IL-15$^{-/-}$ NK cells produced significantly less IFN-γ than did WT cells (Fig. 3*C*). Although it is necessary to maintain NK cell survival in vitro, the addition of rhIL-15 to NK cell cultures may result in the underestimation of the effect of endogenous IL-15 deficiency on NK cell IFN-γ production in vivo. Therefore, we determined the percentage of DX5^{+} NK cells expressing intracellular IFN-γ ex vivo by flow cytometry. As shown in Fig. 3*D*, significantly lower percentages of NK cells from infected IL-15$^{-/-}$ mice expressed intracellular IFN-γ than did NK cells from WT mice. These results indicate that IL-15 is required during the early immune response to blood-stage malaria to support optimal NK cell IFN-γ production.

The inability of soluble rhIL-15 to fully restore IL-12 and IFN-γ production by IL-15$^{-/-}$ DCs and NK cells, respectively, may reflect the fact that the potency of human IL-15 to stimulate mouse spleen cells is lower than that of the murine protein (497); murine rIL-15 was not available at the time our experiments were performed. It may also be explained by the existence of IL-15 as two isoforms with distinct biological activities. The IL-15-SSP isoform is stored intracellularly and mobilized to the plasma membrane following antigenic stimulation, while the IL-15-LSP isoform functions as a secretory cytokine (498,

499). The membrane-bound IL-15-SSP contributes the majority of the biological effects of IL-15, in part due to its ability to function as a receptor to activate cell-signaling pathways that result in cellular adhesion and proinflammatory cytokine production (500-502). Indeed, IL-15 deficiency was associated with lower intracellular IFN-γ expression by $DX5^+$ NK cells during *P. chabaudi* infection. In agreement with our observations, Ohteki et al. (215) also reported that, unlike WT cells, exogenous IL-15 failed to completely rescue the reduced IL-12 and IFN-γ production by lipopolysaccharide-stimulated $IL\text{-}15^{-/-}$ DCs and macrophages. Taken together, these data suggest that IL-15, in the membrane-bound form, may be required during development for maximum type 1 cytokine production by innate immune cells in response to intracellular infection.

Given that antibody-mediated immunity is necessary for parasite clearance (420, 503), we questioned whether the delayed resolution of *P. chabaudi* infection observed in $IL\text{-}15^{-/-}$ mice (Fig. 1*A*) was associated with impaired antibody production. Previous work in our laboratory has shown that Th1-dependent IgG2a and possibly IgG3 are preferentially required for rapid clearance of *P. chabaudi* infection (420). Since IL-15 syngerizes with IL-12 to promote IFN-γ production and Th1-type responses, we expected to observe altered parasite-specific IgG2a and IgG3 responses in $IL\text{-}15^{-/-}$ mice. As shown in Fig. 4, however, $IL\text{-}15^{-/-}$ mice had significantly lower titers of total Ig, IgM, and the IgG subclasses IgG1, IgG2a, IgG2b, and IgG3. The lower levels of malaria-specific antibodies observed in $IL\text{-}15^{-/-}$ mice may explain the inability of $IL\text{-}15^{-/-}$ mice to rapidly resolve the infection as rapidly as WT mice. Moreover, these results suggest that IL-15 supports general antibody production, irrespective of Th1 or Th2 phenotypic affiliation, during blood-stage malaria. Studies have shown that IL-15 stimulates B cell proliferation and Ig production (217) and modulates Ig class switching (218, 246), which supports our observation that IL-15 is important for augmenting antibody responses selectively induced by other immunoregulatory factors.

The results presented here provide the first in vivo evidence that IL-15 contributes to the early control and timely resolution of blood-stage *P. chabaudi* infection. We showed that IL-15 enhanced protective mechanisms involving both innate and adaptive responses. Specifically, IL-15 is required for optimal IL-12 and IFN-γ secretion by DCs, NK cell IFN-γ synthesis, and production of malaria-specific antibodies. Despite impairments in these

immune functions, IL-15 deficiency did not markedly exacerbate mortality or parasitemia during the acute phase. Although the majority of IL-15$^{-/-}$ mice survived the infection, these mice showed significantly higher parasitemia than WT mice during the chronic phase, particularly at 12 to 32 days p.i. following peak parasitemia, and failed to resolve the infection by 32 days p.i. Therefore, IL-15 appears to play a supporting, albeit not indispensable, role in enhancing innate and adaptive immunity necessary to control and completely clear a primary *P. chabaudi* infection in a timely manner.

Previous work in our laboratory has shown that vaccine-induced protection against blood-stage *P. chabaudi* infection requires CD4$^+$ T cells and IFN-γ production (416). Early production of IFN-γ by NK cells and γδT cells is associated with resolution of nonlethal *Plasmodium yoelii* and *P. chabaudi* infections, but is absent in lethal *P. yoelii* and *Plasmodium berghei* infections (378). Moreover, studies in liver-stage *P. yoelii* infection demonstrated the critical role of early IFN-γ production by NK, CD8$^+$ T and γδT cells in protective immunity to sporozoites (373, 374). Accordingly, the most effective vaccination strategies are those that trigger early and strong IFN-γ production, which in turn induces long-lasting, IFN-γ-mediated protective immunity. Although on its own IL-15 is not essential for IFN-γ and antibody production, the results presented in this study and by other investigators (218, 246, 504) demonstrate the benefit of IL-15 as an adjuvant to induce maximal IFN-γ and antibody responses. The potential for IL-15 use in vaccine systems will necessitate further studies that examine how IL-15 interacts with other factors in the complex cytokine network to orchestrate protective immune responses to *Plasmodium* parasites.

Acknowledgements

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Table 1: Peak cytokine levels in serum and spleen cell supernatants following *P. chabaudi* infection in WT and IL-15$^{-/-}$ mice[a]

Cytokine	Serum		Spleen	
	WT	KO	WT	KO
IL-12 p70 (ng/ml)	13.27 ± 0.88	8.04 ± 2.13*	4.98 ± 0.71	4.02 ± 0.74
IFN-γ (ng/ml)	8.06 ± 2.56	2.75 ± 0.49*	34.65 ± 5.16	10.15 ± 1.22*
TNF-α (pg/ml)	105.86 ± 19.32	2.41 ± 1.63**	113.97 ± 6.64	72.46 ± 15.36**
IL-10 (ng/ml)	1.274 ± 0.35	1.176 ± 0.54	1.53 ± 0.15	1.405 ± 0.32

[a] WT and IL-15$^{-/-}$ mice were infected intraperitoneally with 10^6 *P. chabaudi* pRBC. The peak levels of each cytokine during the first week p.i. in vivo and in vitro are presented. Splenocytes were stimulated for 48 h with pRBC (10^6/ml) and supernatants were assayed for cytokines by ELISA. Data are presented as means ± SEM of 10-12 mice pooled from three independent experiments. Comparisons between WT and KO mice were analyzed by ANOVA; *, $P < 0.05$, **, $P < 0.01$.

Figure 1: Course of parasitemia (*A*) and survival rate (*B*) in WT and IL-15$^{-/-}$ (knockout [KO]) mice infected intraperitoneally with 10^6 *P. chabaudi* pRBC. The inset (*A*) shows the course of parasitemia in the chronic stage of infection. Results are pooled from four independent experiments, each containing 8 to 10 mice per group and are presented as means $\pm$ SEM. *, WT versus KO mice, $P < 0.05$ as determined by repeated measures ANOVA. Differences in survival rate were not statistically significant as determined by the chi-square test.

Figure 1

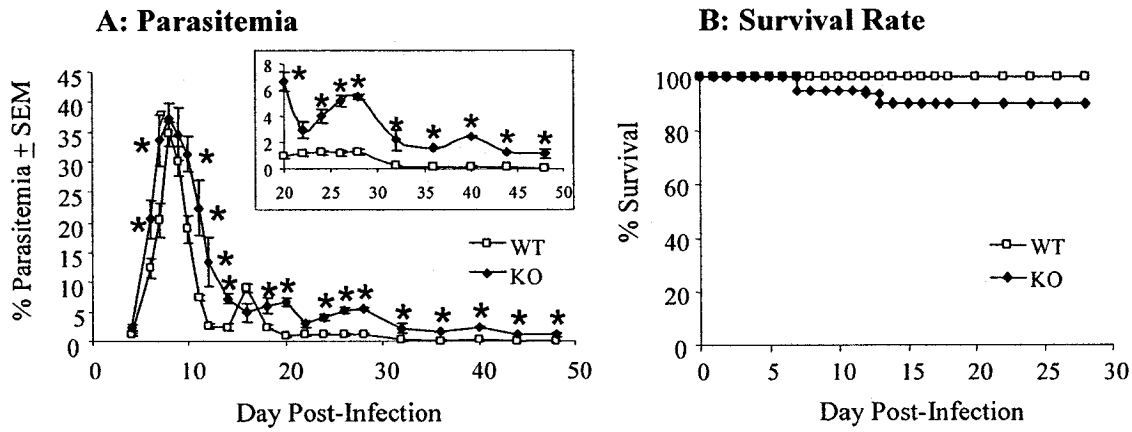

A: Parasitemia
% Parasitemia ± SEM
Day Post-Infection
WT
KO
B: Survival Rate
% Survival
Day Post-Infection
WT
KO

Figure 2: Stimulation with IL-15 in vitro enhances IL-12p70 (*A*) and IFN-γ (*B*) production by splenic $CD11c^+$ DCs from infected WT mice. $CD11c^+$ cells (10^6/well) were cultured in medium alone, or in medium plus pRBC (10^6/ml), rhIL-15 (100 ng/ml), or a combination of pRBC and rhIL-15. After 48 h incubation, supernatants were assayed for IL-12p70 and IFN-γ by ELISA. Data (n=4 per group) are presented as median values ± 95% confidence intervals and are representative of two replicate experiments. ND, non-detectable. *, WT versus knockout (KO) mice, $P < 0.05$, as determined by the Mann-Whitney U test.

Figure 2

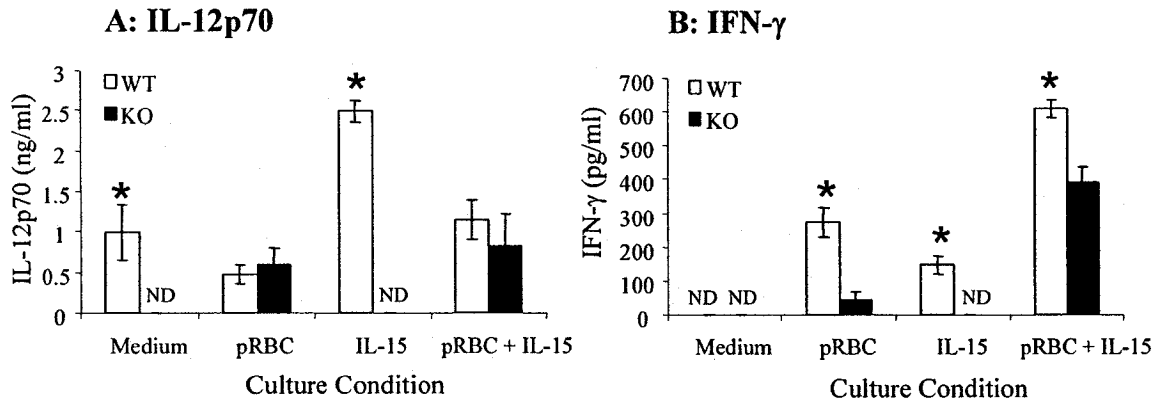

A: IL-12p70
B: IFN-γ
WT
KO
IL-12p70 (ng/ml)
IFN-γ (pg/ml)
ND
Medium
pRBC
IL-15
pRBC + IL-15
Culture Condition

Figure 3: IL-15 deficiency impairs NK cell survival and function, as determined by NK cell apoptosis (*A*), enriched NK cell cytotoxicity (*B*), IFN-γ production (*C*), and intracellular IFN-γ expression (*D*). *A*, NK cells from infected IL-$15^{-/-}$ (or knockout [KO]) mice expressed higher levels of Annexin V, a marker of apoptosis, than did cells from WT mice. *B*, NK cells from KO mice showed impaired cytotoxicity against murine YAC-1 targets compared to cells from WT mice at all time points before and after malaria infection. *C*, NK cells from naive and infected KO mice produced lower levels of IFN-γ than did cells from WT mice. *D*, NK cells from infected KO mice showed lower intracellular IFN-γ expression ex vivo than did cells from WT mice. Representative histograms of gated $DX5^{+}$ cells stained intracellularly for isotype control (outlined peak) or IFN-γ (shaded curve) are shown, with the percentages expressing intracellular IFN-γ. Data (n = 4 per group) are presented as median values ± 95% confidence intervals and are representative of two replicate experiments. *, WT versus KO mice, $P < 0.05$, as determined by the Mann-Whitney U test.

Figure 3

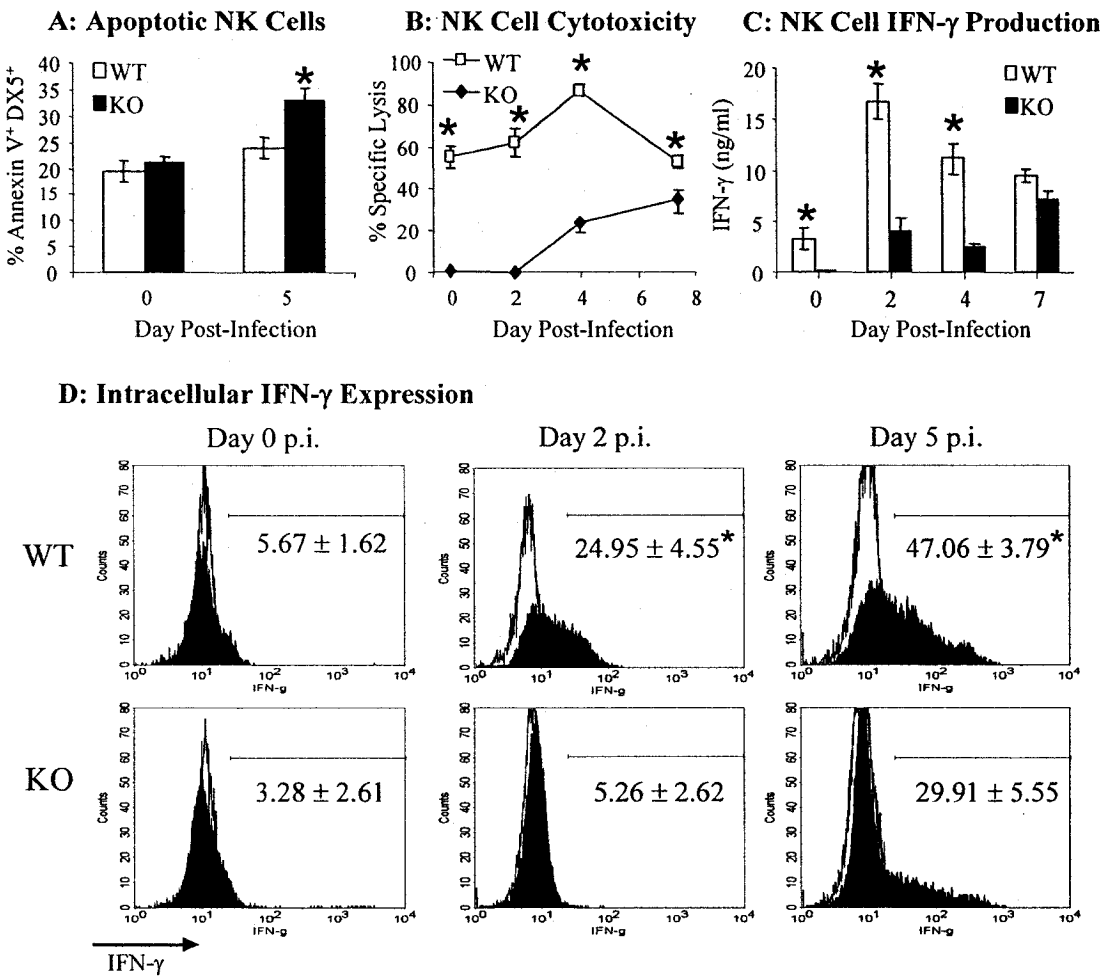
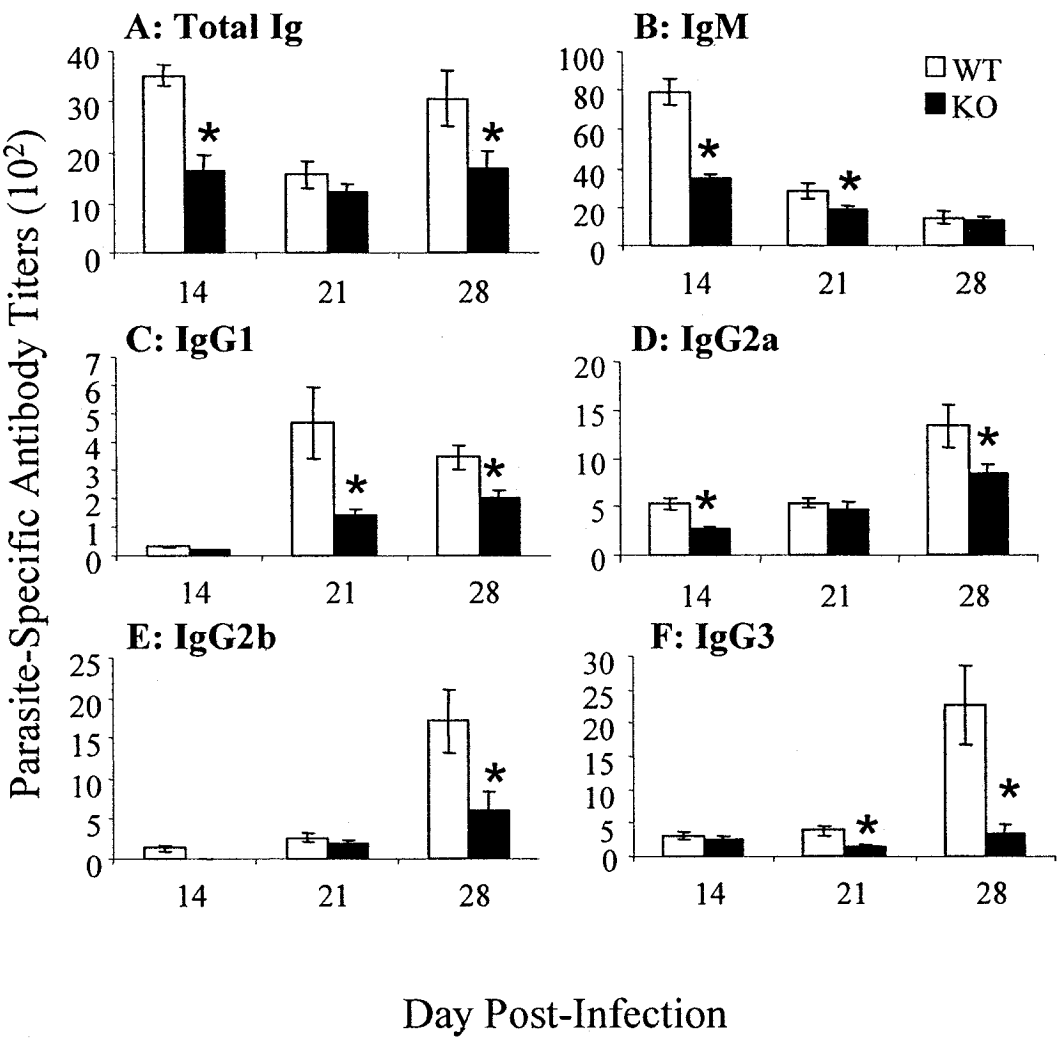

A: Apoptotic NK Cells
% Annexin V+ DX5+
WT
KO
Day Post-Infection
B: NK Cell Cytotoxicity
% Specific Lysis
WT
KO
Day Post-Infection
C: NK Cell IFN-γ Production
IFN-γ (ng/ml)
WT
KO
Day Post-Infection
D: Intracellular IFN-γ Expression
Day 0 p.i.
Day 2 p.i.
Day 5 p.i.
WT
KO
5.67 ± 1.62
24.95 ± 4.55*
47.06 ± 3.79*
3.28 ± 2.61
5.26 ± 2.62
29.91 ± 5.55
Counts
IFN-g
IFN-γ

Figure 4: IL-15$^{-/-}$ mice have defects in Ig production during the chronic stage of blood-stage malaria infection. *P. chabaudi*-specific total Ig (*A*), IgM (*B*), IgG1 (*C*), IgG2a (*D*), IgG2b (*E*), and IgG3 (*F*) were measured in sera of infected WT and KO mice (n = 8 per group). Antibody levels are expressed as endpoint titers, the reciprocal of the highest dilution that yielded the background optical density. *,WT versus KO mice, $P < 0.05$, as determined by ANOVA.

Figure 4


A: Total Ig
B: IgM
C: IgG1
D: IgG2a
E: IgG2b
F: IgG3
WT
KO
Parasite-Specific Antibody Titers (10^2)
Day Post-Infection
14
21
28

CHAPTER 3

INTERACTION OF MOUSE DENDRITIC CELLS AND MALARIA-INFECTED ERYTHROCYTES: UPTAKE, MATURATION AND ANTIGEN PRESENTATION

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Preface

Host resistance to blood-stage *P. chabaudi* infection is associated with Th1-type innate and adaptive immune responses. Based on the results described in Chapter 2, IL-15 appears to play an important, albeit dispensable, role in the regulation of innate immune responses and subsequent Th1-dependent adaptive immunity, especially protective parasite-specific antibody responses. The finding that IL-15 influenced Th1-type cytokine production by splenic $CD11c^+$ DCs is of particular interest because DCs play a seminal role in detecting infection and activating protective immune responses. The focus of experiments described in Chapter 3 was to investigate the pRBC uptake and antigen presentation functions of DCs that result in selective activation of $CD4^+$ T cell responses, predominantly of the Th1 cell phenotype.

Abstract

Consistent with their seminal role in detecting infection, both mouse bone marrow-derived and splenic $CD11c^+$ DCs exhibited higher levels of uptake of *P. chabaudi*-pRBC than of nRBC as determined by our newly developed flow cytometric technique using the dye carboxyfluorescein succinimidyl ester (CFSE) to label RBC prior to co-culture with DCs. To confirm that expression of CFSE by $CD11c^+$ cells following co-culture with CFSE-labeled pRBC represents internalization of pRBC by DCs, we showed co-localization of CFSE-labeled pRBC and PE-labeled $CD11c^+$ DCs by confocal fluorescence microscopy. Treatment of DCs with cytochalasin D significantly inhibited the uptake of pRBC, demonstrating that uptake is an actin-dependent phagocytic process. The uptake of pRBC by splenic $CD11c^+$ DCs was significantly enhanced after infection in vivo and was associated with the induction of DC maturation, IL-12 production, and stimulation of $CD4^+$ T cell proliferation and IFN-γ production. These results suggest that DCs selectively phagocytose pRBC and present pRBC-derived antigens to $CD4^+$ T cells, thereby promoting development of protective Th1-dependent immune responses to blood-stage malaria infection.

Introduction

Malaria, caused by the intracellular parasite *Plasmodium*, is the most important parasitic infection in humans. The global prevalence of malaria continues to rise at an alarming rate and is a major cause of high mortality in children and morbidity in infected adults living in the developing world (2). Despite extensive research, a vaccine is not yet available and many anti-malarial drugs are increasingly ineffective due to widespread drug resistance. In areas where malaria is endemic, individuals acquire partial immunity against malaria only after repeated exposure to the parasite. It may take many years of frequent infection episodes to establish protective immunity capable of preventing clinical disease. Therefore, a better understanding of the mechanisms that induce protective immunity to malaria infection will help identify strategic targets for anti-malaria vaccine development and immunotherapy.

Infections of mice with rodent *Plasmodium* species are useful tools for investigating the immunobiology of blood-stage malaria. In most rodent blood-stage malaria infections, survival depends on the ability to control parasite replication during the acute phase of infection. Immune effector mechanisms that mediate control of parasitemia involve macrophages, NK cells, $CD4^+$ T cells and IFN-γ production, while B cells and Th1-dependent Ab responses are required for elimination and resolution of the chronic stage of infection (reviewed in 8). While several cytokines, such as IL-12 in synergy with IL-15 or IL-18, may result in Th1 cell development (138, 213), IFN-γ is the central effector cytokine mediating protective immunity to blood-stage malaria infection (400). These results highlight the importance of adaptive type 1 immune mechanisms for host resistance to blood-stage malaria and the need to identify critical components of the innate immune response that promote development of IFN-γ-producing $CD4^+$ Th1 cells.

Early interactions between blood-stage parasites and cells of the innate immune system are thought to be important in shaping the adaptive immune response to blood-stage malaria. Compared to other APCs such as B cells and macrophages, DCs are primarily responsible for priming naïve $CD4^+$ T helper cells and stimulating the production of proinflammatory cytokines, which in turn determine the phenotype of an ensuing immune response to invading pathogens. Several studies using mouse models

of malaria have provided evidence for an important role of DCs in inducing protective immunity to blood-stage malaria. *P. chabaudi chabaudi* schizonts were found to induce bone marrow (BM)-derived DCs to express MHC class II and costimulatory molecules and to produce IL-12, TNF-α, and IL-6 (362). In mice infected with *P. chabaudi*, $CD11c^+$ DCs were observed to migrate from the marginal zone of the spleen to the $CD4^+$ T cell-rich periarteriolar lymphoid sheath and to exhibit up-regulated expression of costimulatory molecules as well as increased production of IFN-γ (365). In contrast, macrophage and B cell populations in the spleen expand but remain confined to the red pulp area (365) while $CD11b^+$ macrophages produce a soluble factor(s) that inhibits $CD4^+$ T cell proliferation and production of IL-2 (364, 366). Similarly, splenic $CD11c^+$ DCs, but not $CD11b^+$ macrophages or $B220^+$ B cells, from *P. yoelii* 17X-infected mice stimulate high levels of IL-2, IFN-γ and TNF-α production by naïve $CD4^+$ T cells (364, 366). Importantly, the induction of T cell cytokine production was shown to critically require DC-derived IL-12 (366). Taken together, these results show that DCs activated in vitro and in vivo by rodent *Plasmodium* parasites are able to express costimulatory molecules and produce proinflammatory cytokines to stimulate adaptive type 1 immune responses that are protective against malaria infection.

To initiate an immune response against *Plasmodium* infection, DCs would be required to take up soluble malarial antigens or *Plasmodium*-parasitized RBC (pRBC). Since the *Plasmodium* parasite spends the majority of its erythrocytic life cycle within the RBC, DCs must be capable of recognizing and capturing pRBC circulating in the blood or spleen, and subsequently process and present pRBC-derived antigens to other immune cells. In addition, it would be more efficient if DCs internalized intact pRBC rather than wait for malarial antigens to be released from rupturing pRBC or delivered by other phagocytes such as macrophages and neutrophils. To date, most studies have focused on opsonic and nonopsonic phagocytosis of pRBC by macrophages and demonstrated this process to be an important nonspecific immune defense mechanism in the early control of blood-stage parasite growth (392, 505). By contrast, the uptake of pRBC by DCs and its role in the activation of downstream immune responses, including DC maturation and T-cell stimulatory function, has not been investigated in detail.

It has been reported that DCs internalize *P. falciparum* or *P. yoelii*-infected RBC (359, 371). However, none of the previous studies compared the uptake of malaria-infected RBC by DCs to a physiologically relevant negative control nor measured the level of uptake by DCs before and after malaria infection in vivo. Since DCs have been shown to selectively endocytose apoptotic and allogeneic cells (506), we investigated here whether DCs take up pRBC to a significantly greater extent than nRBC. To do this, we developed a sensitive flow cytometry assay using the dye CFSE to measure the uptake of CFSE-labeled pRBC or nRBC by DCs. Using this technique, we compared the uptake of *P. chabaudi*-infected RBC to that of nRBC by BM-derived and splenic DCs and characterized the kinetics of pRBC uptake by splenic DCs following blood-stage *P. chabaudi* infection in vivo. Both BM-derived and splenic $CD11c^+$ DCs exhibited preferential uptake of pRBC *versus* nRBC and this uptake was dependent in part on actin polymerization, a crucial event in the phagocytosis of large particles. We also hypothesized that the uptake of pRBC by DCs would induce phenotypic and functional maturation as observed with splenic DCs isolated from mice infected with blood-stage *P. chabaudi* (365) or *P. yoelii* (364, 366). Indeed, we observed that the selective uptake of pRBC by splenic DCs was enhanced following infection in vivo and associated with increased expression of MHC class II and costimulatory molecules, IL-12 production, and stimulation of $CD4^+$ T cell proliferation and IFN-γ production. These results confirm the important role of DCs in initiating protective immunity to blood-stage malaria and also provide novel evidence that early interactions between mouse DCs and pRBC result in DC activation and induction of protective Th1 cell-mediated immune responses.

Mice, parasite, and pRBC purification

Female C57BL/6 (B6) mice (Charles River Laboratories), aged 8-12 wk, were maintained in the animal facility of the Montreal General Hospital Research Institute in accordance with the guidelines and policies of the Canadian Council on Animal Care. *P. chabaudi* AS was maintained as previously described (494) and infections were initiated by intraperitoneal injection of 10^6 *P. chabaudi* AS pRBC. To purify pRBC, heparinized blood was obtained via cardiac puncture from *P. chabaudi*-infected B6 mice with 30-50% parasitemia and washed twice with PBS. Blood was diluted with PBS (1-2 ml), loaded onto a 74% Percoll (Sigma-Aldrich) density gradient, and centrifuged at 5000 x *g* for 20 min at room temperature (RT). The top band, containing >96% pRBC as determined by staining with Diff-Quik (American Scientific Products), was collected. For nRBC controls, heparinized blood from naïve mice was loaded onto a 90% Percoll gradient, centrifuged at 5000 x *g* for 20 min at RT, and the top band was collected. RBCs were washed twice with PBS and resuspended in cell culture medium.

Generation of DCs from bone marrow precursors

DCs were cultured from BM precursors of naïve B6 mice using a method adapted from Inaba *et al.* (507) and Lutz *et al.* (508) in the presence of GM-CSF supplied by supernatant from a hypoxanthine, aminopterin, and thymidine (HAT) medium-sensitive Ag8653 myeloma cell line transfected with murine GM-CSF cDNA, which was generated by Brigitta Stockinger (National Institute for Medical Research, Mill Hill, U.K.) (509) and generously provided by George Carayanniotis (Memorial University of Newfoundland, St. John's, Canada). Ag8653 cells were cultured under strict G418 selection in RPMI 1640 medium (Gibco-Invitrogen) with 5% FCS (HyClone Laboratories), 2 mM L-glutamine (Gibco-Invitrogen), 0.5 μM β-mercaptoethanol (Sigma), and 20 μg/ml gentamicin (Sabex). BM cells were flushed from ethanol-sterilized femurs and tibias of mice, passed through a sterile fine wire mesh to remove debris, and washed twice with medium. Red cells were lysed with NH_4Cl lysing buffer. Cells were plated in 6-well plates at a concentration of 2.5 x 10^5 cells/ml in complete

medium consisting of RPMI 1640 (Gibco-Invitrogen) supplemented with 5% FCS (HyClone), 10 mM HEPES (Gibco-Invitrogen), 20 μg/ml gentamicin (Sabex), 2 mM L-glutamine (Gibco-Invitrogen), and 0.5 μM β-mercaptoethanol (Sigma). For the first 3 days of culture, complete medium was also supplemented with 20% Ag8653 culture supernatant. On days 3 and 5 of culture, the plates were swirled gently and 4 ml of supernatant, containing nonadherent cells, was discarded from each well and replaced with 5 ml of fresh complete medium with 10% Ag8653 culture supernatant. To enrich for growing DCs, the top 4 ml of supernatant from each well was discarded on day 7 of culture and the cell clusters were collected for subculture. Cell aggregates were gently dislodged from the plate by repeated pipetting with PBS and 1% FCS (HyClone). Cells were resuspended at 1.5 x 10^6 cells/ml in complete medium supplemented with 10% Ag8653 culture supernatant and cultured for an additional 20-24 h at 37°C. Resulting cells were routinely 84-88% positive for CD11c as determined by flow cytometry.

Purification of CD11c$^+$ DCs and CD4$^+$ T cells from spleen

Spleens from B6 mice were removed aseptically, perfused with PBS and 1% FCS (HyClone), teased gently apart, and pressed through a sterile fine wire mesh. To obtain DCs, cells were suspended in PBS and 5 mM EDTA (Sigma) and separated by loading onto Nycoprep (Axis-Shield) density gradient and centrifuging at 600 x *g* for 20 min at 4°C. Low-density cells at the interphase were collected, washed twice with PBS, and further purified by positive selection using anti-CD11c microbeads (Miltenyi Biotec). The resulting DCs were routinely ≥85-90% positive for CD11c as determined by flow cytometry. Spleen cells were enriched for CD4$^+$ T cells using anti-CD4 microbeads (Miltenyi Biotec) and were ≥85-90% positive for CD4 as determined by flow cytometry.

DC uptake assay and flow cytometric analysis

To stain pRBC or nRBC, cells (10^7 cells/ml) were incubated with 2 mM CFSE (Molecular Probes) in complete medium for 15 min at 37°C. CFSE staining was stopped by adding excess complete medium and washing cells twice with complete medium. BM-derived or splenic CD11c$^+$ DCs (10^6 cells/well) were seeded with CFSE-labeled

pRBC or nRBC in a 1:20 ratio at a final volume of 200 µl for 4 h at 37°C unless otherwise indicated. DCs were pre-treated with cytochalasin D (Sigma) or colchicine (Sigma) dissolved in dimethyl sulfoxide (DMSO; Fischer Scientific) at the indicated concentrations, or with 0.2% DMSO control at 37°C for 30 min prior to the addition of pRBC or nRBC. Treatment with cytochalasin D or colchicine did not decrease DC viability as determined by trypan blue exclusion and 7-amino-actinomycin D (BD Biosciences) staining (data not shown). To determine the effect of cytokines on uptake by DCs, splenic $CD11c^+$ DCs isolated from naïve mice were treated with mouse recombinant (r)IFN-γ (25 ng/ml; R&D Systems) or rIL-10 (200 U/ml; BD Biosciences) in complete medium for 12 h prior to the addition of pRBC or nRBC. Following co-culture, noningested red cells were removed by lysis with NH_4Cl lysing buffer and the remaining DCs were washed twice with PBS, FcR-blocked, and then stained with PE-labeled anti-CD11c mAb (clone HL3; BD Biosciences) in sorting buffer consisting of PBS with 1% FCS (HyClone) and 0.05% sodium azide (Sigma). To control for transfer of CFSE from lysed RBC to DCs during the staining procedure, DCs were added to pRBC or nRBC lysate and analyzed for CFSE expression. The uptake of CFSE-labeled pRBC or nRBC was determined by gating cells on SSC versus FL2 for the $CD11c^+$ population and analyzing CFSE staining on FL1 using FACSCalibur equipped with CellQuest software (BD Biosciences). Uptake of pRBC by splenic DC subsets was determined by staining $CD11c^+$ cells co-cultured with pRBC with PE-labeled mAb to B220 (clone R43-6B2), CD4 (clone GK1.5) and CD8 (clone 53.6.7) and analyzing CFSE expression by these $CD11c^+$ subsets.

Light and confocal fluorescence microscopy

BM-derived DCs co-cultured with CFSE-labeled pRBC were analyzed by light and confocal microscopy. For light microscopy, cells were centrifuged at 600 rpm onto glass slides, fixed by 100% methanol, stained with Diff-Quik, dried, and photographed at 100x magnification using a Zeiss Axioskop 2 plus light microscope (Carl Zeiss). Images were acquired with Bioquant Nova prime software (version 6.710.10MT). For confocal fluorescence microscopy, cells were stained with PE-labeled anti-CD11c Ab and a small aliquot of cells at 10^6 cells/ml were mounted onto glass slides with

fluorescent mounting medium (DakoCytomation), allowed to dry overnight, and observed using a Zeiss Axioskop 2 plus fluorescent microscope. Confocal microscopy images were acquired and analysed using Q capture software (version 1.60).

Splenic DC maturation, cytokine production and Ag presentation to T cells

Following overnight co-culture of splenic $CD11c^+$ DCs with pRBC or nRBC, noningested red cells were removed by lysis with NH_4Cl lysing buffer and DCs were FcR-blocked and then stained with PE-labeled mAb (BD Biosciences) to CD11c, $I\text{-}A^b$ (clone AF6-120.1), CD40 (clone 2/23), CD80 (clone 16-1041), and CD86 (clone GL-1) in sorting buffer. Cells were gated on the $CD11c^+$ and $CFSE^+$ population and the percentages and mean fluorescence intensity (MFI) of cells expressing MHC class II and costimulatory molecules were determined by flow cytometry. To determine DC expression of maturation markers ex vivo before and after *P. chabaudi* infection, splenic $CD11c^+$ DCs purified from naïve and day 5 infected mice were stained with mAb as described above and analyzed by flow cytometry. Supernatants of splenic $CD11c^+$ DCs (10^6 cells/well) incubated with pRBC or nRBC in a 1:10 ratio for 48 h at 37°C were analyzed for cytokine production by ELISA as described previously (400, 415). To determine Ag presentation function of malaria-pulsed DCs, splenic $CD11c^+$ DCs were pulsed with pRBC or nRBC at the ratios indicated or incubated in complete medium only as nonpulsed controls. Following overnight culture, noningested RBC were removed by lysis with NH_4Cl lysing buffer, DCs were washed 3 times with complete medium, and 100 µl of DCs at 5 x 10^6 cells/ml was plated with splenic $CD4^+$ T cells, freshly purified from naïve B6 mice, in varying ratios as indicated at a final volume of 200 µl in 96-well plates for 48 h at 37°C. Proliferation was determined by incorporation of $[^3H]$-thymidine during the last 6 h of incubation. Supernatants from DC and T cell co-cultures were analyzed for IFN-γ production by ELISA as described previously (415). All pRBC and nRBC preparations used in this study tested negative for the presence of endotoxin using a *Limulus* amebocyte lysate gel clot-test (Sigma) with a sensitivity limit of 0.015 EU/ml. In addition, to control for possible endotoxin contamination, pRBC and nRBC were treated with polymixin B (10 µg/ml; Sigma) prior to incubation with DCs. Results obtained with polymixin-treated pRBC or nRBC

were identical to those obtained with untreated red cells (Appendix II *A*), confirming that the pRBC-induced DC responses observed in this study were not attributable to endotoxin contamination.

Statistical analyses

Data are expressed as mean ± SEM. Statistical significance of differences between experimental groups as indicated was analyzed by two-tailed, unpaired Student's *t* test. Statistical significance was defined as follows: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$. All statistical analyses were performed using SAS/STAT software (SAS Institute).

Results

Preferential uptake of malaria-infected RBCs by bone marrow-derived DCs

To better understand the early interactions between DCs and *P. chabaudi*-infected RBC, we developed a flow cytometry technique to measure the uptake of pRBC or nRBC by DCs. The intraerythrocytic location of the *P. chabaudi* AS parasite enabled us to use the dye CFSE to label highly purified pRBC as well as nRBC and then detect CFSE fluorescence expressed by DCs following co-culture. BM-derived DCs were incubated with CFSE-labeled pRBC or nRBC and the percentages of $CD11c^+$ DCs expressing CFSE, representing the population of DCs that had ingested the RBC, were analysed by flow cytometry. BM-derived DCs were able to ingest pRBC to a considerably greater extent (more than 4-fold) than nRBC (Fig. 1*A-B*). The possibility that CFSE inadvertently transferred from lysed RBC to DCs during the RBC lysis and cell surface staining procedures was observed to be minimal (<1% for nRBC; 5% for pRBC; Fig. 1*A right panels*). Lysis controls were routinely performed in all experiments and the values obtained were subtracted from the percentages of uptake presented here. The preferential uptake of pRBC by DCs was observed as early as 2 h of co-culture and peaked at 6 h with no notable increases thereafter at 20 h (Fig. 1*C*). Even after 20 h of co-culture, the percentage of DCs that ingested nRBC was significantly lower than the percentage that ingested pRBC.

DCs internalize malaria-infected RBCs via actin-dependent phagocytosis

Phagocytosis of large particles (usually large than 0.5 μm in diameter) by mammalian cells, including macrophages and DCs, requires actin polymerization at the site of entry and subsequent growth of new actin filaments that facilitate cell membrane motility (510). To determine whether the uptake of pRBC by DCs was mediated by actin-dependent phagocytosis, BM-derived DCs were treated with cytochalasin D, a drug that inhibits actin polymerization and addition of actin monomers to the ends of growing filaments (511), prior to the addition of pRBC or nRBC. As shown in Fig. 2*A*, DCs treated with cytochalasin D had significantly lower uptake of both pRBC (54% *vs.* 13%) and nRBC (7% *vs.* 3%) than untreated DCs. This inhibition of uptake was

observed at all ratios of DC co-cultured with RBC (Fig. 2*B*) as well as at all doses of cytochalasin D tested (Fig. 2*C*). There was no dose response using higher concentrations of cytochalasin D, and the observed reductions were independent of the DMSO used as a diluent, which resulted in similar levels of uptake of pRBC and nRBC as untreated DCs (Fig. 2*C*). Since treatment with cytochalasin D did not completely abrogate the uptake by DCs, some pRBC may have been phagocytosed via nonactin-dependent mechanisms. Treatment with colchicine, a microtubule depolymerizing agent, did not impair uptake of pRBC or nRBC at various concentrations tested (Fig. 2*D*), suggesting that functional microtubules do not play a major role in the uptake of pRBC by DCs.

To demonstrate internalization of pRBC by DCs, cytospins of Diff Quik-stained BM-derived DCs co-cultured with pRBC were examined by light microscopy. Following 2-6 h co-culture and lysis of noningested red cells, intact pRBC were observed inside cytoplasmic compartments of BM-derived DCs that resemble phagosomal vacuoles (Fig. 3*A*). To confirm that the expression of CFSE fluorescence by gated $CD11c^+$ DCs truly represents internalization of pRBC by DCs rather than only attachment of CFSE-labeled cells to the $CD11c^+$ cell surface, we examined co-localization of CFSE-labeled pRBC and PE-stained $CD11c^+$ cells by confocal microscopy (Fig. 3*B-D*). In representative confocal fluorescent microscopy images, CFSE-labeled pRBC are shown to co-localize with three PE-labeled $CD11c^+$ DCs (Fig. 3*D*). Collectively, these microscopy images provide strong evidence that flow cytometric analyses of CFSE expression by gated $CD11c^+$ DCs using the assay we developed were an accurate and objective measurement of internalization by DCs of CFSE-labeled pRBC or nRBC.

Uptake of malaria-infected RBCs by splenic $CD11c^+$ DCs is selectively enhanced following malaria infection in vivo

Next, we used our flow cytometric technique to study the uptake of pRBC by mouse spleen DCs before and during infection with *P. chabaudi*. Although we used BM-derived DCs to standardize our flow cytometric uptake assay and showed that these DCs exhibited preferential uptake of pRBC, it is the DC population in the spleen that is

of major interest because of the central role of this organ in filtering pRBC from the blood and in generating a protective immune response to blood-stage malaria (83). As observed with BM-derived DCs, splenic CD11c$^+$ DCs showed significantly higher (>4-fold) uptake of pRBC than nRBC (Fig. 4*A*) at all time points before and after *P. chabaudi* infection (Fig. 4*B*). Importantly, the percentage of splenic CD11c$^+$ DCs ingesting pRBC increased following *P. chabaudi* infection (47.7% at day 5 post-infection *vs.* 18.6% at day 0; Fig. 4*A*). The uptake of pRBC by splenic CD11c$^+$ DCs increased markedly at days 2-5 post-infection (p.i.) and declined thereafter to pre-infection levels by day 8 p.i. (Fig. 4*B*), indicating that maximal uptake of pRBC ex vivo by splenic DCs preceded peak parasitemia in *P. chabaudi*-infected mice. In contrast, the uptake of nRBC increased only slightly after infection (5% higher at days 5 vs. 0 p.i.) and was significantly lower than the uptake of pRBC at all time points before and after infection. Uptake of pRBC and nRBC by splenic DCs from naïve mice was significantly impaired by treatment with cytochalasin D at all ratios of DCs incubated with pRBC or nRBC (55-65% inhibition; Fig. 4*C*), but was not affected by treatment with colchicine (data not shown). Therefore, splenic DCs selectively recognize and phagocytose pRBC and this preferential uptake of pRBC is enhanced in the early days of blood-stage malaria infection in vivo. These results, while specific to interactions of DCs with malaria-infected RBC, are consistent with the sentinel role of DCs in detecting invading pathogens and capturing foreign antigens during acute infection.

Given that the mouse CD8$^+$ DC subset has been shown to selectively endocytose apoptotic cells (506), we questioned whether the level of pRBC uptake differed among the CD4$^+$, CD8$^+$, and B220$^+$ (plasmacytoid) subsets of splenic CD11c$^+$ DCs. Following co-culture of pRBC with splenic CD11c$^+$ DCs purified from naïve or infected mice, DCs were stained with fluorochrome-labeled antibodies to CD4, CD8 or B220, and the percentage of CD11c$^+$ cells expressing CFSE fluorescence was determined for each DC subset by flow cytometry. All three subsets exhibited phagocytosis of pRBC before and after acute *P. chabaud*i infection (Fig. 4*D*). The CD4$^+$ and CD8$^+$ DC subsets from infected mice showed significantly higher uptake of pRBC than those from naïve mice, while uptake by the B220$^+$ subset was significantly lower after infection.

Effect of cytokines on uptake of malaria-infected RBCs by splenic CD11c$^+$ DCs

Previous work in our laboratory showed that rIFN-γ activates macrophages and increases their phagocytosis of pRBC while rIL-10 has the opposite effect (392). We questioned whether phagocytosis of pRBC by DCs is similarly controlled by cytokines with opposing immunoregulatory effects. Splenic CD11c$^+$ DCs from naïve or day 5 infected mice were treated with rIFN-γ, rIL-10, or not treated (medium) for 12 h prior to the addition of pRBC or nRBC as described previously (392). As shown above, splenic DCs from infected mice demonstrated higher levels of pRBC uptake than DCs from naïve mice (Fig. 4*E*). However, treatment with rIFN-γ or rIL-10 did not significantly affect the uptake of pRBC or nRBC by DCs from either naïve or infected mice, suggesting that uptake of pRBC by DCs is not dependent on the cytokines present in the spleen microenvironment.

Uptake of malaria-infected RBCs by splenic CD11c$^+$ DCs induces maturation, cytokine production, and CD4$^+$ T cell activation

The aforementioned flow cytometric analyses were conducted on total CD11c$^+$ DC populations co-cultured with pRBC and included both phagocytic and nonphagocytic DCs. We then determined whether preferential uptake of pRBC by DCs induces maturation and presentation of antigens to CD4$^+$ T cells. Following an overnight DC uptake assay, splenic CD11c$^+$ DCs that had captured pRBC showed significantly higher expression of MHC class II and costimulatory molecules than DCs that had ingested nRBC as demonstrated by the higher percentages of cells expressing these molecules (Fig. 5*A*) and by the higher levels of mean fluorescence intensity (Fig. 5*B*). The ability of pRBC, but not nRBC, to stimulate maturation of splenic DCs in vitro was consistent with the observation of in vivo maturation after *P. chabaudi* infection (Fig. 5*C*). Since uptake of pRBC by DCs was associated with phenotypic maturation, we assessed whether DCs that had phagocytosed pRBC compared to DCs that had ingested nRBC were also functionally more mature in terms of cytokine production and stimulation of CD4$^+$ T cell responses. Splenic DCs co-cultured with nRBC or in medium alone did not produce detectable levels of IL-12p40 or, importantly, bioactive IL-12p70 compared to

DCs pulsed with pRBC (Fig. 6*A-B*). In addition, splenic DCs pulsed with pRBC produced significantly more IL-2 (Fig. 6*C*) and stimulated significantly higher levels of $CD4^+$ T cell proliferation (Fig. 6*E*) and IFN-γ production (Fig. 6*F*) than did splenic DCs pulsed in medium alone (nonpulsed) or with nRBC. Notably, stimulation of T cell proliferation and IFN-γ production increased in a dose-dependent manner when DCs were pulsed with a higher number of pRBC (Fig. 6*E-F*). The lower stimulation of T cell responses by DCs pulsed in medium alone or with nRBC was not associated with the production of anti-inflammatory IL-10, which was significantly lower than the level produced by DCs pulsed with pRBC (Fig. 6*D*). Several lines of evidence indicate that pRBC is a poor T cell mitogen in the absence of presentation by an APC such as the splenic DCs used in this study: 1) $CD4^+$ T cells incubated with pRBC alone showed minimal proliferation and failed to produce detectable IFN-γ (Fig. 6*E-F*); 2) DCs fixed with 1% paraformaldehyde prior to pulsing with pRBC were unable to stimulate $CD4^+$ T cells (Appendix II *B*); and 3) Th2-type cytokines, IL-4 and IL-13, were not detected in any of the supernatants from DC single cultures or co-cultures with T cells (data not shown). These results suggest that the selective interaction of splenic $CD11c^+$ DCs with pRBC induced DCs to mature, produce IL-12, and promote $CD4^+$ T cell proliferation and differentiation into IFN-γ-secreting cells.

Discussion

Early interactions between DCs and invading pathogens result in activation of DCs and thereby shape the development of adaptive immune responses. In mouse models of malaria, DCs are thought to play an important role in the induction of Th1-dependent immune responses that are protective against blood-stage malaria infection (8, 362, 366). In the present study, we describe a new flow cytometry technique we developed to measure levels of phagocytosis of pRBC and nRBC by mouse BM-derived or spleen DCs in vitro. This is the first report showing that DCs take up and present malaria-infected RBCs in a highly selective manner that is significantly enhanced after infection in vivo. The uptake of pRBC, but not of nRBCs, by splenic $CD11c^+$ DCs was associated with the induction of DC maturation, cytokine production, and stimulation of $CD4^+$ T cell responses. These results provide additional evidence that DCs are important APCs in the innate immune response to blood-stage malaria infection that activate T cells, particularly the $CD4^+$ Th1 cells that produce IFN-γ and mediate type 1 adaptive immune responses such as class switching of B cells to the protective Th1-dependent IgG subclasses during the chronic phase of infection (8, 83).

The uptake of pRBC by splenic $CD11c^+$ DCs increased with acute *P. chabaudi* AS infection and peaked 2-3 days prior to peak parasitemia, which typically occurs at day 7 p.i. (400, 415, 420). This increased phagocytic activity may be due to an expansion of the DC population in the spleen following *P. chabaudi* infection (365, 512). As the parasite replicates, causing the parasite burden to increase in the blood and more pRBC to be deposited in the spleen for removal, blood-borne DCs are recruited to the spleen in increasing numbers. These migratory DCs are likely to be immature and highly efficient in the recognition and capture of foreign antigens. Following peak phagocytic activity at day 5 p.i., the uptake of pRBC by splenic DCs declined to baseline levels, which may reflect down-regulation of DC function and other innate responses once adaptive immunity becomes established. Alternatively, we considered the possibility that DC uptake, similar to macrophage phagocytosis (392), may be modulated by a balance of proinflammatory and anti-inflammatory cytokines. The kinetics of DC uptake of pRBC closely coincided with the level of IFN-γ production observed in *P. chabaudi*-infected B6 mice (415, 420). Notably, the peak level of pRBC uptake by splenic DCs observed

in this study also coincided with peak numbers of splenic CD11c$^+$ DCs expressing IFN-γ at 4 to 5 days following *P. chabaudi* infection (365, 512). For macrophages, levels of nonopsonic phagocytosis also correlate with the ability of infected mice to produce IFN-γ and treatment of macrophages with IFN-γ in vitro enhances phagocytosis of pRBC while treatment with IL-10 inhibits this activity. However, results presented here suggest that the uptake of pRBC by DCs is not modulated by either IFN-γ or IL-10. Given that Ag capture by DCs is essential for DC activation and initiation of innate and adaptive immune responses, it is teleologically reasonable that the uptake of pRBC by DCs is not influenced by immunoregulatory cytokines which in turn are produced as a result of DC-parasite interaction. We therefore favor the view that the increased uptake of pRBC by DCs observed following *P. chabaudi* infection reflects an influx of immature DCs from the periphery into the spleen in response to increasing numbers of pRBC deposited in the spleen during the period of rising parasitemia rather than an up-regulation by proinflammatory cytokines present in situ.

The development of a flow cytometry technique to measure uptake of pRBC by DCs allowed us to investigate whether DCs selectively recognize and capture pRBC in the absence of opsonization by Ab or complement. We used purified pRBC to ensure that pRBC-specific DC uptake and responses were studied. Furthermore, to ensure that the expression of CFSE fluorescence by CD11c$^+$ DCs represented internalization rather than attachment of pRBC to the DC surface, we routinely used a RBC lysis procedure in each DC uptake assay to remove adherent, noningested pRBC and confirmed co-localization of green (CFSE-labeled pRBC) and red (PE-labeled CD11c$^+$ DCs) fluorescence by confocal microscopy. As previously reported for macrophage phagocytosis (513), our flow cytometric analyses consistently showed that DCs ingest pRBC at considerably higher rates than nRBC. Moreover, the uptake of pRBC by DCs required actin polymerization but not functional microtubules, although some pRBC may be ingested by nonactin-dependent mechanisms. Light microscopic images suggested that internalized pRBC are contained within phagosomal compartments in DCs that resemble those that form in macrophages (390, 513). Taken together, these data indicate that uptake of pRBC by DCs occurs via actin-dependent phagocytosis and

provide evidence that our flow cytometry assay offers an objective and accurate method to study DC-mediated phagocytosis of pRBC in human and mouse malaria.

One potential application of our newly developed flow cytometric uptake assay is to investigate the specific components in the DC-parasite interaction in malaria. A key question is the identity of receptors and ligands involved in Ag capture and presentation. Consistent with mechanisms triggering nonopsonic phagocytosis, the process of pRBC internalization by DC most likely occurs through engagement of pRBC-derived ligands with Ag uptake receptors (510). For macrophages, numerous studies have shown that CD36, a class B scavenger receptor, is the main receptor mediating nonopsonic phagocytosis of *P. falciparum*-infected RBC (390, 391, 513). CD36 binds several ligands expressed on the surface of *P. falciparum*-infected RBC, particularly the highly variable but structurally conserved *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1). Although CD36-mediated macrophage phagocytosis may be an important nonspecific defense mechanism for parasite clearance, the interaction between PfEMP-1 and CD36 expressed by endothelial cells has been implicated in *P. falciparum* cytoadherence and sequestration which are associated with severe malaria as well as cerebral malaria (390). Cytoadherent *P. falciparum* parasite lines bind to CD36 and CD54 (ICAM-1) whereas a nonadherent parasite line does not bind these receptors (359). *P. chabaudi*-infected RBCs also adhere to purified CD36 and to endothelial cells in an IFN-γ-dependent manner (514). However, these findings do not conclusively demonstrate that selective recognition and uptake of malaria-infected RBC by DCs are mediated via CD36 or CD54. Other possible candidates include TLR9, which mediates the response of human and mouse plasmacytoid DCs to *P. falciparum*-infected RBC (369), and receptors of the C-type lectin family (e.g. DC-SIGN and DEC-205) involved in receptor-mediated endocytosis (515, 516). Experiments are ongoing in our laboratory to address the role of these and other receptors in uptake of *P. chabaudi*-infected RBC by mouse DCs.

Given the importance of the spleen in host resistance to murine blood-stage *Plasmodium* parasites, the expansion of the splenic DC population (365, 512) and migration of $CD11c^+$ DCs to the T cell-rich zones of the spleen (365) following malaria infection suggest that splenic DCs are in a prime position to initiate protective immune

responses against blood-stage malaria. DCs recognize foreign ligands through pattern recognition receptors, a process that could trigger their maturation as well as the production of proinflammatory cytokines. Indeed, the uptake of pRBC by splenic $CD11c^+$ DCs was linked to activation of DC responses that may play an important role in shaping adaptive T cell-dependent immune responses. Splenic $CD11c^+$ DCs that phagocytosed pRBC were able to present pRBC-derived antigens to $CD4^+$ T cells as demonstrated by the significantly higher levels of proliferation and IFN-γ production induced by DCs pulsed with pRBC compared to DCs incubated alone or with nRBC. Although the extent of pRBC phagocytosis by splenic DCs was comparably lower than that by BM-derived DCs, our results suggest that massive uptake is not required to induce splenic DC activation nor for splenic DCs to present sufficient peptides for T cell activation. Rather, stimulation of T cell responses and, crucially, the induction of Th1 cell development were associated with increased expression of MHC class II and costimulatory molecules as well as production of IL-2 and IL-12. Although $CD11c^+$ DCs enriched from the spleen comprise a heterogeneous population, our results suggest that the uptake of pRBC and subsequent DC activation were not restricted to a specific $CD11c^+$ DC subset. However, since we did not investigate maturation and functional responses among distinct DC subpopulations, we cannot exclude the possibility that cytokine production and T cell stimulatory activity by pRBC-activated DCs were mediated by different DC subsets.

The findings presented here corroborate previous studies demonstrating that DC maturation and function are not impaired following interaction with *Plasmodium* parasites. We show that preferential uptake of *P. chabaudi*-infected RBC by splenic $CD11c^+$ DCs induced their maturation, cytokine production, and activation of $CD4^+$ T cell responses. In vitro maturation of DCs exposed to pRBC but not to nRBC also reflected in vivo maturation following *P. chabaudi* infection. These results are in agreement with other studies showing that splenic $CD11c^+$ DCs from mice infected with *P. chabaudi* exhibit up-regulated expression of CD40, CD54, and CD86 (365) as well as increased production of IFN-γ (365, 512). Similarly, splenic $CD11c^+$ DCs from mice infected with *P. yoelii* 17X stimulate high levels of IL-2, IFN-γ and TNF-α production by responding naïve $CD4^+$ T cells in an IL-12-dependent mechanism (364, 366).

Activation of DCs is not limited to rodent models of malaria: *P. falciparum* schizonts were found to stimulate human plasmacytoid DCs and mouse splenic $B220^+$ DCs to up-regulate CD86 expression, produce IFN-α, and promote γδ T cell proliferation and IFN-γ production through a TLR9-dependent pathway (369). However, these results as well as data presented here contrast those from other studies showing that some isolates of *P. falciparum* (359) as well as rodent *P. yoelii* (370, 371) and *P. chabaudi* (370) inhibit DC maturation in response to LPS stimulation in vitro. Cytoadherent *P. falciparum* parasites were also found to inhibit the ability of DCs to stimulate primary or secondary malaria-specific T cell proliferation (359), while modulation of DC functions by *P. yoelii*-infected RBC was associated with suppressed protective $CD8^+$ T cell responses against liver-stage malaria (371).

There are several possible explanations for the discrepancy among these findings. Studies showing inhibition of DC maturation by *Plasmodium* parasites were conducted with the use of LPS to mature DCs in vitro. However, following initial maturation with LPS, DCs may become exhausted or refractory to further stimulation with malarial antigens (reviewed in 8). Furthermore, LPS induces DC maturation in the absence of CD40 ligation, which has been shown to be required for maximal expression of a mature DC phenotype and for cytokine production (517, 518). Despite the lack of maturation in response to LPS, DCs pulsed with RBC infected with *P. yoelii* or *P. chabaudi* were fully capable of inducing protective immunity against homologous challenge infection, suggesting that DC maturation is merely delayed rather than arrested in vitro (370). Studies showing inhibition of DC maturation and lower T cell stimulatory activity by *Plasmodium* parasites employed DCs derived from human peripheral blood cells (359) or mouse BM precursors (370, 371), while other studies (364-366, 512), including the study presented here, showing up-regulated DC maturation and function employed $CD11c^+$ DCs purified from spleens of naïve or infected mice. DCs grown in culture develop and interact with *Plasmodium spp.* in the absence of the cytokine milieu provided by NK cells, T cells, and other cell types normally present in the spleen microenvironment that support and promote DC maturation. However, the different origins of DCs used among these studies do not fully explain the discrepancy because *P. chabaudi* schizonts were found to induce mouse

BM-derived DCs to up-regulate expression of MHC class II, CD40 and CD86 and to produce IL-12, TNF-α, and IL-6 even in the absence of T cells, NK cells or CD40 ligation (362).

In conclusion, there is growing evidence that DCs play an important role in the host defense against blood-stage malaria infection by linking innate and adaptive immunity. The results presented in this study support the contention that mouse splenic DCs interact with *P. chabaudi*-infected RBC early after infection through preferential uptake, processing, and presentation of malaria-derived antigens to $CD4^+$ T cells. The expression of costimulatory molecules as well as production of IL-2 and IL-12 following uptake of pRBC provide important costimulatory and cytokine signals to support the development and expansion of Th1 cells, which are critically required for protective immunity to blood-stage malaria. A better understanding of the early interactions of DCs with *Plasmodium* parasites will not only provide insights into the immunobiology of malaria infection but may also identify key targets for the future development of vaccines and immunotherapies.

Acknowledgements

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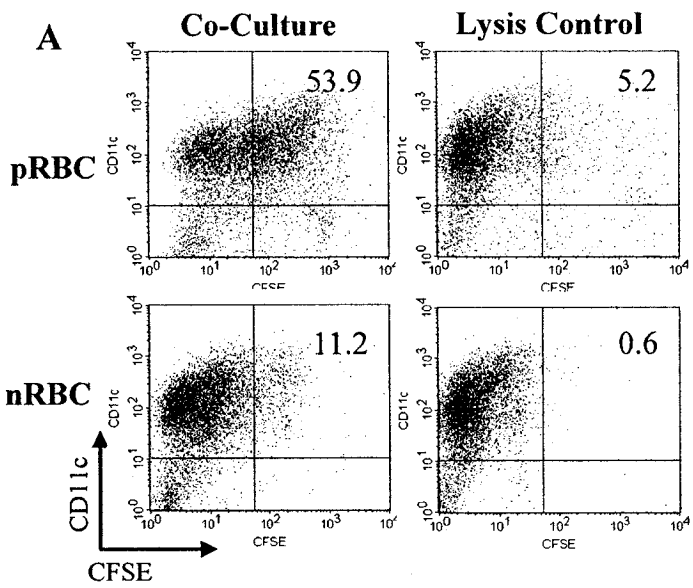
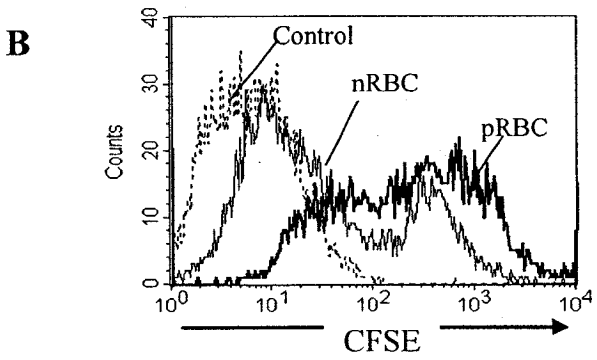
Figure 1: DCs preferentially take up pRBC vs. nRBC. BM-derived DCs were incubated with CFSE-labeled pRBC or nRBC in a ratio of 1:20 for 4 h at 37°C. Following co-culture, noningested RBC were lysed, DCs were stained with PE-labeled anti-CD11c monoclonal Ab, and CFSE fluorescence was determined by flow cytometry. *A,* Percentages of cells expressing both PE-CD11c (FL2) and CFSE (FL1) following co-culture of DCs with intact pRBC or nRBC (*left panels*) or lysis controls (*right panels*) in which DCs were co-cultured with pRBC or nRBC lysate. *B*, Histograms show CFSE expression by $CD11c^+$ DC control (dotted line) or by DCs co-cultured with nRBC (grey line) or with pRBC (black line). *C*, Percentages of gated $CD11c^+$ cells expressing CFSE after incubation with pRBC or nRBC for the time intervals as indicated. Data in *A-B* are representative of 6-8 independent experiments. Data in *C* are expressed as mean ± SEM pooled from three independent experiments. Statistical analyses compared pRBC vs. nRBC at each time point (***, $p<0.001$).

Figure 1


A
Co-Culture
Lysis Control
pRBC
nRBC
53.9
5.2
11.2
0.6
CD11c
CFSE


B
Control
nRBC
pRBC
Counts
CFSE

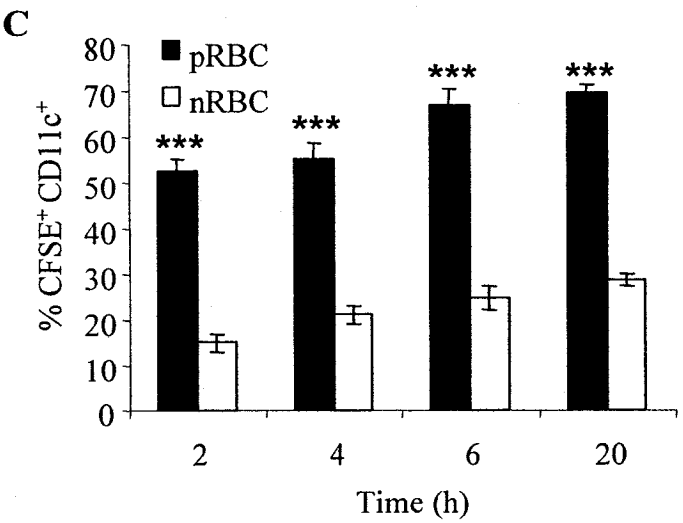

C
pRBC
nRBC
% CFSE+ CD11c+
***
Time (h)

Figure 2: Treatment with cytochalasin D, but not colchicine, inhibits the uptake of pRBC and nRBC by BM-derived DCs. *A*, Percentages of gated $CD11c^+$ cells expressing CFSE following no treatment (*left panels*) or treatment with cytochalasin D (*right panels*). Histograms show CFSE expression by $CD11c^+$ DCs incubated without RBC (line) or with pRBC or nRBC (shaded area). *B*, Treatment with cytochalasin D (CyD; 8 μM) inhibits uptake of pRBC and nRBC at all ratios of DCs incubated with RBC. *C*, Cytochalasin D inhibits the uptake of pRBC and nRBC at all concentrations tested. Percentages of inhibition relative to no treatment (0 μM) are indicated. *D*, Treatment with colchicine does not affect uptake of pRBC or nRBC at any concentration tested. Data are expressed as mean ± SEM pooled from four (*B-C*) or two (*D*) independent experiments. Statistical analyses compared uptake of pRBC with no treatment vs. treatment with cytochalasin D at each DC:RBC ratio (**, $p<0.01$; ***, $p<0.001$).

Figure 2

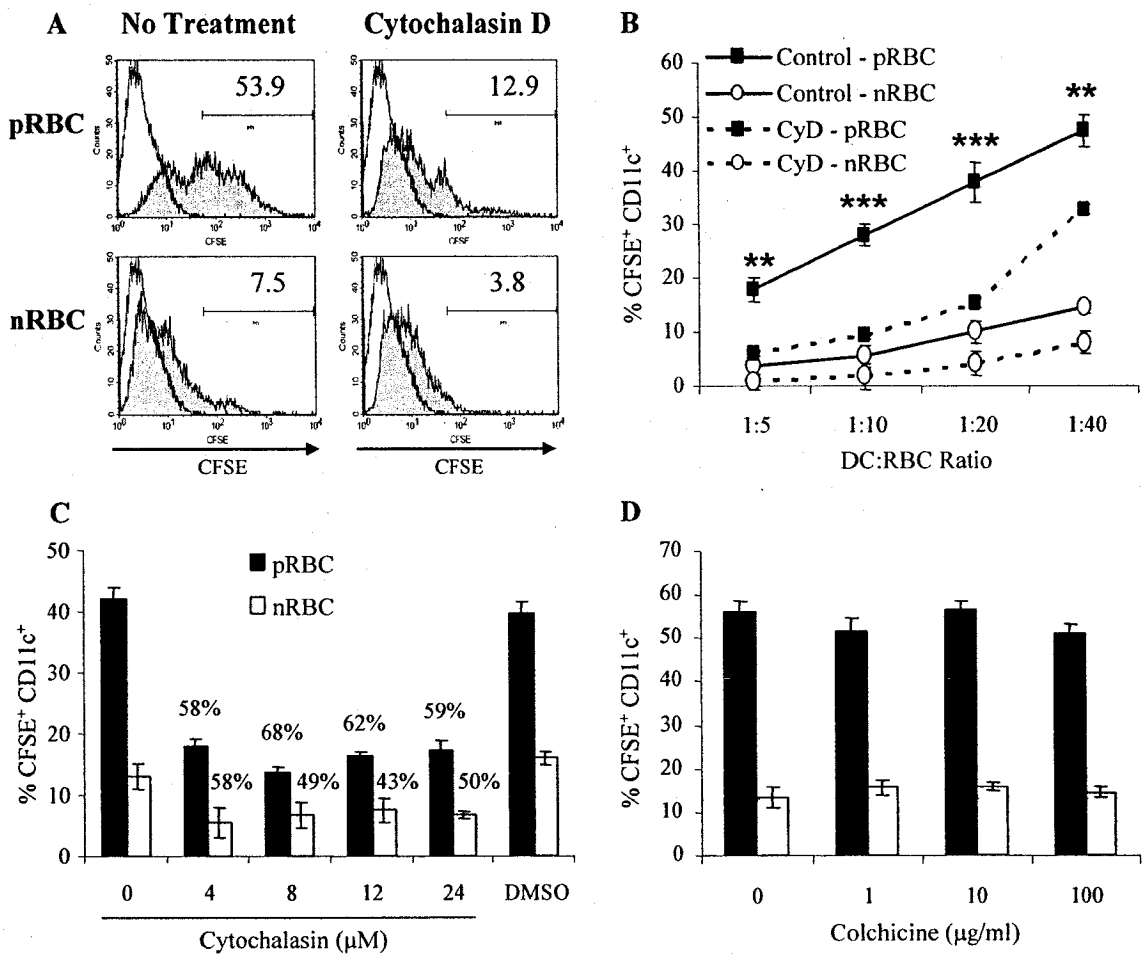
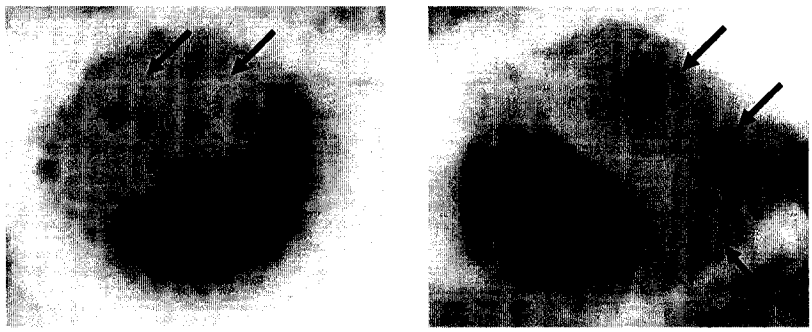

A
No Treatment
Cytochalasin D
pRBC
nRBC
53.9
12.9
7.5
3.8
CFSE
CFSE
B
Control - pRBC
Control - nRBC
CyD - pRBC
CyD - nRBC
% CFSE+ CD11c+
DC:RBC Ratio
1:5
1:10
1:20
1:40
C
pRBC
nRBC
% CFSE+ CD11c+
58%
68%
62%
59%
58%
49%
43%
50%
0
4
8
12
24
DMSO
Cytochalasin (μM)
D
% CFSE+ CD11c+
0
1
10
100
Colchicine (μg/ml)

Figure 3: Internalization of pRBC by BM-derived DCs and co-localization of DCs and CFSE-labeled pRBC. *A*, Diff Quik-stained light microscopic images show pRBC, indicated by arrows, internalized by DCs. *B*, Red fluorescence of PE-labeled $CD11c^+$ DCs. *C*, Green fluorescence of CFSE-labeled cells, representing CFSE-labeled pRBC inside DCs. *D*, Merged confocal microscopic image showing co-localization of CFSE-labeled pRBC and $CD11c^+$ DC. Light microscopic images and confocal fluorescent images were acquired at 100x and 40x magnification, respectively. Data shown are representative of two independent experiments.

Figure 3

A: DC + pRBC



B: CD11c^{+} PE **C: CFSE^{+} Cells** **D: Merge**

Figure 4: Splenic CD11c$^+$ DCs preferentially take up pRBC vs. nRBC at increasing levels in the early days following *P. chabaudi* infection in vivo. *A*, Splenic DCs enriched from naïve (*left panels*) or day 5 infected (*right panels*) B6 mice were incubated with CFSE-labeled pRBC or nRBC and the level of uptake by nongated cells (dot plots) or gated CD11c$^+$ cells (histograms) was determined by CFSE fluorescence as detected by flow cytometry. *B*, Kinetics of pRBC or nRBC uptake by splenic DCs enriched from mice before and after *P. chabaudi* infection, and course of parasitemia in mice infected with *P. chabaudi* during the same experimental period. *C*, Splenic DCs preferentially take up pRBC vs. nRBC at all ratios of DCs incubated with RBC and uptake is inhibited by treatment with cytochalasin D (CyD, dotted lines). *D*, Uptake of pRBC is mediated by CD4$^+$, CD8$^+$ and B220$^+$ subsets of splenic CD11c$^+$ DCs before and after infection. *E*, Pre-treatment of splenic DCs from naïve or day 5 infected mice with rIFN-γ or rIL-10 in vitro does not affect the uptake of pRBC or nRBC compared to untreated DCs. Data in *B-D* were pooled from three independent experiments each containing 3-4 mice per group and are presented as mean ± SEM. Data in *E* are representative of two independent experiments each containing 3-4 mice per group. Statistical analyses in *B-C* compared pRBC vs. nRBC at each time point or DC:RBC ratio and in *D* compared day 0 p.i. vs. day 5 p.i. for each subset (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 4

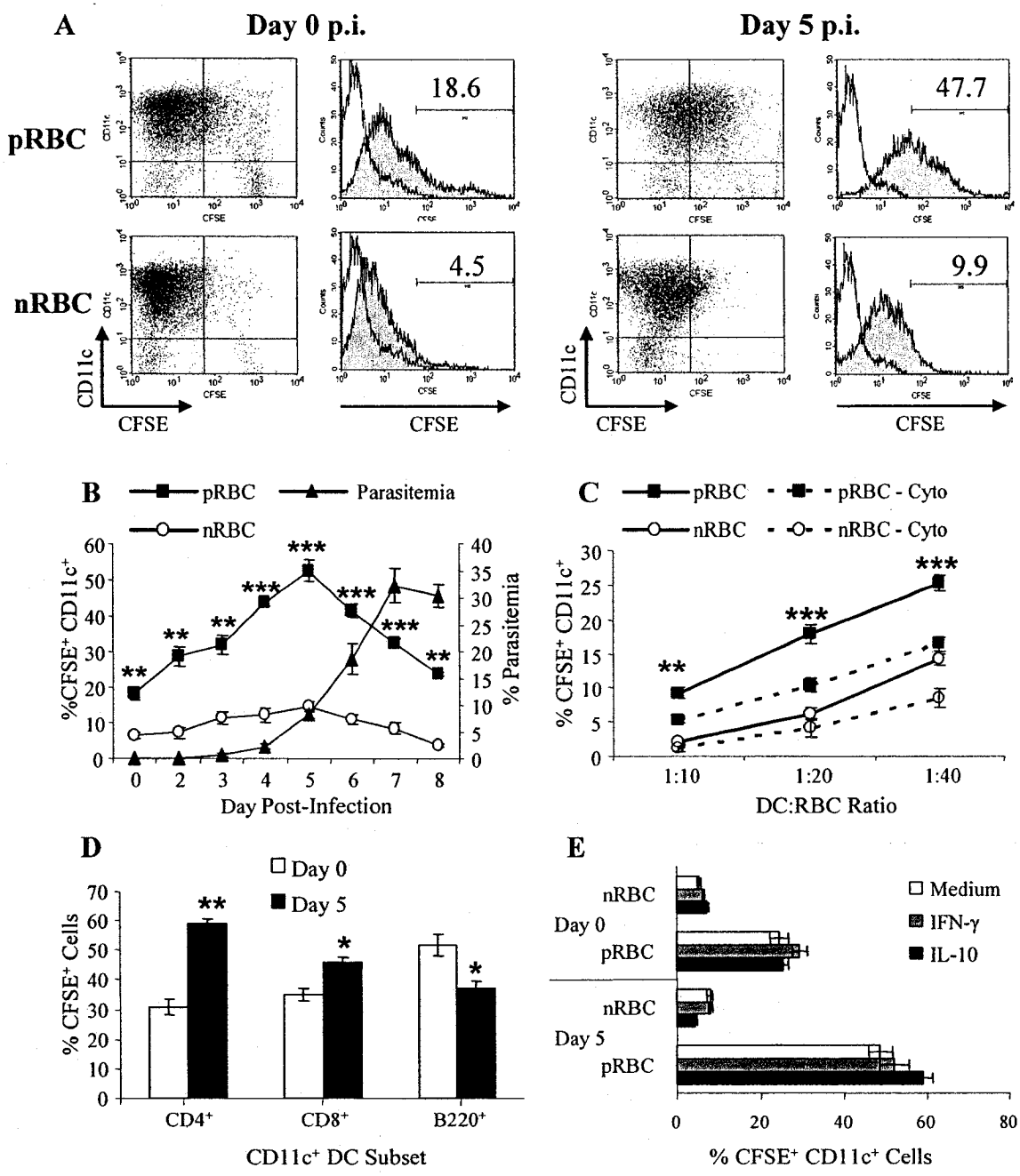

A
Day 0 p.i.
Day 5 p.i.
pRBC
nRBC
18.6
4.5
47.7
9.9
CD11c
CFSE
B
pRBC
Parasitemia
nRBC
%CFSE+ CD11c+
% Parasitemia
Day Post-Infection
C
pRBC
pRBC - Cyto
nRBC
nRBC - Cyto
% CFSE+ CD11c+
1:10
1:20
1:40
DC:RBC Ratio
D
Day 0
Day 5
% CFSE+ Cells
CD4+
CD8+
B220+
CD11c+ DC Subset
E
nRBC
Day 0
pRBC
nRBC
Day 5
pRBC
Medium
IFN-γ
IL-10
% CFSE+ CD11c+ Cells

Figure 5: Uptake of pRBC, but not of nRBC, by splenic DC induces maturation. Splenic $CD11c^+$ DCs from naïve B6 mice were pulsed with CFSE-labeled pRBC or nRBC. Following co-culture overnight, noningested RBC were removed and DCs were stained with PE-labeled Abs to $I\text{-}A^b$ (MHC class II), CD40, CD80, and CD86. *A*, Histograms show PE fluorescence of various markers by gated $CFSE^+$ cells. Higher percentages of DCs co-cultured with pRBC expressed MHC class II and costimulatory molecules compared to DCs co-cultured with nRBC. *B*, Mean fluorescence intensity (MFI) of the expression of MHC class II and costimulatory molecules by gated $CFSE^+$ cells. DCs pulsed with pRBC expressed higher levels of MHC class II and costimulatory molecules than did DCs pulsed with nRBC. *C*, MFI of the expression of maturation markers by splenic $CD11c^+$ DCs ex vivo at days 0 and 5 p.i. Data in *B* and *C* are presented as mean $\pm$ SEM and represent two independent experiments of 3-4 mice per group. Statistical analyses compared pRBC vs. nRBC in *A* and day 0 vs. day 5 p.i. in *B* for each marker MFI (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 5

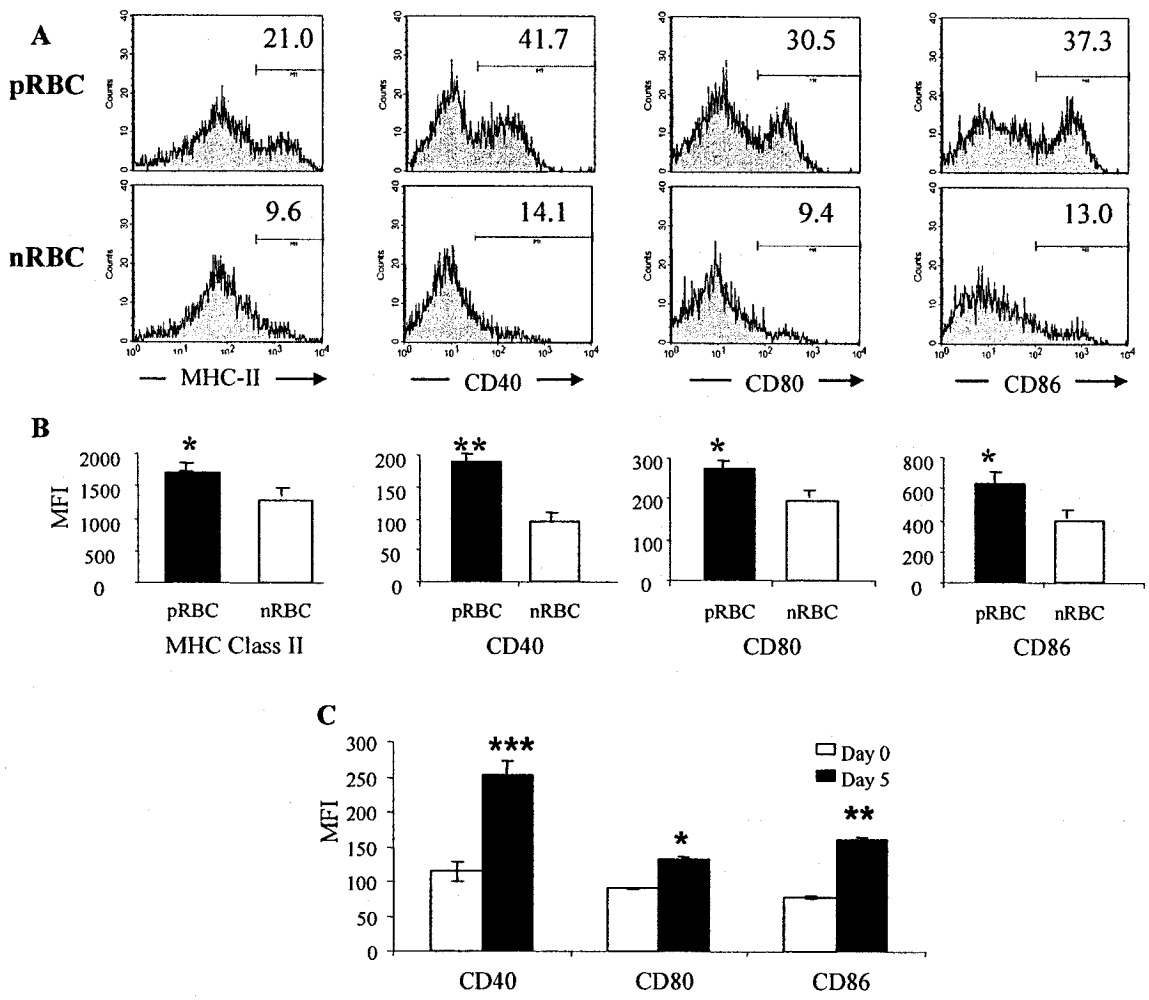
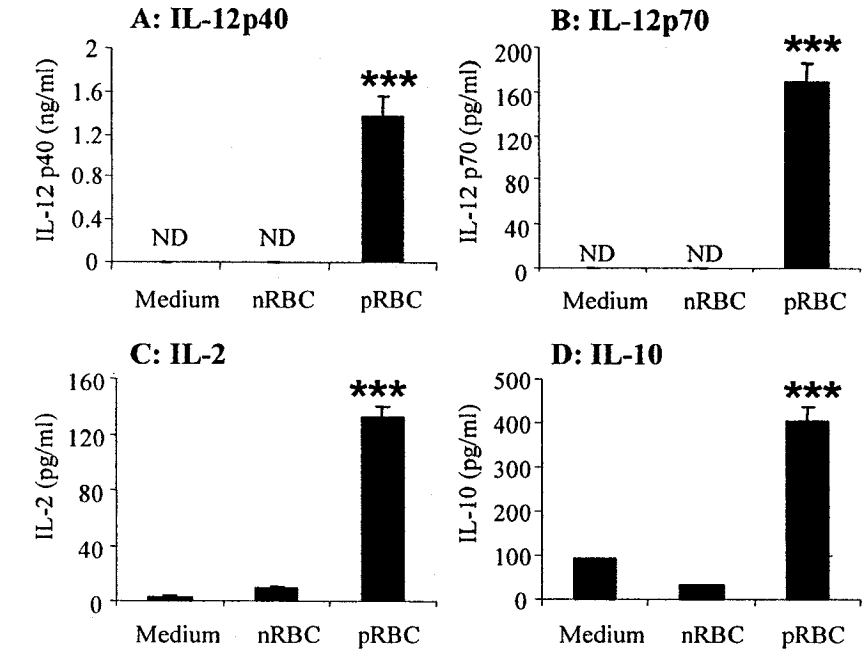
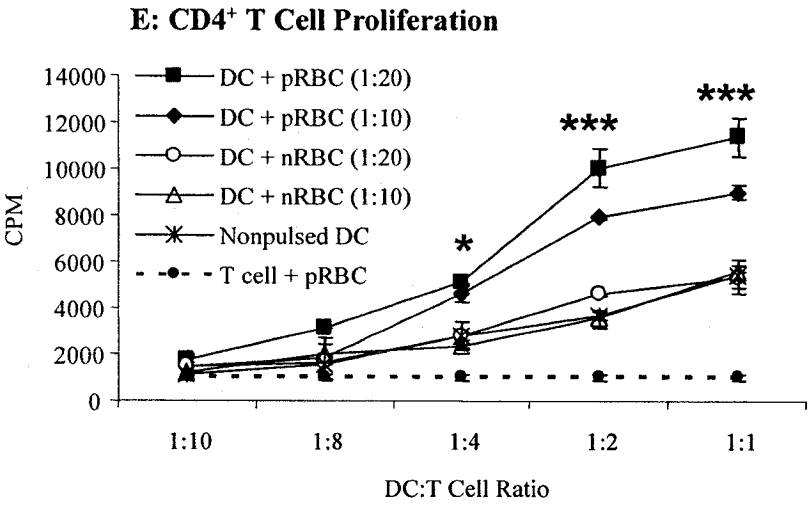
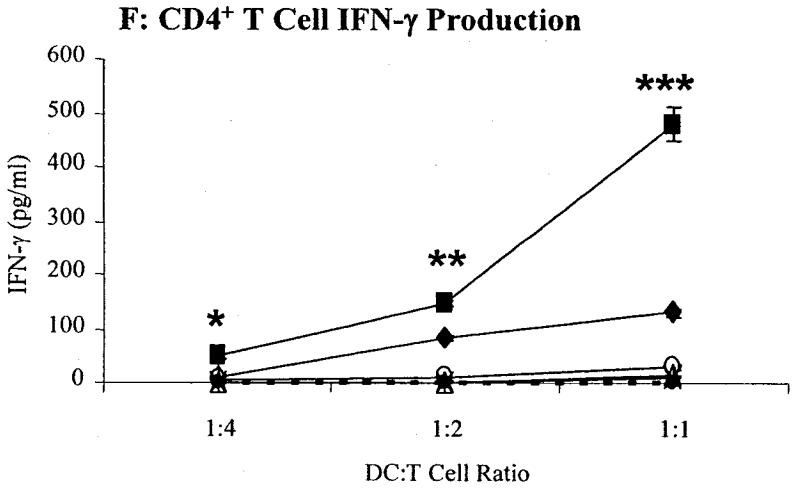

A
pRBC
nRBC
21.0
41.7
30.5
37.3
9.6
14.1
9.4
13.0
MHC-II
CD40
CD80
CD86
B
MFI
pRBC
nRBC
MHC Class II
CD40
CD80
CD86
C
Day 0
Day 5
CD40
CD80
CD86

Figure 6: Splenic $CD11c^+$ DCs exposed to pRBC but not to nRBC produce cytokines (*A-D*) and stimulate $CD4^+$ T cell proliferation (*E*) and IFN-γ production (*F*). $CD11c^+$ DCs were enriched from naïve B6 mice and pulsed overnight with pRBC or nRBC. Following pulsing and removal of noningested RBC, DCs were washed and incubated in medium alone, with nRBC or pRBC, or with freshly purified splenic $CD4^+$ T cells for 48 h at 37°C. DCs pulsed with pRBC produced significantly higher levels of IL-12p40 (*A*), bioactive IL-12p70 (*B*), IL-2 (*C*), and IL-10 (*D*) than DCs pulsed in medium alone or with nRBC as determined by ELISA. *E*, DCs pulsed with pRBC at ratios of 1:10 or 1:20 stimulated significantly higher levels of $CD4^+$ T cell proliferation than nonpulsed DCs or DCs pulsed with nRBC. *F*, DCs pulsed with pRBC stimulated significantly higher levels of IFN-γ production by $CD4^+$ T cells than nonpulsed DCs or DCs pulsed with nRBC. In *E* and *F*, $CD4^+$ T cells cultured with pRBC alone (1:10 ratio) were included as controls. Data are presented as mean ± SEM of 3-4 mice per group and are representative of three independent experiments. Statistical analyses in *A-D* compared pRBC vs. nRBC for each cytokine and in *E-F* compared DC + pRBC (1:20) vs. DC + nRBC (1:20) at each DC:T cell ratio (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Figure 6


A: IL-12p40
IL-12 p40 (ng/ml)
2
1.6
1.2
0.8
0.4
0
***
ND
ND
Medium
nRBC
pRBC
B: IL-12p70
IL-12 p70 (pg/ml)
200
160
120
80
40
0
***
ND
ND
Medium
nRBC
pRBC
C: IL-2
IL-2 (pg/ml)
160
120
80
40
0
***
Medium
nRBC
pRBC
D: IL-10
IL-10 (pg/ml)
500
400
300
200
100
0
***
Medium
nRBC
pRBC


E: CD4+ T Cell Proliferation
CPM
14000
12000
10000
8000
6000
4000
2000
0
DC + pRBC (1:20)
DC + pRBC (1:10)
DC + nRBC (1:20)
DC + nRBC (1:10)
Nonpulsed DC
T cell + pRBC
*
***
***
1:10
1:8
1:4
1:2
1:1
DC:T Cell Ratio


F: CD4+ T Cell IFN-γ Production
IFN-γ (pg/ml)
600
500
400
300
200
100
0
*
**
***
1:4
1:2
1:1
DC:T Cell Ratio

CHAPTER 4

DENDRITIC CELL AND NK CELL CROSSTALK PROMOTES EARLY IFN-γ PRODUCTION AND TH1 IMMUNE RESPONSES TO BLOOD-STAGE MALARIA INFECTION

Rebecca Ing and Mary M. Stevenson

Preface

While IL-15-dependent NK cells constitute one of the first non-specific defenses against invading pathogens, only DCs are able to prime naïve $CD4^+$ T cells and thus initiate adaptive immunity. Activation of NK cells by DCs is potentially a critical event during the innate immune response given that NK cells are major producers of IFN-γ that is critically required for protective immunity to blood-stage malaria. To better understand innate immune responses to blood-stage malaria, experiments described in Chapter 4 aimed to determine whether DCs interact with NK cells after *P. chabaudi* infection to achieve maximal activation of IFN-γ production and Th1 cell responses. An important aspect of this work involves further delineation of the effects of cytokines on DC responses and crosstalk with other immune cells. Therefore, we hypothesized that Th1-promoting cytokines, including IL-12 and IL-15, are critical components of DC responses to blood-stage malaria and are the principal mediators of interactions between DCs and NK cells or $CD4^+$ T cells, leading to development of Th1-type immunity.

Abstract

A Th1-associated innate immune response to blood-stage malaria has been shown to be critical for shaping IFN-γ-dependent adaptive immune responses required for effective control and rapid resolution of infection. In this study, we used a murine model of blood-stage malaria infection to demonstrate that malaria-activated DCs stimulate resting NK cells to secrete high levels of IFN-γ and, reciprocally, activated NK cells induce DC maturation and cytokine production. DCs matured as a result of NK cell stimulation were able to prime $CD4^+$ T cells to proliferate and produce IFN-γ, leading to Th1 adaptive immune responses. DC-mediated activation of NK cell responses was dependent on cell-cell contact as well as IL-2 and IL-12, whereas maximal induction of Th1-type DC responses by NK cells was mediated jointly by cell-cell contact and release of IFN-γ. Therefore, the reciprocal interaction of DCs and NK cells is a critical event in the early innate immune response to blood-stage malaria infection that promotes early IFN-γ production and the induction of protective Th1-associated immune responses.

Introduction

Malaria, caused by *Plasmodium* parasites, is the most important parasitic disease in humans. In the mouse model of *P. chabaudi* AS infection, resistance to blood-stage malaria requires Th1-dependent cellular and antibody responses (8, 400, 420). Extensive research has emphasized the critical role of innate immunity in the polarization of $CD4^+$ Th cells leading to adaptive immunity. During the innate immune response to *P. chabaudi* malaria, infected RBC directly interact with DCs, NK cells, and monocytes/macrophages, inducing their maturation, differentiation and activation of effector functions, particularly the production of immunoregulatory cytokines (139, 365, 392). These early events in host-parasite interactions and the resulting cytokine balance shape adaptive immune responses and determine the course and outcome of infection, including peak parasitemia, parasite clearance, and host survival. To this end, the adaptive immune responses associated with effective control and rapid resolution of blood-stage malaria involve IFN-γ-producing Th1 cells and B cells secreting Th1-associated antibodies (8, 420).

As sentinels of infection, DCs and NK cells perform distinct but mutually dependent functions in the innate immune response to invading pathogens. Early following blood-stage malaria infection, both cell types rapidly accumulate in the spleen, wherein they are primed to undergo maturation and functional activation by infected RBC that were deposited in the spleen for removal (139, 365). DCs are the principal cells responsible for presenting malarial antigens and inducing innate and adaptive immune responses in part through the release of several cytokines including IL-2, IL-12, IL-15, and IFN-γ (364, 366, 519). Activated NK cells lyse target cells and produce proinflammatory cytokines such as IFN-γ and TNF-α, thereby helping to control parasite replication and promote Th1 polarization (139, 379, 512). Although multiple cytokines may result in Th1 cell development, IFN-γ is considered to be the pivotal cytokine mediating protective Th1-type immunity to blood-stage malaria (8, 400). Early production of IFN-γ is a decisive event in the innate immune response to blood-stage malaria because IFN-γ: 1) activates nonopsonic phagocytosis and NK cell-mediated lysis of infected RBC (8, 365); 2) enhances the production of IL-12 by DCs primed by pathogen-derived products, further driving Th1 polarization (260, 520); and

3) synergizes with TCR signals to induce the transcription factor T-bet which regulates both the expression of the IL-12R on naïve T cells and the differentiation of Th1 cells (521). Thus, a better understanding of the mechanisms that maximally promote early IFN-γ production would help identify important targets for the development of effective malaria vaccines.

Recent studies show that crosstalk between DCs and NK cells results in reciprocal activation (323, 381). After exposure to pathogen-derived antigens, DCs mature and migrate to secondary lymphoid tissues where they interact with other immune cells to initiate specific effector cell responses. DCs have been shown to co-localize with NK cells in lymph nodes during infection or antigen challenge (163, 522). Given that NK cells are one of the main producers of IFN-γ in the early immune response to *P. chabaudi* infection, DC-mediated activation of NK cells might be a critical mechanism in triggering Th1 polarization during malaria. Conversely, NK cells may promote the development of Th1-type adaptive immune responses by inducing DC maturation and the production of IL-12. It is not known whether DCs and NK cells interact during the innate immune response to blood-stage malaria, leading to induction of Th1-type responses. It also remains to be determined which cytokines play an important role in supporting crosstalk between DCs and NK cells during the early immune response to blood-stage malaria infection.

Previous studies revealed that IL-12 is a key factor in the development of Th1-type immune responses to blood-stage malaria, mainly due to its potent ability to induce IFN-γ production and Th1 cell differentiation (8, 415, 420). IL-12 production and up-regulated expression of IL-12Rβ2, the receptor subunit mediating IL-12p70 signaling, correlate with resistance to *P. chabaudi* malaria (388, 415). Moreover, IL-12p40$^{-/-}$ mice are susceptible to *P. chabaudi* malaria as indicated by higher levels of mortality and peak parasitemia as well as impaired Th1-type cellular and antibody responses compared to IL-12-sufficient mice (420). IL-15, on the other hand, is important for NK cell survival and proliferation during both homeostasis and the immune response to infection (204, 512). It has been shown that IL-15 synergizes with IL-12 to promote IFN-γ production and Th1 cell development (211, 214), but IL-12 and IL-18 are more effective than IL-15 alone in activating NK cell effector functions (138). Importantly,

IL-15$^{-/-}$ mice show higher parasitemia than wild-type mice only during the chronic stage of infection (512), suggesting that IL-15 likely plays a supporting but limited role in protective immunity to blood-stage malaria. Given the importance of DC-derived IL-12 for the induction of Th1-type immunity and IL-15 for NK cell development, we hypothesized that IL-12 and IL-15 contribute distinct functions in DC responses and interactions with NK cells during blood-stage malaria infection.

In this study, we show that malaria-activated DCs stimulated resting NK cells to secrete high levels of IFN-γ and, reciprocally, activated NK cells induced DCs to mature, produce cytokines, and prime CD4$^+$ T cells to proliferate and release IFN-γ. IL-12 but not IL-15 was required for DC maturation and cytokine production following *P. chabaudi* infection. Moreover, both IL-2 and IL-12 were found to play a critical role in supporting DC-mediated activation of NK cells. Malaria-activated NK cells promoted DC maturation and Th1 cell development in part through the release of soluble mediators, particularly IFN-γ. These results provide novel evidence of a reciprocally activating crosstalk between DCs and NK cells during the early immune response to blood-stage malaria that leads to the induction of protective Th1 adaptive immunity.

Materials and Methods

Mice, parasite, and pRBC purification

IL-12p40$^{-/-}$ (p40$^{-/-}$), IL-15$^{-/-}$ and IFN-$\gamma^{-/-}$ mice, on the C57BL/6 (B6) background, were bred and maintained at the Montreal General Hospital Research Institute (QC, Canada) in accordance with the guidelines of the Canadian Council on Animal Care. IL-15$^{-/-}$ breeder pairs were kindly provided by Dr. Jacques Peschon (Amgen). Age-matched B6 mice (Charles River Laboratories) were used as wild-type (WT) controls. Female mice, aged 8-14 wk, were used in all experiments. *P. chabaudi* AS was maintained as previously described (415) and infections were initiated by i.p. injection of 10^6 pRBC. Purified pRBC and nRBC were obtained using blood from *P. chabaudi*-infected or naïve B6 mice, respectively, as previously described (392, 519). Purified pRBC contained >96% infected RBC.

Purification of CD11c$^+$, DX5$^+$, and CD4$^+$ cells from spleen

Spleen cells were separated using Nycoprep (Axis-Shield) density gradients. Low-density cells at the interphase were collected, washed twice with PBS, and purified by positive selection using anti-CD11c microbeads (Miltenyi Biotec). The resulting DCs were routinely ≥85-90% positive for CD11c as determined by flow cytometry. Spleen cells were enriched for DX5$^+$ NK cells and CD4$^+$ T cells using anti-DX5 and anti-CD4 microbeads (Miltenyi Biotec), respectively, and were ≥85-90% positive for these populations.

DC uptake assay and flow cytometric analysis

The DC uptake assay was performed as recently described (519). Briefly, pRBC or nRBC (10^7 cells/ml) were stained with 2 mM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) in medium consisting of RPMI 1640 (Gibco-Invitrogen), 5% FCS (HyClone Laboratories), 10 mM HEPES (Gibco-Invitrogen), 20 μg/ml gentamicin (Sabex), 2 mM L-glutamine (Gibco-Invitrogen), and 0.5 μM β-mercaptoethanol (Sigma-Aldrich) for 15 min at 37°C. CD11c$^+$ DCs (10^6/well) were seeded with CFSE-labeled pRBC or nRBC in a 1:20 ratio at 200 μl final volume for 4 h at 37°C. After co-

culture, noningested RBC were lysed and DCs were washed, FcR-blocked with anti-CD16/CD32 mAb (2.4G2; BD Biosciences), and stained with PE-labeled anti-CD11c mAb (HL3; BD Biosciences). Uptake was determined by gating cells on SSC versus FL2 for the $CD11c^+$ population and analyzing CFSE staining on FL1 using FACSCalibur equipped with CellQuest Pro software (BD Biosciences).

Splenic DC maturation and cytokine production

Splenic $CD11c^+$ DCs were FcR-blocked and stained with PE-labeled mAb (BD Biosciences) to CD11c, CD40 (2/23), CD80 (16-1041), or CD86 (GL-1). The percentages and mean fluorescence intensity (MFI) of gated $CD11c^+$ cells expressing the above markers were determined by flow cytometry. Supernatants of DCs (10^6/well) incubated in medium alone or with pRBC (1:10 ratio) for 48 h at 37°C were analyzed for cytokine production by ELISA as previously described (400, 420).

DC and NK cell co-cultures

To determine the effect of malaria-activated DCs on resting NK cells, $CD11c^+$ DC purified from spleens of day 5 infected mice were co-cultured with $DX5^+$ NK cells from naïve mice. DCs (10^6/well) were plated with NK cells (10^6/well) in 96-well flat-bottomed plates fitted with a transwell insert (Nalge Nunc) for 24-36 h at 37°C. Single cultures of DCs or NK cells were included as controls. For co-cultures, NK cells (10^6 cells/160 µl) were cultured in the lower wells and DCs (10^6 cells/60 µl) were added either together with the NK cells in the lower wells or separately in the upper compartments of the transwells. In some experiments, co-cultures were stimulated with 20 ng/ml rIL-2 (R&D Systems) or 100 ng/ml rIL-15 (Amgen), or DCs were pre-treated with 250 ng/ml anti-IL-2 mAb (JES6-1A12; R&D Systems) or 250 ng/ml anti-IL-15 mAb (34593.11; Abcam) for 4 h prior to co-culture with NK cells.

To determine the effect of malaria-activated NK cells on resting DCs, $DX5^+$ NK cells enriched from day 5 infected mice were co-cultured with $CD11c^+$ DCs from naïve mice. DCs (10^6/well) were plated with NK cells (10^6/well) in 96-well flat-bottomed plates for 18-24 h at 37°C. Supernatants were analyzed for cytokine levels by ELISA and the cells were stained for $CD11c^+$ and costimulatory molecules as described above.

For T cell assays, DC (2 x 10^5/250 µl) and NK cells (2 x 10^5/250 µl) were co-cultured in 48-well plates for 18-24 h at 37°C. Later, splenic $CD4^+$ T cells (2 x 10^6/500 µl) freshly purified from naïve mice were added to the DC-NK cell co-cultures as described (163). After 2 days, fresh medium containing rIL-2 (10 ng/ml; R&D Systems) was added and the plates were further incubated for 72 h at 37°C. Cells were then collected and 200 µl of 5 x 10^5/ml was plated for 48 h at 37°C in 96-well flat-bottomed plates coated with 5 µg/ml anti-CD3e mAb (145-2C11; BD Biosciences). Proliferation was determined by incorporation of [^{3}H]-thymidine during the last 16 h of incubation. Supernatants were analyzed for cytokine levels by ELISA.

Intracellular IFN-γ expression

Cultured cells were stimulated in vitro with Golgi Stop™ (BD Biosciences), 10 ng/ml of PMA (Sigma) and 250 ng/ml of ionomycin (Sigma) for 2 h, then washed, FcR-blocked, and stained with FITC-conjugated anti-CD11c (BD Biosciences) and/or PE-conjugated anti-CD49b (DX5; BD Biosciences) mAb. Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and stained with APC-conjugated anti-IFN-γ mAb (XMG1.2; BD Biosciences). Cells were gated on the $CD11c^+$ (FL1) or $DX5^+$ (FL2) population and IFN-γ signals on FL3 were generated from the gated cells.

Statistical analyses

Data are expressed as means ± SEM. Statistical significance of differences between two groups was analyzed by two-tailed, unpaired Student's *t* test and multiple comparisons were analyzed using ANOVA. Statistical significance was defined as follows: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$. All statistical analyses were performed using SAS/STAT software (SAS Institute).

Results

IL-12 but not IL-15 is required for optimal DC responses to blood-stage malaria infection

To determine DC responses to blood-stage malaria, we analyzed three components, antigen uptake, co-stimulatory molecule expression and cytokine production, thought to be necessary for effective antigen presentation by DCs. Using our newly developed flow cytometric technique (519), we measured the ability of DCs to capture pRBC by DCs. Purified pRBC and nRBC were labeled with CFSE and then seeded with splenic $CD11c^+$ DCs enriched from naïve and infected mice. Following 4 h incubation, DCs were analysed for expression of CFSE by flow cytometry. As previously reported (519), DCs captured pRBC at higher levels compared to nRBC and this preferential uptake of pRBC was selectively enhanced following infection in vivo (Fig. 1*A* and *B*). Given the different susceptibilities to *P. chabaudi* infection among WT, IL-$15^{-/-}$ and IL-$12p40^{-/-}$ mice (420, 512), we questioned whether these cytokines were important for the ability of DCs to discriminate between pRBC and nRBC and preferentially take up pRBC. As shown in Fig. 1A and B, DCs from IL-$15^{-/-}$ and IL-$12p40^{-/-}$ mice were able to capture pRBC as efficiently as WT DCs before and after *P. chabaudi* infection, indicating that recognition and capture of malarial antigens expressed by infected RBC were not impaired in these cytokine-deficient mice.

To determine whether IL-12 or IL-15 is necessary for DC maturation during malaria, we also analyzed the ex vivo expression of costimulatory molecules by splenic $CD11c^+$ DCs from naïve and infected mice. For WT and IL-$15^{-/-}$ mice, DC expression of CD40, CD80, and CD86 increased following infection (Fig. 1*C*). A deficiency of IL-12p40, but not of IL-15, resulted in lower increases in CD40 and CD86 expression after infection. Therefore, IL-12 but not IL-15 was required for upregulated DC maturation following *P. chabaudi* infection.

DCs have been shown to produce immunoregulatory cytokines in response to blood-stage malaria (362, 366, 392, 512). In this study, splenic $CD11c^+$ DCs from infected WT mice secreted higher levels of cytokines when stimulated in vitro with pRBC than with medium (Fig. 1*D*). WT DCs produced higher levels of IFN-γ than did

IL-15$^{-/-}$ DCs despite equivalent IL-12p70 secretion, a finding that is consistent with results reported previously (512). Importantly, in response to pRBC, IL-12p40$^{-/-}$ DCs produced more IL-2 but less IFN-γ than did WT or IL-15$^{-/-}$ DCs. These results suggest that IL-12 but not IL-15 is essential for pRBC-induced IFN-γ secretion by splenic DCs during blood-stage malaria. IL-10 secretion was enhanced by pRBC stimulation but the levels of production did not differ among the groups. Therefore, despite a normal capacity for pRBC uptake, splenic DCs from malaria-infected IL-12p40$^{-/-}$ mice exhibited lower costimulatory molecule expression and profoundly impaired IFN-γ production.

Malaria-activated DCs stimulate IFN-γ production by NK cells

Given the disparate capacities for DC maturation and cytokine production among WT, IL-15$^{-/-}$ and IL-12p40$^{-/-}$ mice, we questioned the relative contributions of IL-15 and IL-12 to DC and NK cell interactions during the early immune response to blood-stage malaria. To this end, we devised a co-culture system to determine whether DCs from malaria-infected mice induced resting NK cells to produce IFN-γ. Splenic CD11c^{+} DCs were enriched from infected WT mice and co-cultured with splenic NK cells from naïve WT mice. Prior to co-culture, few DCs or NK cells expressed IFN-γ (Fig. 2*A*, *left*). The percentage of DCs expressing IFN-γ increased after co-culture with NK cells compared to DCs cultured alone (Fig. 2*A*, *top right*). Notably, after co-culture with DCs, the percentage of NK cells expressing IFN-γ increased markedly and to a greater extent than that observed in DCs (78.6% vs. 26.4%; Fig. 2*A*, *lower right*), suggesting that NK cells are the major source of IFN-γ in the co-cultures. These results were confirmed in experiments using DCs and NK cells from WT and IFN-γ$^{-/-}$ mice, which showed considerably lower IFN-γ production in co-cultures of WT DCs with IFN-γ$^{-/-}$ NK cells than with WT NK cells (data not shown). Resting DCs from naïve WT mice stimulated lower IFN-γ production in the co-cultures than did malaria-activated DCs from infected WT mice (Fig. 2*B*). Moreover, DC interactions with NK cells were partially dependent on cell-cell contact as demonstrated by the lower production of IFN-γ by NK cells co-cultured with activated DCs in wells equipped with a transwell insert to prevent contact

between the two cell types (Fig. 2*B*). Taken together, these results show that DCs from malaria-infected mice stimulated resting NK cells to produce IFN-γ. Although previous studies suggest that DCs can elicit TNF-α secretion from NK cells (159, 160), very low to no TNF-α production was observed in the DC-NK cell co-cultures (data not shown).

IL-15 is required for NK cell responses but not for DC-mediated stimulation of NK cells

Malaria-infected IL-15$^{-/-}$ mice have severe deficiencies in splenic NK cell expansion and effector function and show impaired DC cytokine production in vitro (512). To determine whether IL-15 contributes to DC–NK-cell crosstalk, DCs from infected WT and IL-15$^{-/-}$ mice were paired and co-cultured in four combinations with NK cells from naïve WT and IL-15$^{-/-}$ mice. NK cells in the syngeneic WT co-cultures showed the highest level (67.5%) of intracellular IFN-γ expression (Fig. 3*A*, *upper left*) and the lowest level (11.2%) in the syngeneic IL-15$^{-/-}$ co-cultures (Fig. 3*A*, *lower right*). Co-culture with WT DCs increased IFN-γ production by IL-15$^{-/-}$ NK cells (19.4% vs. 11.2%; Fig. 3*A*, *upper right*). Notably, IL-15$^{-/-}$ DCs stimulated WT NK cells to produce IFN-γ, albeit to a lesser extent than did WT DCs (52.3% vs. 67.5%; Fig. 3*A*, *lower left*). These results were corroborated by differences in the levels of IFN-γ protein among the various DC and NK cell combinations (Fig. 3*B*). Together, these results suggest that the function of NK cells, but not of DCs, is the principal defect in IL-15$^{-/-}$ mice. In addition to IL-15, DC-derived IL-2 has been shown to be important for DC–NK-cell crosstalk (147). We hypothesized that IL-15$^{-/-}$ DCs are able to stimulate WT NK cells due in part to the compensatory release of IL-2. Although splenic DCs from malaria-infected mice produced very low levels of IL-2 (Fig. 1*D*), the possibility that minute amounts of IL-2 influenced the interactions between DCs and NK cells could not be excluded. Indeed, the addition of anti-IL-2 mAb reduced IFN-γ production in co-cultures of WT NK cells with either WT or IL-15$^{-/-}$ DCs (Fig. 3*C*). These results suggest that while IL-15 may enhance DC-mediated NK cell activation, its main function is likely to support optimal NK cell survival and function as previously reported (512). Furthermore, secretion of IL-2 even at low levels may partially account for the ability of malaria-activated IL-15$^{-/-}$ DCs to stimulate WT NK cells to produce IFN-γ.

IL-2 is more critical than IL-15 in the ability of DCs to elicit IFN-γ production from NK cells

To delineate the relative contributions of IL-2 and IL-15 to DC–NK-cell crosstalk during malaria, DCs from infected mice were co-cultured with resting NK cells in the presence of rIL-2 or rIL-15 with the addition of either anti-IL-15 or anti-IL-2 mAb. The levels of IFN-γ in co-cultures stimulated with rIL-15 (Fig. 4*A*) or rIL-2 (Fig. 4*B*) were higher than those observed in the unstimulated co-cultures. The addition of anti-IL-2 mAb decreased IFN-γ secretion in the co-cultures even in the presence of rIL-15 (Fig. 4*A*). In contrast, the addition of anti-IL-15 mAb did not modulate IFN-γ levels in the IL-2-stimulated co-cultures although it decreased IFN-γ production in the unstimulated co-cultures (Fig. 4*B*). From these data, we concluded that IL-2 is more important than IL-15 for malaria-activated DCs to elicit IFN-γ production by NK cells.

Endogenous IL-12 is required for both DC and NK cell function

Blocking IL-2 decreased but did not completely abolish IFN-γ production in the DC–NK-cell co-cultures (Fig. 3*C* and 4*A*), suggesting that other IFN-γ-promoting cytokines might be involved in NK cell stimulation by malaria-activated DCs. Previous studies demonstrated that IL-12 mediates interactions between DCs and NK cells, leading to abundant IFN-γ production in response to tumor cells (146) or microbial stimuli (152, 323). To determine the role of IL-12 in DC–NK-cell crosstalk during blood-stage malaria, DCs and NK cells from WT and IL-12p40$^{-/-}$ mice were paired in four combinations as described above. Similar to results presented in Fig. 3, the highest level (75.6%) of intracellular IFN-γ expression by NK cells was observed in the syngeneic WT co-cultures (Fig. 5*A*, *upper left*) and the lowest level (21.8%) in the syngeneic IL-12p40$^{-/-}$ co-cultures (Fig. 5*A*, *lower right*). However, WT DCs failed to stimulate IL-12p40$^{-/-}$ NK cells to express IFN-γ above the level observed in the syngeneic IL-12p40$^{-/-}$ co-cultures (Fig. 5*A*, *upper right*) and IL-12p40$^{-/-}$ DCs were unable to activate WT NK cells (Fig. 5*A*, *lower left*). The impaired intracellular IFN-γ expression in co-cultures with IL-12p40$^{-/-}$ DCs and/or NK cells was confirmed by the

low levels of IFN-γ protein secreted (Fig. 5*B*). These results suggest that IL-12p40$^{-/-}$ mice have defects in both DC and NK cell functions following malaria infection.

Consistent with the results obtained with IL-15$^{-/-}$ cells (Fig. 3), the addition of anti-IL-2 mAb decreased IFN-γ production in co-cultures of WT NK cells with either WT or IL-12p40$^{-/-}$ DCs (Fig. 5*C*). Importantly, IFN-γ levels observed in co-cultures of IL-12p40$^{-/-}$ DCs and WT NK cells treated with anti-IL-2 mAb (Fig. 5*C*) were as low as those in syngeneic IL-12p40$^{-/-}$ co-cultures (Fig. 5*B*). Together these data indicate that IL-2 and IL-12, both produced by DCs during the innate immune response, were required for maximal stimulation of resting NK cells by malaria-activated DCs.

Malaria-activated NK cells induce DC maturation and cytokine production

Previous studies showed that NK cells activated by tumor antigens, bacterial products or IL-2 are capable of inducing DCs to mature and produce Th1-promoting IL-12 (163, 323, 523). To examine whether malaria-activated NK cells elicited similar DC responses, splenic NK cells from infected mice were co-cultured with DCs from naïve mice. In preliminary studies, we observed that co-culture of malaria-activated NK cells with resting DCs resulted in reduced recovery of DCs (data not shown), a finding that is consistent with published reports that immature DCs are susceptible to NK cell lysis (145, 524). However, more DCs were recovered when DCs were pre-incubated with purified pRBC for 1 h prior to co-culture with activated NK cells (data not shown) and therefore pRBC-treated DCs were used in subsequent experiments.

DCs cultured with malaria-activated NK cells showed upregulated CD40 and CD86 expression (Fig. 6*B*) compared to DCs cultured alone or with resting NK cells. DCs co-cultured with anti-IFN-γ mAb or with IFN-γ$^{-/-}$ NK cells were equally able to undergo maturation in vitro. Although the percentages of co-cultured DCs expressing each maturation marker were comparable (Fig. 6*A*), DCs co-cultured with malaria-activated NK cells in transwells expressed lower MFI levels of CD40 and CD86 (Fig. 6*B*), suggesting that DC–NK-cell contact but not soluble factors supported maximal NK cell-induced DC maturation.

Malaria-activated NK cells also stimulated DCs to secrete higher levels of IL-12p40, IL-12p70, IL-2, and IL-10 than those observed in DCs cultured alone (Fig. 6*C*).

Importantly, however, DCs co-cultured with WT NK cells and anti-IFN-γ mAb secreted less IL-12p40 and IL-12p70 but more IL-10 than did DCs co-cultured with WT NK cells. Similar results were obtained with DCs co-cultured with malaria-activated IFN-$\gamma^{-/-}$ NK cells, demonstrating that NK cell-derived IFN-γ is required for enhanced IL-12 production. DCs cultured in wells separated from NK cells by a transwell insert showed similar levels of cytokine production as the co-cultures, suggesting that soluble factors released from NK cells are capable of inducing DC cytokine production. Taken together, these results suggest that cell-cell contact and soluble mediators, particularly IFN-γ, act in concert to promote optimal DC activation by malaria-activated NK cells, leading to phenotypic maturation and IL-12 production.

DCs activated by malaria-primed NK cells are able to induce Th1 cell development

Based on our findings that malaria-activated NK cells stimulated resting DC to upregulate costimulatory molecule expression and secrete Th1-promoting cytokines, we further investigated whether DCs matured and capable of producing cytokines as a result of interaction with NK cells could prime $CD4^+$ T cell responses. Using a modified protocol (163), $CD4^+$ T cells from naïve WT mice were added to co-cultures of malaria-activated NK cells and resting DCs, and these cells were then transferred to wells coated with anti-CD3 mAb for analysis of T cell-specific proliferation and cytokine responses. Control cultures of anti-CD3-stimulated DCs and NK cells alone did not proliferate nor produce cytokines above background levels (data not shown), and T cells cultured alone or with either DCs or NK cells proliferated and secreted cytokines at significantly lower levels than did T cells cultured with NK cell-activated DCs (Fig. 7*A* and *B*). DCs activated by WT NK cells from infected mice stimulated higher levels of T cell proliferation than did DCs activated by IFN-$\gamma^{-/-}$ NK cells (Fig. 7*A*). Importantly, DCs activated by IFN-$\gamma^{-/-}$ NK cells stimulated T cells to produce significantly lower levels IFN-γ but higher levels of both IL-4 and IL-10 compared to DCs activated by WT NK cells (Fig. 7*B*). Thus, malaria-activated NK cells stimulated DCs to induce T cell proliferation and Th1 cell development through an IFN-γ-dependent mechanism.

Discussion

DCs and NK cells contribute distinct regulatory and effector functions to innate resistance to blood-stage malaria infection (139, 366, 379, 512, 519). Recent studies have revealed that DCs and NK cells not only activate innate and adaptive immune responses but also regulate these responses through cellular crosstalk (381). In the present study, we show that splenic DCs and NK cells interact during the early immune response to blood-stage malaria, resulting in reciprocal activation and potent IFN-γ production. Early after *P. chabaudi* infection, splenic DCs were observed to selectively capture infected RBC, mature and produce immunoregulatory cytokines, such as IL-2, IL-12, IL-10, and IFN-γ. Malaria-activated DCs elicited high levels of IFN-γ production by resting NK cells in a mechanism that required IL-2 and IL-12 for DC-mediated activation and IL-15 for optimal NK cell function. Reciprocally, malaria-activated NK cells stimulated DCs to mature, secrete IL-12, and prime resting $CD4^+$ T cells to proliferate and secrete Th1-polarizing cytokines. The ability of NK cells to trigger DCs to produce IL-12 and promote IFN-γ-secreting $CD4^+$ T cells was dependent on NK cell-derived IFN-γ production. These results show for the first time the important interactions between DCs and NK cells during early blood-stage malaria infection and define the cytokines involved in the reciprocal activation of these cell types, leading to the induction of protective Th1 adaptive immune responses.

DCs play an important role in the initiation of immune responses early after blood-stage malaria infection. The ability of splenic DCs to upregulate costimulatory molecule expression and produce immunoregulatory cytokines, as shown here, are in agreement with previous studies which concluded that DCs are phenotypically and functionally mature during early blood-stage malaria infection (362, 364-366, 369, 519). DCs activate and shape immune responses in part through the production of cytokines and, in turn, DC responses to invading pathogens are regulated by a complex cytokine network. To this end, IL-12 activates DC responses required for priming Th1 cell development (525), while IL-15 promotes IL-12 production and synergizes with IL-12 to increase Th1-type cytokine production by DCs and NK cells (138, 211, 214). Also, IL-2 activates NK cells and induces IFN-γ synthesis (147, 154), and its production by DCs in response to inflammatory stimuli is critically regulated by IL-15 (526). This early

cytokine cascade results in the production of IFN-γ, a key factor mediating protective immunity to blood-stage malaria (400). IFN-γ produced during the innate response activates APCs (520) and anti-malarial cytolytic mechanisms (139, 392), enhances IL-12 synthesis (260, 520), and promotes Th1 cell development (525).

Earlier work in our laboratory showed that IL-12p40$^{-/-}$ and IL-15$^{-/-}$ mice differ in their capacity to control and survive an acute *P. chabaudi* infection (420, 512). Since IL-12p40$^{-/-}$ mice are generally more susceptible than IL-15$^{-/-}$ mice, we hypothesized that IL-12 and IL-15 contribute differently to DC responses to blood-stage malaria. Results shown here indicate that IL-12, but not IL-15, is necessary for optimal DC maturation and Th1-type cytokine production following *P. chabaudi* infection. The impaired DC functions observed in IL-12p40$^{-/-}$ mice were not related to differences in uptake of malarial antigens because DCs from IL-12p40$^{-/-}$ and IL-15$^{-/-}$ mice were equally able to preferentially capture infected RBC both before and after infection. IFN-γ production was severely impaired in IL-12p40$^{-/-}$ DCs compared to WT DCs, consistent with the well-documented role of IL-12 in stimulating IFN-γ production by APCs (262). Given that an early burst of IFN-γ primes DCs for both high IL-12 production and strong Th1-polarizing capacity (260, 520) and that IL-12 stimulates NK cells to produce abundant IFN-γ and, in a positive feedback loop, to induce further DC maturation (527), defects in IFN-γ and IL-12 production by DCs may impair the activation of downstream innate and adaptive immune responses to blood-stage malaria infection.

Since DCs have been shown to activate NK cells, we investigated whether DCs from malaria-infected mice are able to stimulate NK cells to produce IFN-γ and determined the relative contributions of DC-derived cytokines, namely IL-12, IL-15 and IL-2, to DC–NK-cell interactions. Upon capture of pathogen-derived antigens, DCs secrete chemokines to recruit circulating NK cells to inflamed tissues or draining lymph nodes (522). Indeed, both DCs and NK cells colocalize in human lymph nodes under steady-state conditions, providing evidence for their interaction in vivo (522). The specific mechanisms by which DCs activate NK cells are under intense investigation. DCs prime resting NK cells through cell-cell contact (159, 160) and release of soluble mediators, such as type 1 IFN, IL-2, IL-12, IL-15, and IL-18 (146-148, 151, 323). Some cytokines, namely IL-2 and IL-15, act as potent growth factors for NK cell

development and expansion. After their recruitment to secondary lymphoid organs, DCs and T cells secrete IL-2, which induces colocalized NK cells to produce IFN-γ and mediate effective antimicrobial and antitumor activity (522, 528). Indeed, DC-derived IL-2 is required for activation of NK cell lysis and IFN-γ production in response to bacteria and tumor cells (147). Furthermore, DC-derived IL-12, IL-15 and IL-18 have been shown to contribute significantly to DC-mediated activation of NK cells (151, 152, 323). Initially, it was difficult to reconcile the need for both cell-cell contact and cytokines for DC-mediated NK cell activation. Recent studies, however, showed that IL-12 is mobilized to the DC surface and presented to NK cells via an immunostimulatory synapse (150), DC-derived IL-15 is presented in trans to NK cells via DC-bound IL-15Rα (152, 153), and IL-18 secretion is confined to the DC–NK-cell synaptic cleft (151). Although we did not study the role of IL-18 in DC–NK-cell crosstalk during *P. chabaudi* infection, IL-18 has been shown to be important for optimal IFN-γ production during infections with rodent *Plasmodium yoelii* or *Plasmodium berghei* (398). NK cells can trigger immature DCs to release IL-18 (151), which in turn activates a subset of effector NK cells that support the ability of DCs to produce IL-12 and promote Th1 cell differentiation (149). However, in contrast to the IFN-γ-secreting NK cells induced by malaria-activated DCs in vitro as shown in this study or by *P. chabaudi* infection in vivo (139, 512), the IL-18-primed NK cells produce very low levels of IFN-γ upon interaction with DCs (149).

Previous studies have reported conflicting findings regarding the relative contributions of IL-2, IL-12 and IL-15 to DC–NK-cell crosstalk. Although most studies agree that DC-derived cytokines help mediate DC activation of NK cells, the results are inconclusive as to which factor(s) are required for these interactions (147, 152, 153, 381). These differences may be explained in part by the use of disparate stimulants, in vitro culture systems, and cells of human versus murine origin. In this study, we show that splenic DCs from malaria-infected mice were able to stimulate resting NK cells to produce high levels of IFN-γ in a mechanism that critically required IL-2 and IL-12 for optimal DC-mediated activation of NK cells. Conversely, IL-15 may enhance DC-mediated NK cell activation but was mainly involved in supporting NK cell survival and function, as previously reported (204, 512). Our results show that IL-15$^{-/-}$ DCs have

normal IL-12 production during blood-stage malaria and that both IL-2 and IL-12 may act to compensate for IL-15 deficiency, thus enabling malaria-primed IL-$15^{-/-}$ DCs to activate NK cells. In contrast, IL-$12p40^{-/-}$ NK cells were not responsive to stimulation with malaria-activated WT DCs, suggesting that endogenous IL-12 deficiency may impair both NK cell function directly and/or indirectly through failing to support DC-mediated NK cell activation in vivo. The importance of IL-12, but not IL-15, for DC functions and DC–NK-cell crosstalk correlates with the greater susceptibility of IL-$12p40^{-/-}$ mice to blood-stage malaria compared to WT and IL-$15^{-/-}$ mice. These results corroborate previous studies regarding the ability of DCs to activate NK cells via cell-cell contact and DC-derived cytokines, and further extend these observations by providing novel evidence for DC–NK-cell interaction in the context of an intracellular parasitic infection. Although our in vitro culture system cannot fully replicate cellular events occurring in vivo, the use of DCs and NK cells isolated from naïve or malaria-infected mice may help identify innate mechanisms that contribute to protective immune responses to malaria infection in vivo.

NK cells provide an early line of defense against invading pathogens through their ability to lyse infected cells and secrete large quantities of IFN-γ. Notably, NK cell IFN-γ production, but not cytotoxicity, plays a major role in mediating protective immunity to *P. chabaudi* infection (139). Recent evidence suggests that NK cells are important not only in early innate resistance to invading pathogens but also in promoting an effective immune response, particularly Th1-type responses. Activated NK cells induce DC maturation and cytokine production through a mechanism that is dependent on cell-cell contact and, to a lesser extent, on TNF-α and IFN-γ secreted by NK cells (159, 160, 163). While it is unclear how TNF-α acts to stimulate DCs, NK cell-derived IFN-γ has been shown to upregulate expression of the DC costimulatory molecule, inducible T cell antigen (4-IBB), which promotes autocrine or paracrine DC expansion, maturation and migration (161). In this manner, NK cells may provide contact-dependent and soluble signals for Th1-type polarization of DCs during the early stages of malaria infection. Following activation by NK cells at the site of infection, DCs migrate to the lymph nodes or spleen and carry NK cell-derived signals for the development of Th1 cell responses (163). In the present study, we showed that splenic NK cells from

malaria-infected mice were able to stimulate DCs to upregulate expression of the maturation markers, CD40 and CD86, and to secrete IL-2 and IL-12. Direct contact between DCs and NK cells contributed to optimal NK cell-induced DC maturation, while NK cell-derived soluble factors, namely IFN-γ, were required for the release of IL-12 by DCs. These findings are consistent with a previous study showing that soluble factors released by virally-activated NK cells induce DC maturation and enhance IL-12p70 production (163). Importantly, in this study, malaria-activated IFN-$\gamma^{-/-}$ NK cells not only failed to induce high IL-12 production by DCs but also promoted secretion of Th1-suppressing IL-10. We cannot exclude a possible contribution of TNF-α in NK cell-mediated DC maturation (159, 160, 162), although previous studies have shown that TNF-α activity is not required for early protective Th1 responses against a primary *P. chabaudi* infection (479, 480). Nevertheless, our results provide strong evidence supporting the notion that NK cells play a critical immunoregulatory role in shaping the phenotype of DC responses and consequently influence the development of adaptive immune responses. Indeed, $CD4^+$ T cells primed by co-cultures of DCs and malaria-activated WT NK cells showed high proliferative responses and preferential IFN-γ production. In contrast, DCs stimulated by malaria-activated IFN-$\gamma^{-/-}$ NK cells primed $CD4^+$ T cells to produce lower levels of IFN-γ but higher levels of IL-4 and IL-10. Taken together, our data suggest that NK cells may play an important role in regulating anti-malarial immunity by inducing DC maturation and IL-12 production, leading to efficient priming of Th1 cell expansion and differentiation. Experiments are in progress in our laboratory to evaluate whether DC–NK-cell crosstalk benefits the control of blood-stage malaria in vivo.

Based on our results, we propose that splenic DCs and NK cells interact in the early phase of blood-stage malaria infection to induce reciprocal activation and abundant IFN-γ production, which favors the development of protective Th1-type adaptive immune responses. The outcome of DC–NK-cell interactions is shaped by a complex interplay of physical and cytokine factors; direct contact with DCs as well as DC-derived cytokines contributed significantly to NK cell activation, while NK cell contact and NK cell-derived IFN-γ acted in concert to induce Th1-promoting DC responses. Furthermore, the observations that DCs triggered NK cells to secrete IFN-γ in part

through DC-derived IL-12 and, reciprocally, NK cells activated DC maturation and IL-12 production suggest that DC–NK-cell crosstalk provides an important amplification loop in the regulation of innate immunity to blood-stage malaria. Enhanced production of IL-12 and IFN-γ as a result of DC–NK-cell crosstalk efficiently primed Th1 polarization of $CD4^+$ T cells. Activated Th1 cells, in the later stage, may result in additional IFN-γ production, further NK cell and macrophage activation, and secretion of Th1-dependent antibody isotypes associated with effective control and rapid resolution of *P. chabaudi* infection (8, 400, 420). In conclusion, the results presented here provide novel insights into the mechanisms and regulation of innate immunity to blood-stage malaria and thus may help identify relevant immunological targets for the development of effective malaria vaccines and immunotherapies.

Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of MiFong Tam for maintaining the parasite, performing flow cytometry, and critically reviewing the manuscript.

Figure 1: IL-12 is required for DC maturation and IFN-γ production but not for uptake of pRBC early after blood-stage malaria infection. *A*, Uptake of pRBC by splenic DCs at day 0 and day 4 post-infection (p.i.). Histograms represent CFSE expression by gated $CD11c^+$ cells cultured alone (solid grey area) or with CFSE-labeled pRBC (lines). Splenic $CD11c^+$ DCs were enriched from WT (—), $IL\text{-}15^{-/-}$ (---), and $IL\text{-}12p40^{-/-}$ (···) mice. *B*, Kinetics of pRBC and nRBC uptake by splenic DCs from naïve and *P. chabaudi*-infected mice. Data were pooled from 3 experiments each containing 3-4 mice per group. *C*, Mean fluorescence intensity (MFI) of the expression of costimulatory molecules by gated $CD11c^+$ cells from naïve or infected mice. Data are representative of 2 experiments each containing 3-4 mice per group. *D*, Splenic $CD11c^+$ DCs from infected mice were stimulated without (medium) or with pRBC in vitro. Supernatants were analyzed for IFN-γ, IL-2, IL-12p70, and IL-10 by ELISA. Data are representative of 2 experiments each containing 3-4 mice per group.

Figure 1

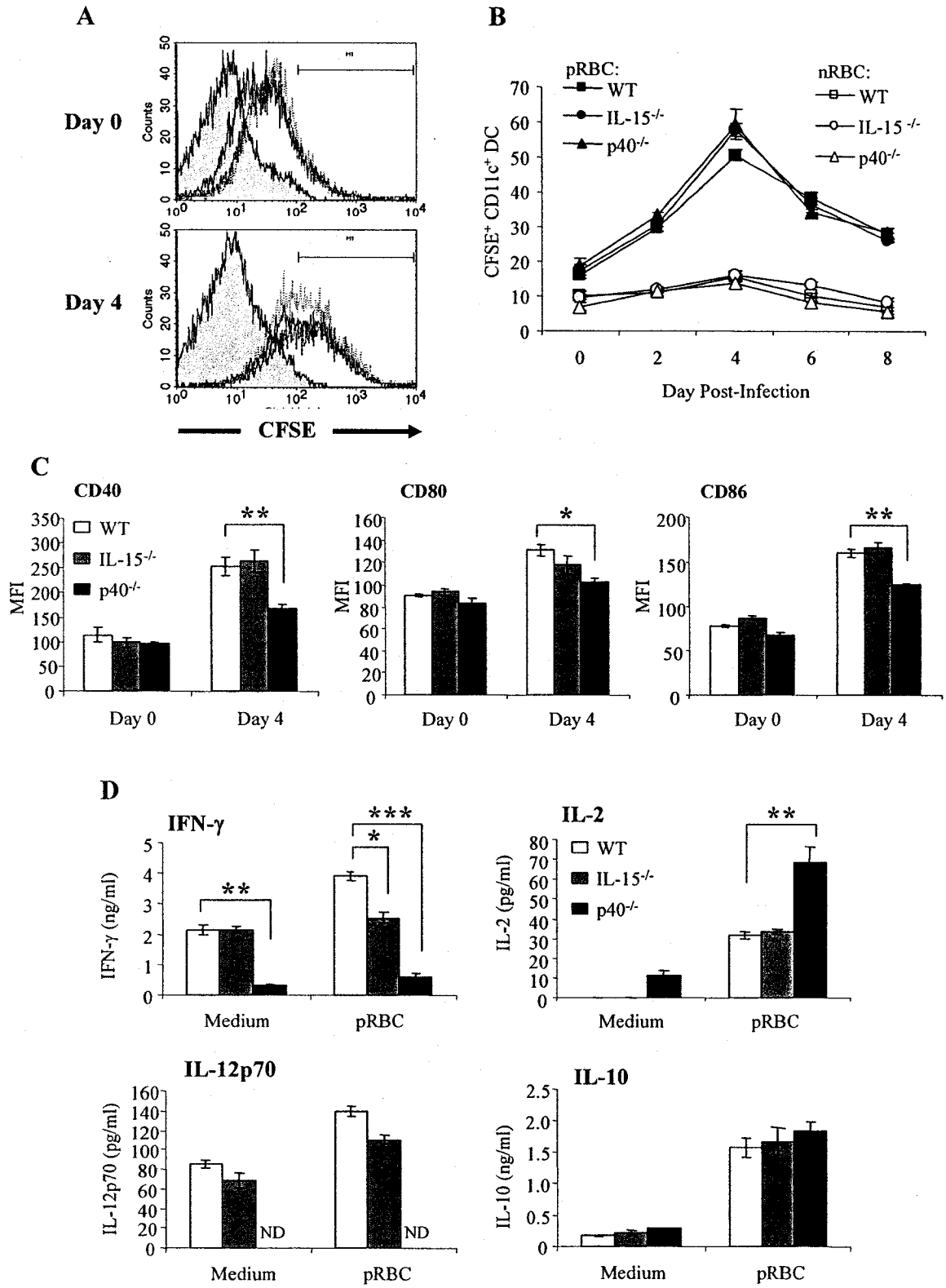

A
Day 0
Day 4
Counts
CFSE
B
pRBC:
WT
IL-15-/-
p40-/-
nRBC:
WT
IL-15 -/-
p40-/-
CFSE+ CD11c+ DC
Day Post-Infection
C
CD40
CD80
CD86
MFI
WT
IL-15-/-
p40-/-
Day 0
Day 4
D
IFN-γ
IFN-γ (ng/ml)
IL-2
IL-2 (pg/ml)
IL-12p70
IL-12p70 (pg/ml)
IL-10
IL-10 (ng/ml)
ND
Medium
pRBC

Figure 2: Malaria-activated DCs stimulate resting NK cells to produce high levels of IFN-γ. $CD11c^+$ DCs from infected mice and $DX5^+$ NK cells from naïve mice were cultured separately or together and analyzed for IFN-γ production. *A*, Left panels show IFN-γ expression by DCs and NK cells isolated from infected and naïve mice, respectively, prior to co-culture. Right panels show IFN-γ expression by DCs and NK cells cultured alone (lines) or together (shaded areas). Data are representative of 4 experiments showing similar results. *B*, IFN-γ protein levels as determined by ELISA in single cultures of NK cells from naïve mice or DCs from infected mice (Day 5 DC) and in co-cultures of NK cells with DCs from naïve mice (Day 0 DC), Day 5 DC, or Day 5 DC separated by a transwell insert. Data are representative of 4 experiments each containing 3-4 replicates per group.

Figure 2

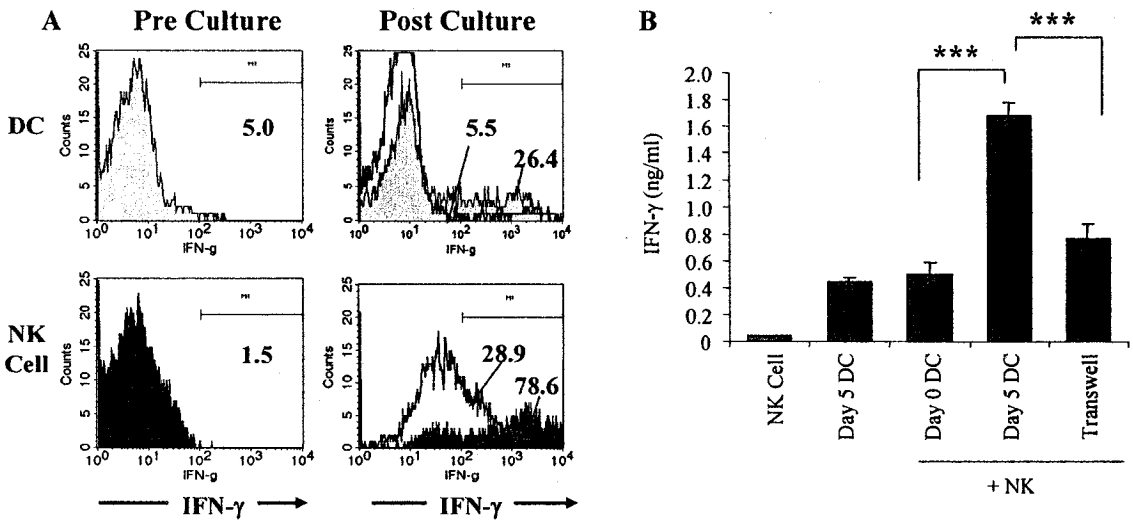

A
Pre Culture
Post Culture
DC
NK Cell
5.0
5.5
26.4
1.5
28.9
78.6
Counts
IFN-g
IFN-γ
B
IFN-γ (ng/ml)
***
***
NK Cell
Day 5 DC
Day 0 DC
Day 5 DC
Transwell
+ NK

Figure 3: IL-15 is required for NK cell function but not for DC-mediated stimulation of NK cells. Splenic DCs from infected mice and NK cells from naïve mice were cultured together in mixed and matched combinations. *A*, Intracellular IFN-γ expression by DCs and NK cells from WT and IL-$15^{-/-}$ mice after 24 h of co-culture. Each panel displays composite histograms of IFN-γ signals from gated $CD11c^{+}$ or $DX5^{+}$ cell populations. Shaded histograms represent DCs from WT or IL-$15^{-/-}$ mice, lines represent NK cells, and the percentages of DCs or NK cells expressing IFN-γ are shown. Data are representative of 4 experiments showing similar results. *B*, IFN-γ levels in the co-culture supernatants as quantified by ELISA. Data are representative of 4 experiments each containing 3-4 replicates per group. *C*, WT and IL-$15^{-/-}$ DCs were co-cultured with WT NK cells and IL-2 activity was blocked using mouse anti-IL-2 mAb. IFN-γ levels were quantified by ELISA after 36 h of co-culture. Data are representative of 2 experiments each containing 3-4 replicates per group.

Figure 3

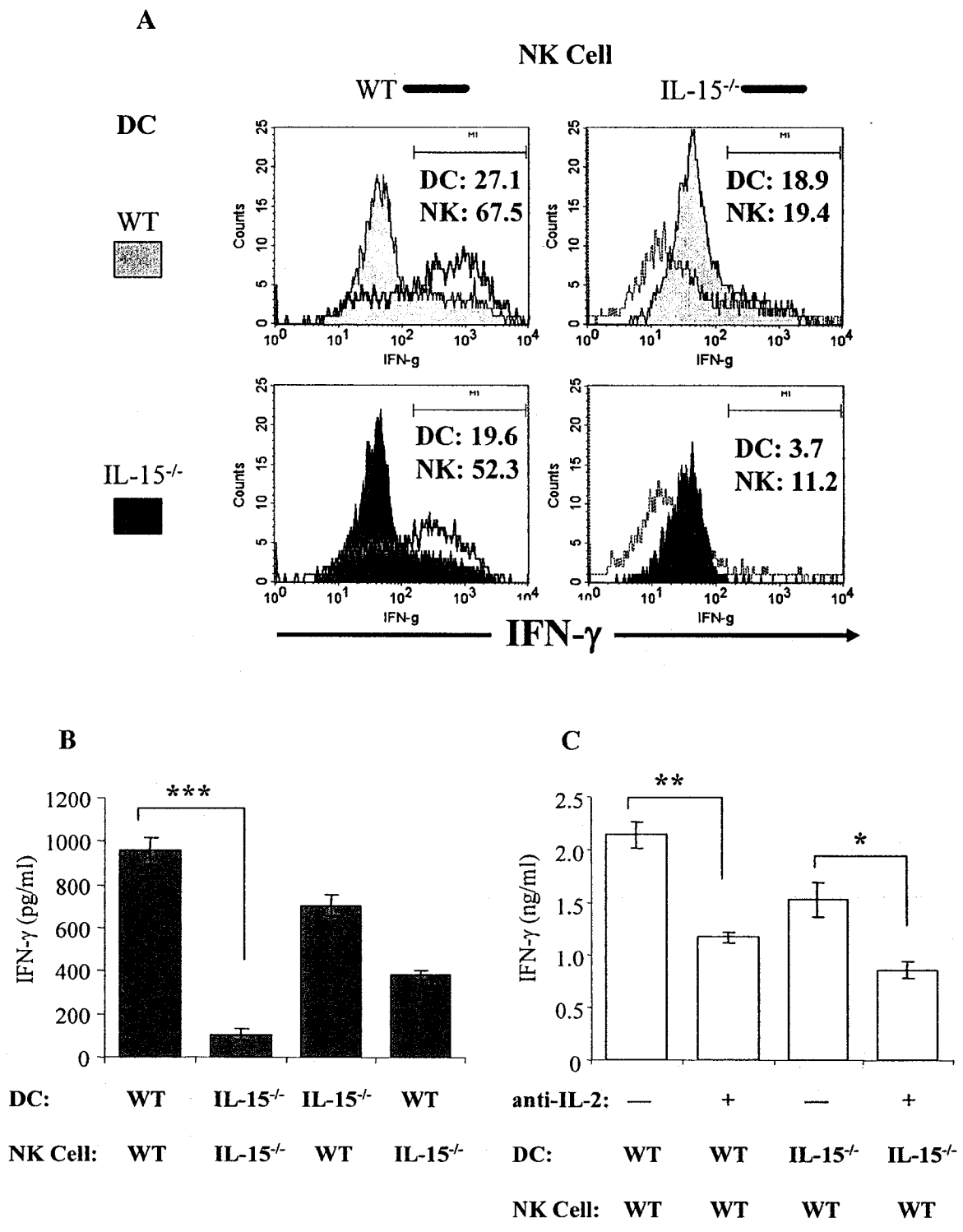

A
NK Cell
WT
IL-15-/-
DC
WT
IL-15-/-
DC: 27.1
NK: 67.5
DC: 18.9
NK: 19.4
DC: 19.6
NK: 52.3
DC: 3.7
NK: 11.2
Counts
IFN-g
IFN-γ
B
IFN-γ (pg/ml)
1200
1000
800
600
400
200
0
***
DC: WT IL-15-/- IL-15-/- WT
NK Cell: WT IL-15-/- WT IL-15-/-
C
IFN-γ (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
**
*
anti-IL-2: — + — +
DC: WT WT IL-15-/- IL-15-/-
NK Cell: WT WT WT WT

Figure 4:. IL-2 is superior to IL-15 in supporting the ability of malaria-activated DCs to stimulate IFN-γ production by NK cells. $CD11c^+$ DCs from infected WT mice and $DX5^+$ NK cells from naïve WT mice were cultured together in the presence of rIL-15 or rIL-2 and treated with anti-IL-2 or anti-IL-15 mAb. IFN-γ levels in the supernatants were quantified by ELISA after 36 h of co-culture. *A*, WT DCs and NK cells were co-cultured with or without rIL-15 and anti-IL-2 mAb. *B*, WT DCs and NK cells were co-cultured with or without rIL-2 and anti-IL-15 mAb. Data are representative of 3 experiments each containing 3-4 replicates per group.

Figure 4

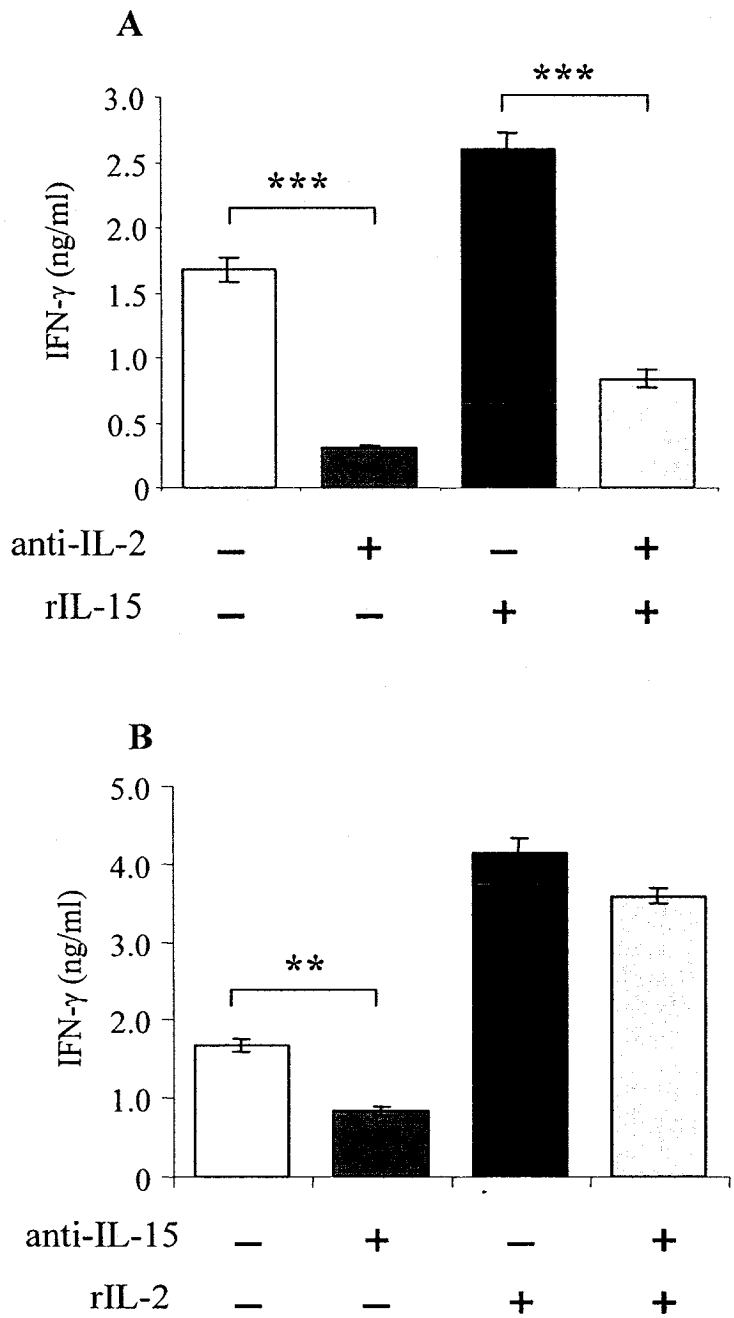

A
***
***
3.0
2.5
2.0
1.5
1.0
0.5
0
IFN-γ (ng/ml)
anti-IL-2 – + – +
rIL-15 – – + +
B
**
5.0
4.0
3.0
2.0
1.0
0
IFN-γ (ng/ml)
anti-IL-15 – + – +
rIL-2 – – + +

Figure 5: IL-12 is required for both DC-mediated NK cell activation and maximal NK cell responsiveness to DC stimulation. Splenic DCs from infected mice and NK cells from naïve mice were cultured together in mixed and matched combinations. *A*, IFN-γ expression by DCs and NK cells from WT and IL-12p40$^{-/-}$ mice after 24 h of co-culture. Each panel displays composite histograms of IFN-γ signals from gated CD11c^{+} or DX5^{+} cell populations. Shaded histograms represent DCs from WT or IL-12p40$^{-/-}$ mice, lines represent NK cells, and the percentages of DCs or NK cells expressing IFN-γ are shown. Data are representative of 4 experiments showing similar results. *B*, IFN-γ levels in the co-culture supernatants as quantified by ELISA. Data are representative of 4 experiments each containing 3-4 replicates per group. *C*, WT and IL-12p40$^{-/-}$ DCs were co-cultured with WT NK cells and IL-2 activity was blocked using mouse anti-IL-2 mAb. IFN-γ levels were quantified by ELISA after 36 h of co-culture. Data are representative of 2 experiments each containing 3-4 replicates per group.

Figure 5

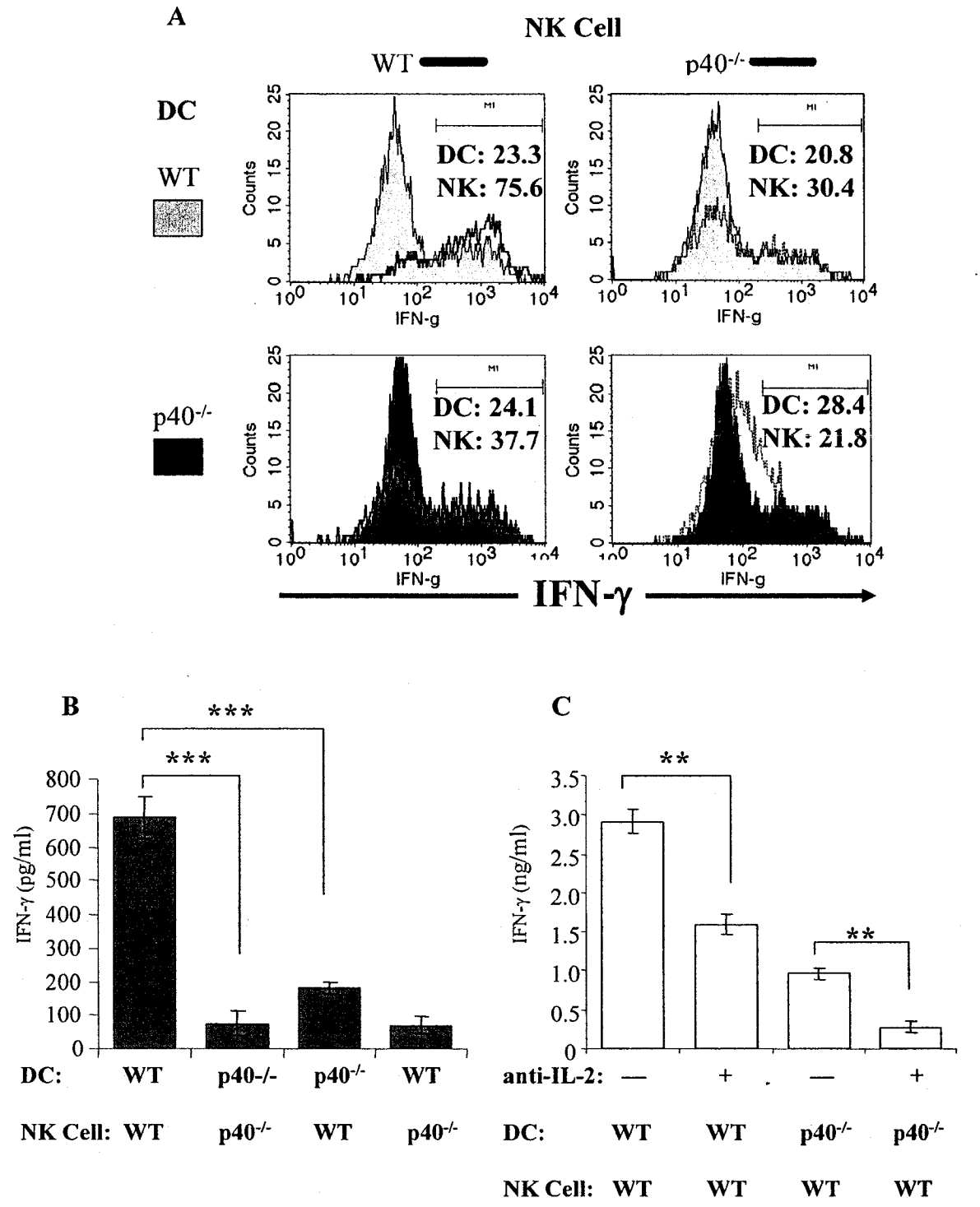

A
NK Cell
WT
p40-/-
DC
WT
p40-/-
DC: 23.3
NK: 75.6
DC: 20.8
NK: 30.4
DC: 24.1
NK: 37.7
DC: 28.4
NK: 21.8
Counts
IFN-g
IFN-γ
B
***
***
IFN-γ (pg/ml)
800
700
600
500
400
300
200
100
0
DC: WT p40-/- p40-/- WT
NK Cell: WT p40-/- WT p40-/-
C
**
**
IFN-γ (ng/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
anti-IL-2: — + — +
DC: WT WT p40-/- p40-/-
NK Cell: WT WT WT WT

Figure 6: Malaria-activated NK cells stimulate resting DCs to mature and secrete cytokines. Splenic NK cells from infected mice were co-cultured with DCs from naïve mice and DCs were then analyzed for expression of maturation markers (*A-B*) and cytokine production (*C*). To determine the role of IFN-γ in these interactions, DCs were co-cultured with WT NK cells and anti-IFN-γ mAb or with IFN-$\gamma^{-/-}$ NK cells. DCs were also co-cultured with NK cells in wells with a transwell insert to prevent cell-cell contact. *A*, Composite histograms show expression of maturation markers by DC control (---) and DCs co-cultured in transwells (—) or with WT (▬) or IFN-$\gamma^{-/-}$ (▬) NK cells. *B*, Cells were gated on $CD11c^{+}$ signal and analyzed for CD40, CD80 and CD86 expression (MFI) after co-culture with medium or NK cells from naïve (Day 0 NK) or infected (Day 5 NK) mice. *C*, Cytokine levels in supernatants of DCs cultured alone or with Day 0 or Day 5 NK cells as detected by ELISA. Data are representative of 3 experiments each containing 3-4 replicates per group.

Figure 6

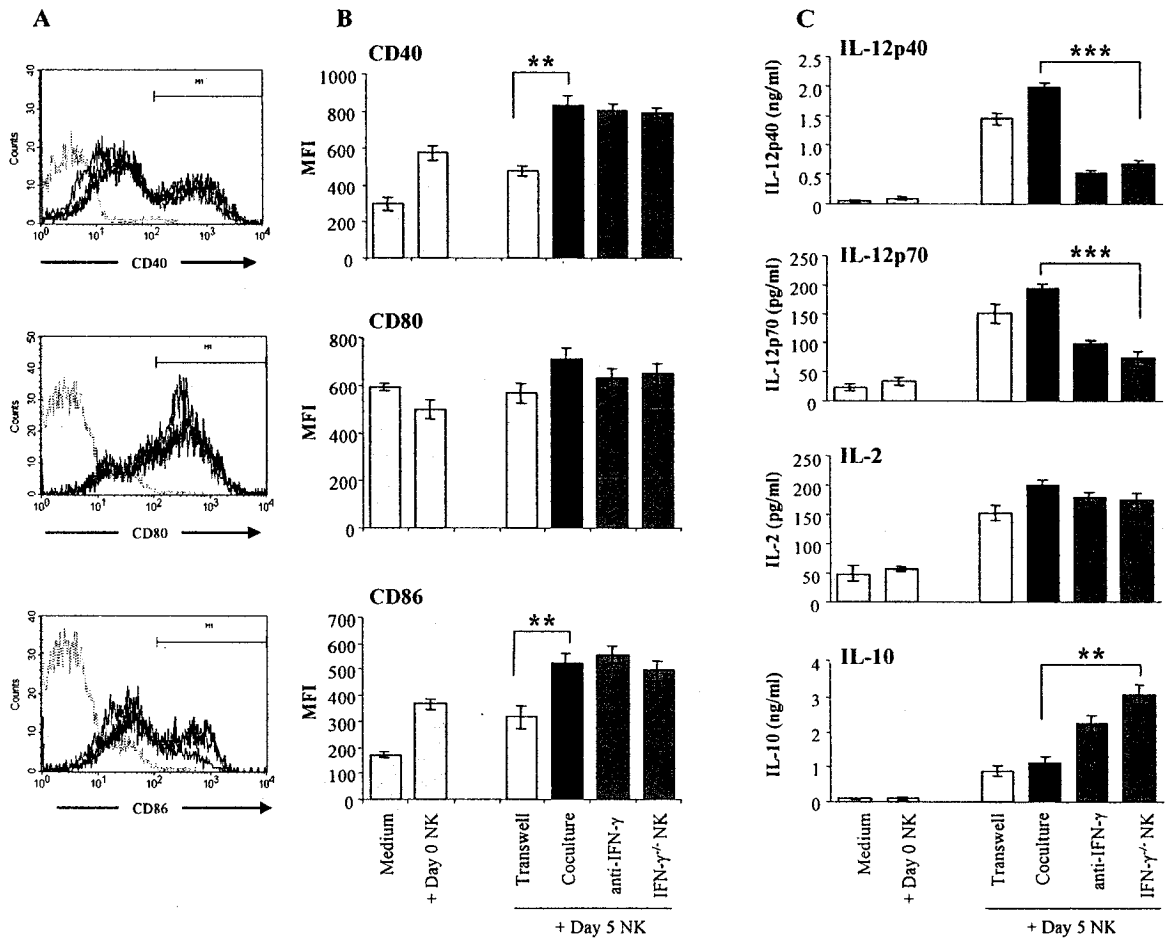
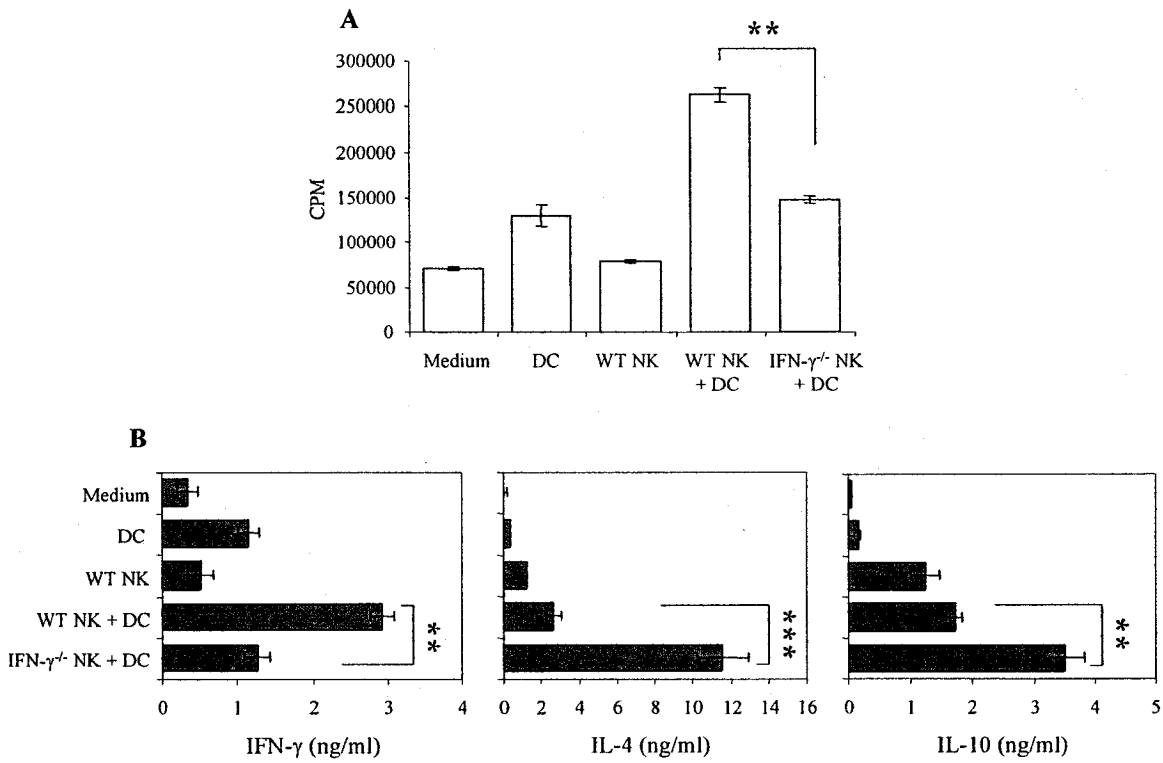

A
B
C
CD40
CD80
CD86
MFI
IL-12p40
IL-12p70
IL-2
IL-10
IL-12p40 (ng/ml)
IL-12p70 (pg/ml)
IL-2 (pg/ml)
IL-10 (ng/ml)
Counts
Medium
+ Day 0 NK
Transwell
Coculture
anti-IFN-γ
IFN-γ-/- NK
+ Day 5 NK

Figure 7: NK cell-activated DCs are able to stimulate $CD4^+$ T cells to proliferate and produce cytokines in an IFN-γ-dependent mechanism. Splenic NK cells from infected WT or IFN-$\gamma^{-/-}$ mice were co-cultured with DCs from naïve mice for 24 h and then plated with freshly isolated $CD4^+$ T cells from naïve mice. After 5 days, cells were harvested, restimulated in wells coated with anti-CD3 mAb for 48 h, and analyzed for proliferation (*A*) and cytokine production (*B*). $CD4^+$ T cells cultured alone (medium) or with DCs or WT NK cells separately were included as controls. *A*, DCs activated by WT NK cells induced higher levels of T cell proliferation than did DCs cultured alone or with IFN-$\gamma^{-/-}$ NK cells. *B*, DCs activated by WT NK cells induced higher levels of T cell-specific cytokine production than did DCs or NK cells alone. DCs activated by IFN-$\gamma^{-/-}$ NK cells stimulated T cells to secrete lower levels of IFN-γ but higher levels of IL-4 and IL-10 than did DCs activated by WT NK cells. Data are representative of 3 experiments each containing 3-4 replicates per group.

Figure 7


A
**
300000
250000
200000
150000
100000
50000
0
CPM
Medium
DC
WT NK
WT NK + DC
IFN-γ-/- NK + DC
B
Medium
DC
WT NK
WT NK + DC
IFN-γ-/- NK + DC
**
***
**
0 1 2 3 4
IFN-γ (ng/ml)
0 2 4 6 8 10 12 14 16
IL-4 (ng/ml)
0 1 2 3 4 5
IL-10 (ng/ml)

CHAPTER 5

DENDRITIC CELLS REGULATE INDUCTION OF EFFECTOR OR REGULATORY T CELL RESPONSES TO BLOOD-STAGE MALARIA INFECTION

Rebecca Ing, Jessica St. Pierre, Ciarico Piccirillo, and Mary M. Stevenson

Preface

In the mouse model of *P. chabaudi* infection, resistant B6 mice resolve a primary infection within 4 to 5 weeks and show a strong Th1-type response early in the spleen, whereas susceptible A/J mice succumb to the infection and show impaired Th1-type immunity. Given that DC responses are important for induction of protective Th1 immunity, the study described in Chapter 5 investigated whether the susceptibility of A/J mice is associated with altered DC phenotype or function during blood-stage malaria. Experiments were designed to compare the functional status of splenic $CD11c^+$ DCs from infected A/J versus B6 or H-2 congenic B10.A mice with regard to pRBC uptake, maturation, cytokine secretion and stimulation of $CD4^+$ T cell proliferation and cytokine production. Consistent with findings reported in previous chapters, data from this study indicate that DC-derived cytokines, such as IL-12 and possibly IL-2, are key determinants of whether an effector Th1 cell or a regulatory T cell response develops, thereby resulting in resistance or susceptibility, respectively, to blood-stage malaria.

Abstract

DCs play an important role in initiating type 1 immune responses critical for resistance to blood-stage malaria infection. In this study, we investigated DC function among mouse strains with divergent capacities to control and survive a primary *P. chabaudi* infection. Following infection, compared to resistant B6 or B10.A mice, splenic $CD11c^+$ DCs from susceptible A/J mice exhibited greater uptake of infected erythrocytes, expressed higher levels of costimulatory molecules, and stimulated higher levels of $CD4^+$ T cell proliferation. Despite a more mature phenotype, DCs from infected A/J mice produced less IL-12 and IFN-γ in response to pRBCs in vitro, suggesting a lower capacity to induce type 1 immune responses. Indeed, A/J DCs elicited lower IFN-γ production from NK cells as well as lower IFN-γ, but higher IL-10, secretion from $CD4^+$ T cells, possibly indicating selective induction of IL-10-producing regulatory T cells. In agreement with this notion, higher percentages of $Foxp3^+CD4^+CD25^+$ T cells were observed in infected A/J versus resistant mice. These data indicate that despite normal antigen uptake and phenotypic maturation, DCs from susceptible mice were unable to produce Th1-polarizing cytokines required to activate NK cells and $CD4^+$ T cells for IFN-γ-dependent protective immunity to blood-stage malaria infection.

Introduction

Protective immunity to blood-stage malaria infection requires $CD4^+$ T cells as well as Th1-associated cytokines and antibodies. Early control of parasitemia is mediated by IL-12-dependent production of IFN-γ, which activates macrophages, NK cells, and other cells to carry out host defenses that limit parasite growth during acute infection (400, 420, 477). In the chronic phase, Th1 cells and cytokines direct B cells to produce Th1-dependent antibodies required for parasite clearance and resolution of the infection (420, 435). Failure to maintain early Th1 responses can result in rapid increases in parasite load (378), although excessive or uncontrolled proinflammatory responses in turn may lead to increased disease severity and mortality (72, 73). While anti-inflammatory responses are beneficial during the chronic phase to prevent immune-mediated pathology, the presence of anti-inflammatory or regulatory factors during T cell priming may inhibit the generation of a Th1 response, leading to impaired protective immunity to blood-stage malaria. Indeed, the presence of IL-10 and absence of IFN-γ downregulate antigen-specific Th1 responses in neonates whose mothers had *P. falciparum*-induced placental malaria (484). Similarly, high levels of TGF-β impair IFN-γ- and NO-dependent resistance to blood-stage *P. chabaudi* infection (487), and the inhibition of an early TGF-β response promotes IFN-γ and TNF-α production as well as rapid resolution of *P. yoelii* infection (82). Evidence for a possible role of Treg cells in immune suppression during malaria infection was first shown in a study reporting that deletion of $CD4^+CD25^+$ T cells protects mice from infection with a lethal *P. yoelii* strain (434). In humans experimentally infected with *P. falciparum*, upregulation of TGF-β production and Foxp3-expressing $CD4^+CD25^+$ T cells is associated with higher levels of parasite growth in vivo (238). The immune mechanisms responsible for the increased TGF-β and Treg cell responses in cases of severe malaria are not known. Also not well understood is the role of innate immune responses, particularly those regulated by DCs, in the induction of T cell immunity versus tolerance during blood-stage malaria infection.

DCs are specialized APCs that initiate and regulate immune responses to self and nonself antigens. Immature DCs are highly efficient at capturing and processing antigens for subsequent priming of naïve $CD4^+$ T cells. After an encounter with invading pathogens, DCs receive instructive signals via pattern recognition receptors, such as the TLRs,

cytokines and other inflammatory mediators in the microenvironment to undergo diverse maturation processes that will influence the initiation of immune responses. In this manner, DCs control the type and magnitude of innate and adaptive immune responses to an infection. Activation of T cells by DCs depends mainly on efficient antigen processing and presentation, expression of costimulatory molecules, and production of Th cell-polarizing cytokines (109). In response to antigenic stimuli, DCs express defined cell surface molecules and cytokines that determine the outcome of adaptive immunity, such as whether a Th1 or Th2 effector response develops.

Although DCs are major stimulators of T cell effector responses, it has been proposed that DCs can also induce the development of Treg cells that suppress immune responses and maintain tolerance (108). Earlier studies proposed that the maturational stage of the DC determines the induction of T cell immunity or tolerance (101, 103). Immature DCs were believed to induce T cell anergy or development of Treg cells, whereas DCs matured by pathogen-driven activation stimuli were thought to be immunogenic DCs that can induce strong primary T cell responses (529). However, this concept has been challenged by recent studies demonstrating that antigen-bearing, phenotypically mature DCs are able to induce and expand Treg cells (330, 334, 530). In the new paradigm, tolerance-inducing DCs must first undergo partial maturation, which includes uptake and processing of antigen, expression of stable peptide-MHC complexes, and upregulation of costimulatory molecules (106). In the absence of inflammatory signals provided by TLR ligation, however, DCs do not produce IL-12 and other Th cell-polarizing cytokines and their development is arrested at a semi-mature stage (110). These semi-mature DCs are unable to prime differentiation of effector Th cells and instead are thought to trigger generation of Treg cells.

It is controversial whether *Plasmodium* parasites activate or inhibit DC maturation. Early studies reported that pRBCs suppress DC maturation and impair DC-mediated T cell priming in response to LPS stimulation (359, 371). By contrast, data from human and mouse model systems support the notion that DCs play an important role in early host-parasite interactions and subsequent induction of protective immunity to blood-stage malaria (366, 369, 370, 519).The experimental model of *P. chabaudi* infection has been useful for elucidating protective host immune responses to blood-stage malaria. The ability to control and eliminate a primary blood-stage *P. chabaudi* infection differs among inbred

strains of mice: C57BL/6 (B6) mice show moderate levels of acute parasitemia, resolve the infection by 4-5 wk post-infection (p.i.), and mount an early Th1 response in the spleen with increased production of Th1-type cytokines, whereas A/J mice develop fulminating parasitemia and severe anemia, succumb to infection by day 10-12 p.i., and show impaired Th1-type immune responses (388, 415, 531, 532). Given the divergent Th cell responses and outcomes of infection between these two strains of mice, we hypothesized that the ability to effectively control and resolve blood-stage malaria infection is determined by DC-mediated activation of innate and adaptive immune mechanisms.

To address this, we compared the functional status and possible role of DCs in the generation of a Th1 immune response to a primary *P. chabaudi* infection among genetically resistant and susceptible mice. We also determined which DC function—antigen uptake, costimulation, or cytokine production—is associated with induction of protective immunity to blood-stage malaria. Results showed that DCs from susceptible A/J mice were able to capture pRBCs and express costimulatory molecules at high levels, but these DCs were unable to produce Th1-polarizing cytokines and induce IFN-γ-secreting NK cells and Th1 effector cells compared to DCs from resistant B6 or H-2 congenic B10.A mice. Importantly, DCs from infected A/J mice stimulated high numbers of IL-10-secreting $CD4^+$ T cells, which might indicate selective induction of Treg cells by these DCs. Indeed, higher percentages of $Foxp3^+CD4^+CD25^+$ Treg cells were observed in A/J mice than in B6 or B10.A mice following *P. chabaudi* infection in vivo. These findings suggest functional differences in DCs that initiate and control $CD4^+$ T cell responses to blood-stage malaria infection, resulting in either immunity or tolerance.

Materials and Methods

Mice, parasite, and parasitized RBC purification

Female C57BL/6 (Charles River Laboratories), H-2 congenic B10.A (Jackson Laboratories) and A/J (Jackson Laboratories) mice, aged 8-14 wk, were maintained in the animal facility of the Montreal General Hospital Research Institute in accordance with the guidelines of the Canadian Council on Animal Care. *P. chabaudi* AS was maintained as previously described (494) and infections were initiated by i.p. injection of 10^6 pRBCs. Purified pRBCs and nRBCs were obtained using blood from *P. chabaudi*-infected or naïve B6 mice, respectively, as previously described (519). Purified pRBC contained >96% infected RBC.

Purification of $CD11c^+$, $DX5^+$, and $CD4^+$ cells from spleen

To obtain DCs, spleen cells were separated using Nycoprep (Axis-Shield) density gradients. Low-density cells at the interphase were collected, washed twice with PBS, and purified by positive selection using anti-CD11c microbeads (Miltenyi Biotec). The resulting DCs were routinely ≥85-90% positive for CD11c as determined by flow cytometry. Spleen cells were enriched for $DX5^+$ NK cells and $CD4^+$ T cells using anti-DX5 and anti-CD4 microbeads (Miltenyi Biotec), respectively, and were ≥85-90% positive for these populations.

DC uptake assay and flow cytometric analysis

The DC uptake assay was performed as recently described (519). Briefly, pRBC or nRBC (10^7 cells/ml) were stained with 2 mM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) in medium consisting of RPMI 1640 (Gibco-Invitrogen), 5% FCS (HyClone Laboratories), 10 mM HEPES (Gibco-Invitrogen), 20 μg/ml gentamicin (Sabex), 2 mM L-glutamine (Gibco-Invitrogen), and 0.5 μM β-mercaptoethanol (Sigma) for 15 min at 37°C. $CD11c^+$ DCs (10^6/well) were seeded with CFSE-labeled pRBC or nRBC in a 1:20 ratio at a final volume of 200 μl for 4 h at 37°C. After co-culture, noningested RBC were lysed with NH_4Cl and DCs were washed, FcR-blocked with anti-CD16/CD32 mAb (2.4G2; BD Biosciences), and stained with PE-labeled anti-CD11c mAb

(HL3; BD Biosciences). Uptake was determined by gating cells on SSC versus FL2 for the $CD11c^+$ population and analyzing CFSE staining on FL1 using a FACSCalibur equipped with CellQuest Pro software (BD Biosciences).

Splenic DC maturation and cytokine production

Splenocytes and enriched splenic $CD11c^+$ DCs were FcR-blocked with anti-mouse CD16/CD32 mAb (2.4G2; BD Biosciences) and stained with PE-labeled mAb (BD Biosciences) to CD11c, $I\text{-}A^b$ (AF6-120.1), anti-$I\text{-}A^b$ (AF6-120.1) or anti-$I\text{-}A^k$ (11-5.2), CD40 (2/23), CD80 (16-1041), or CD86 (GL-1). The percentages and mean fluorescence intensity (MFI) of gated $CD11c^+$ cells expressing the above markers were determined by flow cytometry. Supernatants of DCs (10^6/well) incubated in medium alone or with pRBC (1:10 ratio) for 48 h at 37°C were analyzed for cytokine production by ELISA as previously described (415).

DC and NK cell co-cultures

To determine the effect of malaria-activated DCs on resting NK cells, $CD11c^+$ DC purified from spleens of day 5 infected mice were co-cultured with $DX5^+$ NK cells from naïve mice in four combinations as previously described (Chapter 4; 533). Briefly, DCs (10^6/well) were plated with NK cells (10^6/well) in 96-well flat-bottomed plates for 24-36 h at 37°C. Single cultures of DCs or NK cells were included as controls. Cells were analyzed for intracellular IFN-γ expression and supernatants for IFN-γ levels by ELISA.

Intracellular cytokine expression

Following DC and NK cell co-culture, cells were stimulated in vitro with Golgi Stop™ (BD Biosciences), 10 ng/ml of PMA (Sigma) and 250 ng/ml of ionomycin (Sigma) for 2 h, then washed, FcR-blocked, and stained with FITC-conjugated anti-CD11c (BD Biosciences) and/or PE-conjugated anti-CD49b (DX5; BD Biosciences) mAb. Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and stained with APC-conjugated anti-IFN-γ mAb (XMG1.2; BD Biosciences). Cells were gated on the $CD11c^+$ (FL1) or $DX5^+$ (FL2) population and IFN-γ signals on FL4 were generated from the gated cells.

$Foxp3^+CD25^+$ expression by $CD4^+$ T cells

Splenocytes from naïve and infected mice were analyzed for Foxp3, CD4 and CD25 expression by flow cytometry. Splenocytes were prepared as previously described (512), FcR-blocked and stained with PE-conjugated Foxp3 (eBioscience), Cy-conjugated CD4 (BD Biosciences), and APC-conjugated CD25 (BD Biosciences) overnight. Signals for Foxp3 (FL2) and CD25 (FL4) were generated from cells gated on the $CD4^+$ population (FL3).

$CD4^+$ T cell assays

To determine T cell stimulatory function of malaria-activated DCs, splenic $CD11c^+$ DCs (5×10^5 cells) from naïve or infected mice were plated with splenic $CD4^+$ T cells, freshly purified from naïve B10.A mice, in varying ratios as indicated at a final volume of 200 µl in 96-well plates for 48 h at 37°C. Proliferation was determined by incorporation of [^{3}H]-thymidine during the last 16 h of incubation. Supernatants from DC and T cell co-cultures were collected and analyzed for cytokine production by ELISA. In some experiments, DCs were pre-treated with 250 ng/ml anti-IL-2 mAb (JES6-1A12; R&D Systems), 250 ng/ml anti-IL-15 mAb (34593.11; Abcam), or 100 ng/ml anti-IL-12 mAb (C17.8; BD Biosciences) for 4 h prior to co-culture with $CD4^+$ T cells. These mAb concentrations were determined to be optimal for suppressing T cell proliferation and corresponded to the ND_{50} as indicated by the manufacturer.

Statistical analyses

Data are expressed as means ± SEM. Statistical significance of differences between groups was analyzed by two-tailed, unpaired Student's *t* test and multiple comparisons were analyzed using ANOVA. Statistical significance was defined as follows: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$. All statistical analyses were performed using SAS/STAT software (SAS Institute).

Results

DCs from different strains of mice show divergent phenotypic and cytokine responses to blood-stage malaria infection

Previous work in our laboratory has shown that malaria-susceptible A/J mice exhibit impaired splenomegaly following a primary *P. chabaudi* infection compared to resistant B6 mice (388, 532). Similarly, in this study, as shown in Table 1, *P. chabaudi*-infected A/J mice had lower numbers of total splenocytes as well as lower numbers and percentages of splenic $CD11c^+$ cells than did B6 or B10.A mice. Although the percentages of different $CD11c^+$ DC subsets were generally similar among the groups, A/J mice showed lower numbers of these subsets as a consequence of their lower total spleen cellularity.

To determine whether DCs from A/J mice were functionally impaired, splenic $CD11c^+$ DCs purified from naïve or infected mice were analyzed for the ability to capture pRBCs and to provide costimulatory and cytokine signals for the activation of innate and adaptive immune responses. Using flow cytometry to quantify the uptake of CFSE-labeled RBCs, we have previously demonstrated that splenic $CD11c^+$ DCs preferentially recognize and internalize pRBC over nRBC before and after *P. chabaudi* infection in vivo (519). Indeed, as shown in Fig. 1, splenic $CD11c^+$ DCs from both H-2 congenic B10.A and A/J mice captured pRBC at higher levels than nRBC. Following *P. chabaudi* infection in vivo, the uptake of pRBC and, to a significantly lesser extent, nRBC was selectively enhanced (Fig. 1*B*) and correlated with upregulated costimulatory molecule expression (Fig. 1*C*). Surprisingly, however, significantly higher percentages of DCs from A/J mice captured pRBC compared to DCs from B10.A mice at day 4 to 8 post-infection (Fig. 1*A* and *B*). Consistent with the higher levels of pRBC uptake, splenic DCs from infected A/J mice also expressed higher levels of costimulatory molecules CD40, CD80 and CD86 than did DCs from infected B6 or B10.A mice (Fig. 1*C*).

Although splenic DCs from infected A/J mice exhibited a higher capacity for pRBC uptake and expression of costimulatory molecules, these DCs produced significantly lower amounts of Th1-polarizing cytokines IL-12 and IFN-γ compared to DCs from infected B6 or B10.A mice following pRBC stimulation in vitro (Fig. 2). These results are in agreement with previous reports of markedly lower IL-12 and IFN-γ production in the sera and

spleens of A/J versus B6 mice (387, 388, 415, 531, 532). Despite impaired production of Th1-type cytokines, DCs from A/J mice secreted more IL-2, albeit at relatively low concentrations compared to other cytokines, than did DCs from resistant mice. IL-10 production by splenic DCs increased after malaria infection but did not differ significantly among the mouse strains, although it is important to note that high production of IL-10 by DCs from infected A/J mice coincided with low production of IL-12. In this study, cytokine production by DCs in vitro was measured at day 5 post-infection (p.i.), a time point that is associated with increasing parasitemia, pRBC uptake and significant upregulation of costimulatory molecule expression. In this regard, the differential cytokine responses observed might reflect selective disparities in DC responses to blood-stage malaria among genetically resistant and susceptible mice.

Malaria-activated DCs from susceptible A/J mice fail to stimulate resting NK cells to produce IFN-γ

Previous studies have demonstrated lower NK cell cytotoxicity and IFN-γ production in A/J versus B6 mice following *P. chabaudi* infection (139). We confirmed that highly enriched $DX5^+$ NK cells from naïve and malaria-infected A/J mice exhibited lower cytotoxicity and IFN-γ production than did NK cells from B6 or B10.A mice (Appendix III). Recent work in our laboratory (Chapter 4; 533) has shown that splenic $CD11c^+$ DCs from malaria-infected B6 mice are able to activate resting splenic $DX5^+$ NK cells through the expression of surface molecule and cytokine signals to produce high levels of IFN-γ in vitro. Accordingly, we hypothesized that the lower NK cell responses observed in A/J mice may be indicative of not only impaired NK cell functions but also perturbations in DC-mediated activation of NK cells. To address this, DCs from infected A/J and B10.A mice were paired and co-cultured in four combinations with NK cells from naïve A/J and B10.A mice. NK cells in the syngeneic B10.A co-cultures showed the highest level (50.8%) of intracellular IFN-γ expression (Fig. 3*A*, *upper left*) and the lowest level (14.2%) in the syngeneic A/J co-cultures (Fig. 3*A*, *lower right*). However, B10.A DCs failed to stimulate A/J NK cells to express IFN-γ above the level observed in the syngeneic A/J co-cultures (Fig. 3*A*, *upper right*) and, more importantly, A/J DCs were unable to fully activate B10.A NK cells (Fig. 3*A*, *lower left*). The impaired intracellular IFN-γ expression in co-cultures

with A/J DCs and/or NK cells was confirmed by the significantly low levels of IFN-γ protein secreted (Fig. 3*B*). These results suggest that A/J mice have defects in both DC and NK cell functions following *P. chabaudi* infection.

DCs from susceptible A/J mice have normal capacity to stimulate $CD4^+$ T cell proliferation in an IL-2-dependent mechanism

In a previous study, we demonstrated that preferential uptake of pRBC by splenic $CD11c^+$ DCs is associated with concomitant induction of high levels of $CD4^+$ T cell proliferation and IFN-γ production (519). To determine whether DCs from the different strains of mice show disparate capacities for activation of resting $CD4^+$ T cells, particularly induction of Th1 cells, we co-cultured splenic $CD11c^+$ DCs from infected A/J or B10.A mice with $CD4^+$ T cells from naïve B10.A mice and measured T cell proliferation and cytokine responses in vitro. DCs from naïve A/J mice stimulated lower levels of $CD4^+$ T cell proliferation than did DCs from B10.A mice (Fig. 4*A*). However, and unexpectedly, this response is reversed following *P. chabaudi* infection: DCs from day 4 or 7 infected A/J mice stimulated significantly higher levels of $CD4^+$ T cell proliferation than did DCs from infected B10.A mice (Fig. 4*A*).

To delineate the relative contributions of IL-2, IL-12, and IL-15 to DC-primed T cell proliferation, antibodies blocking the activity of these cytokines were added to the DC and T cell co-cultures. Neutralization of IL-12 and IL-15 in vitro had no effect on the distinct patterns of T cell proliferative responses induced by DCs from infected A/J or B10.A mice (Fig. 4*B*). In contrast, as shown in Fig. 4*C*, blocking IL-2 in vitro reduced T cell proliferation stimulated by DCs from either A/J mice ($P < 0.05$ A/J DC compared to A/J DC + anti-IL-2) or B10.A mice ($P < 0.05$ B10.A DC compared to B10.A DC + anti-IL-2), suggesting that DC-derived IL-2 mediates activation of $CD4^+$ T cell proliferation.

DCs from susceptible A/J mice selectively induce IL-10-secreting $CD4^+$ T cells while DCs from resistant B10.A mice induce IFN-γ-secreting $CD4^+$ T cells

Production of IFN-γ by $CD4^+$ T cells is characteristic of Th1-committed cells (534), while production of IL-10 is generally restricted to Th2 and/or Treg cells (535). DCs play a major role in activating $CD4^+$ T cell differentiation and, in part through the secretion of

immunoregulatory cytokines, may determine the phenotype of the $CD4^+$ T cell response. Thus far, our results indicate that DCs from infected mice were able to capture pRBC, express costimulatory molecules and stimulate IL-2-dependent $CD4^+$ T cell proliferation, but showed notable defects in Th1-type cytokine production. We questioned whether DCs from different mouse strains were equally able to induce IFN-γ-secreting Th1 cells that are required for protective immunity against blood-stage malaria.

Unfractionated splenocytes from infected A/J mice showed lower IFN-γ but higher IL-10 production when stimulated without or with anti-CD3 mAb in vitro (Fig. 5*A*). These results are indicative of differences in cytokine production by spleen cells from A/J versus B10.A mice infected with *P. chabaudi* and are consistent with previous studies showing that malaria-infected A/J mice produce lower Th1-type cytokines but higher levels of IL-4 and IL-5 than do B6 mice (478, 532). To determine the ability of DCs from A/J versus B10.A mice to induce $CD4^+$ T cells to secrete functionally divergent cytokines, DCs from infected mice were co-cultured with $CD4^+$ T cells from naïve B10.A mice as described above. As shown in Fig. 5*B*, DCs from infected A/J mice stimulated lower IFN-γ but higher IL-10 production by $CD4^+$ T cells than did DCs from B10.A mice, suggesting that DCs from malaria-susceptible mice selectively skew $CD4^+$ T cells toward an IL-10-dominant Treg cell phenotype. Notably, DCs from the two mouse strains stimulated equivalent production of IL-4 from $CD4^+$ T cells, indicating an absence of DC-mediated deviation toward Th2 cell differentiation despite coincident impaired IFN-γ and upregulated IL-10 responses in cultures containing DCs from A/J mice. As expected, IL-12 and, to a lesser extent, IL-2 were required for optimal T cell IFN-γ production induced by B10.A DCs ($P < 0.05$ compared to no antibody treatment; Fig. 5*C*). Of the three cytokines, only IL-2 was observed to support high levels of $CD4^+$ T cell IL-10 production induced by DCs from infected A/J mice ($P < 0.05$ compared to no antibody treatment; Fig. 5*D*). It is important to note that blocking IL-12 in vitro failed to impair T cell proliferation and therefore IL-12 is critically required for priming $CD4^+$ T cells for IFN-γ production in a manner that is unrelated to proliferation. In contrast, the lower IFN-γ and IL-10 production observed following IL-2 neutralization could be attributed in part to concurrent decreases in T cell proliferation as shown in Fig. 4*C*. Taken together, these suggest that DCs from susceptible

A/J mice selectively induce IL-10-producing $CD4^+$ T cells while DCs from resistant mice activate IFN-γ-producing $CD4^+$ T cell responses.

$CD4^+$ T cells induced in malaria-susceptible mice show regulatory phenotype

To determine whether the $CD4^+$ T cells stimulated with DCs from A/J mice show a regulatory phenotype, the expression of CD25 by enriched $CD4^+$ T cells was analyzed following co-culture with DCs from naïve B10.A mice (D0 DC) or from infected A/J or B10.A mice (D5 DC). As shown in Fig. 6*A*, higher percentages of $CD4^+$ T cells expressed CD25 following co-culture with DCs from infected A/J mice compared to DCs from naïve or infected B10.A mice. Moreover, the percentages of $CD4^+$ T cells expressing CD25 after co-culture with DCs from naïve or infected B10.A mice were comparable to those of $CD4^+$ T cells cultured alone (Fig. 6*A*). Experiments are in progress to determine whether these $CD4^+CD25^+$ cells preferentially induced by DCs from infected A/J mice also express higher levels of the Treg cell marker Foxp3. Consistent with the finding that DCs from A/J mice have tolerogenic potential for the induction of Treg cells, we observed significantly higher percentages of $Foxp3^+CD4^+CD25^+$ T cells in A/J mice than in B6 or B10.A mice following *P. chabaudi* infection (Fig. 6*B* and *C*). It is important to note that the percentages of these Treg cells decreased in B6 and B10.A mice after infection, but infected A/J mice continue to exhibit the same percentage of Treg cells as their naïve counterparts. Together, these results suggest that DCs with tolerogenic features might play a role in inducing and maintaining Treg cells in malaria-susceptible mice following *P. chabaudi* infection.

Discussion

DCs are thought to play an important role in initiating and shaping innate and adaptive immune responses to blood-stage malaria infection. In this study, we demonstrated that DCs from genetically resistant and susceptible strains of mice are functionally distinct and induce different immune responses to a primary blood-stage *P. chabaudi* infection. Different pathways of T cell priming by DCs may be responsible for the divergent outcomes of blood-stage malaria among these mice. DCs from resistant B6 or B10.A mice were shown to produce high levels of the Th1-polarizing cytokines IL-12 and IFN-γ and to stimulate resting NK cells and $CD4^+$ T cells to secrete abundant IFN-γ, thus leading to the development of protective Th1 immunity. By contrast, DCs from susceptible A/J mice showed marked functional differences, including low IL-12 but high IL-10 production, failure to stimulate IFN-γ production by NK cells and $CD4^+$ T cells, and selective induction of IL-10-secreting $CD4^+$ T cells in vitro. This tolerogenic potential of DCs from malaria-susceptible mice was not attributable to altered capacities for antigen uptake or costimulatory molecule expression, and correlated with higher percentages of Foxp3-expressing $CD4^+CD25^+$ T cells in these mice following *P. chabaudi* infection. These results provide important evidence that genetic differences in DCs may determine whether T cell immunity or tolerance develops following blood-stage malaria.

Host resistance to blood-stage malaria requires IL-12-dependent production of IFN-γ during two distinct phases of infection: in the acute phase, IFN-γ, in concert with other proinflammatory mediators such as IL-12, IL-15 and TNF-α, activate anti-parasite defenses that control parasite growth (392, 400, 512), while IFN-γ-secreting Th1 cells are critical in the later phase to help B cells produce Th1-associated antibodies that mediate parasite clearance and eliminate the infection (420). A high level of IFN-γ production by NK cells, macrophages and $CD4^+$ T cells as well as high titers of Th1-associated antibodies are associated with protection against blood-stage malaria in humans and mouse models (139, 420, 512, 531, 532). In contrast to IFN-γ, the primary function of IL-10 appears to be inhibition of inflammatory immune responses (265, 273). Although previous studies showed that IL-10 is necessary in late infection to dampen inflammation and thereby prevent immune-mediated pathology (78), the presence of anti-inflammatory mediators

during the early stage of $CD4^+$ T cell priming may impair the generation of protective immune responses to blood-stage malaria.

In this study, we showed that splenic $CD11c^+$ DCs purified from resistant B6 and H-2 congenic B10.A mice early after *P. chabaudi* infection were able to express costimulatory and cytokine signals needed to stimulate resting splenic $CD4^+$ T cells. Anti-CD3-stimulated splenocytes from infected A/J mice produced less IFN-γ and more IL-10 than cells from B10.A mice, suggesting IL-10-dominant spleen cell responses in malaria-susceptible mice. To determine the role of DCs in regulating $CD4^+$ T cell differentiation, we analyzed the cytokine profile of $CD4^+$ T cells co-cultured with DCs from *P. chabaudi*-infected mice. Malaria-activated DCs from different mouse strains stimulated distinct types of $CD4^+$ T cell responses that were associated with divergent outcomes of infection. DCs from resistant B10.A mice stimulated high IFN-γ production by NK cells as well as high IFN-γ and low IL-10 production by $CD4^+$ T cells in a mechanism that was critically dependent on DC-derived IL-12. In contrast, DCs from susceptible A/J mice failed to induce significant IFN-γ production by NK cells but retained the capacity to prime $CD4^+$ T cells for proliferation and, importantly, for high IL-10 production coincident with low IFN-γ production. These results suggest that DCs from susceptible mice can selectively induce Treg cell differentiation. Indeed, infected A/J mice exhibited higher percentages of $Foxp3^+CD4^+CD25^+$ T cells than did B6 or B10.A mice, indicating a possible role of Treg cells in the impaired development of protective Th1 immunity to blood-stage malaria in genetically susceptible mice. Consistent with our results, high numbers of $CD4^+CD25^+$ T cells expressing Foxp3 are associated with decreased IFN-γ responses and more rapid parasite growth in humans infected with *P. falciparum* (238).

Although DCs have been shown to activate and expand Treg cells in other model systems (327, 329), it is not known whether tolerogenic DCs are involved in Treg cell-associated suppression of immunity to blood-stage malaria. Previous studies have shown that DCs isolated from mice early after infection with blood-stage parasites are phenotypically and functionally mature as assessed by the expression of costimulatory molecules (365, 366, 519), production of type 1 IFN, IL-2, IL-12 and IFN-γ in response to pRBC or parasite byproducts such as hemozoin (58, 366, 369, 536), potent stimulation of $CD4^+$ T cells (366, 367, 536), and immunizing protection against challenge infection

conferred by adoptive transfer (370). As the infection progresses, however, DCs downregulate their production of IL-12 and TNF-α and support less IFN-γ production by $CD4^+$ T cells (536). Coincident with the decrease in proinflammatory responses, DCs from late infection have diminished capacity for pRBC uptake (519) and upregulate IL-10 production as well as IL-10-dominated T cell responses (536). Together these results suggest that DC function switches from an immunogeneic to a tolerogenic phenotype as a mechanism to elicit anti-inflammatory or regulatory immune responses that may prevent immune-mediated disease during chronic infection (536). In early infection, however, IL-12-producing DCs are required for the development of IFN-γ-dependent innate and adaptive immune responses (366, 519, 536), which are known to protect against blood-stage malaria in both humans and mouse models (140, 400). Here, we provide novel evidence that DCs may play an important role in the induction of IL-10-secreting T cells in the acute immune response to *P. chabaudi* infection, leading to tolerance, not immunity, and thus enhanced susceptibility to blood-stage malaria. We demonstrated that splenic DCs that produced high levels of Th1-polarizing cytokines, such as IL-12 and IFN-γ, were also able to stimulate resting NK cells and $CD4^+$ T cells to secrete abundant IFN-γ, resulting in protective Th1 immunity. In contrast, DCs from susceptible A/J mice have tolerogenic function as assessed by the preferential induction of IL-10-secreting $CD4^+$ T cells during the early immune response to *P. chabaudi* infection. DC-mediated induction of IL-10-secreting T cells in A/J mice may represent an immune mechanism underlying the inability of these mice to control and survive *P. chabaudi* infection. In A/J mice, which show high peak parasitemias and normally succumb to a primary *P. chabaudi* infection within 10-12 days, it is likely that induction of Treg cells by IL-10-producing DCs acts not to prevent immunopathology but to suppress immunity to blood-stage malaria. Further research is required to delineate the precise function of Treg cells during blood-stage malaria.

The finding that splenic $CD11c^+$ DCs from infected A/J mice were able to express abundant costimulatory molecules but failed to produce IL-12 and IFN-γ demonstrate the need to distinguish between phenotypic and functional maturation. Higher levels of costimulatory molecule expression and IL-2 secretion by DCs from infected A/J mice were associated with stimulation of T cell proliferation, while the inability to produce Th1-promoting cytokines correlated with lower induction of IFN-γ-secreting NK and $CD4^+$ T

cells. Thus, phenotypic maturation of DCs as indicated by the expression of maturation markers is not consistently accompanied by functional maturation with regard to cytokine secretion and initiation of protective immune responses. Moreover, DCs with tolerogenic potential retain the capacity to discriminate between pRBC and nRBC and to undergo maturation following *P. chabaudi* infection. Studies in other systems have supported the view that mature DCs can induce divergent $CD4^+$ T cell responses with different immune outcomes. Mature DCs bearing antigen in lymph nodes can induce $CD4^+$ T cell proliferation but not effector responses (537). MyD88-deficient DCs are able to express costimulatory molecules, stimulate $CD4^+$ T cell proliferation, and recruit monocytes following LPS stimulation in vitro or *Listeria* infection in vivo (538, 539). However, these MyD88-deficient DCs do not produce cytokines, including IL-12 and TNF-α, in response to microbial stimuli and are unable to drive $CD4^+$ T cell differentiation into effector Th cells (110, 538, 539). Moreover, DCs that are activated solely by proinflammatory stimuli in the absence of pathogen-specific TLR signaling can mature normally and support T cell clonal expansion but fail to produce IL-12 and to induce Th1 cell differentiation and IgG2a production (110). Collectively, these data suggest that susceptibility to blood-stage malaria may involve DCs that are phenotypically mature but are unable to induce T cell-mediated immunity in the absence of the production of Th1-polarizing cytokines.

The specific mechanisms involved in DC-mediated induction of Treg cells have not been clearly defined. Evidence from diverse model systems has implicated a role for IL-2, IL-10 and TGF-β in the differentiation, function and expansion of Treg cells (293, 298, 299, 303, 337). In agreement with previous studies (334, 336), the results in this study suggest that the cytokine profile of the DC may influence the type of $CD4^+$ T cell response generated. In the early immune response to *P. chabaudi* infection, IL-12-producing DCs from B10.A mice stimulated high numbers of IFN-γ-secreting NK and $CD4^+$ T cells, whereas DCs from A/J mice produced high IL-10 but low IL-12 and selectively induced IL-10-secreting $CD4^+$ T cells in the absence of IFN-γ production by NK cells and $CD4^+$ T cells. Moreover, the higher IL-2 production by DCs from A/J mice was associated with increased T cell proliferation and IL-10 production. Although previous studies have shown that high levels of TGF-β early after *P. chabaudi* or *P. falciparum* infection impair IFN-γ and NO-dependent immunity to blood-stage malaria (238, 487), it is not known whether

upregulated TGF-β production is responsible for defects in the immune responsiveness of A/J mice. Experiments are ongoing to determine the kinetics of TGF-β production in vivo and by DCs in vitro among the different mouse strains during the acute phase of *P. chabaudi* infection as well as the possible role of DC-derived TGF-β in the induction or maintenance of $Foxp3^+CD4^+CD25^+$ T cells in malaria-susceptible mice. It will also be important to demonstrate whether neutralizing IL-10 can reverse the tolerogenic status of DCs from A/J mice in favor of activation of Th1 cell differentiation and potent stimulation of IFN-γ production. Lastly, to confirm a role for tolerogenic DCs in the induction of Treg cells during blood-stage malaria, it will be necessary to determine whether the IL-10-dominant $CD4^+$ T cells induced by DCs from A/J mice express upregulated Foxp3 and exhibit suppressive activity. It will also be of interest to characterize the kinetics of $Foxp3^+CD4^+CD25^+$ T cell responses in different strains of mice during acute *P. chabaudi* infection, and to correlate these data with the functional status of IL-10-secreting $CD4^+$ T cells induced by DCs in vitro. These studies are in progress in our laboratory.

In conclusion, the results obtained in this study provide novel evidence for an important role of DCs in the initiation of effector versus regulatory $CD4^+$ T cell responses during blood-stage malaria infection. Depending on the genetic background of the host, DCs from *P. chabaudi*-infected mice possess much of the machinery, such as expression of costimulatory molecules and production of Th cell-skewing cytokines, required to modulate the differentiation of $CD4^+$ T cells into Th1 or Treg cells that mediate divergent immune outcomes to blood-stage malaria infection. Future experiments will further delineate the cytokines and other signals expressed by DCs for priming different T cell responses to blood-stage malaria. Understanding the mechanisms by which DCs regulate the balance between T cell immunity and tolerance during blood-stage malaria will likely advance the development of novel therapeutic approaches that boost the host immune response to *Plasmodium* parasites while minimizing the potential for disease.

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Table 1: Numbers of splenic $CD11c^+$ DCs and subsets in mice infected with *P. chabaudi*[a]

Mouse Strain	Total Spleen Cells ($\times 10^7$)	Total $CD11c^+$ ($\times 10^6$)	$CD40^+$ $CD11c^+$ ($\times 10^6$)	$CD80^+$ $CD11c^+$ ($\times 10^6$)	$CD86^+$ $CD11c^+$ ($\times 10^6$)	$I\text{-}A^+$ $CD11c^+$ ($\times 10^6$)[b]
C57BL/6	23.2 ± 3.64	19.05 ± 1.47 (8.21)	15.14 ± 1.96 (6.52)	14.82 ± 1.37 (6.39)	8.94 ± 1.24 (3.85)	13.75 ± 3.02 (5.93)
B10.A	19.6 ± 1.91	14.87 ± 0.69 (7.59)	12.00 ± 1.22 (6.12)	12.11 ± 0.88 (6.18)	8.15 ± 0.62 (4.16)	12.46 ± 2.77 (6.36)
A/J	8.24 ± 0.91*	4.46 ± 0.18* (5.41)*	3.83 ± 0.98* (4.65)	3.87 ± 0.37* (4.70)	2.45 ± 0.46* (2.97)	3.73 ± 0.98 (4.53)

[a] Spleen cells were collected from *P. chabaudi*-infected mice at day 7 post-infection and analyzed by flow cytometry for expression of CD11c and costimulatory molecules. Data are presented as absolute numbers (mean ± SEM) and the percent for each cell population is indicated in the parentheses.

[b] C57BL/6 cells were stained with FITC-conjugated anti-$I\text{-}A^b$ mAb and B10.A and A/J cells were stained with FITC-conjugated anti-$I\text{-}A^k$ mAb

*, $P < 0.05$ comparing C57BL/6 vs. A/J mice.

Figure 1. Splenic DCs from A/J mice show higher uptake of pRBC (*A* and *B*) and express higher levels of costimulatory molecules (*C*) than do DCs from B6 or B10.A mice following *P. chabaudi* infection. *A*, Splenic $CD11c^+$ DCs enriched from day 5 infected B10.A (*left panels*) or A/J (*right panels*) mice were incubated with CFSE-labeled pRBC or nRBC and the level of uptake by nongated cells (dot plots) or gated $CD11c^+$ cells (histograms) was determined by CFSE fluorescence as detected by flow cytometry. *B*, Kinetics of pRBC or nRBC uptake by splenic DCs enriched from B10.A or A/J mice before and after *P. chabaudi* infection. Data were pooled from 3 experiments each containing 3-4 mice per group. *C*, Mean fluorescence intensity (MFI) of the expression of costimulatory molecules by gated $CD11c^+$ cells from naïve or infected mice. Data are representative of 2 experiments each containing 3-4 mice per group. Statistical analyses compared A/J to B10.A in *B* and compared A/J to B6 and B10.A in *C*. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 1

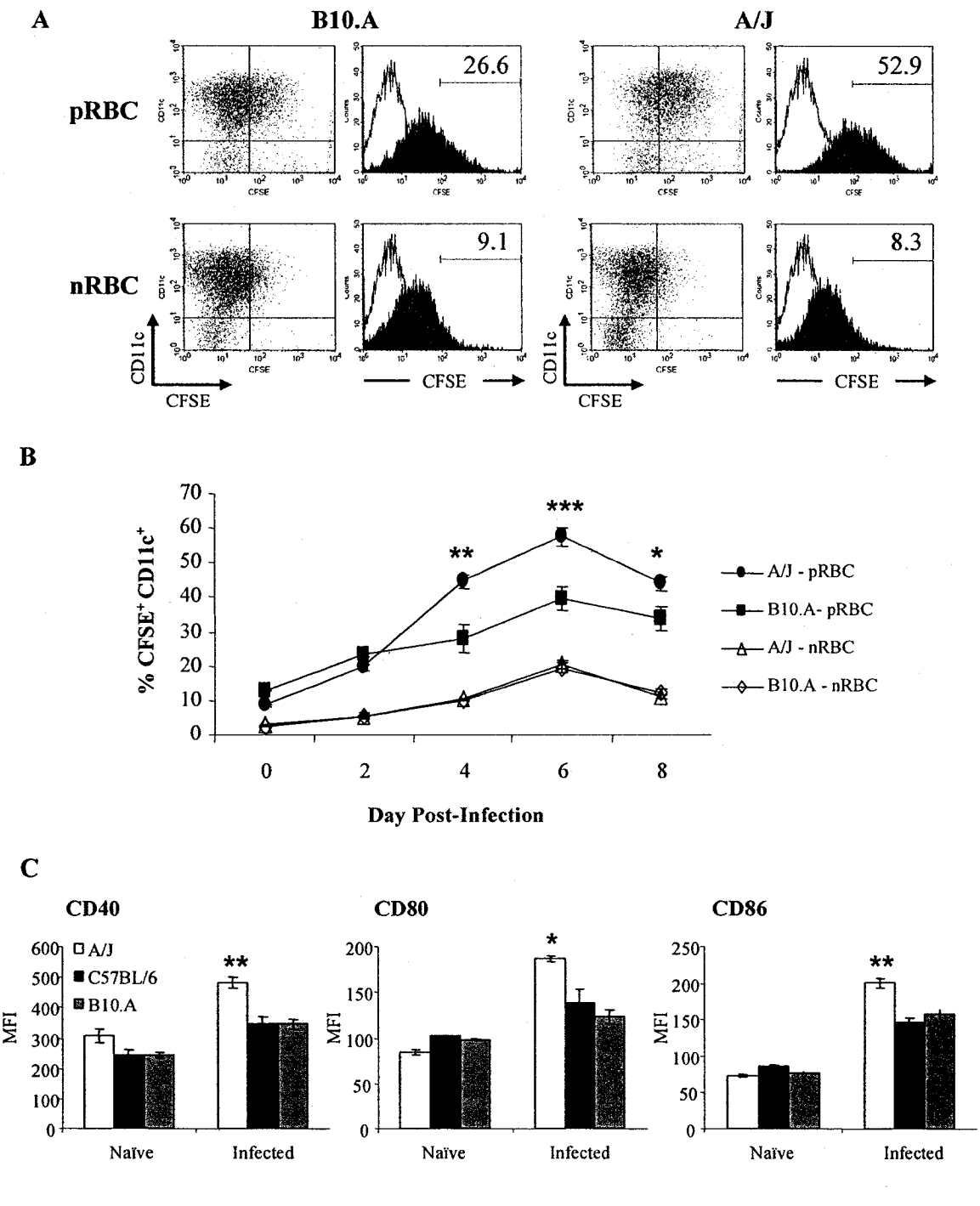
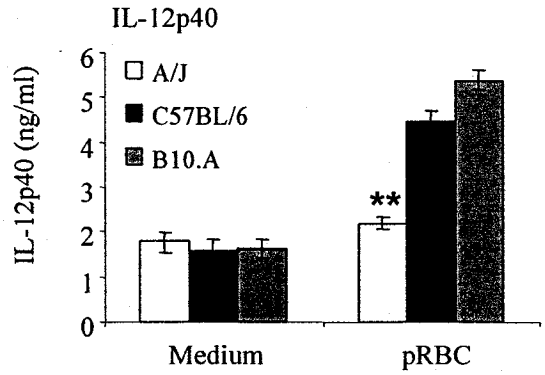
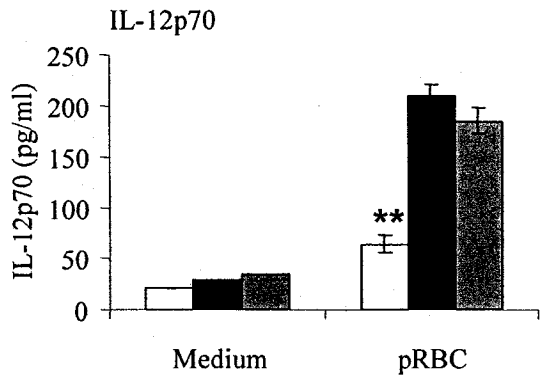
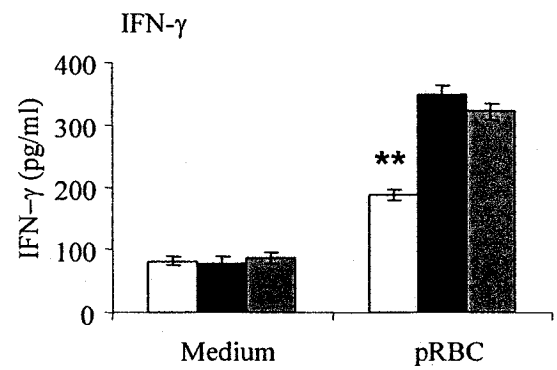

A
B10.A
A/J
pRBC
nRBC
26.6
52.9
9.1
8.3
CD11c
CFSE
B
% CFSE+ CD11c+
Day Post-Infection
A/J - pRBC
B10.A- pRBC
A/J - nRBC
B10.A - nRBC
C
CD40
CD80
CD86
A/J
C57BL/6
B10.A
MFI
Naïve
Infected

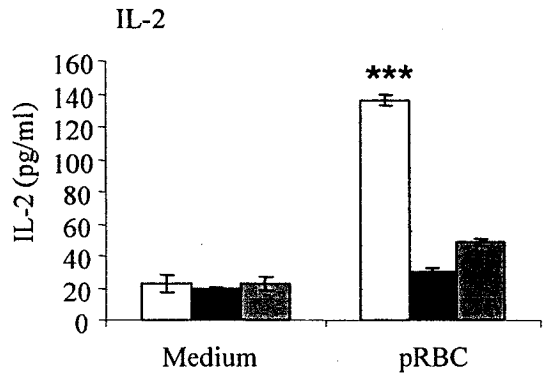
Figure 2. DCs from A/J mice produce lower levels of IL-12 and IFN-γ but higher levels of IL-2 compared to DCs from B6 or B10.A mice. Splenic $CD11c^+$ DCs enriched from infected mice were cultured without (medium) or with pRBC for 48 h. Supernatants were analyzed for IL-12p40, IL-12p70, IFN-γ, IL-2 and IL-10 by ELISA. Data are representative of 3 experiments each containing 3-4 mice per group. Statistical analyses compared A/J to B6 and B10.A. (**, $P < 0.01$; ***, $P < 0.001$).

Figure 2


IL-12p40
A/J
C57BL/6
B10.A
IL-12p40 (ng/ml)
0
1
2
3
4
5
6
**
Medium
pRBC


IL-12p70
IL-12p70 (pg/ml)
0
50
100
150
200
250
**
Medium
pRBC


IFN-γ
IFN-γ (pg/ml)
0
100
200
300
400
**
Medium
pRBC


IL-2
IL-2 (pg/ml)
0
20
40
60
80
100
120
140
160
***
Medium
pRBC

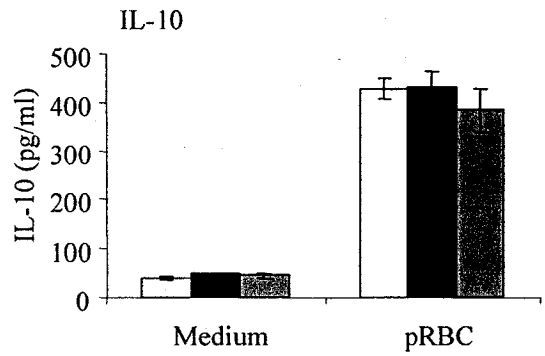
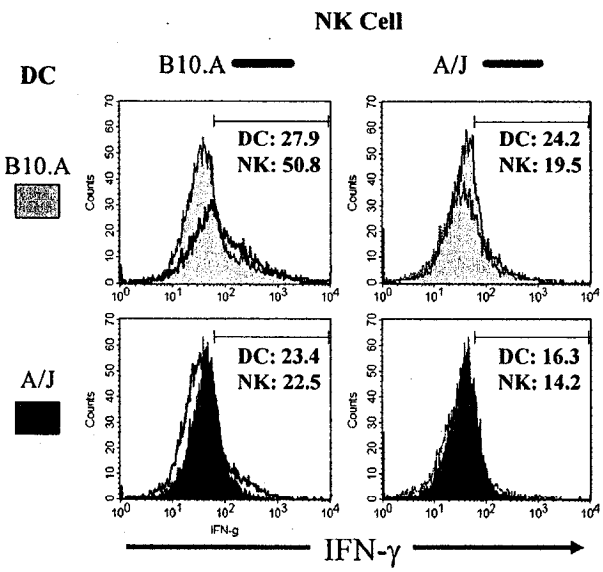

IL-10
IL-10 (pg/ml)
0
100
200
300
400
500
Medium
pRBC

Figure 3. A/J mice show impaired crosstalk between malaria-activated DCs and resting NK cells. Splenic $CD11c^+$ DCs from infected mice and NK cells from naïve mice were cultured together in mixed and matched combinations. *A*, Intracellular IFN-γ expression by DCs and NK cells from B10.A and A/J mice after 24 h of co-culture. Each panel displays composite histograms of IFN-γ signals from gated $CD11c^+$ or $DX5^+$ cell populations. Shaded histograms represent DCs from B10.A or A/J mice, lines represent NK cells, and the percentages of DCs or NK cells expressing IFN-γ are shown. Data are representative of 3 experiments showing similar results. *B*, IFN-γ levels in the co-culture supernatants as quantified by ELISA. Data are representative of 3 experiments each containing 4 replicates per group. (***, $P < 0.001$).

Figure 3


NK Cell
DC
B10.A
A/J
B10.A
A/J
DC: 27.9
NK: 50.8
DC: 24.2
NK: 19.5
DC: 23.4
NK: 22.5
DC: 16.3
NK: 14.2
Counts
IFN-g
IFN-γ

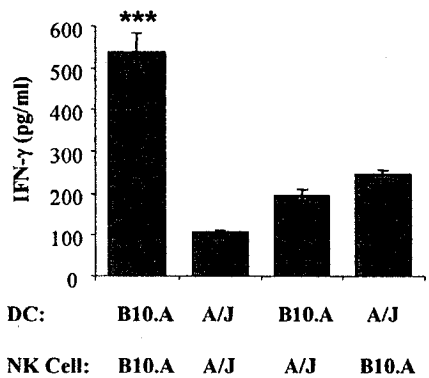

***
IFN-γ (pg/ml)
600
500
400
300
200
100
0
DC: B10.A A/J B10.A A/J
NK Cell: B10.A A/J A/J B10.A

Figure 4. DCs from infected A/J mice have normal capacity to stimulate $CD4^+$ T cell proliferation in an IL-2-dependent mechanism. DCs from naïve or infected B10.A or A/J mice were co-cultured with $CD4^+$ T cells from naïve B10.A mice. *A*, Following *P. chabaudi* infection, DCs from A/J mice stimulated higher levels of $CD4^+$ T cell proliferation than did DCs from B10.A mice. *B*, IL-15 and IL-12 were not required for DC-activated $CD4^+$ T cell proliferation. DCs from infected B10.A or A/J mice were treated with anti-IL-15 or anti-IL-12 mAbs prior to co-culture with $CD4^+$ T cells. *C*, IL-2 is necessary for optimal stimulation of $CD4^+$ T cell proliferation by malaria-activated DCs. DCs from infected B10.A or A/J mice were treated with anti-IL-2 mAb prior to prior to co-culture with $CD4^+$ T cells. Data are representative of 3 experiments each containing 3-4 replicates per group. In *B* and *C*, $CD4^+$ T cells cultured without DCs were included as negative controls. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 4

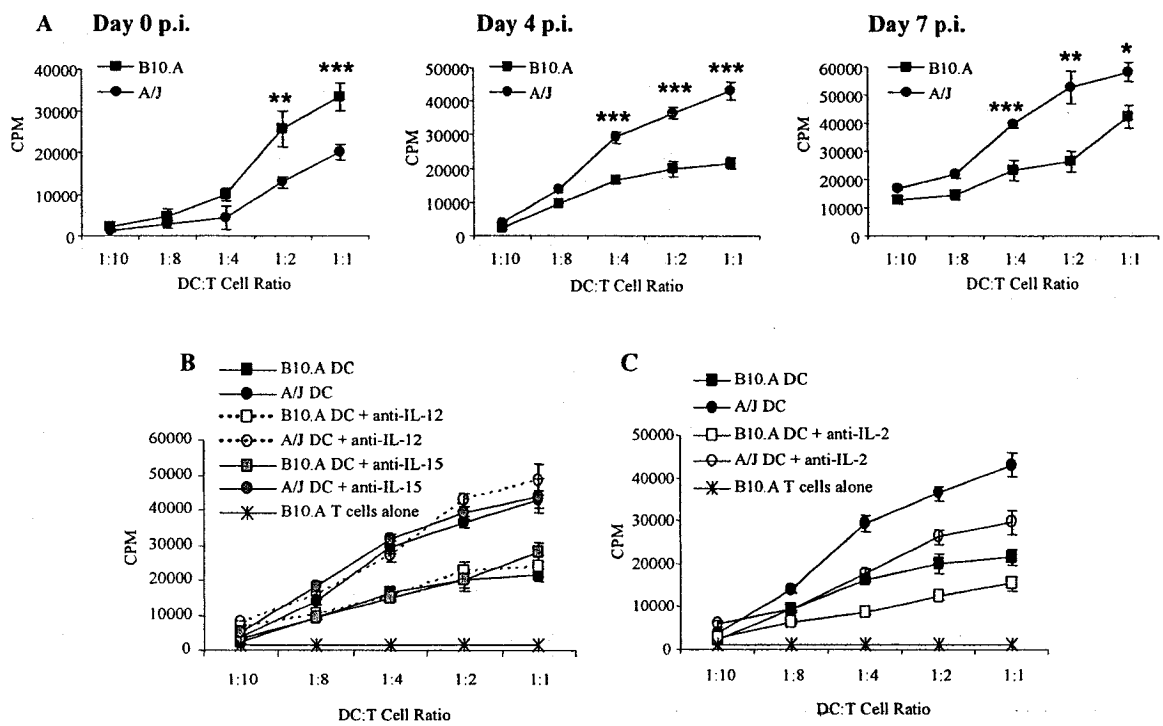

A
Day 0 p.i.
Day 4 p.i.
Day 7 p.i.
B10.A
A/J
CPM
DC:T Cell Ratio
1:10
1:8
1:4
1:2
1:1
B
C
B10.A DC
A/J DC
B10.A DC + anti-IL-12
A/J DC + anti-IL-12
B10.A DC + anti-IL-15
A/J DC + anti-IL-15
B10.A T cells alone
B10.A DC + anti-IL-2
A/J DC + anti-IL-2

Figure 5. DCs from infected A/J mice induce resting $CD4^+$ T cells to produce less IFN-γ but more IL-10. *A*, Unfractionated spleen cells from infected A/J mice stimulated without (medium) or with anti-CD3 mAb produced less IFN-γ but more IL-10 than did cells from B10.A mice. *B*, DCs from infected A/J mice stimulated $CD4^+$ T cells to produce lower levels of IFN-γ but higher levels of IL-10 than did DCs from B10.A mice. Levels of IL-4 produced were comparable between the two co-cultures. *C*, To determine the role of different cytokines in DC-induced IFN-γ production by $CD4^+$ T cells, DCs from infected B10.A mice were treated with blocking mAb against IL-2, IL-12, or IL-15 prior to co-culture with $CD4^+$ T cells. *D*, Similarly, to determine the role of different cytokines on DC-induced IL-10 production by $CD4^+$ T cells, DCs from infected A/J mice were treated with blocking mAb against the specific cytokine prior to co-culture with $CD4^+$ T cells. Data are representative of 3 experiments each containing 3-4 mice per group. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 5

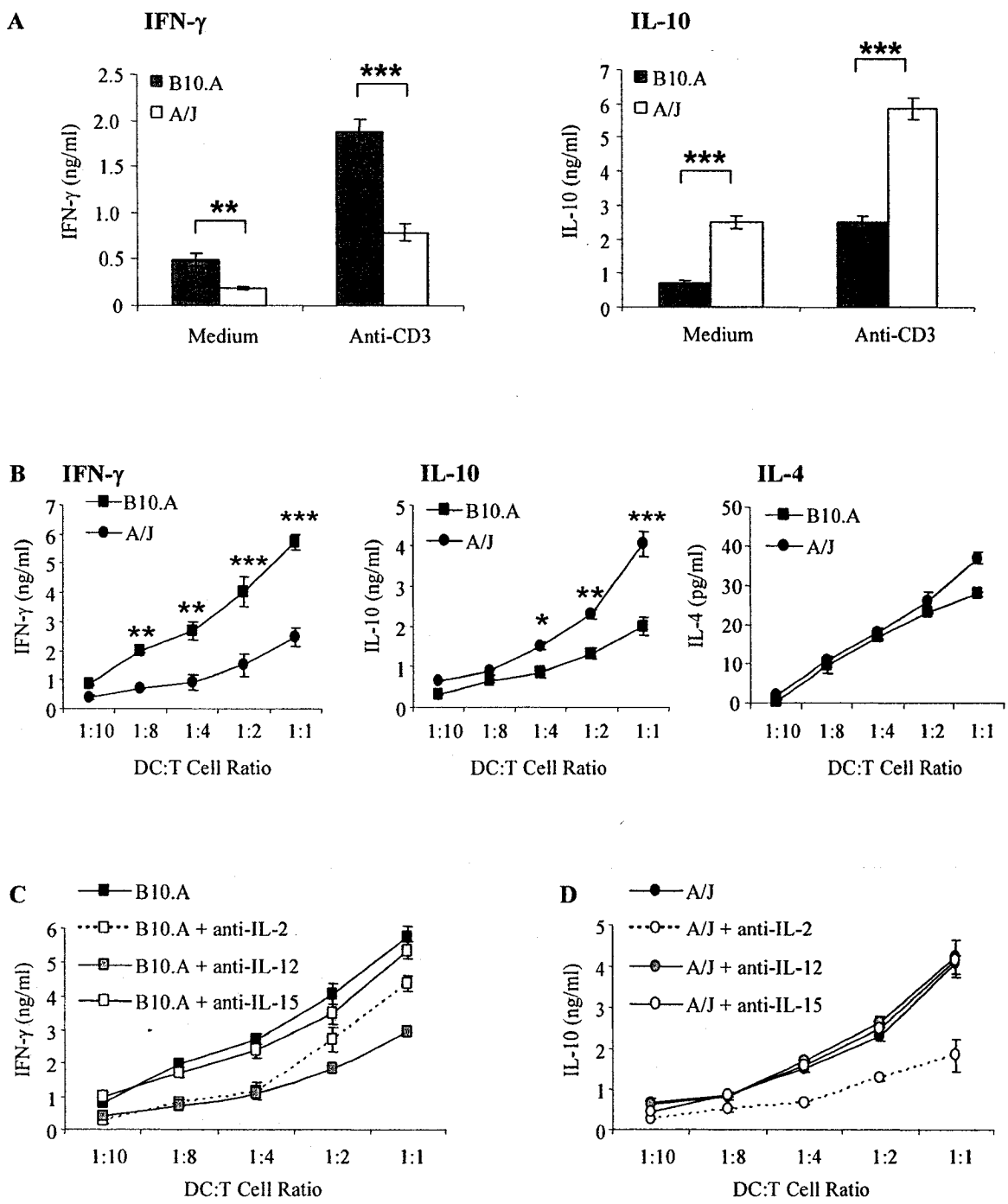
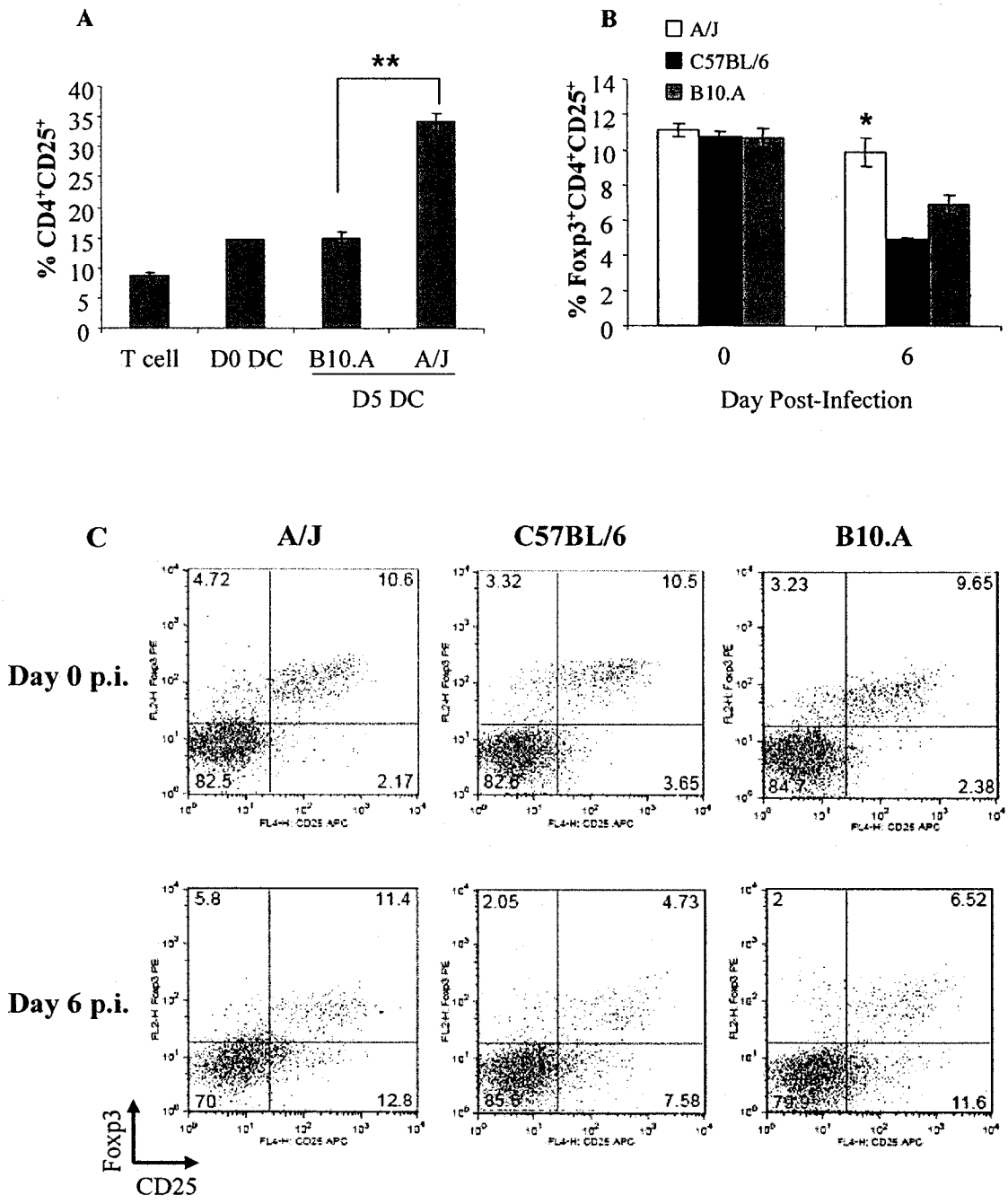

A
IFN-γ
IL-10
B10.A
A/J
IFN-γ (ng/ml)
IL-10 (ng/ml)
Medium
Anti-CD3
B
IFN-γ
IL-10
IL-4
IL-4 (pg/ml)
DC:T Cell Ratio
1:10
1:8
1:4
1:2
1:1
C
B10.A + anti-IL-2
B10.A + anti-IL-12
B10.A + anti-IL-15
D
A/J + anti-IL-2
A/J + anti-IL-12
A/J + anti-IL-15

Figure 6. DCs from infected A/J mice have tolerogenic potential for inducing $CD4^+$ T cells with a regulatory phenotype (*A*) and this correlates with increased Treg cells in vivo (*B* and *C*). *A,* Splenic $CD11c^+$ DCs from naïve B10.A mice or day 5 infected A/J or B10.A mice were co-cultured with $CD4^+$ T cells enriched from naïve B10.A mice for 48 h at 37°C. Following culture, the percentage of gated $CD4^+$ T cells expressing CD25 was determined by flow cytometry. Single cultures of T cells alone were included as controls. Data are representative of two independent experiments containing 3-4 mice per group. *B,* Unfractionated splenocytes were analyzed for CD4, CD25, and Foxp3 expression by flow cytometry. Percentages of gated $CD4^+$ T cells expressing both CD25 and Foxp3 are shown for naïve and day 6 infected mice in one experiment with 3 mice per group. *C,* Representative dot plots of gated $CD4^+$ T cells expressing CD25 and/or Foxp3 are shown before and after *P. chabaudi* infection. The percentage in each quadrant is indicated. (*, $P < 0.05$; **, $P < 0.01$).

Figure 6


A
**
% CD4+CD25+
40
35
30
25
20
15
10
5
0
T cell
D0 DC
B10.A
A/J
D5 DC
B
A/J
C57BL/6
B10.A
% Foxp3+CD4+CD25+
14
12
10
8
6
4
2
0
*
0
6
Day Post-Infection
C
A/J
C57BL/6
B10.A
Day 0 p.i.
4.72
10.6
82.5
2.17
3.32
10.5
82.6
3.65
3.23
9.65
84.7
2.38
Day 6 p.i.
5.8
11.4
70
12.8
2.05
4.73
85.5
7.58
2
6.52
79.9
11.6
Foxp3
CD25

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Malaria is the most important parasitic disease in humans. Despite extensive efforts to eradicate the disease, including chemotherapy, insecticides and the use of insecticide-treated bed nets, malaria continues to afflict an increasing number of individuals worldwide and cause substantial morbidity and mortality in sub-Saharan Africa. The development and implementation of an effective anti-malarial vaccine that at the very least diminishes parasite densities and prevents clinical disease is widely regarded as the most promising strategy to control human malaria. A better understanding of mechanisms underlying protective immunity would greatly facilitate vaccine development and improve treatment modalities. The complexity of the plasmodial life cycle, involvement of many host tissues and antigenic components, and multi-faceted interactions with the host immune system all present formidable challenges to the elucidation of immune responses that effectively eliminate the infection with minimum pathology to the host. The overall objective of this thesis was to determine the role of DCs in host-parasite interactions and subsequent induction of protective immunity to blood-stage malaria. Importantly, the studies described herein emphasize the critical contributions of key cytokines to DC responses and interactions with other cells of the innate and adaptive immune system during blood-stage malaria.

The experimental model of *P. chabaudi* AS infection has been useful for elucidating protective host immune responses against blood-stage malaria. Inter-stain differences in the ability to resolve and survive a primary *P. chabaudi* infection have led to genetic mapping of the control of blood-stage malaria to a locus (*Char*2) on mouse chromosome 8. This region accounts for 10% of variance in peak parasitemia between A/J and C57BL/6 mice (87, 88) and contains several possible candidate genes. IL-15 is one candidate found in the genetic interval for *Char2* that may modulate host resistance to blood-stage malaria.

Interleukin 15 as a candidate modulator of host resistance to blood-stage malaria

Given that IL-15 was identified as a candidate for genetically-regulated variability in peak parasitemia, it is important to define a functional basis for this cytokine in host resistance to blood-stage malaria. To this end, the study described in Chapter 2 investigated the role of endogenous IL-15 in innate immune responses and development

of adaptive immunity to *P. chabaudi* AS malaria. Compared to wild-type B6 mice, IL-$15^{-/-}$ mice were unable to resolve a primary *P. chabaudi* infection in a timely manner and also showed impairments in NK cell expansion and IFN-γ production, optimal synthesis of IL-12 and IFN-γ by DCs, and protective Th1-dependent Ab responses. Importantly, the defect in IFN-γ synthesis by both DCs and NK cells from IL-$15^{-/-}$ mice could not be restored fully by addition of exogenous IL-15 in vitro. These results indicate that IL-15 is required for early control and timely resolution of blood-stage malaria through regulation of innate responses and subsequent activation of type 1 adaptive immunity.

A notable limitation of this study is that we did not determine the level or kinetics of IL-15 production following *P. chabaudi* infection or among mice with differential abilities to control blood-stage malaria. Techniques and reagents to measure levels of IL-15 protein were not readily available at the time these experiments were performed, and later studies showed that the majority of functions attributed to IL-15 are mediated by its membrane-bound isoform (500-502). Nonetheless, preliminary studies using gene array RT-PCR and northern blot analysis showed weak expression of IL-15 mRNA in the spleen after *P. chabaudi* infection and no differences in mRNA expression levels between B6 and A/J mice (data not shown).

Based on the results reported here and in other studies, we propose that IL-15 plays an important but dispensable role in a two-stage progression of the immune response to blood-stage malaria infection. In the first stage, malarial antigens may stimulate macrophages and DCs to express IL-15 and IL-15Rα through direct cell contact and/or DC-derived cytokines such as IFN-α/β (496). IL-15 then potentiates IL-12 and IFN-γ synthesis by APCs, which in turn enables them to become further activated via a positive autocrine feedback loop. IL-15, alone or in synergy with other Th1 cytokines, stimulates NK cells, and possibly $CD8^+$ T cells and γδT cells, to produce large quantities of IFN-γ, thereby enhancing the priming of naïve $CD4^+$ T cells toward Th1 cell differentiation and subsequent initiation of type 1 adaptive immune responses. Activated Th1 cells, in the later stage, would result in additional IFN-γ production, further macrophage and NK cell activation, and B cell differentiation. For B cells, IL-15 is a key cytokine promoting B cell proliferation and Ig class switching, resulting in strong parasite-specific antibody

responses. This combination of cellular and antibody-mediated effector mechanisms is beneficial for controlling parasitemia and resolving chronic infection.

Antigen uptake and presentation functions of dendritic cells during blood-stage malaria

The finding that IL-15 enhances Th1-type cytokine production by splenic $CD11c^+$ DCs was of particular interest because DCs play a seminal role in detecting infection and activating protective immune responses. RBCs, wherein malaria parasites undergo asexual reproduction, do not express MHC molecules and therefore are unable to directly stimulate naïve T cells. Initiation of adaptive immunity must be generated through malaria-activated APCs, such as DCs. Studies on the role of DCs in immunity to blood-stage malaria have produced conflicting results. An early study showed that *P. falciparum*-infected RBCs suppress the maturation of immature blood DCs in response to LPS stimulation in vitro (359). However, the numbers of circulating DCs expressing CD83, a marker of DC maturation, were found to be comparable between healthy and malaria-infected children in Kenya, suggesting that *P. falciparum* does not suppress DC maturation in vivo (361). Similarly, several studies using mouse malaria models have shown that DCs exposed to pRBC are able to mature normally, secrete cytokines, and prime $CD4^+$ T cell responses (364-367). However, it is not known whether DCs selectively recognize and capture pRBC, and whether uptake of pRBC by DCs results in autocrine activation and the initiation of adaptive immunity such as $CD4^+$ T cell proliferation and IFN-γ production.

The study described in Chapter 3 investigated the antigen uptake and presentation functions of DCs that lead to activation of $CD4^+$ T cell responses during blood-stage malaria. A sensitive flow cytometry assay was devised to measure and compare the uptake of pRBC versus nRBC by DCs in vitro. In this assay, purified pRBC or nRBC were labeled with fluorescent CFSE and then co-cultured with bone marrow-derived DCs or splenic $CD11c^+$ DCs from naïve or *P. chabaudi*-infected mice. The percentage of $CFSE^+$ DCs as determined by flow cytometry is indicative of the level of uptake of pRBC or nRBC. Confocal fluorescence microscopy confirmed the colocalization of fluorescent signals from ingested CFSE-labeled pRBC and DCs, thus validating the use of this assay to quantify pRBC or nRBC uptake by DCs. Consistent with their seminal

role in detecting infection, both bone marrow-derived and splenic DCs exhibited higher uptake of pRBC than nRBC. Uptake of pRBC by splenic DCs was selectively enhanced after infection and was accompanied by upregulated expression of MHC class II and costimulatory molecules and production of cytokines, particularly IL-12. By contrast, these responses were low or absent in DCs co-cultured with nRBCs. Importantly, DCs loaded with pRBC but not nRBC were able to stimulate high levels of $CD4^+$ T cell proliferation and IFN-γ production. Together, these data provide strong evidence that early interactions between DCs and intraerythrocytic malaria parasites result in activation of three key DC functions—antigen uptake, costimulation and cytokine production—needed to prime naïve or resting $CD4^+$ T cells to proliferate and differentiate into IFN-γ-secreting Th1 cells.

These results support and extend other findings that DCs exposed to RBCs infected with human or rodent *Plasmodium* or purified from infected mice are fully functional APCs capable of expressing costimulatory molecules, producing Th1-polarizing cytokines, and stimulating $CD4^+$ T cells. However, there are other reports from human or mouse systems of immune suppression by *Plasmodium*-infected RBCs (359, 371). As discussed in Chapter 3, there are several possible explanations for the discrepancy between findings of functional DC responses observed in mice infected with rodent *Plasmodium* strains and those of studies with isolates of the human parasite or with *P. yoelii* infection in mice. Findings from recent studies may further help resolve this issue. Several studies used LPS as a maturation stimulus to demonstrate impaired maturation or cytokine production by DCs after exposure to pRBC. It must be noted that the production of IL-12 by DCs is tightly regulated and transient. Once stimulated, DCs may become paralyzed or exhausted and unable to produce IL-12 upon further stimulation with microbial products (317, 318). In some of these studies that showed pRBC-induced maturation defects in DCs (359, 370, 371), LPS was added before pRBC to the DC cultures. These LPS-stimulated DCs may respond very differently to a second stimulation, such as pRBC, than unstimulated DCs. Indeed, preincubation of bone marrow-derived DCs with *P. chabaudi*-infected RBC did not inhibit subsequent LPS-induced maturation (394). Despite pRBC-induced defects in maturation, DCs are able to prime $CD4^+$ T cells in vivo (367, 370) and to restimulate recall $CD8^+$ T cell responses

(367), indicating that the observed suppression of DC responses by pRBC is short-lived and reversible in vivo.

The kinetics of DC responses may determine whether suppression or activation is observed after in vivo infection or in vitro exposure to pRBC. Early after *P. yoelii* infection in mice (12-24 h), interactions between splenic $CD11c^+$ DCs and pRBCs result in DC phenotypic and functional maturation (536), as shown in other studies. As malaria infection progresses, DCs produce less IL-12 and TNF-α in response to TLR ligands, produce higher levels of IL-10, and support less IFN-γ production by $CD4^+$ T cells. This switch from proinflammatory to anti-inflammatory or regulatory responses by DCs in the later stages of malaria infection might reflect a beneficial homeostatic process to control inflammation and prevent immunopathology. Taken together, the immune activation versus suppression reported by different studies may reflect distinct stages of the same response. More studies are required to clarify the potential immunomodulatory effects of pRBCs on DCs during distinct stages of malaria infection.

The finding, reported in this thesis, that DCs selectively recognize and phagocytose pRBCs but not nRBCs raises the question of what pathogen-uptake receptors on DCs are involved in pRBC uptake. It is well documented that the scavenger receptor CD36 mediates phagocytosis of pRBC by macrophages and thus contributes to parasite clearance (391, 513, 540). Although *P. chabaudi* and cytoadherent strains of *P. falciparum* bind to purified CD36 (359, 360, 514), there is presently no evidence for a direct role of CD36 in the uptake of pRBC by DCs. To investigate this, we used our flow cytometry assay to determine whether uptake of pRBC by DCs is mediated via CD36. Bone marrow-derived and splenic $CD11c^+$ DCs were treated with anti-CD36 mAb (clone 63, Cascade Biosciences) to block this receptor prior to co-culture with CFSE-labeled pRBC. Flow cytometric analyses showed high binding of the anti-CD36 mAb to DCs, but there were no differences in the level of pRBC uptake between mAb-treated and untreated DCs (unpublished data). Although these results need to be repeated and confirmed with DCs from $CD36^{-/-}$ mice, they suggest a minimal role for CD36 in pRBC uptake by DCs. Studies using other systems suggest that the uptake of pathogens, tumor cells or apoptotic cells by DCs involves receptors of the C-lectin family, such as DEC-205 and DC-SIGN (515, 541). In particular, DCs use DC-SIGN to capture bacteria,

fungi, viruses and intracellular parasites such as *Leishmania*, to migrate from blood into tissues through interactions with endothelial ICAM-2, and to form an immunological synapse with T cells via ICAM-3 (515, 542, 543). Certain viruses such as HIV and bacteria such as mycobacteria enter the DC, normally a nonpermissive host cell, via DC-SIGN, and the infected DC then efficiently transmits the pathogen to neighboring permissive target cells such as T cells (544, 545). Thus, DC-SIGN might be a potential candidate in receptor-mediated uptake of pRBC by DCs. It will also be of interest to determine whether DCs, like macrophages (392), are able to capture free merozoites, and subsequently mature and acquire the capacity to prime naïve T cells, as demonstrated in Chapter 3 with DCs that had phagocytosed pRBC. Further experiments are necessary to identify the receptor(s) and precise mechanisms for recognition and uptake of pRBC and merozoites by DCs.

Dendritic cell and NK cell interactions during blood-stage malaria

In addition to antigen uptake and presentation, DCs may initiate immune responses through cognate interactions with other cells of the innate immune system. Although prior to our work there have been no studies of DC–NK-cell interactions during blood-stage malaria, there is preliminary evidence that such interactions occur in vivo and are important for determining the outcome of malaria infection. During the innate immune response to *Plasmodium* infection, DCs may express several cytokines, including IL-2, IL-12, IFN-γ, TNF-α (Chapters 2 and 3; 364, 366, 536) and, in humans, IFN-α (369). Some of these cytokines are known to activate macrophages and NK cells to secrete cytokines and to become phagocytic or cytolytic (146, 147, 392). Activation of NK cells by DCs might be a critical event in the innate immune response because NK cells are one of the main producers of IFN-γ that is absolutely required for protective immunity to blood-stage malaria (139, 140, 400). The work presented in Chapter 4 indicates that splenic $CD11c^+$ DCs purified from *P. chabaudi*-infected mice were able to stimulate resting splenic NK cells to secrete high levels of IFN-γ in vitro. In agreement with studies in other systems, NK cells from infected mice were able to induce DCs to mature and produce Th1-type cytokines. DCs matured as a result of stimulation by malaria-activated NK cells were able to prime resting $CD4^+$ T cells to proliferate and produce

high levels of IFN-γ. These results suggest that the reciprocal interaction of DCs and NK cells is a critical event in the early innate immune response to blood-stage malaria infection that promotes early IFN-γ production and the induction of protective Th1 immune responses.

As IL-15 is an integral cytokine for NK cell development and activation as well as for DC cytokine synthesis (Chapter 2), we postulated that IL-15 is one of the factors that promote DC-mediated activation of NK cells. However, IL-15$^{-/-}$ mice show higher parasitemia only during the chronic stage of *P. chabaudi* malaria and were eventually able to resolve the infection without significantly higher mortality than wild-type B6 mice. By contrast, IL-12p40$^{-/-}$ mice show a severe course of infection including high parasitemia and mortality (420). Given the importance of DC-derived IL-12 for induction of Th1 immunity and IL-15 for NK cell function, we delineated the relative contributions of IL-12 and IL-15 to DC responses and interactions with NK cells during blood-stage malaria. IL-12, but not IL-15, was found to be critical for DC maturation, Th1-type cytokine production and stimulation of maximal IFN-γ production by NK cells. Defects in DC–NK-cell interactions observed in IL-12p40$^{-/-}$ mice correlated with higher parasitemia and mortality compared to WT and IL-15$^{-/-}$ mice. These results suggest that IL-12, but not IL-15, is critically required for DC-mediated IFN-γ production by NK cells, whereas the role of IL-15 is likely to promote NK cell development and survival.

A conceptual model, shown in Fig. 6.1, is proposed for the pivotal position of DC–NK-cell interactions in the induction of early IFN-γ production and subsequent Th1 cell development. After contact with pRBCs, DCs mature and secrete immunoregulatory cytokines. Mature DCs activate NK cells by providing critical contact-dependent signals, which may include surface-bound cytokines, such as IL-12, IL-15 and IL-18, as well as soluble mediators, such as IL-2. In our study, we observed that DC-derived IL-2 and IL-12 are required for activation of NK cell IFN-γ production, while IL-2 and IL-15 are important for NK cell development and function. Once activated directly by pRBCs (141) or indirectly via crosstalk with DCs, NK cells secrete high levels of IFN-γ, which in turn may contribute to further maturation of DCs and enhance DC-mediated polarization of CD4$^+$ T cells to the Th1 phenotype. After interaction with malaria-activated NK cells, DCs become highly efficient at priming CD4$^+$ T cells to secrete

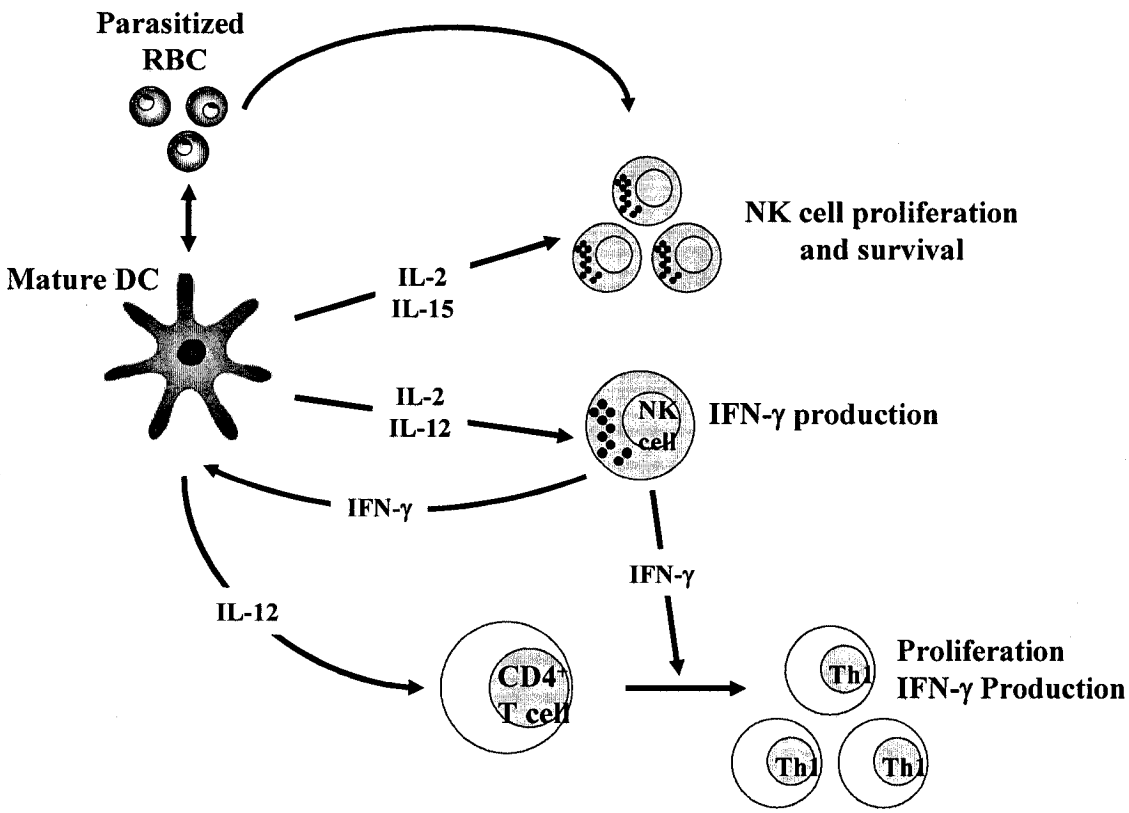


Figure 6.1: Summary of DC and NK Cell Interactions in Blood-Stage Malaria

IFN-γ, thus propagating a robust Th1 immune response to blood-stage malaria. Together, the results provide novel insights into reciprocal DC–NK-cell interactions during blood-stage malaria, as well as the roles of key mediators of this crosstalk.

There are other cytokines not evaluated in our study that may also play an important role in malaria-specific DC–NK-cell crosstalk. TNF-α has been shown to be a key cytokine in NK cell-mediated DC maturation (156, 160, 162, 546). Our transwell experiments suggest that soluble mediators, such as TNF-α, released by NK cells are not as crucial as cell-cell contact in the ability of malaria-activated NK cells to induce DC maturation. Although we cannot exclude a possible contribution of TNF-α in NK cell-mediated DC maturation (159, 160, 162), previous studies have demonstrated that TNF-α activity is not required for protective Th1 immunity against a primary *P. chabaudi* infection (388, 480). In addition, IL-18 has been shown to direct DC-mediated activation of NK cells in response to viral or bacterial stimuli (149, 151, 155) and to contribute significantly to protective immunity against human and rodent malaria (398, 475). Additional experiments will be necessary to determine the role(s) of these mediators in DC–NK-cell crosstalk during blood-stage malaria.

Lastly, a major limitation of our study is the lack of data on the benefit of DC–NK-cell crosstalk for control of blood-stage malaria in vivo. Our studies comparing inbred strains of mice with differential susceptibilities to blood-stage malaria (Chapter 5) may provide initial evidence that efficient DC–NK-cell crosstalk is integral to the ability of the host to overcome and survive a primary *P. chabaudi* infection. Specifically, DCs from susceptible A/J mice were unable to stimulate resting NK cells to secrete IFN-γ compared with DCs from resistant H-2 compatible B10.A mice. Recent data provide evidence that NK cells are required in vivo for optimal DC responses to blood-stage malaria. Mice treated with anti-asialo-GMI Ab to deplete NK cells developed higher parasitemia after primary *P. chabaudi* infection than mice treated with control rabbit Ig (139, unpublished data). Moreover, splenic $CD11c^+$ DCs from NK cell-depleted mice showed lower levels of costimulatory molecule (CD40 and CD86) expression and IL-12p70 production in vitro as well as impaired stimulation of $CD4^+$ T cell proliferation and IFN-γ secretion than DCs from control mice (unpublished data). Together, these results indicate that NK cells support DC maturation, cytokine production and induction

of IFN-γ-secreting Th1 cells in vivo. To further confirm the in vivo relevance of DC–NK-cell crosstalk, another potential experiment is to devise a DC-based malaria vaccine and adoptively transfer the malaria-loaded DCs into IL-15$^{-/-}$ mice which lack functional NK cells or into B6 mice which have been depleted of NK cells in vivo. Appropriate controls include nonvaccinated mice (vaccine control) and vaccinated wild-type or untreated mice (NK cell depletion control). A proposed protocol for this DC-based malaria vaccine is described below. Mice capable of high levels of NK cell IFN-γ production effectively control primary blood-stage malaria infection (139, 378). Having demonstrated that malaria-activated DCs stimulate IFN-γ production by NK cells in vitro (Chapter 4), the DC transfer experiment aims to determine whether malaria-loaded DCs can activate NK cells in vivo and whether this interaction protects mice from challenge infection with blood-stage parasites.

Role of dendritic cells in induction of immune activation versus tolerance

In Chapter 5, we investigated whether the susceptibility of A/J mice is associated with altered DC response profiles during blood-stage malaria. To dissect the specific functional responses of DCs required for Th1 induction, we analyzed and compared the capacity for antigen uptake, costimulation, and cytokine production by DCs from inbred strains of mice with differential abilities to control and survive a primary *P. chabaudi* infection. Although A/J mice had lower numbers of CD11c^{+} DCs in the spleen, these DCs were able to take up pRBC, express costimulatory molecules, and stimulate CD4^{+} T cell proliferation at higher levels than did DCs from B6 or B10.A mice after *P. chabaudi* infection. Despite a more mature phenotype, DCs from infected A/J mice secreted less IL-12 and IFN-γ in vitro, suggesting these DCs may be unable to induce protective type 1 immune responses. Indeed, A/J DCs stimulated lower IFN- γ production from NK cells as well as lower IFN-γ, but higher IL-10, secretion from CD4^{+} T cells than did DCs from B10.A mice. IL-2 was necessary for DC-mediated T cell proliferation, whereas IL-12 was critical for T cell IFN-γ production. That A/J DCs secreted more IL-2 and less IL-12 in response to pRBC in vitro than did B6 or B10.A DCs may explain their greater capacity to induce T cell proliferation but not IFN-γ production. These data suggest that DCs from A/J mice can capture pRBC, mature phenotypically, and present malarial

antigens to $CD4^+$ T cells for proliferative responses. Importantly, however, DCs from susceptible mice are defective in producing Th1-type cytokines required to activate NK cells and induce preferential Th1 cells required for protective immunity to blood-stage *P. chabaudi* infection.

Although the two-stage progression of DC development—immature (tolerance) to mature (immunity)—has been useful to understand DC biology, recent studies have provided evidence that maturation may not result in immune activation. The findings that splenic DCs from infected A/J mice were able to express abundant costimulatory molecules but failed to produce IL-12 and IFN-γ and to induce IFN-γ-secreting NK and Th1 cells demonstrate that phenotypic maturation is not consistently accompanied by functional maturation required for initiation of protective immunity. As discussed in Chapter 5, evidence from studies in other systems support the notion that mature DCs bearing antigen and costimulatory molecules can induce $CD4^+$ T cell proliferation but not effector responses (110, 537-539). Collectively, these data suggest that susceptibility to blood-stage malaria may involve DCs that develop a mature surface phenotype but are unable to induce appropriate immunity in the absence of malaria-induced production of Th cell-polarizing cytokines. Instead, DCs that produce high IL-10 but low IL-12 and IFN-γ have a tolerogenic potential to induce Treg cells, which have been shown to suppress immunity and maintain tolerance in the steady state and following infection.

The inability of DCs from malaria-susceptible mice to prime Th1 cell-mediated immunity reveals several new potential avenues for research into the role of DCs in immunity to blood-stage malaria. First, the defects in Th1-type cytokine production and Th1 cell induction observed in DCs from A/J mice are similar to those reported in mice deficient in the Th1-specific transcription factor T-bet. In addition to activating IFN-γ gene expression in T cells and NK cells and initiating Th1 lineage commitment (225), T-bet is expressed at high levels in DCs and is required for optimal production of IFN-γ and activation of antigen-specific Th1 cells by DCs (547). Moreover, T-bet increases IL-12R expression in naïve $CD4^+$ T cells, thus rendering them more responsive to IL-12 stimulation for IFN-γ synthesis and Th1 development (521). Mice lacking T-bet are unable to control infections with *Salmonella* or *Mycobacterium tuberculosis*, and exhibit reduced IFN-γ but increased IL-2 and IL-10 production (548, 549). It is intriguing that

T-bet-deficient mice do not develop a fully polarized Th2 response. Similarly, our study showed that DCs from different inbred strains were able to stimulate comparable but low levels of IL-4 production by $CD4^+$ T cells, indicating that impaired Th1 immunity to blood-stage malaria is not due to deviation toward Th2 cell differentiation. Further experiments are warranted to determine whether differential expression of T-bet by DCs among resistant and susceptible mice controls the development of protective Th1 immunity to blood-stage malaria.

Another possibility is that susceptibility of A/J mice to blood-stage malaria reflects the preferential induction of Treg cells by DCs. Although there were no differences in IL-10 production by DCs among the groups of mice, only the DCs from infected A/J mice showed high IL-10 production coupled with low to absent IL-12 production. This pattern is similar to DCs previously described to have tolerogenic potential for immunosuppression and induction of Treg cells (336). In our study, we showed that compared to the Th1 cell-inducing DCs from resistant mice, DCs from infected A/J mice favored the development of IL-10-secreting $CD4^+$ T cells. Naturally occurring and inducible Treg cells secreting IL-10 and/or TGF-β have been implicated in the regulation of immunity to infection. Although Treg responses during chronic infection help to dampen inflammation and prevent tissue damage, activation of Treg cells in the early stage of immune response may impair effective pathogen control. In A/J mice, which show high peak parasitemias and normally succumb to a primary *P. chabaudi* infection within 10-12 days, it is likely that DC-mediated induction of Treg cells during the acute phase of infection acts not to prevent immunopathology but to increase susceptibility to blood-stage malaria. It would therefore be important to characterize the phenotype and function of the IL-10-secreting $CD4^+$ T cells induced by DCs from A/J mice. A key question is whether these putative Treg cells express Foxp3, a marker of $CD4^+CD25^+$ Treg cells, and are activated in A/J mice following *P. chabaudi* infection in vivo. Experiments are in progress to address this issue and two possible outcomes are predicted: 1) IL-10-secreting $Foxp3^+$ Treg cells are induced preferentially in A/J mice and contribute to poor early control of blood-stage malaria; 2) the IL-10-secreting $CD4^+$ T cells observed in A/J mice do not express $Foxp3^+$ but have comparable suppressive activity, as previously demonstrated in other systems (234). Furthermore, it will be of

interest to determine the specific mechanisms by which malaria-activated DCs favor Treg cell activity or survival. Antigen-processing DCs can directly expand functional $CD4^+CD25^+$ Treg cells (330). DC-derived cytokines such as IL-2 and TGF-β may convert conventional T cells into $CD4^+CD25^+$ or $Foxp3^+$ Treg cells that mediate suppressive effects in an IL-10-dependent manner (298, 299, 339). Given the higher levels of IL-2 secreted by DCs from infected A/J mice whereas TGF-β production by DCs was not detectable, it might be proposed that DC-derived IL-2 contributes more to the induction or activation of Treg cells in malaria-susceptible mice. Furthermore, based on previous findings that IL-15 promotes the growth and activity of antigen-specific $CD4^+CD25^+$ Treg cells (301-303) as well as the results presented in Chapter 2 that IL-15 is not essential for anti-malarial immunity, it will be important to reconsider the role of IL-15 in the immune response to blood-stage malaria. To this end, further research is warranted to determine whether IL-15 supports the expansion and maintenance of peripheral Treg cells, leading to tolerance and impaired Th1 immunity in *P. chabaudi*-susceptible mice. Data from these studies will help to advance our understanding of the mechanisms that regulate the balance between effector and regulatory T cells, thereby influencing both parasite and host survival strategies.

As antigen uptake by DCs is the first critical step in the initiation of immune responses, uptake receptors might be involved in the decision-making process of T cell differentiation. Accordingly, a third potential area of research concerns the type of antigen receptors expressed by DCs from different mouse strains that can lead to the induction of either immunity or tolerance. Previous studies have shown that some C-type lectin receptors, integrins and Fc receptors are involved in tolerance induction (550, 551). Upon binding of antigens, these receptors may activate signaling pathways that block maturation of the DC or induce inhibitory signals that render the DC tolerogenic (550, 551). Candidate tolerance-inducing receptors in malaria include CD36 and some integrins, which mediate uptake of apoptotic material, cellular debris and other exogenous antigens and play an important role in cross-tolerization of $CD8^+$ T cells by DCs (552, 553). Studies in mice, however, have shown that CD36, $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin are not required for cross-presentation of tissue antigens by $CD8^+$ murine DCs (554). Alternatively, DEC-205 mediates endocytosis of antigens targeted for

presentation on MHC class II molecules. Unlike other receptors which are degraded following uptake, DEC-205 recycles to the DC surface and mediates additional rounds of antigen uptake without further activation of DCs. Although this mechanism of antigen uptake can greatly enhance presentation of peptide-MHC complexes, repetitive stimulation of $CD4^+$ and $CD8^+$ T cells with immature DCs have been shown to induce tolerance (103, 541). Moreover, a subset of DEC-205^+CD8^+ DCs in mouse spleens have been shown to inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells in vitro (555, 556), and liver-derived and pulmonary DEC-205^+ DCs are poor stimulators of T cell proliferation and can induce T cells that produce IL-10 (334, 557). Thus, an important goal of future studies might be to determine whether DEC-205 and/or other antigen uptake receptors play a role in the tolerogenic function of DCs from malaria-susceptible mice.

Proposal for dendritic cell-based malaria vaccine

Given that DCs in resistant strains of mice are fully functional APCs during blood-stage malaria, we propose that the antigen capture and immunostimulatory activities of DCs could be exploited to create a DC-based immunization against infection with *P. chabaudi*. Antigen-pulsed DCs have been used to effectively protect immunized mice from *Toxoplasma, Listeria* and *Leishmania* infection (558-560). To date, there is only one published study in blood-stage malaria: mice immunized with bone marrow-derived DCs pulsed with the respective intact pRBCs survive lethal infections with blood-stage *P. yoelii* YM or *P. chabaudi* (370). Transfer of antigen-pulsed DCs also induces cross-strain protection. Despite these encouraging results, however, it is important to note that while pRBC-pulsed DCs enhanced survival to lethal *Plasmodium* infections, the surviving mice continued to develop high levels of parasitemia. Moreover, the high rates of mortality ($\geq$80%) reported in this study with *P. chabaudi* infections in normal untreated B6 mice have not been observed in other laboratories, including that of the author, using identical mouse and parasite strains (384, 363, 532). More research is required to confirm and define the induction of acquired immunity to blood-stage malaria by immunizing DCs, particularly in genetically susceptible strains of mice.

As described in Chapter 3, splenic DCs pulsed with pRBC overnight were able to upregulate costimulatory molecule expression and to stimulate high levels of T cell

proliferation and IFN-γ production compared to nonpulsed DCs or DCs pulsed with nRBC. The ability of pRBC-pulsed DCs to induce IFN-γ-secreting $CD4^+$ T cells was critically dependent on DC-derived IL-12 although this cytokine was not required for DC-stimulated $CD4^+$ T cell proliferation (Appendix IV), as previously shown in Chapter 5. To determine the efficacy of using malaria-loaded DCs as a vaccine, we propose to adoptively transfer pRBC-pulsed DCs to naïve B6 or A/J mice and then monitor parasitemia and survival after challenge with *P. chabaudi*. To determine the relative roles of IL-15, IL-12 and IFN-γ in DC-mediated immunization, the adoptive transfer experiments can be performed in two phases. The first phase will determine whether endogenous Th1 cytokines are required in the recipient for effective DC-mediated immunization by transferring WT DCs to mice deficient in Th1 cytokines. The second phase will determine whether Th1 cytokines expressed by donor DCs are required for protective immunization by transferring donor DCs from cytokine-deficient mice to WT mice. Furthermore, this DC vaccine may be used to study the functional relevance of DC–NK cell interactions in vivo during blood-stage malaria (discussed above). Data from these DC vaccine studies may help identify DC-mediated immune mechanisms that generate sterile or long-lasting protective immunity to blood-stage malaria, thereby advancing the development of effective anti-malarial vaccines for use in humans.

Final Conclusions

In summary, the studies presented in this thesis investigated the role of DCs in the initiation and control of immune responses to blood-stage *P. chabaudi* infection. Data from these studies revealed specific mechanisms by which DCs control innate and adaptive immunity to blood-stage malaria, including preferential pRBC uptake, costimulatory signals, cytokine production, and activation of NK cells and $CD4^+$ T cells. Moreover, cytokines produced by malaria-activated DCs or by other immune cells after activating crosstalk with DCs play key roles in regulating the induction, maintenance and suppression of immune responses to blood-stage malaria. It is apparent that the type, timing and magnitude of DC responses, in concert with other immune cells and soluble or surface-bound mediators interacting in a tightly regulated immunological cascade, are critically important in determining the outcome of malaria infection.

To summarize the results described in this thesis and place them in context of other previously reported findings, a synopsis is presented in Fig. 6.2 of the cascade of immune responses to a primary blood-stage malaria infection. The course of blood-stage *P. chabaudi* infection in resistant C57BL/6 mice is characterized by three stages of early (acute) infection, peak parasitemia, and late (chronic) infection, which induce and are modulated by distinct but coordinated innate and adaptive immune responses. Innate immunity is mediated by APCs such as DCs, B cells and macrophages, as well as by effector cells such as macrophages, NK cells and γδ T cells which perform important phagocytic, cytotoxic and cytostatic host defenses to control acute infection. Cytokines, including IL-12, IFN-γ, TNF-α, IL-2, and IL-15, are produced by malaria-primed DCs and can activate other innate immune cells, mainly macrophages and NK cells, to exert their parasite inhibitory functions and to secrete proinflammatory cytokines. Activated macrophages also secrete NO and reactive oxygen species that mediate additional cytotoxic effects on malaria parasites and infected RBCs. During the early immune response to blood-stage malaria, DCs play an important role in detecting infection by recognizing and capturing pRBCs (Chapter 3), undergoing maturation and presenting costimulatory signals (Chapters 3-5), secreting inflammatory and immunoregulatory cytokines (Chapters 2-5), and interacting with NK cells to induce potent, reciprocal activation (Chapter 4). As the infection progresses, DCs lose their capacities for pRBC uptake (Chapter 3) and cytokine production (536). Although DCs and other inflammatory responses diminish with time, the presence of Th1-promoting cytokines and contact-dependent signals early in the immune response is critical for the development of protective Th1 immune responses (Chapters 2-5; 400, 420), which are maximally induced before or at the time of peak parasitemia (Chapter 2 & 5; 420, 536). Impaired production of Th1-promoting cytokines by DCs may lead to induction of IL-10-secreting $CD4^+$ T cells with suppressor activity, resulting in more severe course of infection and higher mortality, as demonstrated in susceptible A/J mice (Chapter 5).

Adaptive immunity against blood-stage malaria is mediated primarily by IFN-γ-secreting Th1 cells and activated B cells that secrete Th1-dependent antibodies, including IgG2a (in mice) and IgG1 (in humans). Antibody-dependent cell cytotoxicity (ADCC) and cellular inhibition (ADCI) contribute to parasite clearance and resolution of

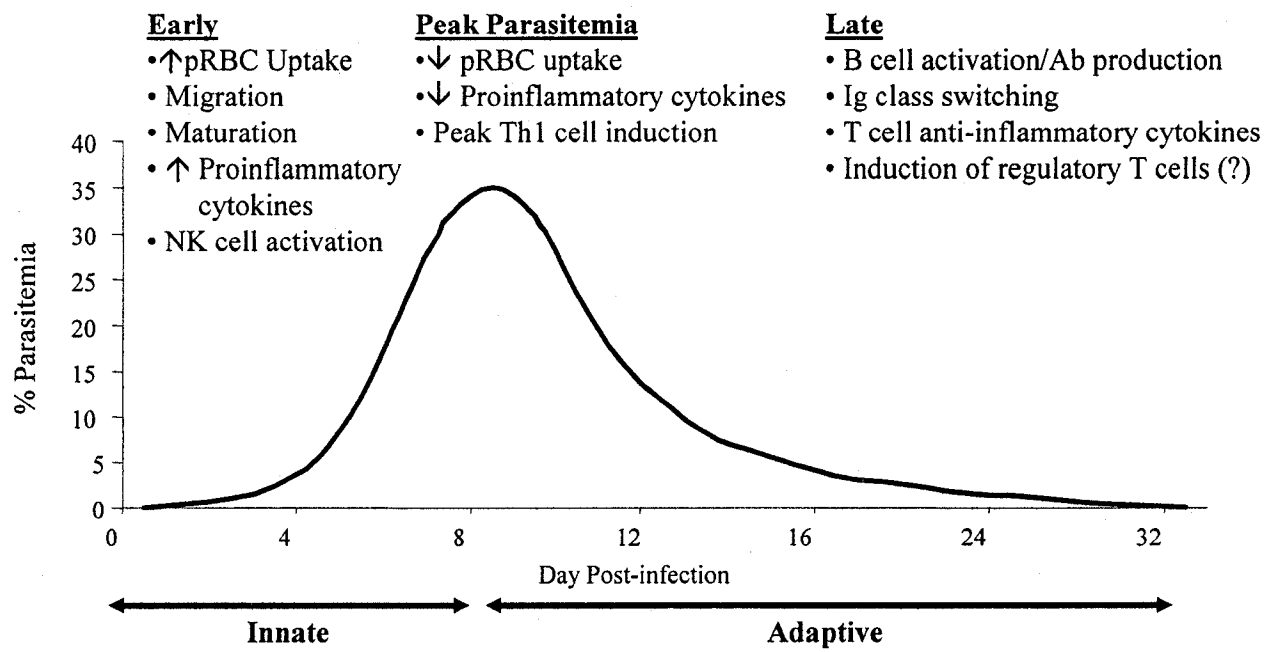
chronic infection. Although Th2 cells are activated during the chronic stage, Th1-promoting cytokines such as IFN-γ are responsible for promoting Ig class switching to produce Th1-dependent protective antibodies (420). Regulatory or anti-inflammatory cytokines such as IL-10 and TGF-β mediate downregulation of inflammatory responses during the later stages of infection, likely as an attempt to prevent immune-mediated malarial disease (78, 82, 488, 536). DCs have been shown to interact with B cells to stimulate their activation, antibody production, and Ig class switching. For these responses, DCs may provide B cell growth and class switching factors, such as IL-15 (Chapter 2; 218), and ligands for B cell activating receptors, such as BLys and April (218). DCs present during chronic infection may induce regulatory T cells, which secrete IL-10 and TGF-β that in turn dampen inflammatory responses (536). Taken together, DCs play an important role in promoting immune mechanisms that allow resolution of both the infection and the inflammatory response, thus influencing a balance between health and disease in blood-stage malaria infection.

Implications for the Control and Eradication of Human Malaria

Effective control strategies for human malaria are hampered by the complexity of the malaria parasite's life cycle and by the fact that most individuals do not develop natural immunity after a single infection. Antigenic variation, poor immunogenecity of individual parasite antigens, parasite-mediated interference with the development of effector responses such as induction of regulatory mechanisms and/or deletion of malaria-specific immune cells, involvement of diverse tissue compartments and cell types, toxicity of parasite-derived products such as hemozoin and GPI, and interactions of malaria parasites with maternal and neonatal immunity all contribute to the slow onset of immunity which is typically incomplete, short-lived in the absence of continual exposure and not protective against different *Plasmodium* variants. In areas of stable transmission, older children gradually develop clinical immunity to severe malaria but remain susceptible to high rates of infection. On the other hand, the progression to and death from severe anemia or CM is associated with high, uncontrolled proinflammatory immune responses in young children, pregnant women, and nonimmune adults. Thus, a major challenge for malaria eradication strategies is to design a vaccine, likely in combination with a stimulatory adjuvant or

immunotherapy, that will induce many types of immune responses against multiple antigenic targets and thus provide broad protection against heterologous parasite strains. Moreover, a successful vaccine will need to induce different classes of immune responses in a sequential manner: 1) strong inflammatory responses that can control parasite growth and thus minimize parasitemia during the acute phase of infection, and 2) regulatory or anti-inflammatory mechanisms during the chronic or curative phase to limit or resolve the anti-parasite immune responses so as to prevent immune-mediated pathology.

As important APCs that provide contact and soluble signals for the development of immune responses, DCs are pivotally positioned to initiate, regulate and potentiate anti-malarial immunity. Cytokines, in particular, are key mediators of important interactions with other immune cells, such as NK and $CD4^+$ T cells, and also determine the type of immune response that develops. Data from this thesis work demonstate that an optimal immune response to blood-stage malaria involves DC activation, proinflammatory cytokines and type 1 effector mechanisms early after infection. As the infection progresses, DC function is downregulated, clearance of remaining parasites is mediated primarily by antibodies, and anti-inflammatory cytokines are needed to resolve the inflammation and prevent immunopathology. A successful outcome of malaria infection therefore hinges on a coordinated progression from one type of immune response to another. An impaired proinflammatory response will lead to excessive parasite growth, whereas a lack of anti-inflammatory mechanisms or tolerance will result in excessive pathology. Immunogenic DCs initiate pro-inflammatory mechanisms that can help control parasite growth, but in excess may cause pathology. Alternatively, tolerogenic DCs promote anti-inflammatory mechanisms that resolve inflammation and prevent pathology, but may suppress protective immunity if the timing and magnitude of this response are inappropriate. An effective immunization strategy must ensure that vaccine components can be recognized, processed and presented by DCs, thereby enabling the activation of vaccine-induced immune responses that mediate protection against natural infection. Importantly, an effective vaccine must also be able to elicit both the immunogenic and tolerogenic properties of DCs in a balanced, coordinated succession so that one can achieve maximum immunity with minimum pathology.



Cellular Effectors:	DC, macrophage, NK cell, γδ T cell	$CD4^+$ T cell, B cell, T regulatory cell
Soluble Effectors:	IL-12, IL-18, IL-2, IL-15, IFN-γ, TNF-α, NO	IFN-γ, TNF-α (immunity) IL-15 (?), IL-10, TGF-β (regulatory) IgG2a, IgG2b, IgG3 (mice); IgG1 (humans)
Mechanisms:	macrophage phagocytosis, NK cell cytotoxicity, cytostasis and parasite killing by nitrogen and oxygen radicals	parasite clearance by ADDC and ADCI modulation of immune-mediated pathology

Figure 6.2: Summary of Innate and Adaptive Immune Responses to Blood-Stage Malaria Infection

STATEMENT OF ORIGINALITY

Chapter 2 describes the course and outcome of a primary *P. chabaudi* AS infection in IL-15$^{-/-}$ mice. Based on genetic linkage studies, IL-15, a key cytokine in the development and activation of innate immune cells and memory CD8$^+$ T cells, was identified as a positional candidate for the control of parasitemia in mice. In this study, comparisons between wild-type and IL-15$^{-/-}$ mice revealed an important, but not indispensable, role for IL-15 in the early control and timely resolution of blood-stage malaria via regulation of innate immune mechanisms and Th1-dependent adaptive immune responses. These experimental results are the first to show that IL-15 is required for both optimal IFN-γ synthesis by DCs and NK cells as well as production of Th1-associated antibodies.

Chapter 3 demonstrates that BM-derived and splenic CD11c$^+$ DCs are able to differentiate between pRBC and nRBC, resulting in preferential uptake of pRBC, maturation and cytokine production. Moreover, uptake of pRBC by splenic CD11c$^+$ DCs was selectively enhanced after infection to a significantly greater extent than the uptake of nRBC. Splenic DCs activated following uptake of pRBC, but not of nRBC, were able to stimulate CD4$^+$ T cells to proliferate and produce high levels of IFN-γ. We developed a new flow cytometric technique which was used to determine levels of pRBC versus nRBC uptake by DCs and to demonstrate that pRBC uptake by DCs is an actin-dependent phagocytic process. These data provide novel evidence that early after malaria infection DCs are important APCs that recognize and interact with pRBC in a highly selective manner to induce activation of Th1-type immune responses required for protection against blood-stage malaria.

Chapter 4 investigated the reciprocal interaction between splenic DCs and NK cells during the innate immune response to blood-stage malaria infection. The experimental results presented in this chapter show that malaria-activated DCs stimulate resting natural killer (NK) cells to secrete high levels of IFN-γ and, reciprocally, activated NK cells induce DC maturation and cytokine production. DCs matured as a result of NK cell stimulation were able to prime CD4$^+$ T cells to proliferate and produce IFN-γ, leading to

Th1 adaptive immune responses. This study also dissected the relative roles of IL-2, IL-15, IL-12 and IFN-γ in mediating DC–NK-cell crosstalk and subsequent production of IFN-γ and Th1 cell differentiation. These data provide novel insights into cytokine-mediated regulation of innate immunity to blood-stage malaria.

Chapter 5 investigated whether host susceptibility to blood-stage malaria is associated with altered splenic DC phenotype or function. The data presented here represent the first analysis of differential splenic DC responses among strains of mice resistant or susceptible to *P. chabaudi* infection. DCs from susceptible A/J mice were able to capture pRBC in vitro, mature following infection in vivo, and stimulate proliferation of $CD4^+$ T cells in vitro. Importantly, however, DCs from A/J mice were defective in producing type 1 cytokines and failed to activate IFN-γ production from NK or $CD4^+$ T cells required for protective immunity to blood-stage malaria. The ability of DCs from infected A/J mice to induce high levels of IL-10-secreting $CD4^+$ T cells suggests DC-mediated activation of regulatory T cells. Indeed, infected A/J mice showed higher percentages of $Foxp3^+CD4^+CD25^+$ T cells in the spleen than did resistant C57BL/6 or B10.A mice. These results provide evidence for a role of tolerogenic DCs in the induction of regulatory T cells in the early immune response, leading to suppression of protective Th1 immunity in malaria-susceptible mice.

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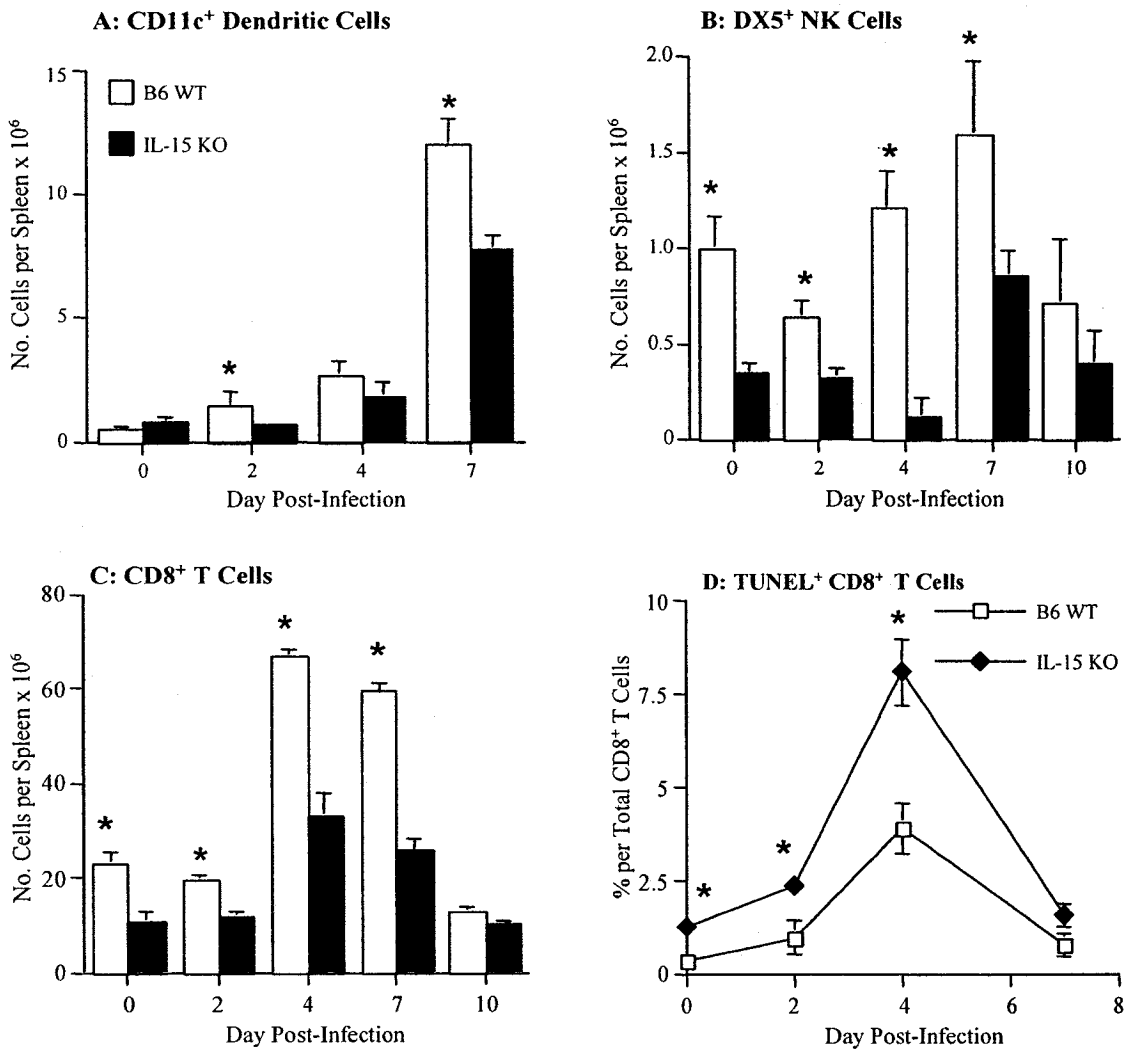
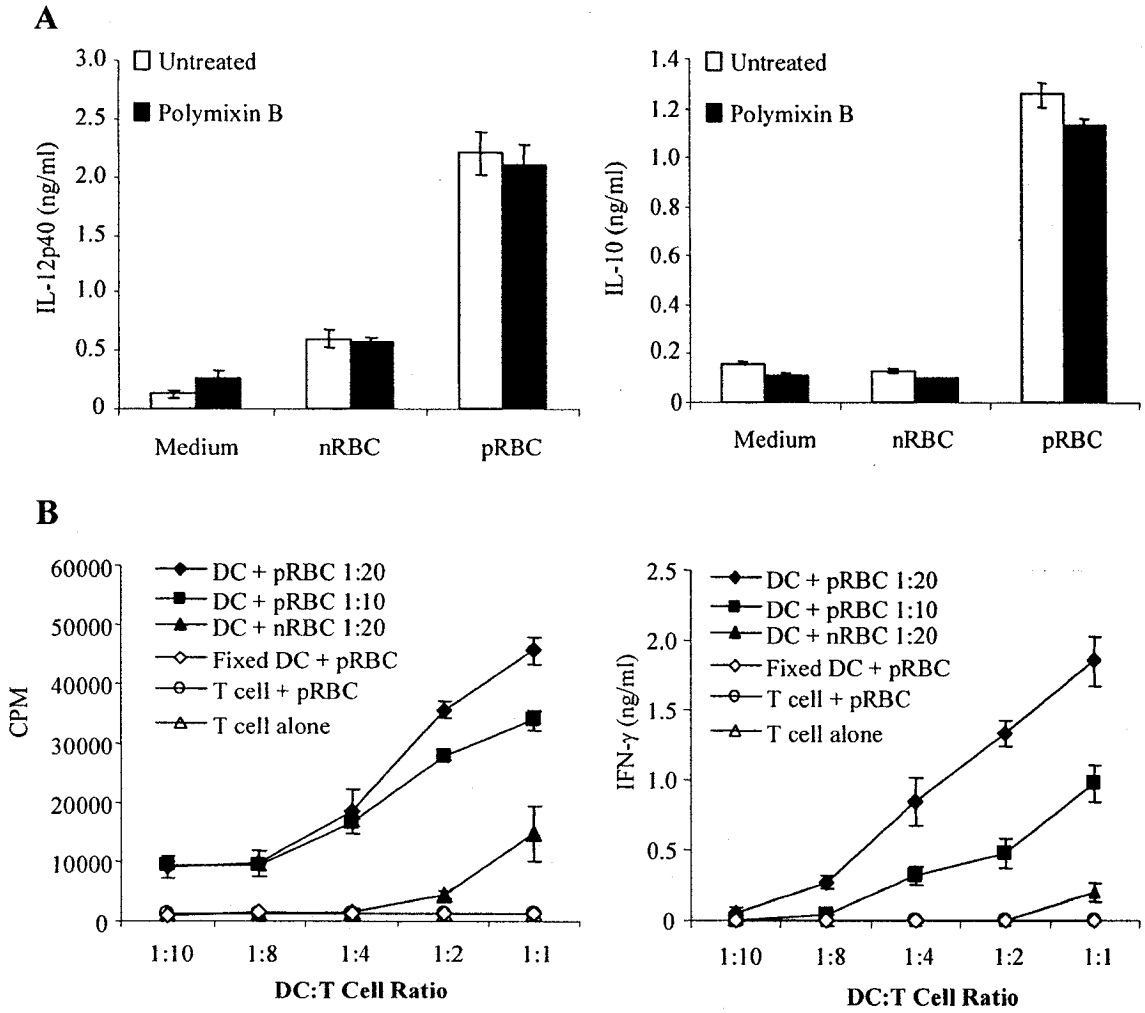
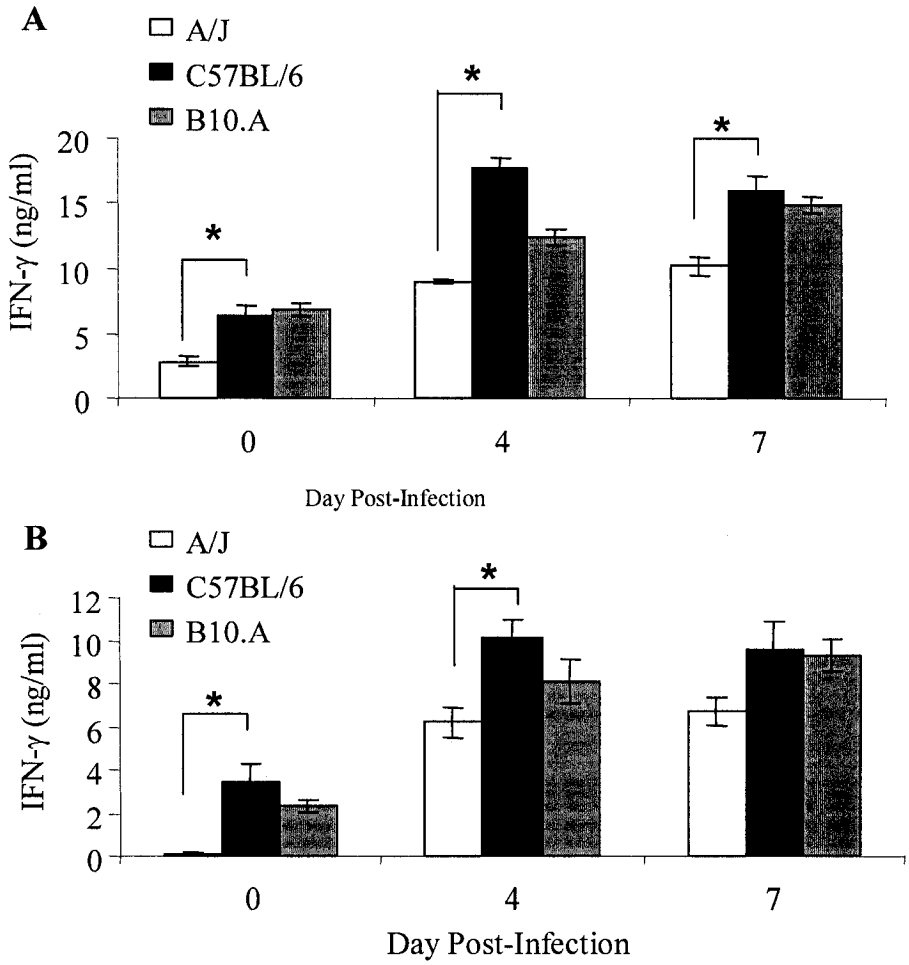
APPENDICES



Appendix I: IL-15 deficiency impairs expansion of DCs (*A*), NK cells (*B*) and $CD8^+$ T cells (*C*) as well as survival of $CD8^+$ T cells (*D*) in mice following blood-stage malaria infection. Single cell suspensions of spleen cells were stained for cell markers on various days p.i. (3-4 mice per group) and analyzed by flow cytometry. Spleen cell numbers are expressed as total numbers x 10^6 per spleen (mean ± SEM), as indicated. The percentages (± SEM) of apoptotic $CD8^+$ T cells were determined from the number of cells staining positive for both TUNEL, which labels DNA strand breaks generated during apoptosis, and CD8 compared to the total number of $CD8^+$ cells per spleen at each time point. Data are representative of two replicate experiments. *, B6 WT versus IL-15 KO mice, $P < 0.05$, as determined by the Mann-Whitney U test.

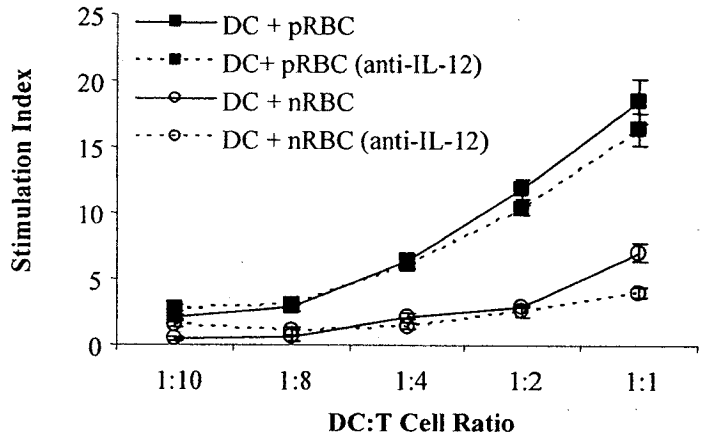


Appendix II: The ability of pRBC-pulsed DCs to stimulate $CD4^+$ T cells was not attributable to endotoxin contamination (A) or mitogenic properties of pRBC (B). *A*, DCs pulsed with polymixin B-treated pRBC produced identical levels of IL-12p40 (*left*) and IL-10 (*right*) as DCs pulsed with untreated pRBC. Splenic $CD11c^+$ DCs isolated from naïve B6 mice were incubated with no red cells (medium), with nRBC, or with pRBC. Red cells were untreated or pre-treated with 10 μg/ml polymixin B for 30 min prior to incubation with DCs. After 48 h co-culture, supernatants were analyzed for cytokine production by ELISA. *B*, pRBC-pulsed DCs, but not fixed DCs or nRBC-pulsed DCs, stimulated high levels of $CD4^+$ T cell proliferation (*left*) and IFN-γ production (*right*). To fix DCs, splenic $CD11c^+$ DCs enriched from naïve B6 mice were treated with 1% paraformaldehyde for 30 min at room temperature, washed extensively, and incubated in culture medium overnight to allow leaching of fixative. Normal and fixed DCs were pulsed with pRBC or nRBC overnight and then plated with freshly purified $CD4^+$ T cells for 48 h at 37°C. Proliferation was determined by incorporation of $[^3H]$-thymidine during the last 12 h of incubation. IFN-γ levels in culture supernatants were determined by ELISA.

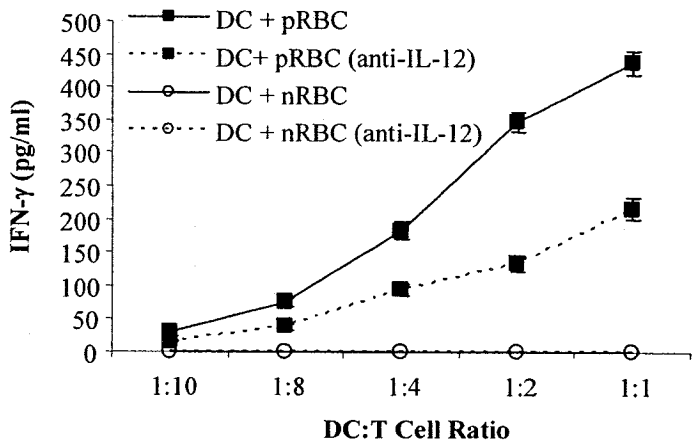


Appendix III: Impaired IFN-γ production by NK cells from A/J mice before and early after *P. chabaudi* infection compared to C57BL/6 and congenic B10.A mice. Splenic $DX5^+$ NK cells were enriched from naïve and infected mice at the days indicated and 5 x 10^5 cells/well were cultured in vitro with 10 ng/ml of rIL-2 (*A*) or 100 ng/ml of rIL-15 (*B*) for 72 h. IFN-γ levels in culture supernatants were determined by ELISA. Data are representative of 2 independent experiments each with 3-4 mice per group. *, $P < 0.05$ as determined by Student's *t* test.

A: Proliferaton



B: IFN-γ Production



Appendix IV: DC-derived IL-12 is required for DC-mediated stimulation of $CD4^+$ T cell IFN-γ production (*B*) but not proliferation (*A*). DCs pulsed with pRBC or nRBC were pre-treated or not with anti-IL-12 antibody for 2 h prior to co-culture with $CD4^+$ T cells from naïve B6 mice. *A*, Proliferation of $CD4^+$ T cells as determined by $[^3H]$-thymidine incorporation after 48 h co-culture. Stimulation index is relative to unstimulated T cell controls. *B*, IFN-γ production in co-cultures as determined by ELISA. Data are representative of 3 independent experiments.