Coinfection with Blood-Stage *Plasmodium* Promotes Systemic Type I Interferon Production during Pneumovirus Infection but Impairs Inflammation and Viral Control in the Lung

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Acute lower respiratory tract infections (ALRTI) are the leading cause of global childhood mortality, with human respiratory syncytial virus (hRSV) being a major cause of viral ALRTI in young children worldwide. In sub-Saharan Africa, many young children experience severe illnesses due to hRSV or *Plasmodium* infection. Although the incidence of malaria in this region has decreased in recent years, there remains a significant opportunity for coinfection. Recent data show that febrile young children infected with *Plasmodium* are often concurrently infected with respiratory viral pathogens but are less likely to suffer from pneumonia than are non-*Plasmodium*-infected children. Here, we hypothesized that blood-stage *Plasmodium* infection modulates pulmonary inflammatory responses to a viral pathogen but does not aid its control in the lung. To test this, we established a novel coinfection model in which mice were simultaneously infected with pneumovirus of mice (PVM) (to model hRSV) and blood-stage *Plasmodium chabaudi* chabaudi AS (PcAS) parasites. We found that PcAS infection was unaffected by coinfection with PVM. In contrast, PVM-associated weight loss, pulmonary cytokine responses, and immune cell recruitment to the airways were substantially reduced by coinfection with PcAS. Importantly, PcAS coinfection facilitated greater viral dissemination throughout the lung. Although *Plasmodium* coinfection induced low levels of systemic interleukin-10 (IL-10), this regulatory cytokine played no role in the modulation of lung inflammation or viral dissemination. Instead, we found that *Plasmodium* coinfection drove an early systemic beta interferon (IFN-β) response. Therefore, we propose that blood-stage *Plasmodium* coinfection may exacerbate viral dissemination and impair inflammation in the lung by dysregulating type I IFN-dependent responses to respiratory viruses.

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trointestinal tract (7). Whether such regulatory processes might also inhibit immune responses to concurrent respiratory viral infection remains unclear. Interestingly, recent data from a region of Tanzania where malaria is endemic showed that febrile young children infected with *Plasmodium* were often concurrently infected with respiratory viral pathogens but were 50% less likely to suffer from pneumonia than were non-*Plasmodium*-infected children (11). These epidemiological data support our hypothesis that blood-stage *Plasmodium* infection modulates pulmonary inflammatory responses during respiratory viral infection but does not itself facilitate viral control.

In this paper, we present a novel mouse model of RSV-*Plasmodium* coinfection. Using this model, we demonstrate that blood-stage *Plasmodium* coinfection reduces clinical symptoms and inflammatory responses associated with viral infection of the lung, but, importantly, also impairs viral control in this organ. We show that *Plasmodium*-induced immunosuppression in the lung does not depend on IL-10, as was previously reported for systemic coinfection with other *Plasmodium* species, or gastrointestinal infection with *Salmonella* but is associated instead with an accelerated systemic beta interferon (IFN-β) response.

**MATERIALS AND METHODS**

**Plasmodium and viral infections.** Specific-pathogen-free female C57BL/6 mice, age 6 to 10 weeks, were used for all experiments. All procedures were approved by The University of Queensland Animal Ethics Committee. To initiate viral respiratory tract infection, each mouse was intranasally (i.n.) inoculated with 5 to 10 PFU of pneumovirus of *PVM* (strain J 3666) (12) or vehicle (Dulbecco’s modified Eagle’s medium [DMEM] plus 10% fetal calf serum) under light isoflurane anesthesia. To initiate blood-stage *Plasmodium* infection, *Plasmodium chabaudi* chabaudi AS (PcAS) parasites were used after one *in vivo* passage in wild-type C57BL/6 mice. Each mouse was infected intravenously (i.v.) via a lateral tail vein injection with 10⁵ freshly prepared packed red blood cells (pRBC). To establish coinfection, the mice were first inoculated with *Plasmodium*, lightly anesthetized, and then inoculated with PVM.

**Assessing peripheral blood parasitemia.** Blood parasitemia was measured in Diff Quik-stained (Lab Aids, Narrabeen, NSW, Australia) thin blood smears obtained from tail bleeds only when the passage mice were being assessed for the presence of parasites. For an analysis of parasitemia in the experimental mice, a previously established flow cytometric method (13–16) was employed to measure parasitemia more rapidly. Briefly, a single drop of blood from a tail bleed or cardiac puncture was diluted in 250 μl of RPMI containing 5 U/ml heparin sulfate. The diluted blood was simultaneously stained with 5 μM Syto84 (Life Technologies) to detect RNA/DNA and 10 μM Hoechst 33342 (Sigma) to detect DNA for 30 min in the dark at room temperature. The staining was quenched with 10 volumes of ice-cold RPMI, and the samples were immediately analyzed by flow cytometry using an LSRFortessa fluorescence-activated cell sorter (FACS) analyzer (BD Biosciences) and FlowJo software (Tree Star, CA, USA). pRBC were readily detected as being Hoechst 33342⁺ Syto84⁺. Reticulocytes were readily distinguished from pRBC in PcAS-infected mice, even though they contained slightly more DNA/RNA than that seen in reticulocytes in naive or PVM-infected mice (Fig. 1A).

**Sample extraction and processing.** After the mice were euthanized by pentobarbitone overdose, blood was obtained by cardiac puncture and centrifuged at 13,000 x g and 4°C for 15 min twice to collect serum, which was stored at −80°C. Bronchoalveolar lavage (BAL) was performed, as described previously (17), the BAL fluid (BALF) was centrifuged at 5,000 x g and 4°C for 5 min, and the supernatant was stored at −80°C until cytokine analysis. Red blood cells were removed from the cell pellet with Gey’s lysis buffer. The cells were washed twice in phosphate-buffered saline (PBS) and a cytospin prepared (StatSpin CytoFuge). The cells were air-dried, fixed with 100% (vol/vol) methanol for 15 min, and stained with May-Grünwald Giemsa stain. Typically, 300 to 400 cells were counted for each cytospin sample. The right lobe of the lung was used for histology (fixed in 10% formalin neutral buffer overnight before storage in 70% ethanol). The serum and BALF samples were assessed for IL-6, tumor necrosis
factor (TNF), IL-10, monocyte chemoattractant protein-1 (MCP-1), IFN-γ, and IL-12p70 using cytometric bead arrays (CBA) (mouse inflammation kit; BD) or IFN-α or IFN-β using a ProcartaPlex Lumexin xMAP technology-based bead array (eBioscience), according to the manufacturers’ instructions.

**Assessing viral load via lung immunohistochemistry.** Paraffin-embedded sections were prepared, as previously described (12). Briefly, 5-μm-thick tissue sections were pretreated with 10% normal goat serum for 30 min. Primary antibodies against PVM (1:8,000 dilution; the anti-serum against PVM protein G was kindly provided by Ulla Buchholz) were added and incubated overnight at 4°C. The sections were washed three times in PBS–0.05% Tween 20 before incubation with biotinylated anti-mouse polyclonal antibody (Invitrogen). This step was then repeated but with streptavidin–alkaline phosphatase. After 60 min of incubation at room temperature, the sections were washed three times and immunoreaction development was performed with Fast Red (Sigma-Aldrich). The sections were counterstained with hematoxylin before mounting with Permount.

**In vivo IL-10 receptor blockade.** Monoclonal antibody-secreting hybridomas were grown in 5% (vol/vol) fetal calf serum and RPMI containing 10 mmol/liter L-glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin. Purified antibody was prepared from the culture supernatants by protein G column purification (Amersham, Uppsala, Sweden), followed by endotoxin removal (Mustang membranes; Pall, East Hills, NY). Anti-IL-10 receptor (anti-IL-10R) blocking monoclonal antibody (1B1.3a) and control IgG were administered in 0.25-mg doses via intraperitoneal (i.p.) injection in 200 μl of 0.9% sodium chloride (Baxter) per mouse on days 0, 3, and 6 postinfection.

**Statistical analysis.** Comparisons between the two groups were performed using nonparametric Mann-Whitney tests, unless stated otherwise, in which case Student t tests for Gaussian distributions were employed. A P value of <0.05 was considered significant. All statistical analyses were performed using the GraphPad Prism 6 software.

**RESULTS**

PVM coinfection does not alter the course of blood-stage PeAS infection. To model the possible interplay between viral respiratory infection and blood-stage *Plasmodium* infection in vivo, we established a murine coinfection model in which C57BL6/J mice were first infected intravenously with blood-stage *P. chabaudi chabaudi* AS (PeAS) parasites and then immediately infected intranasally with pneumovirus of mice (PVM). This combination of pathogens was chosen because both replicate well in mice, and neither causes lethal disease when administered alone, thus offering multiple advantages for investigating interaction between coinfection and PVM infection. As peripheral blood parasitemia was essentially identical in *P. chabaudi* AS alone (Fig. 1A).

Moreover, the degree of splenomegaly observed in these two groups of mice, a surrogate marker for an active immune response to blood-borne or systemic pathogens, was equivalent at days 7 and 14 postinfection (p.i.) (Fig. 1B). Together, these data suggest that pulmonary PVM infection had not impacted the ability of the host to respond to and control primary PeAS infection.

PeAS coinfection abrogates PVM-induced weight loss and pulmonary inflammatory responses. We next assessed the impact of coinfection on PVM-induced pulmonary immune responses and disease symptoms. First, mice infected with PVM alone lost approximately 15% of their starting body weight by day 8 p.i. (Fig. 2A), an outcome consistent with previous published data (12). Strikingly, weight loss was significantly reduced in coinfected mice and was absent in uninfected mice and those infected with PeAS alone (Fig. 2A). Second, inflammatory cytokine levels, particularly those of IL-6, MCP-1, IL-10, TNF, and IFN-γ, were highly upregulated in the BALF samples from the mice infected with PVM alone but were significantly reduced in the coinfected mice (Fig. 2B). Also noteworthy, PeAS infection alone induced almost no BALF cytokine responses, suggesting that the movement of blood-borne parasites through lung microvasculature was not sufficient to drive such local immune responses (Fig. 2B).

Finally, while PVM infection alone induced a significant recruitment of immune cells, such as lymphocytes, eosinophils, and neutrophils, into the airways (Fig. 2C), this did not occur during PeAS infection alone or, more importantly, during coinfection (Fig. 2C). Together, these data indicate that PVM-associated weight loss and pulmonary cytokine and cellular inflammatory responses were substantially suppressed by coinfection with blood-stage PeAS parasites.

**PeAS coinfection increases PVM dissemination into lung parenchyma.** Since PeAS coinfection had suppressed PVM-dependent lung inflammation, we next assessed the impact of coinfection on viral loads in this organ by immunostaining for the virus. At the peak of PeAS infection, we observed a trend toward reduced PVM infection in airway epithelial cells (Fig. 3A and B). However, we found evidence of more PVM in the lung parenchyma of coinfected mice than in those infected with PVM alone (Fig. 3A and C). These data suggest that coinfection with PeAS parasites facilitated a greater dissemination of PVM into the parenchyma of the lung. We also noted that PVM was undetectable in the lungs of singly or coinfected mice by day 14 p.i., as assessed by immunostaining (data not shown). These data indicate that although pulmonary viral burdens and dissemination were exacerbated during coinfection, the mice were capable of ultimately clearing PVM while experiencing blood-stage PeAS infection.

**Systemic IL-10 during PeAS coinfection does not regulate PVM-induced pulmonary immune responses.** We next sought to determine a mechanism by which *Plasmodium* infection prevented virally induced lung inflammatory responses. Recent reports indicate that *Plasmodium* suppresses immune responses to concurrent infections via IL-10, both during systemic infection with a second lethal *Plasmodium* species (9, 10) and locally in the gastrointestinal tract during nontyphoidal salmonellosis (7, 8). Therefore, we hypothesized that *Plasmodium*-induced systemic IL-10 inhibits immune responses to respiratory viral coinfection.

First, we found that IL-10 was expressed systemically at moderate levels during coinfection and in PeAS infection alone but not in mice infected with PVM alone (Fig. 4A). Interestingly, this contrasted with the localized IL-10 responses in the lung, which were evident more in PVM-infected mice than in either PeAS-infected or coinfected mice (Fig. 2B). Next, we studied the impact of a systemic blockade of IL-10R signaling upon coinfection (Fig. 4B to D). This treatment significantly reduced splenomegaly (Fig. 4B)
and reduced peripheral blood parasitemia (Fig. 4C) compared to those effects in coinfected mice given control IgG. However, we observed no restoration of immune cellular recruitment to the airways in coinfected mice upon IL-10R blockade (Fig. 4D), while it was apparent that this treatment exacerbated BALF responses in the mice infected with PVM alone (Fig. 4D). Finally, we noted no effect of IL-10R blockade on PVM dissemination throughout the lung parenchyma in the coinfected mice (data not shown). These data suggest that coinfection with blood-stage \( Pc \) AS exacerbates PVM dissemination and suppresses inflammatory immune responses in the lung independently of IL-10.

**PcAS coinfection drives an early systemic IFN-\( \beta \) response.** It has been shown that IFN-\( \alpha/\beta \) transcriptional upregulation occurs transiently toward the end of the first week of infection in the lungs of PVM-infected mice (19). This coincides, at least in neonatal mice, with enhanced plasmacytoid dendritic cell (pDC) numbers, which depend upon IFN regulatory factor 7 (IRF7), Toll-like receptor 7 (TLR7), and MyD88 signaling (12). These type I IFN-dependent immune processes mediate PVM control and cellular inflammatory responses in the lung, as well as drive weight loss (12). Therefore, we hypothesized that PcAS coinfection might dysregulate this transient and highly regulated type I IFN response. To test this, we studied IFN-\( \alpha \) and IFN-\( \beta \) protein levels during the early stages of coinfection, when the level of PcAS parasitemia was low but detectable (~0.5%). Although we saw no increase in IFN-\( \alpha/\beta \) levels in the lung tissues of the coinfected mice compared to those in the singly infected mice (data not shown), we did observe an early systemic IFN-\( \beta \) (but not IFN-\( \alpha \)) response in the coinfected mice, which was absent from the singly infected mice (Fig. 5). These data provide evidence that coinfection with PcAS drives a unique systemic type I IFN response that does not normally occur during infection with PVM alone.

**DISCUSSION**

In this study, we established a murine coinfection model with which to explore possible competition and interplay between a respiratory virus and blood-stage *Plasmodium* parasites in a mammalian host. In designing this model, we specifically chose two pathogens known to infect and replicate efficiently in inbred C57BL/6J mice. We chose these species to avoid infection with a large bolus of a poorly replicative pathogen, which might be expected to stimulate the innate immune system nonphysiologically and thus perturb subsequent pathogenesis. Second, we chose two pathogens with similar growth rates in vivo to avoid the chance that either pathogen would dominate due to a much higher replication rate. We believe our model has been instructive, because...
both pathogens were clearly able to replicate simultaneously in the host. Indeed, the blood-stage *Plasmodium* parasite growth appeared unaffected, while PVM growth was moderately increased during coinfection. Therefore, we believe this model may be of general utility for exploring the interactions between respiratory viruses and blood-stage *Plasmodium* parasites.

A key aspect of our model is the simultaneous nature of the two infections. However, it is clearly more likely that there is a certain time period, perhaps days, between RSV and *Plasmodium* infections in regions where malaria is endemic. Therefore, it would be of interest in the future to determine if infection timing and/or parasite density affects the capacity of *Plasmodium* to exert its modulatory effects against pulmonary viral infections. Interestingly, recent data suggest that the density of *Plasmodium* parasites in the bloodstream does not significantly affect the incidence of coinfection in children (11). Whether parasite density affects susceptibility to pulmonary disease caused by respiratory pathogens remains untested.

In this study, we found that *Plasmodium* coinfection substantially suppressed inflammatory cytokine production and cellular recruitment to the lung in response to a respiratory virus and furthermore that this suppression did not require IL-10. Impor-
**FIG 5** Coinfection with PcAS and PVM drives an early systemic IFN-β response. Wild-type mice were coinfected with PVM (i.n., 10 PFU) and PcAS (i.v., 10^7 pRBC) or were infected singly with each pathogen (n = 3 to 5). The serum IFN-β protein levels were measured when the PcAS parasitemias were at 0.5%. The data are representative of two independent experiments and show the mean ± SEM (one-way analysis of variance [ANOVA], Dunnett’s multiple-comparison test). *, P < 0.05; **, P < 0.01.

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