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Interferon-Mediated Innate Immune Responses against Malaria Parasite Liver Stages

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SUMMARY

Mosquito-transmitted malaria parasites infect hepatocytes and asymptomatically replicate as liver stages. Using RNA sequencing, we show that a rodent malaria liver-stage infection stimulates a robust innate immune response including type I interferon (IFN) and IFNγ pathways. Liver-stage infection is suppressed by these infection-engendered innate responses. This suppression was abrogated in mice deficient in IFNγ, the type I IFN α/β receptor (IFNAR), and interferon regulatory factor 3. Natural killer and CD49b$^+$CD3$^+$ natural killer T (NKT) cells increased in the liver after a primary infection, and CD1d-restricted NKT cells, which secrete IFNγ, were critical in reducing liver-stage burden of a secondary infection. Lack of IFNAR signaling abrogated the increase in NKT cell numbers in the liver, showing a link between type I IFN signaling, cell recruitment, and subsequent parasite elimination. Our findings demonstrate innate immune sensing of malaria parasite liver-stage infection and that the ensuing innate responses can eliminate the parasite.

INTRODUCTION

Plasmodium parasites, the causative agents of malaria, infect approximately 300–500 million people each year, which results in the deaths of 267,000 people annually (World Health Organization, 2013). Mammalian infection is initiated following the bite of a mosquito carrying infectious sporozoites, which are inoculated into the dermis. These sporozoites actively enter the bloodstream and are transported to the liver, where they invade a hepatocyte within which they grow and develop as liver stages. Each parasite undergoes replication, leading to an enormous increase in parasite biomass and culminating in the release of 10,000–50,000 infectious exoerythrocytic merozoites into the blood (reviewed in Lindner et al., 2012). These merozoites initiate cyclical asexual intraerythrocytic replication responsible for the pathological manifestations of blood-stage malaria infection.

Innate immune responses are the first line of defense against invading pathogens and are integral in shaping the adaptive immune response necessary for long-term antigen-specific protection against pathogens. Blood-stage *Plasmodium* parasite infection generates robust innate immune responses that have been extensively studied and are predominantly mediated by interferon γ (IFNγ), tumor necrosis factor α, and interleukin-12 (reviewed in Riley and Stewart, 2013). However, innate immune responses generated during pre-erythrocytic (sporozoite and liver stages) infection remain undefined. Understanding if and how the host detects and responds to liver-stage parasites is important, because relatively few parasites establish a productive infection in hepatocytes under natural transmission conditions. Thus, an effective innate immune response against a small number of parasites during this point in the infection cycle could inhibit the onset of disease.

A small number of previous studies in rodent models of malaria have examined the induction of innate immune responses by pre-erythrocytic *Plasmodium* parasites (reviewed in Liehl and Mota, 2012). Histopathology on livers infected with either *P. yoelii* (Py) or *P. berghei* sporozoites revealed the presence of cellular infiltrates that form foci late during liver-stage development around the time of exoerythrocytic merozoite release (Khan and Vanderberg, 1991; Mack et al., 1978). These studies initially identified these cellular infiltrates as being predominantly eosinophils and neutrophils (Khan and Vanderberg, 1991, 1992). Other studies have shown that natural killer (NK) cells (Pied et al., 2000; Roland et al., 2006) and natural killer T (NKT) cells (Soulard et al., 2007) infiltrate the liver following infection, but this is after the liver-stage infection has progressed to a blood-stage infection. It is difficult to study the effect of innate immune responses on primary pre-erythrocytic infection, because the peak of the innate immune response occurs at the end of liver-stage development, just prior to or concurrent with exoerythrocytic merozoite release. Thus, the antiparasitic effects of innate immune responses engendered by pre-erythrocytic parasites remain unknown.

Here, we analyze the innate immune response induced by Py parasites in the liver using wild-type (WT) parasites and attenuated parasites that are incapable of progressing to blood-stage infection. Analysis of RNA sequencing (RNA-seq) gene expression data from the livers of infected mice revealed that pre-erythrocytic stage parasites induce an innate immune response that is mediated by type I IFNs and IFNγ resulting in the reduction of parasites in the liver. Primary liver infections with attenuated parasites suppress a secondary liver-stage parasite infection with WT parasites, providing a robust method to measure the functional consequences of the liver-stage innate immune responses

in vivo. Suppression of infection was abrogated in mice deficient in type I IFN signaling pathways and in the effector cytokine IFNγ. We further show that this innate immune response is dependent upon NKT cells, which secrete IFNγ and increase in the liver following parasite infection in WT mice, but not type I interferon-α/β receptor (IFNAR)-deficient mice.

RESULTS

Comparative RNA-Seq Reveals an Induction of IFN-Mediated Signaling Pathways following *Plasmodium* Liver-Stage Infection

Gene expression analysis has been effectively utilized in multiple models to identify innate immune responses to infection (Diercks and Aderem, 2013; Liehl et al., 2014). The few previous studies conducting histological analysis of innate immune cell responses to *Plasmodium* pre-erythrocytic infection have reported that immune cells infiltrate the liver between 48 and 72 hr postinfection (hpi) with rodent malaria sporozoites (Khan and Vanderberg, 1991, 1992; Liehl et al., 2014). Thus, we initially examined changes in gene expression during liver-stage infection in mice at 72 hpi. Infection with WT Py sporozoites progresses to the blood stage after about 50 hpi. In order to ensure that measured responses at 72 hpi are only caused by liver infection and not by the ensuing blood-stage infection, we utilized an attenuated *P. yoelii* parasite (PyA) that develops to late liver-stage forms but does not progress to blood-stage infection (Vaughan et al., 2009). Gene expression data derived from both C57BL/6 and BALB/cJ mouse livers (n = 4) 72 hpi following mock infection with salivary gland material from uninfected mosquitoes (mock) or attenuated PyA sporozoite infection was analyzed using RNA-seq (Tables S1 and S2). Transcript levels that changed by at least a factor of four ($\log_2$ fold change of ±2) with a p value < 0.01 were considered significant. A total of 75 transcripts were significantly up- or downregulated in C57BL/6 mice following PyA infection (69↑, 6↓) (Figures 1A and 1B). Comparatively, 200 transcripts in BALB/cJ mice were differentially regulated (198↑, 2↓) (Figures 1A and 1C). A total of 42 transcripts were upregulated in both mouse strains (Figure 1A), many of which encode factors involved in innate immune responses (Figure 1D). Pathway analysis of differentially expressed genes was carried out using the Ingenuity IPA software package and the Upstream Regulator Analysis tool to identify the molecular regulators that explain the observed gene expression signatures. Interestingly, IFNγ and IFNβ were significant regulators of the observed innate immune response to infection in both mouse strains (Figures 1E and 1F). Furthermore, STAT1, a transcription factor shared by both type I and type II IFN signaling pathways (Stark and Darnell, 2012), was also a major regulator of the observed transcriptional changes (Figures 1E and 1F). Gene expression was additionally carried out at an earlier time point, 24 hpi, and also revealed that IFN-mediated pathways were upregulated, although fewer genes were differentially regulated at this time point (BALB/cJ: 125; C57BL/6: 51) (Figure S1; Tables S3 and S4). To confirm the gene expression data, we monitored IFNβ and IFNγ protein levels in mice 72 hpi by ELISA. IFNβ was not measurable in the livers of mock-infected mice but was detected in the livers of PyA-infected mice (Figure 1G). Similarly, IFNγ was detectible in the sera of PyA-infected mice, but not in mock-infected mice (Figure 1H). Importantly, the liver-stage burden following infection with 50,000 WT Py sporozoites in IFNγ$^{-/-}$ mice was approximately twice that of infected WT C57BL/6 mice as determined by quantitative PCR (qPCR) (Figure S2). This suggests that the innate upregulation of IFNγ following pre-erythrocytic infection leads to a reduction in liver-stage burden.

Primary *Plasmodium* Liver-Stage Infection Inhibits a Secondary Liver-Stage Infection

Having established that *Plasmodium* pre-erythrocytic stage parasites induce an IFN-driven innate immune response in the liver, we next sought to generate a robust model that would enable us to measure the induction of this innate immune response and determine the functional significance of the response on liver-stage parasites. To this end, we infected mice after the primary infection for a second time with WT Py sporozoites in order to determine if the impact of the innate response on this secondary infection could be used as a functional measure (Figure 2A). Because PyA does not complete liver-stage development and does not transition to the blood-stage infection (Vaughan et al., 2009), its use in the primary infection allowed us to evaluate the effect of a liver-stage-engendered innate immune response on a secondary infection at time points greater than 48 hr after the primary infection. Conversely, primary infection with WT Py sporozoites leads to blood-stage infection after approximately 50 hr. This blood-stage infection engenders its own innate immune response, which would mask the liver-stage-specific innate immune response. BALB/cJ mice were mock infected or infected with 100,000 WT Py or PyA sporozoites on day −1 (primary infection). On day 0, the mice were infected with 50,000 Py sporozoites (secondary infection) that constitutively express a GFP-luciferase fusion protein (*PyGFP-luc*) (Miller et al., 2013). Liver-stage burden of *PyGFP-luc*-infected mice was monitored at 24 and 43 hpi by bioluminescent imaging (Figure 2A). Mock-infected mice supported a robust *PyGFP-luc* secondary infection at 24 and 43 hpi, whereas the secondary infection in both Py- and PyA-infected mice was reduced by approximately 70-fold at both time points (Figures 2B and 2C). The reduction of *PyGFP-luc* liver-stage burden observed at 24 hpi in mice given either a Py or PyA primary infection can be attributed to the effects of the innate immune response generated by the primary pre-erythrocytic infection. This reduction of *PyGFP-luc* secondary infection was further confirmed by qPCR analysis of infected livers at 43 hpi (Figure 2D) and by examining the time to blood-stage patency. Reduced time to patency is indicative of a reduction of liver-stage burden that leads to a lower number of exoerythrocytic merozoites produced and thus delayed occurrence of detectable blood-stage infection. In these types of studies, a 1-day delay in time to patency correlates to an approximate 90% reduction in liver-stage burden (Gantt et al., 1998). To this end, mock- or PyA-infected BALB/cJ mice were administered a secondary infection with either 50,000 or 1,000 *PyGFP-luc* sporozoites, and subsequent blood-stage infection was monitored by microscopic evaluation of Giemsa-stained blood smears (Table 1). After infectious sporozoite challenge of mice that