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PAD4 controls chemoattractant production and neutrophil trafficking in malaria

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Abstract

Peptidylarginine deiminase 4 (PAD4) is a key regulator of inflammation but its function in infections remains incompletely understood. We investigate PAD4 in the context of malaria and demonstrate a role in regulation of immune cell trafficking and chemokine production. PAD4 regulates liver immunopathology by promoting neutrophil trafficking in a *Plasmodium chabaudi* mouse malaria model. In human macrophages, PAD4 regulates expression of CXCL chemokines in response to stimulation with TLR ligands and *P. falciparum*. Using patient samples, we show that CXCL1 may be a biomarker for severe malaria. PAD4 inhibition promotes disease tolerance and may represent a therapeutic avenue in malaria.

KEYWORDS
chemokine, malaria, neutrophil, PAD4

1 | INTRODUCTION

Peptidylarginine deiminase 4 (PAD4) belongs to a 5-membered family of calcium-dependent enzymes that catalyze protein citrullination—the posttranslational conversion of arginine residues to citrulline.[1] PAD4 is abundantly expressed in myeloid cells such as neutrophils and macrophages,[2] where it can regulate chromatin structure and gene expression.[3] The physiological function of the enzyme is only partially understood; however, it is clear that PAD4 strongly promotes proinflammatory responses in vivo, enhancing disease and tissue damage in models of lupus,[4] rheumatoid arthritis,[5] thrombosis,[6] atherosclerosis,[7] and cancer.[8] Pharmacologic inhibitors of PAD4 have been proposed as potential therapeutics in these diseases.[3]

Most PAD4 studies to date have focused on neutrophils, abundant myeloid cells with both protective and detrimental roles in inflammation. Neutrophils respond to infectious or inflammatory stimuli by phagocytosis, degranulation, or extrusion of chromatin in the form of neutrophil extracellular traps (NETs).[9] NETs trap and prevent dissemination of microbes but can also propagate inflammation via their immunostimulatory properties.[9] In neutrophils, PAD4-mediated citrullination of histones regulates chromatin structure[1] and has been implicated in both enhancement of NET immunogenicity[10] and direct control of NET release,[6,11] although the latter has been contested.[12,13]

The function of PAD4 in other myeloid cells is less clear. Citrullination is not restricted to histones but can also modulate activity of various transcription factors[14,15] and apoptosis regulators such as p53.[16] In macrophages, citrullination was reported to regulate the assembly of the NLRP3 inflammasome.[17]

To better characterize the function of PAD4 in systemic inflammation, we examined the role of the enzyme in malaria, a disease

Abbreviations: AST, aspartate aminotransferase; BMDM, bone-marrow-derived macrophage; NE, neutrophil elastase; NETs, neutrophil extracellular traps; PAD4, peptidylarginine deiminase type 4.

caused by replication of *Plasmodium* parasites in red blood cells (RBCs). Malaria annually affects over 200 million people and leads to approximately 400,000 deaths.[18] The majority of individuals infected with *P. falciparum*, the most lethal parasite species, suffer from uncomplicated malaria. A portion of patients, primarily children in sub-Saharan Africa, develop severe symptoms such as metabolic acidosis, severe anemia, or cerebral malaria. Organ failure in severe malaria is initiated by cytoadhesion of infected RBCs (iRBCs) in the microvasculature, causing endothelial activation and recruitment of inflammatory cells, which leads to vascular leak or occlusion.[18]

Neutrophils and CD8+ T cells both contribute to malaria immunopathology. NET release in malaria promotes iRBC cytoadhesion,[19,20] while neutrophil and CD8+ T cell infiltration of organs causes tissue damage.[19,21–24] Understanding chemoattractant regulation is therefore essential for understanding malaria immunopathology.

2 | MATERIALS AND METHODS

2.1 | Reagents

Chemicals used were GSK484 (Cayman), LPS (*E. coli* O127:B8; Sigma), R848 (Sigma), MCSF (Peprotech), and Pam3CSK4 (Invivogen).

2.2 | *Plasmodium chabaudi* infections

Mouse experiments were approved by *Landesamt für Gesundheit und Soziales* (Germany) and the Home office (UK). Animals were bred at the Max Planck Institute for Infection Biology or purchased from Charles River. PAD4−/− mice were a kind gift from Prof. Denisa Wagner. PAD4−/− and WT C57BL/6J were confirmed to be 99.66% identical by single nucleotide polymorphism analysis.

Plasmodium chabaudi AS was obtained from Prof. Jean Langhorne. Male mice aged 8–15 weeks were infected by intravenous injection of 1×10^4 viable *P. chabaudi*. The same passage of *P. chabaudi* was used in all experiments.

2.3 | Human samples

Collection of blood samples was approved by the Comité d'Ethique Régional Indépendent de Lambaréné (Gabon) and NHS REC 18/EE/0265. All participants provided written informed consent. Patient characteristics are described in Ref. [19].

2.4 | Histology

Livers were harvested, stained, and scored as previously described.[19] Neutrophils were detected using an in-house antibody directed against neutrophil calgranulin A.[19] CXCL1 was immunostained with Proteintech 12335-1-AP and macrophages with anti-F4/80 antibody Proteintech 28463-1-AP primary antibodies, followed by anti-rabbit IgG secondary antibody (ThermoFisher A-11012). CD8+ T cells were stained with monoclonal antibody SP16 (Thermo Fisher MA5-16345) and anti-rabbit AP-Red conjugated secondary. Image acquisition was performed on Leica DMI 6000D at 20× magnification, with LAS X software. The total CXCL1 area was calculated in Fiji by manually thresholding the image, followed by normalization to total DAPI area.

2.5 | Quantification of sequestration

Mice were infected with a luciferase-expressing strain of *P. chabaudi*.[24] Mice were culled at nighttime, which is the time of maximum schizont sequestration and perfused systemically by injection of 10 ml of PBS into the heart. Organs were removed and processed for luminescence as previously described.[19]

2.6 | Flow cytometry

Quantification of circulating neutrophils was performed as previously described.[19] Neutrophils were counted as CD45+, CD115−, and Ly6G/C+ cells. Antibodies were all from BD Biosciences: V500 anti-CD45 (561487), PE anti-CD115 (565249), PerCP-Cy5.5 anti-Ly6G/C (561103), BV421 anti-Ly6G (127627). For isolated neutrophil staining, we used APC anti-mouse CXCR2/CD182 (Biolegend 149312) and PE anti-mouse CD117/c-Kit (Biolegend 105807).

2.7 | In vitro macrophages and neutrophils

Mouse bone marrow-derived macrophages (BMDMs) were prepared with 10% final volume L929 cell-conditioned medium. Mouse neutrophils were isolated from bone marrow as previously described,[25] using EasySep™ Mouse Neutrophil Enrichment Kit (Stem Cell). Human macrophages were prepared as previously described,[19] from peripheral blood monocytes isolated by positive selection (CD14 microbeads; Miltenyi). Monocytes were differentiated into macrophages for 7 days in RPMI 1640 containing penicillin/streptomycin, glutamine, and 5 ng/ml human MCSF.

Mouse or human macrophages were plated in 6-well plates at 0.9 million per well and incubated with stimuli (at indicated concentrations) or with iRBCs in a 1:5 ratio for 24 h in RPMI supplemented with 10% FCS.

2.8 | *P. falciparum* culture

NF54 parasites were a kind gift of Dr Robert Sauwerwein. Parasites were cultured in O+ human RBCs at 5% hematocrit in RPMI 1640 with hypoxanthine (50 μg/ml), HEPES (25 mm), 0.225% sodium bicarbonate, and 10 mg/ml gentamicin, supplemented with 10% heat-inactivated

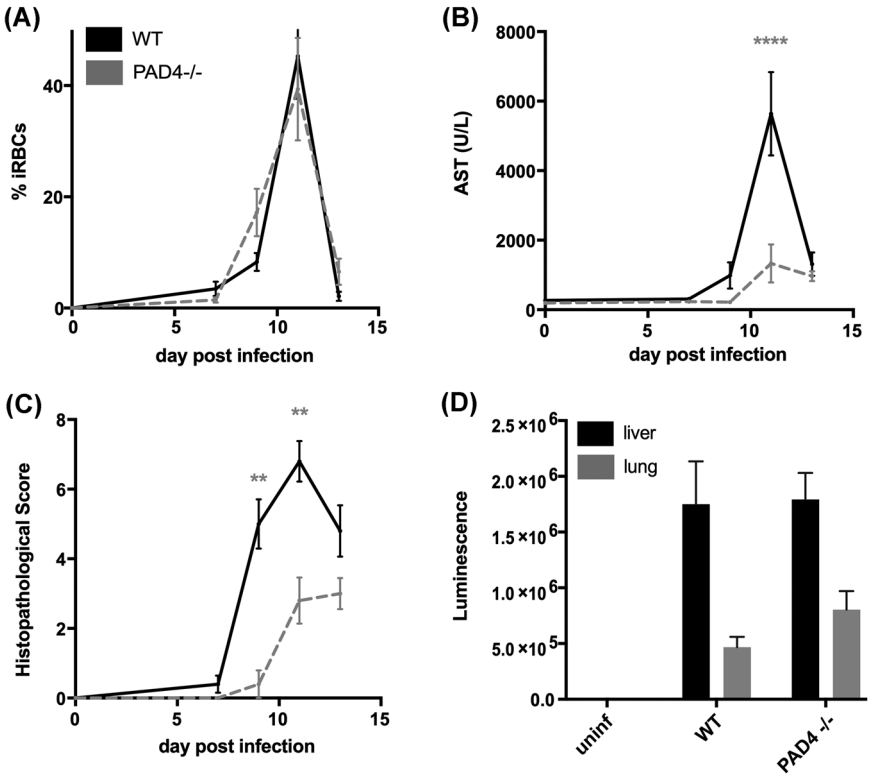

FIGURE 1 PAD4 promotes liver damage independently of parasitemia in *P. chabaudi* infections
(A) Quantification of parasitemia from Giemsa-stained blood smears; (B) plasma concentration of AST; (C) blinded histopathology scores of livers; (D) parasite sequestration in liver and lung determined from luminescence of luciferase-expressing transgenic parasites at peak parasitemia. $n = 6$ mice, 2 independent experiments, graphs show mean ± SEM. Asterisks indicate significance:
$^{**}p < 0.01$ and
$^{****}p < 0.0001$ by 2-way ANOVA comparison.

human serum. Parasites were kept at 37°C, in 2% O_2, 5% CO_2, and 93% N_2. Late stage parasites were purified using Percoll–sorbitol gradients as previously described.[26]

2.9 | Biomarker quantifications

Aspartate aminotransferase (AST) was quantified in mouse plasma by the veterinarian service laboratory at SYNLAB.vet GmbH (Berlin, Germany). NETs were quantified as before,[19] by combining the Cell Death Detection ELISA Kit (Roche) and anti-MPO antibody (1 μg/ml final concentration, HM1051BT; Hycult). Human and mouse CXCL1 and CXCL2 were quantified using DuoSet ELISA (R&D Systems).

2.10 | Quantitative PCR

RNA was extracted with RNeasy kit (Qiagen). cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Bioscience). The following primers were used to quantify mouse transcript abundance: 18s (house keeping gene): 5- GTAA CCCGTTGAACCCCATT-3 and 5-CCATCCAATCGGTAGTAGCG-3;

CXCL1: 5-CTTGACCCTGAAGCTCCCTT-3 and 5-GTTGTCAGAA GCCAGCGTTC-3; CXCL2: 5-TTGCCTTGACCCTGAAGCC-3 and 5-CCCGGGTGCTGTTTGTTTT-3;

CXCL5: 5-CCTCCTTCTGGTTTTTCAGTTTAGC-3 and 5-GCATTT CTGTTGCTGTTCACGCTG-3;

CXCR2: 5-AGCAAACACCTCTACTACCCTCTA-3 and
5-GGGCTGCATCAATTCAAATACCA-3.

Real time PCR was carried out with SYBR green (Thermo) on QuantStudio 3 machine. Relative transcript abundance was calculated using the 2-ΔΔCT method.

3 | RESULTS/DISCUSSION

We infected wildtype and PAD4−/− mice with asexual stage *P. chabaudi*, which predominantly sequesters in the liver, inducing inflammation and hepatocyte necrosis.[19,24] Parasites proliferated at similar rates in wildtype and knockout animals, indicating that PAD4 does not control the host antiparasitic response (Figure 1(A)). On the other hand, liver damage was strikingly reduced in PAD4−/− animals, as measured by both circulating AST (Figure 1(B)) and by histopathologic analysis (Figure 1(C)).

Liver damage in the *P. chabaudi* model results from accumulation of both parasites and inflammatory cells.[19,24] We tested which of these is affected in the PAD4 knockout. Quantification of *P. chabaudi* cytoadherence using a luciferase expressing parasite revealed no difference in sequestration rates or patterns (Figure 1(D)). On the other hand, trafficking of neutrophils (Figures 2(A) and 2(B)) and CD8+ T cells (Figure S1(A) and S1(B)) was significantly reduced in PAD4−/− livers, compared with controls. In summary, PAD4 promotes malaria immunopathology by enhancing immune cell trafficking without affecting parasite sequestration patterns. This is consistent with findings in a lupus model, where renal neutrophil infiltration and pathology were also reduced in PAD4−/− mice.[27]

To understand the mechanistic basis of the observed reduction in neutrophil accumulation in the liver, we analyzed abundance of

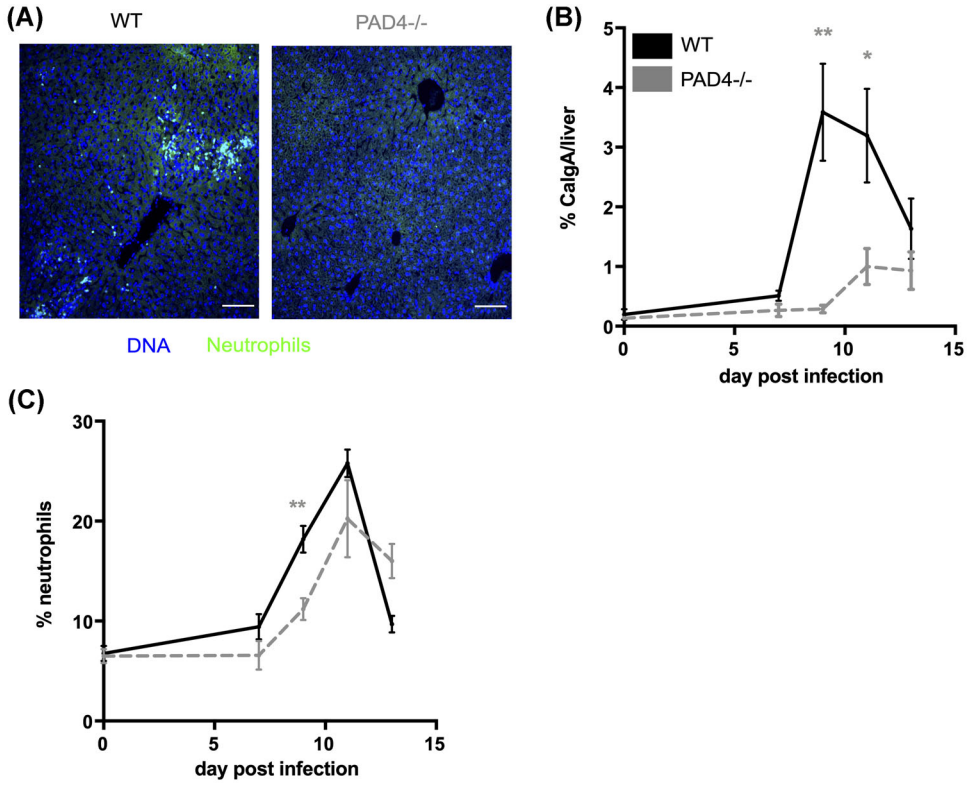

FIGURE 2 PAD4 promotes trafficking of neutrophils into infected livers
(A) Representative immunofluorescence image of neutrophils (green) in livers (DAPI, blue) at peak parasitemia; (B) quantification of (A), $n = 3$ noninfected and 5 infected mice; (C) flow cytometric enumeration of neutrophils in blood, $n = 9–10$ animals, 3 independent experiments. (A–C) Black = WT and gray = PAD4−/−, graphs show mean ± SEM. Asterisks indicate significance: $^{*}p < 0.05$, $^{**}p < 0.01$ by 2-way ANOVA comparison (B) and unpaired *t*-test (C)

circulating neutrophils in peripheral blood. Circulating neutrophils were reduced at day 9 postinfection in PAD4−/− mice (Figure 2(C)), although there were no significant reductions at other time points. We also found no difference in basal granulopoiesis, as demonstrated by equivalent numbers of both mature and immature neutrophils in naïve wildtype and PAD4−/− bone marrow (Figures S2(A)–S2(C)).

The twofold reduction in circulating neutrophil counts in PAD4−/− animals could not fully account for the nearly tenfold difference in liver neutrophil accumulation, leading us to postulate an additional trafficking delay. We therefore investigated other mechanisms by which PAD4 regulates immune cell trafficking.

We found no difference in levels of circulating nucleosomes (Figure S2(D)) or NET components (MPO–DNA complexes; Figure S2(E)) showing that NET formation is not altered. This is consistent with a previous report of unchanged in vitro NET formation by PAD4−/− neutrophils in response to heme/TNF,[19] although conflicting findings have also been reported for macrophage inhibitory factor-induced NETosis in malaria.[11]

Neutrophil migration is controlled by gradients of chemokines such as CXCL-1, -2, -5, and -8.[28–31] We quantified CXCL1 by immunofluorescence in liver sections and found reduced levels in PAD4−/− mice compared with controls (Figures 3(A) and Figure S3(A)). Importantly, macrophage numbers did not differ significantly between WT and PAD4−/− mice (Figure S3(B)). CXCL1 concentration in plasma was also reduced in PAD4−/− mice (Figure 3(B)). On the other hand, surface expression of CXCR2, the main receptor for CXCL1, did not differ significantly between circulating neutrophils from infected WT and PAD4−/− mice (Figure 3(C)), indicating a defect in chemokine production, rather than the ability to detect them. Normal or slightly elevated CXCR2 expression levels were confirmed in bone marrow-isolated neutrophils (Figures S3(C)–S3(F)). This is in agreement with studies showing normal in vitro migration capacity of PAD4−/− neutrophils.[27]

To test for a cell intrinsic PAD4 role in macrophage chemoattractant production, we quantified CXCL1 release by mouse BMDMs. *Plasmodium* is sensed via TLRs-4, -7, and -2,[32] so we stimulated BMDMs with corresponding agonists. After 24 h, PAD4−/− BMDM showed a small but statistically significant reduction in CXCL1 protein release in response to all stimuli: the TLR7 agonist R848, the TLR2 agonist Pam2CSK, and the TLR4 agonist LPS (Figure 3(D)). Interestingly, the difference was much more prominent at 48 h poststimulation (Figure 3(D)), showing that wildtype, but not PAD4−/− macrophages, continue secreting CXCL1 for longer periods. We similarly found reduced secreted quantities of a second chemokine—CXCL2, at 48 h (Figure 3(E)) but not at 24 h poststimulation (Figure S4(A)). We next assayed chemokine mRNA abundance and found transcript levels of *Cxcl1*, *2*, and *5* to be strongly induced by R848 without significant

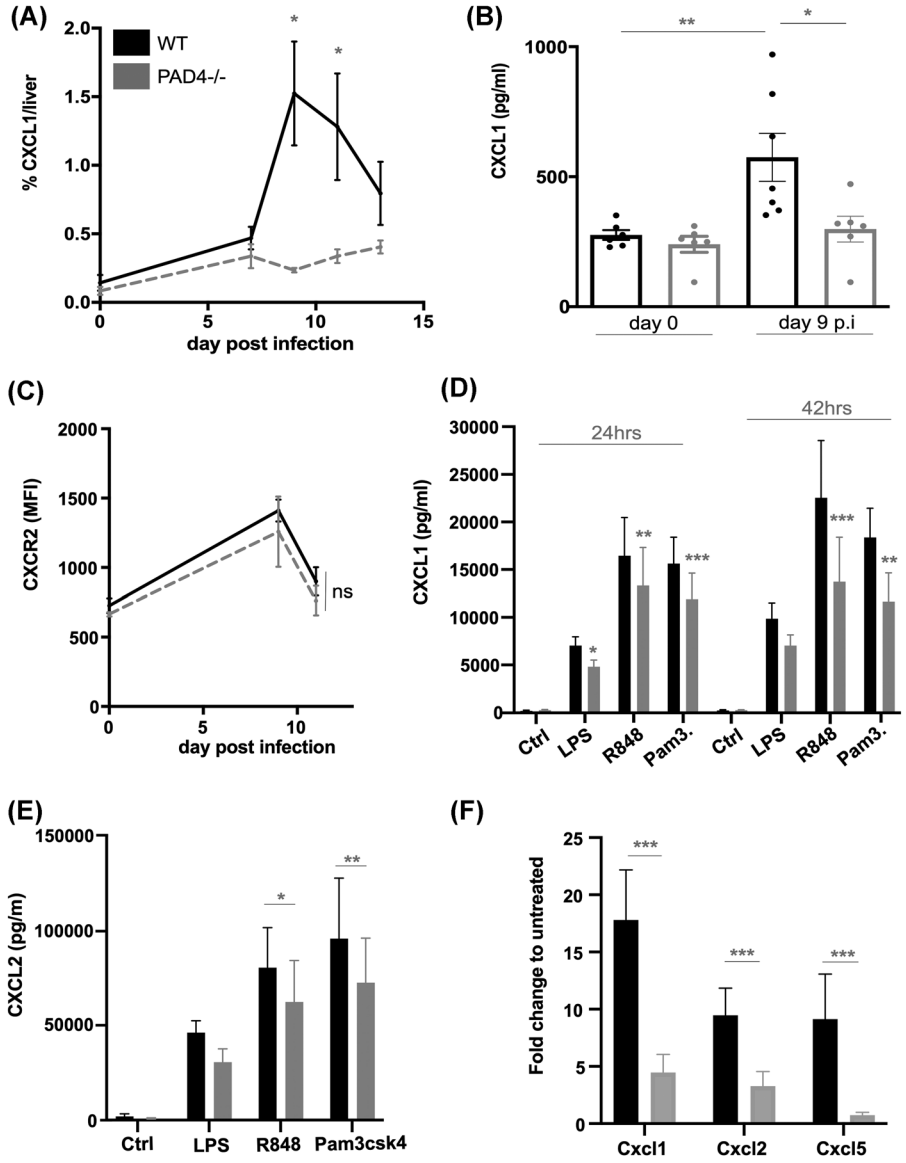

FIGURE 3 PAD4 regulates chemokine production

(A) Quantification of CXCL1 immunofluorescence in liver sections ($n = 5$); (B) plasma CXCL1 levels in WT and PAD4−/− mice, $n = 6$–7 mice; (C) CXCR2 MFI in WT and PAD4−/− circulating neutrophils ($n = 6$ mice); (A–C): 2 independent experiments; (D and E) ELISA supernatant concentrations of CXCL1 (D) at 24 and 48 h, and CXCL2 (E) at 48 h poststimulation of mouse BMDM supernatants ($n = 8$ biologic replicates) with LPS (200 ng/ml), resiquimod/R848 (5 μM) and Pam3CSK (100 ng/ml); (F) relative abundance of CXCL1, -2, and -5 mRNA levels from mouse BMDM, stimulated with resiquimod/R848 (5 μM) for 42 h. Chemokine transcript abundances were normalized to 18s levels and are presented as fold change relative to untreated; ($n = 4$ biologic replicates). Graphs show mean ± SEM. Asterisks indicate: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, by 2-way ANOVA (A, C, D, E, and F) and 1-way ANOVA comparison (B)

differences in WT and PAD4−/− at 2 h poststimulation (Figure S4(B)). In contrast, mRNA levels of all 3 chemokines were significantly reduced in PAD4−/− macrophages at 24 h (Figure S4(C)) and 48 h (Figure 3(F)) poststimulation. In summary, PAD4 regulates sustained, long-term transcription of *Cxcl1*, *2*, and *5* in macrophages.

To test if PAD4 controls chemokine production in human cells, we treated monocyte-derived macrophages (n = 5–6 donors) with GSK484, a PAD4 pharmacologic inhibitor. As with mouse macrophages, PAD4 inhibition significantly reduced CXCL1 (Figure 4(A)) and CXCL2 (Figure 4(B)) secretion in TLR7-stimulated human macrophages (Figure 4(A)), consistent with a reported role for PAD4 in TLR7 responses in lupus.[4,27] We obtained similar results with human monocytes (Figure S4(D)). Finally, we isolated *P. falciparum* trophozoite and schizont iRBCs from in vitro culture and used them to stimulate human macrophages in the presence of GSK484. PAD4 inhibition led to reduced CXCL1 secretion in response to malaria parasites, demonstrating a key role for PAD4 in regulation of this chemoattractant (Figure 4(B)).

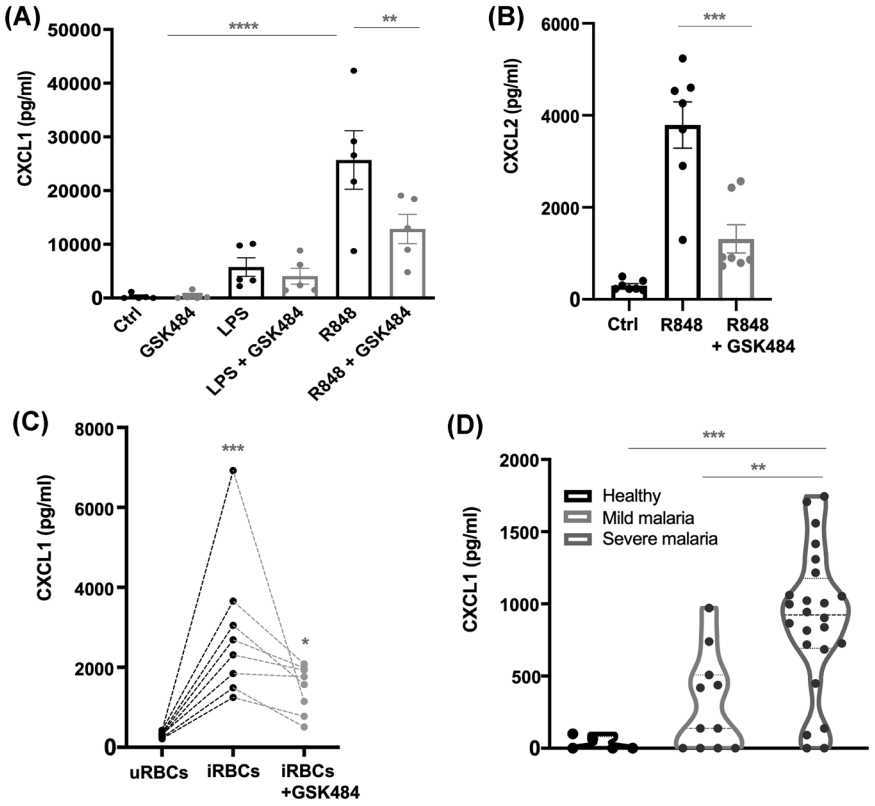

FIGURE 4 PAD4 regulates chemokine production in human macrophages CXCL1 (A and C) and CXCL2 (B) concentrations in human monocyte-derived macrophage supernatants, stimulated with resiquimod (5 μM) and GSK484 (10 μM) (A and B) and *P. falciparum* iRBC (C), for 24 h, measured by ELISA, $n = 5$–8 healthy donors (D) CXCL1 concentration in plasma of mild ($n = 11$) and severe ($n = 24$) malaria patients and controls ($n = 5$), determined by ELISA. Graphs show mean ± SEM. Stars indicate: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, by 1-way ANOVA comparison (A, C, and D) and paired *t*-test analysis (B)

CXCL1 has been shown in other disease models to orchestrate immune cell trafficking into various organs affected by malaria, including the brain.[33] CXCL1 levels in malaria patients have not previously been reported. To test if circulating levels of this chemoattractant are linked to malaria severity, we quantified CXCL1 in plasma from Gabonese pediatric patients with uncomplicated and severe *P. falciparum* malaria. We found significantly higher CXCL1 in severe malaria compared with mild or uninfected controls (Figure 4(C)), demonstrating that this chemokine may be a biomarker of severe disease.

Our findings confirm the importance of immune cells in inflammatory damage of the liver parenchyma in the *P. chabaudi* model and demonstrate that liver injury is not mediated by parasite factors alone. Similar results were obtained by antibody-mediated depletion of neutrophils[22] and by neutralization of the neutrophil growth factor GCSF.[19] Neutrophils are also pathogenic in the *P. berghei* lung injury model.[21] In humans, NETs are specifically detected in the retinal neurovasculature of cerebral malaria patients.[19] Neutrophil infiltration is also observed in placentas of women suffering from pregnancy associated malaria.[34,35] It remains unclear, however, to what extent neutrophils directly damage hepatocytes. CD8 T cell trafficking was also reduced in PAD4−/− mice (Figure S1) and both cell types carry a high load of proteolytic and cytotoxic molecules. CD8 T cells have been implicated in organ damage in other malaria models[36] and in humans.[37] Further investigations are required to decipher the interplay of neutrophils and T cells in malaria immunopathology.

We demonstrate that the role of PAD4 in inflammation is not limited to cell-intrinsic regulation of neutrophils but includes control of chemokine production in macrophages. Although the effect of PAD4 inhibition on individual chemokines was modest, a cumulative effect on multiple chemokines may explain the prominent delay in liver trafficking. The reduction in immunopathology in PAD4−/− mice is likely to be caused by a combination of reduced granulopoiesis, impaired chemokine production as well as previously described neutrophil-intrinsic defects. The latter include dampened p38 MAPK signaling in response to TLR7 ligands[27] as well as altered immunostimulatory properties of NETs.[10]

Several mechanisms could explain the effect of PAD4 inhibition on chemokine production. PAD4 citrullinates multiple transcription factors that are important for inflammatory gene expression, including NFkB.[15] PAD4 also interacts with the apoptotic regulator p53[16] and may potentially regulate macrophage apoptosis, which could explain the delayed CXCL1 and 2 phenotype in PAD4−/− macrophages. Finally, it remains possible that PAD4 regulation of CXCL1 is not direct but rather mediated by citrullination of an intermediate secreted factor.

Our study has several limitations. Experiments were performed in the *P. chabaudi AS* infection model, which only induces mild pathology, despite demonstrating true iRBC cytoadhesion.[24] It would be of value to test the role of PAD4 in more virulent models, such as *P. berghei*, where neutrophils also have a detrimental effect on pathology.[21] Similarly, we only analyzed neutrophil trafficking up to day 13

postinfection, making it unclear to what extent neutrophil infiltration is impaired versus delayed.

Our study suggests that targeting PAD4 may be a viable therapeutic opportunity in malaria, where adjunctive therapies to treat inflammatory pathology are badly needed. Investigation of PAD4 in other rodent malaria models, in which brain and lung inflammation are prominent, would help clarify the therapeutic potential of PAD4 inhibition. Further studies are warranted to fully explore the fundamental and complex role of PAD4 in myeloid cell biology.

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AUTHORSHIP

B. A., D. C., and S. L. K. conceived the experiments. D. C., S. L. K., B. A., S. G., C. R., and J. K. performed the experiments. B. M. contributed with the clinical samples. B. A. and D. C. wrote the paper. D. C. and S. L. K. contributed equally to this work.

DISCLOSURE

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

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