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Report

Suppression of Host p53 Is Critical for *Plasmodium* Liver-Stage Infection

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SUMMARY

Plasmodium parasites infect the liver and replicate inside hepatocytes before they invade erythrocytes and trigger clinical malaria. Analysis of host signaling pathways affected by liver-stage infection could provide critical insights into host–pathogen interactions and reveal targets for intervention. Using protein lysate microarrays, we found that *Plasmodium yoelii* rodent malaria parasites perturb hepatocyte regulatory pathways involved in cell survival, proliferation, and autophagy. Notably, the prodeath protein p53 was substantially decreased in infected hepatocytes, suggesting that it could be targeted by the parasite to foster survival. Indeed, mice that express increased levels of p53 showed reduced liver-stage parasite burden, whereas p53 knockout mice suffered increased liver-stage burden. Furthermore, boosting p53 levels with the use of the small molecule Nutlin-3 dramatically reduced liver-stage burden in vitro and in vivo. We conclude that perturbation of the hepatocyte p53 pathway critically impacts parasite survival. Thus, host pathways might constitute potential targets for host-based antimalarial prophylaxis.

INTRODUCTION

Parasites of the genus *Plasmodium* are the causative agents of the deadly disease malaria, afflicting 350–500 million people annually and causing 800,000 deaths worldwide (Snow et al., 2005). After transmission by an infected *Anopheles* mosquito, the parasite travels quickly through the bloodstream to the liver and infects hepatocytes. The parasite then grows and replicates within hepatocytes, presumably evading detection by the host, and ultimately spawns tens of thousands of daughter merozoites, which are released into the bloodstream and infect red blood cells, leading to symptomatic infection (Vaughan et al., 2008).

One feature of host manipulation that has been previously suggested is the ability of *Plasmodium berghei* rodent malaria parasites to render their host hepatocyte partially resistant to artificial induction of apoptosis in vitro, both early (van de Sand et al., 2005) and late (Leirião et al., 2005) during liver-stage development. It remains unclear, however, how broadly the host hepatocyte responds to infection and if the parasite attempts to counteract responses that might impact its survival. One potential mechanism that could explain how parasitized hepatocytes become resistant to apoptosis involves activation of the hepatocyte growth factor receptor, but this mechanism appears unique to the rodent malaria parasite *P. berghei* (Carrolo et al., 2003; Kaushansky and Kappe, 2011). Furthermore, some studies have measured transcriptional changes that occur in *P. yoelii* and *P. berghei*-infected hepatocytes (Albuquerque et al., 2009; Tarun et al., 2008), yet perturbations in the translational and posttranslational host cell environment that occur upon parasite liver-stage infection have not been elucidated.

RESULTS

It remains technically challenging to study protein level cellular responses to liver-stage infection because infection rates are low, and thus, infected cells can only be isolated in limited quantities. To circumvent this roadblock, we used reverse-phase protein microarray technology, which enables broad but targeted proteomic investigations on small sample sizes (Sevecka et al., 2011). The platform uses cellular lysates deposited in nanoliter droplets on nitrocellulose-coated glass slides in which levels of specific proteins or their posttranslational modifications can be detected by probing the lysates with appropriate antibodies (Figure 1A). We assembled a diverse set of antibodies, many of which have been previously validated for use in reverse-phase arrays (Sevecka et al., 2011). These antibodies recognize proteins involved in numerous cellular outcomes, including survival, apoptosis, proliferation, cell-cycle control, and autophagy. Approximately 10,000 parasite-infected HepG2/CD81 hepatoma cells as well as uninfected cells were isolated by fluorescence-activated cell sorting (FACS), making use of GFP-tagged *P. yoelii* parasites (Tarun et al., 2006). Protein extracts from each sample were prepared and printed in quadruplicate on 48 separate nitrocellulose pads followed by probing the arrays with the selected set of antibodies to obtain quantitative information

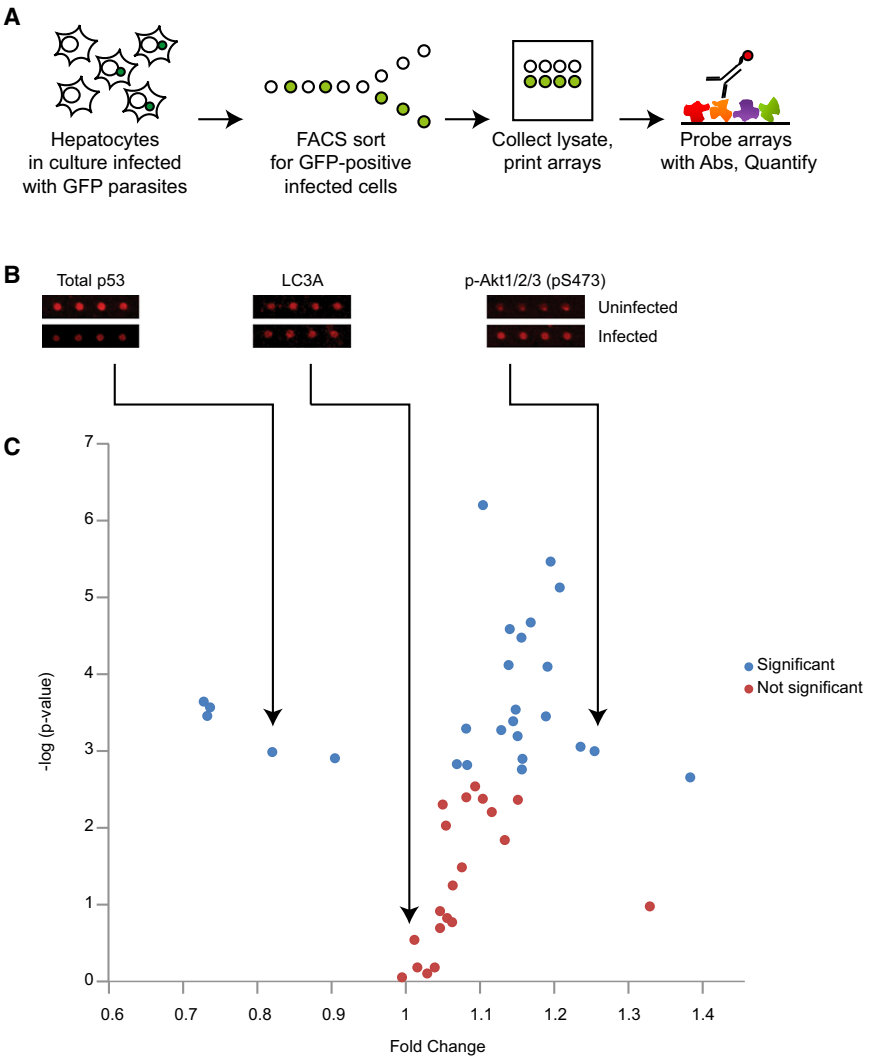

Figure 1. The Use of Protein Microarrays to Study Host Signaling during Liver-Stage Malaria Infection

(A) Schematic representing steps required to obtain lysate microarray data from HepG2/CD81 cells either infected or uninfected with *P. yoelii*-GFP liver stages. Liver stages were allowed to develop for 24 hr. Abs, antibodies.

(B) Representative array images from three antibodies: total p53, LC3A, and p-Akt1/2/3 (pS473).

(C) Graph representing the ratio of infected (GFP-positive) cells to uninfected (GFP-negative) host cells for signals obtained for 46 separate antibodies, plotted against the log of the p value obtained. Each point represents a single antibody. Significant differences that pass multiple hypothesis testing (Holm-Bonferroni method) are shown in blue; nonsignificant differences are shown in red. See also Figure S1.

on changes in host cell protein abundance and/or modifications (Table S1; Figure 1C).

Strikingly, the resulting data showed that numerous signaling proteins are perturbed in parasite-infected cells (Table S1; Figure 1B). Multiple pathways were simultaneously impacted at significant but varying levels, indicating that the liver-stage parasite drives a multipronged approach to modulate signaling by the infected host cell. When we examined which signals were most significantly changed in infected cells, we found pronounced increases in the antiapoptotic-signaling proteins p-Bcl-2 ($p = 0.001$) and p-Akt/PKB ($p = 0.0008$ and $p = 0.000003$ for two separate antibodies) and the proproliferative phosphorylated states of the mammalian target of rapamycin (mTor) ($p = 0.000008$) and Retinoblastoma (Rb) ($p = 0.003$) (Table S1). Furthermore, we identified decreases in phosphorylated forms of the proapoptotic proteins p53 ($p = 0.0004$) and Bad ($p = 0.001$ and 0.0002 for two separate antibodies) as well as a decrease in total abundance of p53 ($p = 0.0003$ and $p = 0.001$ for two separate antibodies) (Table S1; Figure 2A).

Remarkably, these data are consistent with the hypothesis that a cohesive network of parasite-mediated-signaling changes render the infected host cell more hospitable for the parasite. The increases of activated Akt and Bcl-2 along with the decrease in Bad indicate a multifaceted survival response, which assists the parasite in protecting its host cell. The increase in mTor suggests protection against autophagy, which could severely impede intracellular parasite development. Activation of Rb suggests that the infected hepatocyte is pushed toward a proliferative state. Finally, the decrease in p53 levels fits into both the proliferative and antiapoptotic framework (Figure 2A; Table 1). Together, the observed perturbations are consistent with a general antiapoptotic, proliferative, antiautophagic environment within the infected host cell.

Because any screen must be validated with alternative approaches, we next sought to confirm the major pathways that were impacted by parasite infection. Both Akt antibodies have been previously validated in a variety of cell lines, including HepG2 cells (Luckert et al., 2012). Encouragingly, signal for all four antibodies in the Akt/mTor pathway recorded increased in response to parasite infection (Figure S1C). In order to further validate additional screen hits, we first explored reproducibility between biological samples, and scalability with data obtained from immunoblotting. We found that one p53 antibody was highly reproducible across biological replicates (Figure S1A). To determine whether this antibody also gave a linear relationship between western blotting and lysate array measurements, we generated cellular lysates with variable levels of p53 using a range of concentrations of the MDM-2 inhibitor Nutlin-3. By monitoring p53 levels in these lysates by both western blot and lysate array, we determined that the relationship between