Anaplasma phagocytophilum Dihydrolipoamide Dehydrogenase 1 Affects Host-Derived Immunopathology during Microbial Colonization

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Anaplasma phagocytophilum is a tick-borne rickettsial pathogen that provokes an acute inflammatory response during mammalian infection. The illness caused by *A. phagocytophilum*, human granulocytic anaplasmosis, occurs irrespective of pathogen load and results instead from host-derived immunopathology. Thus, characterizing *A. phagocytophilum* genes that affect the inflammatory process is critical for understanding disease etiology. By using an *A. phagocytophilum* Himar1 transposon mutant library, we showed that a single transposon insertion into the *A. phagocytophilum* dihydrolipoamide dehydrogenase 1 gene (*lpda1* [APH_0065]) affects inflammation during infection. *A. phagocytophilum* lacking *lpda1* revealed enlargement of the spleen, increased splenic extramedullary hematopoiesis, and altered clinicopathological abnormalities during mammalian colonization. Furthermore, LPDA1-derived immunopathology was independent of neutrophil infection and correlated with enhanced reactive oxygen species from NADPH oxidase and nuclear factor (NF)- κ B signaling in macrophages. Taken together, these findings suggest the presence of different signaling pathways in neutrophils and macrophages during *A. phagocytophilum* invasion and highlight the importance of LPDA1 as an immunopathological molecule.

Anaplasma phagocytophilum is an obligate intracellular pathogen that replicates inside myeloid and nonmyeloid cells, causing human granulocytic anaplasmosis (HGA)—an important tick-borne disease in the United States and Europe (14, 47). HGA clinical and laboratory findings are fever, myalgia, headache, malaise, thrombocytopenia, leukopenia, anemia, mild hepatic injury, and splenomegaly. Symptoms vary from none to mortality and may include septic shock-like syndrome, acute respiratory distress, and opportunistic infections (14). Infection results in hospitalization for 36% of patients, whereas 7% of clinical cases lead to intensive care unit admission and 0.6% to death (14).

A. phagocytophilum inflammatory response is induced by host innate immune mechanisms and does not directly correlate with pathogen load (6, 35, 53, 58). The importance of gamma interferon (IFN- γ) for *A. phagocytophilum* immunity is well documented in IFN- γ -deficient mice. Hence, IFN- γ produced by Natural Killer (NK) and NKT cells contributes to pathogen defense (4, 43) and mice deficient in IFN- γ are more susceptible to *A. phagocytophilum* (2, 53). During *A. phagocytophilum* infection, elevated IFN- γ levels are observed in the peripheral blood of severely ill patients (15). Reactive nitrogen species cause damaging inflammatory histopathology (6), and commonly observed pathological features are decreased bone marrow function and changes in hematopoietic progenitor cells in the spleen, most likely due to aberrant CXCL12/CXCR4 signaling (26, 27, 53). Interleukin (IL)-12/23p40, IL-18, and CD4⁺ T cells are critical for pathogen elimination from the host (44, 45). Conversely, mice deficient in Toll-like receptor 2 (TLR2), TLR4, inducible nitric oxide synthase (iNOS), myeloid differentiation primary response gene 88 (MyD88), tumor necrosis factor (TNF), NADPH oxidase, perforin, and Fas/FasL are capable of clearing *A. phagocytophilum* in-

fection but these molecules play a role in host-derived immunopathology (4, 58).

The primary site of *A. phagocytophilum* infection in the mammalian host is neutrophils (47, 48). However, increasing evidence suggests that neutrophils do not play a major role in *A. phagocytophilum* innate immunity. Neutrophils are significantly decreased in numbers during *A. phagocytophilum* infection and do not efficiently clear *A. phagocytophilum* from the blood (14). Furthermore, *A. phagocytophilum* colonization of neutrophils leads to impaired signaling and polarization (50), reduced binding to endothelial cells and IFN- γ signaling (7), transmigration (10, 42), and lowered phagocytosis and shedding of cell surface adhesion molecule receptors (21). Macrophages are not a site of infection; however, these cells are important for combating *A. phagocytophilum* infection. *A. phagocytophilum* triggers proinflammatory responses in macrophages via nuclear factor (NF)- κ B signaling through TLR2 (11), and animal models show increased macrophage infiltration and hemophagocytosis in tissues infected with *A. phagocytophilum* (28, 34). HGA clinical and histopathological features in patients also suggest macrophage activation (15).

This dichotomy between pathogen eradication and inflamma-

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tion suggests that different immune cells regulate cytokine secretion or pathogen survival during *A. phagocytophilum* infection. These data also argue for the presence of molecules that regulate inflammation but not bacterial infection. In this report, we show that the *A. phagocytophilum* molecule dihydrolipoamide dehydrogenase 1 (LPDA1) affects inflammation in mice. Mice infected with *A. phagocytophilum* lacking *lpda1* revealed blood abnormalities, enlargement of the spleen, and altered splenic extramedullary hematopoiesis. Furthermore, LPDA1-derived immunopathology positively correlated with macrophage but not neutrophil activation. These findings suggest that LPDA1 acts as an immunopathological molecule during *A. phagocytophilum* infection.

MATERIALS AND METHODS

Bioinformatics analysis. Dihydrolipoamide dehydrogenase domain structures were obtained from GenBank and the comprehensive microbial resource (CMR) genome database (<http://cmr.jcvi.org>). Phenogram analysis of dihydrolipoamide dehydrogenase proteins was conducted using MEGA version 4.

Ethics statement. Blood samples were obtained from healthy, non-pregnant adults. This procedure was approved by the Human Research Review Board at the University of California—Riverside. Animals were housed in the Animal Resources Facility according to the guidelines described under the federal Animal Welfare Regulations Act. Food and water were provided *ad libitum*, and all animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California—Riverside. Mouse strains *nlr4*^{-/-} and *asc*^{-/-} (from Millennium Pharmaceuticals) and *caspase-1*^{-/-} (from Richard Flavell at Yale University) were previously described (57). C57BL/6, *nox2* (*gp91*^{phox})^{-/-}, and *il1r1*^{-/-} mice were purchased from Jackson Laboratories. We used mice 6 to 10 weeks of age.

Bacterial strains. The University of California—Riverside approved the use of *A. phagocytophilum* strains. The *A. phagocytophilum* wild-type HZ and the mutant *lpda1*::TnHimar1 strain were grown in HL-60 cells (ATCC CCL-240). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) with L-glutamine and hydroxyethyl piperazineethanesulfonic acid (HEPES) (Thermo Scientific) and 20% heat-inactivated fetal bovine serum (FBS) in 5% CO₂ and humidified air at 37°C. Cell lines were transformed to express mCherry and spectinomycin resistance under the control of the *amtr* promoter and flanked by the transposase recognition sequences using the Himar1 transposon system. Construction of the transposase expression plasmid, bacterial transformation, selection, and rescue cloning assays were performed as previously described (20). A single transposon insertion into the *A. phagocytophilum* *lpda1* sequence was detected by inverse PCR. Sequence annotation was determined by using the genome browser software Artemis (9).

Mouse infection and bacterial load. C57BL/6 mice were infected by intraperitoneal injection with the *A. phagocytophilum* wild-type HZ or the mutant *lpda1*::TnHimar1 strain (1×10^7 or 1×10^8 bacteria). To quantify the *A. phagocytophilum* load in the peripheral blood, DNA from anticoagulated peripheral blood was extracted with a DNeasy tissue kit (Qiagen), according to the manufacturer's recommendation. Quantitative reverse transcriptase PCR (qRT-PCR) was performed using iQ SYBR green Supermix and a Bio-Rad iQ5 optical system. Primer sequences for *A. phagocytophilum* were as follows: for 16S-F, 5'-CAGCCACACTGGAA CTGAGA-3'; and for 16S-R, 5'-CCCTAAGGCCTTCCTCACTC-3'. Gene expression was normalized to β -actin F (5'-CGCATCCTCTTCCT CCCT-3') and β -actin R (5'-TGGAATCCTGTGGCATCC-3') by using the absolute-quantification method (51).

Total RNA from organs, cells, or tissues was isolated with TRIzol reagent from Invitrogen. *A. phagocytophilum*-infected salivary glands and midguts from *Ixodes scapularis* nymphs were provided by Erol Fikrig (Yale University). For these experiments, nymphs were reared at 26°C with 85% relative humidity and a daily photoperiod of 14 h light and 10 h dark. A

total of 10 to 12 nymphal *I. scapularis* ticks were placed on infected C57BL/6 mice and allowed to feed for 72 h. Nymphs were gently removed, and midguts and salivary glands were dissected and processed for analysis. Mouse organs were collected at day 4 postinfection. Bone-marrow-derived cells and human peripheral blood mononuclear cells (PBMCs) were collected 24 h poststimulation at a multiplicity of infection (MOI) of 50, and HL-60 cells were harvested 42 h postcolonization (MOI of 10). cDNA was prepared with a Verso cDNA synthesis kit (Thermo Scientific). Primer sequences for *A. phagocytophilum* *lpda1* were as follows: for F, 5'-ATCGTCAACAGTGGCACAAA-3'; and for R, 5'-CTGATGTTTGGC ACATACCG-3'. Gene expression was normalized to β -actin.

Gentamicin protection assays. Freshly isolated peritoneal neutrophils were infected with the *A. phagocytophilum* strain HZ or the mutant *lpda1*::TnHimar1 strain at the indicated time and MOI. Neutrophils were then treated with gentamicin (50 μ g/ml) for 30 min before harvesting from the culture plate and then washed with phosphate-buffered saline (PBS) by centrifugation at $200 \times g$ for 10 min at room temperature. qRT-PCR was performed as described above, and expression was shown according to the relative-quantification ($2^{\Delta\Delta CT}$) method (51).

Complete blood count and clinical chemistry. C57BL/6 mice were infected by intraperitoneal injection with the *A. phagocytophilum* wild-type HZ or the mutant *lpda1*::TnHimar1 strains (1×10^8 bacteria). Blood was collected at day 5 postinfection, and complete blood count and clinical chemistry analyses were performed in a commercial laboratory (Antech Diagnostics). Neutrophils, platelets, lymphocytes, hemoglobin, hematocrit, red blood cells, thyroxine (T4), alkaline phosphatase, and blood urea nitrogen (BUN) were measured.

Cytology and histopathology. C57BL/6 mice were infected by intraperitoneal injection with the *A. phagocytophilum* wild-type HZ or the mutant *lpda1*::TnHimar1 strains (1×10^8 bacteria). Spleens were harvested and weighed at day 15, normalized to the body weight, and contrasted to those of noninfected mice. Spleens were gently dried, and freshly cut sections were blotted onto glass slides to make impression smears for cytological evaluation. Smears were fixed in absolute methanol for 1 min prior to staining with modified Wright-Giemsa (Sigma-Aldrich). All cytology smears were scanned initially for staining uniformity. A 250-cell differential count was performed for each impression smear, with cells categorized as immature erythroid; mature (postmitotic) erythroid; immature myeloid; mature (postmitotic) myeloid, with mature neutrophils and mature eosinophils further separated out; lymphocytes; plasma cells; or macrophages. Other cell types were noted but in numbers too low to affect differential counting.

Spleens were fixed with 10% neutral buffered formalin and routinely embedded in paraffin wax. Sections (5 μ m) were obtained and stained with hematoxylin and eosin. All histopathology slides were initially scanned prior to development of a grading system. Spleen sections were scored as follows: lymphoid hyperplasia was rated from 0 to 3, with a score of 0 indicating typical splenic lymphoid follicles from a normal spleen and scores of 1, 2, and 3 indicating mild, moderate, and marked hyperplasia, respectively. Extramedullary hematopoiesis (EMH) was rated 0 to 4, with a score of 1 indicating the slight to mild degree of EMH that is typically seen in a mouse spleen, a score of 0 for any spleen lacking evidence of EMH, and scores of 2, 3, and 4 indicating mild, moderate, and marked increases in EMH, respectively. All pathological evaluation of samples was performed in a blinded fashion by a board-certified veterinary pathologist.

Isolation of peritoneal neutrophils and culture. Neutrophil isolation was performed as previously described (30). Briefly, C57BL/6 mice were intraperitoneally administered 1 ml of 3% sterile casein followed by a repeat of the injection the next morning. Three hours after the second injection, animals were sacrificed and peritoneal lavage was performed. The cells in the lavage fluid were washed three times with PBS followed by centrifugation at $200 \times g$ for 10 min at room temperature. Neutrophils were isolated using a continuous-density gradient with Percoll (Sigma) by ultracentrifugation at $60,650 \times g$ at 4°C for 20 min. Neutrophils were

washed with PBS and cultured in an ultra-low-cluster 24-well plate (Corstar) with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C in a 5% CO₂ cell culture incubator. Neutrophil viability was determined as 91% to 98% by trypan blue exclusion, and the purity was determined as 82% to 86% by cytospin cytocentrifugation and Diff-Quik staining. Cell death was assayed by the quantification of lactate dehydrogenase (LDH) using an LDH cytotoxicity detection kit (TaKaRa) and an iMark microplate absorbance reader (Bio-Rad), according to the manufacturer's instructions.

Generation of bone-marrow-derived cells and peripheral blood mononuclear cells. The generation of bone-marrow-derived macrophages (BMDMs) and bone-marrow-derived dendritic cells (BMDDCs) has been previously described (18, 31). For BMDMs, femurs and tibias of mice were flushed with a 25-gauge needle and DMEM (Thermo Scientific) and then spun for 10 min at 4°C at 1,500 rpm. Pellets were resuspended in DMEM supplemented with 30% L929 cell-conditioned media and 10% FBS. Cells were then cultured in 10-cm-diameter petri dishes at 37°C in a 5% CO₂ tissue culture incubator for 5 to 6 days, with fresh media added on day 3 in the presence of penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). For the generation of BMDDCs, RPMI 1640 containing L-glutamine was supplemented with 10% FBS, 50 µM β-mercaptoethanol, and 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech) in the presence of penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). Peripheral blood mononuclear cells were isolated by using the Polymorphprep protocol (Axis Shield).

Dual-luciferase reporter assay. Human embryonic kidney 293T (HEK293T) cells were transfected with the NF-κB firefly and renilla luciferase reporter gene plasmids using the calcium phosphate method overnight (29). The renilla luciferase gene was used as an internal control for transfection efficiency. Thirty-six hours after transfection, cells were treated with *A. phagocytophilum* HZ and the mutant *lpda1::TnHimar1* strains (MOI of 50). Luciferase activity was measured 24 h later using a dual-luciferase reporter assay system (Promega) with a Perkin Elmer Victor 2 1420 multilabel counter according to the manufacturer's instruction. Luciferase activity was normalized to that of noninfected cells.

Confocal microscopy. Cells were stimulated with *A. phagocytophilum* strains. After two washes with PBS, cells were stained with 1 µg/ml cholera toxin B (Molecular Probes) at 4°C for 10 min and fixed with methanol. Cells were blocked with 5% bovine serum albumin (BSA)-PBS and stained for 60 min with primary polyclonal antibodies—either *A. phagocytophilum* (raised in rabbits at Yale University) (1:100) or the nuclear localization signal (NLS) of the p65 subunit of NF-κB (Millipore) (1:100)—at room temperature. Cells were then washed with PBS and stained with fluorescence-conjugated secondary antibodies for 30 min at room temperature. Cells were mounted with Vectashield mounting media containing 4',6-diamidino-2-phenylindole (DAPI). Confocal microscopy was done by using a Leica SP2 microscope.

ROS detection. Rotenone, antimycin, and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma. We assessed reactive oxygen species (ROS) using the ROS-specific fluorescence probe 2',7'-dichlorofluorescein diacetate (H2DCFDA) (Invitrogen), as described previously (13). Fluorescence was recorded in 96-well plates over time with a Perkin Elmer Victor 2 1420 multilabel counter using a fluorescein isothiocyanate (FITC) filter (excitation, 485 nm; emission, 538 nm). Mitochondrion-associated ROS levels were measured by staining cells with MitoSOX (Molecular Probes) (2.5 µM) for 30 min at 37°C. Cells were washed with fluorescence-activated cell sorter (FACS) buffer and resuspended for analysis. Cells were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences) and FCS Express (De Novo) software.

Immunoblot analysis. Proteins were extracted in radioimmunoprecipitation assay (RIPA) buffer with complete protease and phosphatase inhibitor cocktails from Roche. The protein concentration was measured using a protein assay kit from Bio-Rad. Total cell lysates were separated using sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and

transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked and incubated with primary and secondary antibodies. Development was assayed using an enhanced chemiluminescence (ECL) Western blot analysis system from Pierce. Rabbit anti-β-actin antibody was purchased from Thermo Scientific. Rabbit anti-mouse IκB-α and mouse anti-mouse p-IκB-α were purchased from Cell Signaling. Densitometry was performed with ImageJ open-source software (National Institutes of Health).

Enzyme-linked immunosorbent assay (ELISA). Mouse tumor necrosis factor (TNF)-α, IL-1β, and IL-6 were measured with a BD OptEIA set from BD Biosciences. Mouse IFN-γ, macrophage inflammatory protein 2 (MIP-2), and IL-12p40 were measured with capture and detection antibodies from eBioscience.

Statistical analysis. Data were expressed as means ± standard errors of the means (SEM). Gaussian distribution was determined by the D'Agostino and Pearson normality test. For data points that followed a Gaussian distribution, the following parametric analyses were used: unpaired Student's *t* test (two-group comparisons); one-way analysis of variance (ANOVA) (comparisons of three or more groups; one variable); two-way ANOVA (comparisons of three or more groups; two variables); and three-way ANOVA (comparisons of three or more groups; three variables). Kruskal-Wallis ANOVA was used for data points that did not follow a Gaussian distribution. Bonferroni (parametric) and Dunn's (non-parametric) *post hoc* multiple-comparison tests were used following ANOVA. All statistical calculations were performed by using GraphPad Prism version 5.04 and Minitab 16 (three-way ANOVA). Graphs were made by using GraphPad Prism version 5.04. *P* < 0.05 was considered statistically significant.

RESULTS

***A. phagocytophilum* *lpda1* expression during pathogen infection.** Phenogram analysis revealed that *lpda* genes are present across many members of the families *Anaplasmataceae* and *Rickettsiaceae*, including *A. phagocytophilum* (Fig. 1A). The *A. phagocytophilum* HZ genome carries three *lpda* genes, two of which are 100% similar (APH_0065 and APH_1070), and it is unclear whether they are paralogues (Fig. 1B). A third gene (APH_0393) is clustered together with the *lpda* sequence from the closely related species *A. marginale* (AM_797) (Fig. 1B). Further investigation of *lpda1* (APH_0065) showed that this gene carries three domains: two Rossmann-fold NAD(P)⁺-binding protein domains (NADB Rossmann) at positions 2 to 40 (e-value = 3.73 e⁻⁵) and 185 to 243 (e-value = 3.89 e⁻⁹) and one pyridine nucleotide-disulfide oxidoreductase dimerization domain (pyridine oxidoreductase) at positions 352 to 461 (e-value = 4.26 e⁻³³) (Fig. 1C). *lpda1* expression was low in *I. scapularis* ticks, mouse neutrophils, and human PBMCs during bacterial colonization (Fig. 1D). However, *lpda1* was highly expressed in the human promyelocytic leukemia cell line HL-60 and mouse dendritic cells (BMDDC) and macrophages (BMDMs).

***A. phagocytophilum* LPDA1 affects host-induced immunopathology.** To determine the *lpda1* function *in vivo*, we constructed and screened an *A. phagocytophilum* Himar1 transposon mutant library using clonal strain HZ (Fig. 2A). Among the different mutants tested, inverse PCR revealed a single transposon insertion into the *A. phagocytophilum* dihydrolipoamide dehydrogenase 1 (*lpda1* [APH_0065]) gene. By using two different doses (1 × 10⁷ or 1 × 10⁸ bacteria), we did not detect any pathogen load differences when the *A. phagocytophilum* *lpda1::TnHimar1* mutant was used to infect mice (Fig. 2B and C).

Clinicopathological abnormalities, however, were observed when C57BL/6 mice were infected with the mutant *lpda1::TnHi-*

FIG 1 Characterization of the *A. phagocytophilum* *lpda1* gene. (A) Phenogram displaying similarities among many *lpda* genes from the families Anaplasmataceae and Rickettsiaceae. (B) Alignment of LPDA pyridine nucleotide-disulfide oxidoreductase dimerization domains from *A. phagocytophilum* (APH) and *A. marginale* (AM). Conserved regions are shown in yellow. (C) Diagram illustrating the LPDA1 domains. Data represent Rossmann-fold NAD(P)⁺-binding proteins (NADB Rossmann; positions 2 to 40 and 185 to 243) and the pyridine nucleotide-disulfide oxidoreductase dimerization domain (Pyridine oxidoreductase; positions 352 to 461). (D) *lpda1* transcription in cells or organs of *I. scapularis* ticks, mice, and humans measured by qRT-PCR and normalized to *A. phagocytophilum* infection (*lpda1*/16S). Data are presented as means $\pm$ SEM. BMDMs—bone-marrow derived macrophages; BMDDCs—bone-marrow derived dendritic cells; PBMC—peripheral blood mononuclear cells.

mar1 strain at day 5 postinfection (Fig. 3) and day 15 postinfection (data not shown). Peripheral blood neutropenia and lymphopenia occurred in animals infected with the mutant *lpda1*::TnHimar1 strain compared to naïve animals (Fig. 3A and C). Mice infected with the mutant *lpda1*::TnHimar1 strain had decreased neutropenia only compared to animals infected with the wild-type *A. phagocytophilum* HZ strain (Fig. 3A). Thrombocytopenia (Fig. 3B) and anemia (as judged by the hematocrit and red blood cell counts) were observed only in mice infected with the wild-type *A. phagocytophilum* HZ strain compared to naïve mice (Fig. 3E and F). Anemia also occurred in mice infected with the wild-type *A. phagocytophilum* HZ strain compared to animals infected with the mutant *lpda1*::TnHimar1 strain. Both groups of infected mice had decreased total serum T4 levels compared to noninfected mice with a more substantial decrease in wild-type bacterial infection (Fig. 3G). Wild-type-infected mice had decreased serum alkaline phosphatase (Fig. 3H), and both groups of infected mice had decreased blood urea nitrogen (BUN) levels compared to noninfected mice, but no difference was observed between infected groups (Fig. 3I).

The mechanisms that drive hematological abnormalities during Anaplasmataceae mouse infection are not clearly understood. However, the myelosuppressive properties of IFN- γ may be involved (32). We noticed that mice infected with the mutant *lpda1*::TnHimar1 strain had higher levels of IFN- γ in the peripheral blood than wild-type-infected animals (Fig. 4A). These findings correlated with increased spleen weight and splenic extramedullary hematopoiesis in *lpda1*-infected mice compared to wild-type-infected animals (Fig. 4B and C). Increased splenic extramedullary hematopoiesis in *lpda1*-infected mice consisted of expanded populations of immature hemic precursor cells of all lineages (erythroid, myeloid, and megakaryocytic) (Fig. 4C and data not shown). Mice infected with the *A. phagocytophilum* *lpda1*::TnHimar1 strain had peripheral neutropenia (Fig. 3A) but no substantial increase in mature splenic neutrophil numbers (Fig. 4D). On the other hand, mice infected with the wild-type *A. phagocytophilum* HZ strain had normal neutrophil counts in the peripheral blood but higher levels of splenic neutrophilia (Fig. 4D and E). Taken together, our findings suggest that LPDA1 affects the hemato-

FIG 2 *A. phagocytophilum lpda1* does not affect bacterial load in mice. (A) Insertion diagram representing the location of the Himar1 transposon in the *A. phagocytophilum* HZ genome. The Himar1 transposon was inserted into the *lpda1* gene (APH_0065), generating the *A. phagocytophilum* mutant *lpda1*::TnHimar1. *A. phagocytophilum* was transformed to express the red fluorescence protein mCherry (Cherry) and the spectinomycin (Spec) antibiotic resistance gene under the control of the *amtr* promoter. The Himar end indicates transposon boundaries. (B and C) C57BL/6 mice were infected with the wild-type *A. phagocytophilum* strain HZ (WT) ($n = 20$) or the *lpda1*::TnHimar1 mutant (*lpda1*) ($n = 20$). Mice were infected by intraperitoneal injection with either (B) 10^7 or (C) 10^8 *A. phagocytophilum* bacteria, and bacterial load was measured by the absolute quantification qRT-PCR method at the indicated time points. Data represent the results of two-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. NS—not significant. $P < 0.05$.

logical profile and spleen hematopoiesis during *A. phagocytophilum* infection.

LPDA1 affects *A. phagocytophilum* colonization of mouse neutrophils. Since *A. phagocytophilum* primarily infects neutrophils, we investigated whether colonization of the *A. phagocytophilum lpda1*::TnHimar1 strain correlated with host-derived immunopathology. Neither the wild-type nor the mutant *A. phagocytophilum* strains affected cell death (Fig. 5A). The wild-type *A. phagocytophilum* infected neutrophils, as previously described (47). However, the mutant *A. phagocytophilum lpda1*::TnHimar1 strain was not able to effectively colonize neutrophils after 2 h postinfection (Fig. 5B). These findings prompted us to perform gentamicin protection assays to quantify the ability of the mutant *A. phagocytophilum lpda1*::TnHimar1 strain to invade mouse neutrophils. This method has been previously used to explain *A. phagocytophilum* tropism for these cells (22). Similar to our results observed with confocal microscopy (Fig. 5B), the *A. phagocytophilum lpda1*::TnHimar1 mutant did not effectively invade neutrophils compared to the wild-type HZ strain 2 h postinfection (Fig. 5C).

LPDA1 also affected cytokine and chemokine production by neutrophils. The wild-type *A. phagocytophilum* HZ strain triggered higher levels of TNF- α and MIP-2 secretion than the mutant *lpda1*::TnHimar1 strain (Fig. 5D and E). Next, we investigated whether the *A. phagocytophilum lpda1*::TnHimar1 defect in neutrophil colonization would affect signaling. NF- κ B is an important signaling molecule for *A. phagocytophilum* immune response (11). In nonstimulated cells, inhibitors of κ B (I κ Bs) sequester NF- κ B dimers in the cytoplasm by masking the nuclear localization signals (NLS) of NF- κ B protein p65. Activation of

NF- κ B is initiated by phosphorylation and subsequent degradation of I κ Bs by a proteasome complex (40). Degradation of I κ Bs leads to translocation of the NF- κ B complex to the nucleus and expression of inflammatory genes (40). In a time course experiment, we noticed that phosphorylation of I κ B peaked at 5 min when the wild-type *A. phagocytophilum* HZ strain infected neutrophils, whereas the mutant *lpda1*::TnHimar1 induced maximum phosphorylation at 30 min (Fig. 5F and G). The delayed effect of the mutant *lpda1*::TnHimar1 on neutrophil signaling was also observed for I κ B degradation. The presence of I κ B was reduced to 50% at 4 h postinfection with the wild-type *A. phagocytophilum* HZ strain, whereas degradation of I κ B during infection with the *lpda1*::TnHimar1 mutant never achieved more than 25% in neutrophils (Fig. 5H and I).

***A. phagocytophilum* LPDA1 modulates macrophage NF- κ B-driven cytokine secretion.** *A. phagocytophilum*-induced immunopathology is associated with macrophage activation (11, 15, 28, 34). Therefore, we investigated whether LPDA1 had any effect on BMDM stimulation. The mutant *lpda1*::TnHimar1 strain caused increased secretion of TNF- α and IL-12p40 compared to wild-type strain HZ (Fig. 6A and B). Complementation studies for *A. phagocytophilum* are not available (47). Therefore, we could not rescue the wild-type phenotype using classical bacterial genetics studies. However, the effect observed for the *A. phagocytophilum* mutant *lpda1*::TnHimar1 was not due to a transposon artifact. First, infection of the *A. phagocytophilum* harboring a Himar1 insertion in an irrelevant intergenic region did not affect secretion of NF- κ B cytokines (data not shown). Second, secretion of IL-1 β —a cytokine that is regulated by the inflammasome (5)—was not affected by the Himar1 transposon in the *A. phagocytophilum*

FIG 3 *A. phagocytophilum* LPDA1 causes hematological changes during pathogen infection of mice. C57BL/6 mice were infected with the wild-type *A. phagocytophilum* strain HZ (WT) ($n = 4$ to 6) or *lpda1::TnHimar1* ($n = 5$) (*lpda1*) and contrasted to noninfected mice (-). Blood was collected at day 5 postinfection, and levels of (A) neutrophils, (B) platelets, (C) lymphocytes, (D) hemoglobin, (E) hematocrit, (F) red blood cells, (G) T4, (H) alkaline phosphatase, and (I) BUN were measured and results compared using an unpaired Student's *t* test (*lpda1*, WT). NS—not significant. Data represent means of the results. *, $P < 0.05$.

mutant *lpda1::TnHimar1* strain compared to the wild-type strain (Fig. 6C), suggesting that the cytokine effect observed in macrophages was specific for NF- κ B signaling. Third, the mutant *A. phagocytophilum lpda1::TnHimar1* strain showed phenocopy activation of the inflammasome during macrophage stimulation (Fig. 6D to F and data not shown) (44). *A. phagocytophilum lpda1::TnHimar1* triggered reduced IL-1 β secretion during pathogen stimulation of *asc-* and *caspase-1*-deficient macrophages compared to wild-type cells (Fig. 6D). NLRC4 was only partially required for IL-1 β secretion by macrophages during *A. phagocytophilum lpda1::TnHimar1* stimulation, as shown for *nlrc4*-deficient macrophages (Fig. 6D). The IL-1 β receptor did not play a major role during *A. phagocytophilum* stimulation of macrophages, as mice deficient in the IL-1 β receptor (*il1r1*) did not show any differences in IL-1 β secretion compared to wild-type cell results during pathogen stimulation (Fig. 6D). The effect of the inflammasome is restricted to IL-1 β secretion (36). Indeed, the secretion of IL-6 and IL-12p40 was not affected during infection of macrophages deficient in components of the inflammasome (Fig. 6E and F).

***A. phagocytophilum* LPDA1 inhibits NF- κ B activation in macrophages via ROS from NADPH oxidase.** The regulatory ef-

fect of *A. phagocytophilum* LPDA1 on NF- κ B in macrophages was confirmed by Western blot analysis and confocal microscopy. Time course series showed increased and faster NF- κ B activation of BMDMs (as judged by faster phosphorylation, degradation of the inhibitor protein I κ B- α , and p65 translocation) when macrophages were stimulated with the *lpda1::TnHimar1* mutant in contrast to the wild-type *A. phagocytophilum* HZ strain (Fig. 7A to D). Phosphorylation of I κ B during stimulation with the *lpda1::TnHimar1* mutant strain was twice the amount seen with non-stimulated BMDMs at 6 h (Fig. 7C). However, at its peak, phosphorylation of I κ B increased only 50% when the wild-type *A. phagocytophilum* HZ strain stimulated BMDMs (Fig. 7C). The increased effect of the mutant *lpda1::TnHimar1 A. phagocytophilum* on macrophage signaling also correlated with I κ B degradation. The presence of I κ B was reduced to 50% at its lowest level when the mutant *lpda1::TnHimar1* strain stimulated macrophages, whereas there was only a 25% reduction in I κ B degradation for BMDMs stimulated with the wild-type *A. phagocytophilum* HZ strain (Fig. 7B and D). Consistent with these findings, the *lpda1::TnHimar1* mutant exhibited a 2-fold increase in NF- κ B activation when HEK293T cells were transfected with an NF- κ B

FIG 4 *A. phagocytophilum* LPDA1 affects pathogen-derived immunopathology in mice. C57BL/6 mice were infected by intraperitoneal injection with either wild-type *A. phagocytophilum* strain HZ (WT) (10^8 bacteria) ($n = 5$) or the *lpda1::TnHimar1* mutant (*lpda1*) (10^8 bacteria) ($n = 5$). (A) IFN- γ was measured in the blood at day 2 by ELISA. Comparisons were performed by one-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. (B) Spleens were harvested and weighed at day 15. Spleen weight of C57BL/6 mice infected with either the WT strain ($n = 18$) or the *lpda1* mutant ($n = 11$) was normalized to the animal's body weight and contrasted to those of noninfected mice ($n = 5$) at day 15 postinfection. Comparisons were performed by one-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means of the results. (C) Extramedullary hematopoiesis was scored in histopathology sections of spleen from control mice and mice infected with WT *A. phagocytophilum* strain HZ or the *lpda1* mutant. Comparisons were performed by Kruskal-Wallis one-way ANOVA—Dunn's (*lpda1*, WT); data are presented as means of the results. (D) Cytological impression smears of spleen from control mice (-) and mice infected with either the WT *A. phagocytophilum* strain HZ or the *lpda1* mutant. Neutrophils were counted and are shown as a percentage of the total differential count. Comparisons were performed by one-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. *, $P < 0.05$. (E) Splenic extramedullary hematopoiesis from control (-), WT, and *lpda1*-infected mice, as judged by increased hemic precursors (cytological images; left panels—30 μ m) and trilineage hemic precursors (histopathological images; right panels—150 μ m).

promoter-driven luciferase reporter construct and compared to the wild-type *A. phagocytophilum* HZ strain (Fig. 7E). Enhanced NF- κ B activation in macrophages during stimulation with the *lpda1::TnHimar1* mutant correlated well with increasing numbers of the *lpda1::TnHimar1* mutant inside the cells at 2 and 6 h post-stimulation (Fig. 7F). These results suggested that the *A. phagocytophilum* *lpda1::TnHimar1* mutant was eliminated faster by BMDMs than the wild-type *A. phagocytophilum* HZ strain. At 24 h, most if not all *A. phagocytophilum* bacteria were degraded by macrophages (data not shown).

Next, we tested the ability of *A. phagocytophilum* to induce ROS during macrophage stimulation. ROS is widely linked to

inflammation and is important for NF- κ B activation (38). In contrast to neutrophils, where *A. phagocytophilum* suppresses ROS, BMDMs induced ROS in response to *A. phagocytophilum* (Fig. 7G). Among the many sources of ROS inside the cells, mitochondria and NADPH oxidases are the most common (38). To determine the source of ROS during *A. phagocytophilum* stimulation of BMDMs, we used *nox2* (*gp91^{phox}*)-deficient mice. *Nox2*-deficient mice do not produce ROS from NADPH oxidase, which can be used as a mechanism of defense against microbial infection. Although ROS in *nox2*-deficient mice were not completely abolished during pathogen stimulation, wild-type *A. phagocytophilum* stimulation produced significantly

FIG 5 *A. phagocytophilum* colonization of the *lpda1*::TnHimar1 mutant is impaired in neutrophils. (A) Cell death in peritoneal neutrophils, as judged by LDH assays after 2 h of stimulation with either the wild-type HZ strain (WT) or the *lpda1*::TnHimar1 (*lpda1*) *A. phagocytophilum* strain (MOI, 10 or 50). (B) Peritoneal neutrophils (1×10^6) from C57BL/6 mice were stimulated with the WT strain or the *lpda1* mutant (MOI, 50) for 2 h and stained with cholera toxin subunit B (green) and an antibody against *A. phagocytophilum* (red) for confocal microscopy. Bar = 10 μ m. (C) Peritoneal neutrophils (1×10^6) were stimulated with the WT strain or the *lpda1* mutant (MOI, 50) for 2 h. Thirty minutes before cell harvesting, gentamicin (50 μ g/ml) was added to the medium to eliminate extracellular bacteria. Cells were washed extensively with PBS, and the *A. phagocytophilum* load was measured by qRT-PCR, as judged by the 16S/actin ratio. Comparisons were performed using an unpaired Student's *t* test (*lpda1*, WT); data are presented as means $\pm$ SEM. (D and E) Peritoneal neutrophils (1×10^6) from C57BL/6 mice were stimulated with the WT and the *lpda1* *A. phagocytophilum* strains (MOI, 50). Supernatants were collected, and (D) TNF- α and (E) MIP-2 were measured by ELISA. Comparisons were performed by one-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. *, $P < 0.05$. (F) Cell lysates were collected and immunoblotted (IB) with p-I κ B- α and I κ B- α antibodies at the indicated time points. β -Actin was used as a normalizing control. Densitometry was performed for (G) p-I κ B- α and (H) I κ B- α .

less ROS in *nox2*-deficient cells than in wild-type macrophages (Fig. 7G).

Our observation was further confirmed by measuring ROS in mitochondria. In contrast to rotenone and antimycin (two compounds that produce ROS by inhibiting complex I and III in the mitochondria), *A. phagocytophilum* did not induce any detectable ROS in *nox2*-deficient BMDMs (Fig. 7H). Phorbol-12-myristate-13-acetate (PMA), which induces cells to undergo the NADPH oxidase-dependent respiratory burst, was used as a negative control for *nox2*-deficient cells and did not increase ROS levels. To determine whether the *lpda1*::TnHimar1 *A. phagocytophilum* mutant produces more ROS than wild-type *A. phagocytophilum* during BMDM stimulation, we measured total ROS. We noticed a consistent and statistically significant increase in the ROS levels during BMDM stimulation with the *lpda1*::TnHimar1 mutant compared to the wild-type *A. phagocytophilum* HZ strain (Fig. 7I). Taken together, our data show that LPDA1 inhibits BMDM ROS production by NADPH oxidase, leading to reduced NF- κ B activation and cytokine production during stimulation with *A. phagocytophilum*.

DISCUSSION

A. phagocytophilum studies have defined the contribution of innate and adaptive immunity during infection. On the one hand, infection-induced immunopathology is due to innate immunity (6, 12, 17, 28, 35, 52, 53). On the other hand, pathogen eradication is obtained by adaptive immunity (4, 44, 55, 58). This clear-cut dichotomy suggests that some genes in the *A. phagocytophilum* genome may contribute to pathogen colonization while others may be directly associated with the disease state in mammals. The latter are clinically relevant because cytopenias and splenomegaly—common HGA symptoms—are largely due to host inflammation (16, 26, 27, 56). In this study, we characterized the molecule LPDA1 during *A. phagocytophilum* colonization of the mammalian host. LPDA1 proved important to the disease state, because animals infected with the mutant *lpda1*::TnHimar1 developed more-pronounced splenomegaly, extramedullary hematopoiesis, and an altered hematological profile compared to mice infected with the wild-type *A. phagocytophilum* HZ strain.

A. phagocytophilum infection results in bone marrow dysfunction and hematopoietic stem and progenitor cell mobilization

FIG 6 *A. phagocytophilum* LPDA1 inhibits NF- κ B-mediated cytokine secretion in macrophages. (A to C) BMDMs (1×10^6) from C57BL/6 mice were stimulated with the wild-type HZ (WT) and *lpda1*::TnHimar1 (*lpda1*) *A. phagocytophilum* strains (MOI, 50) for 18 h. Supernatants were collected, and (A) TNF- α , (B) IL-12p40, and (C) IL-1 β were measured by ELISA. Comparisons were performed by one-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. (D to F) BMDMs (8×10^5) from wild-type, *nlrc4*^{-/-}, *asc*^{-/-}, *caspase-1*^{-/-}, and *il1r1*^{-/-} mice were stimulated with the *lpda1* *A. phagocytophilum* strain at an MOI 50 for 18 h. (D) IL-1 β , (E) IL-6, and (F) IL-12p40 were measured by ELISA. Experiments were repeated at least twice. Comparisons were performed by one-way ANOVA—Bonferroni (knockout cells, WT); data are presented as means $\pm$ SEM. *, $P < 0.05$.

(27)—raising the possibility that the *lpda1*::TnHimar1 mutant triggered altered granulopoiesis in mice. Accordingly, peripheral neutrophil populations were quantitatively lower in mice infected with the *A. phagocytophilum* *lpda1*::TnHimar1 mutant than in mice infected with the wild-type HZ strain. Mice infected with the *lpda1*::TnHimar1 mutant strain had higher levels of IFN- γ in the serum than animals infected with the wild-type *A. phagocytophilum* strain. Studies in a mouse model of infection with a related pathogen, *Ehrlichia muris*, found changes of hematopoiesis in IFN- γ -deficient mice (32), supporting a role for IFN- γ in granulopoiesis regulation during *A. phagocytophilum* infection.

CD4⁺ lymphocytes play a primary role in systemic IFN- γ production and *A. phagocytophilum* eradication (1, 4). However, relative lymphopenia and circulating IFN- γ levels displayed an inverse relationship to peripheral lymphocyte counts in mice infected with the *A. phagocytophilum* *lpda1*::TnHimar1 mutant. Quantitation of peripheral lymphocyte counts and lymphocyte populations in lymphoid tissues is complex, as lymphopenia can reflect total body lymphocyte depletion or lymphocyte sequestration in lymphoid tissues (19, 54). Given the presence of marked splenomegaly with lymphoid hyperplasia in both groups of infected mice, it is likely that infection-induced lymphopenia arose due to mechanisms such as lymphocyte sequestration and altered recirculation. Increased systemic IFN- γ levels during infection presumably reflect the pathogen-induced stimulation of NK and NKT cells. These cells are known to produce IFN- γ during early stimulation with *A. phagocytophilum* (4, 43).

We observed an effect of LPDA1 on *A. phagocytophilum* colonization of neutrophils. However, pathogen survival was not influenced by LPDA1 during *in vivo* infection. We reconcile these findings by suggesting that extrapolation of the *A. phagocytophilum* load in neutrophils should be interpreted with caution, be-

cause neutrophil load does not correlate with disease etiology. Furthermore, *A. phagocytophilum* has been shown to colonize a variety of cells other than neutrophils and independent laboratories have demonstrated that *A. phagocytophilum* infects the microvascular endothelium (24, 39), cells of megakaryocytic lineage (23), and mast cells (41).

The lack of correlation between bacterial load and immunopathology observed in our studies implies tolerance as a potential strategy of host defense during *A. phagocytophilum* infection. Tolerance mitigates the negative impact of infection irrespective of pathogen burden (37). Our definition of tolerance should not be confused with immunological tolerance, which is classically defined as unresponsiveness to self-antigens. We use this word as described by Medzhitov and colleagues (37) and Schneider and colleagues (3), who define tolerance as the ability to withstand pathogen colonization independently of pathogen load. We speculate that recognition of *A. phagocytophilum* LPDA1 by the immune system would avoid the deleterious effects of overarching inflammation, thereby mitigating the health impact of *A. phagocytophilum*. Conversely, in the absence of LPDA1, the immune system would develop a more pronounced inflammation, causing exacerbated splenomegaly and hematological changes. Typically, tolerance occurs in tissues of high cell renewal and autonomous capacity such as the spleen (3). Increased splenomegaly and extramedullary abnormalities were observed for mice infected with the mutant *lpda1*::TnHimar1 strain.

An alternative explanation for these findings is that control of inflammation by *A. phagocytophilum* via LPDA1 could change hematopoietic progenitor and lineage-committed cells. Inflammation-mediated changes would increase the release of myeloid and nonmyeloid cells for pathogen colonization. This line of thinking is supported by recent studies showing that *A. phagocytophilum* causes hematopoietic alterations in the spleen, with an

FIG 7 LPDA1 inhibits NF- κ B nuclear translocation in macrophages via NADPH ROS. (A) BMDMs (1×10^6) from C57BL/6 mice were stimulated for 6 h with either the wild-type HZ (WT) or *lpda1::TnHimar1* (*lpda1*) *A. phagocytophilum* strains (MOI, 50) and stained with cholera toxin subunit B (green), DAPI (blue), and an antibody against the nuclear localization signal of the p65 subunit of NF- κ B (red; white arrows). Bar = 10 μ m. (B) Cell lysates were collected and immunoblotted (IB) with p-I κ B- α and I κ B- α antibodies. β -Actin was used as a normalizing control. (C and D) Densitometry was performed for (C) p-I κ B- α and (D) I κ B- α . (E) HEK293T cells were transfected with an NF- κ B luciferase reporter plasmid. At 36 h posttransfection, cells were stimulated with the WT or the *lpda1* *A. phagocytophilum* strains (MOI, 50) for 24 h. Luciferase activity was measured and normalized to that of noninfected cells, and the renilla luciferase gene was used as an internal control. Comparisons were performed using an unpaired Student's *t* test (*lpda1*, WT); data are presented as means $\pm$ SEM. (F) BMDMs (1×10^6) from C57BL/6 mice were stimulated with the WT or the *lpda1* *A. phagocytophilum* strains (MOI, 50) and stained with cholera toxin subunit B (green), DAPI (blue), and an antibody against *A. phagocytophilum* (red) for confocal microscopy. Bar = 20 μ m. (G) BMDMs (2×10^5) from wild-type and *nox2*^{-/-} mice were stimulated with the WT *A. phagocytophilum* strain (MOI, 50). ROS production was monitored using the fluorescent probe H2DCFDA at the indicated time points. Comparisons were performed by three-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. (H) BMDMs (1×10^6) from *nox2*^{-/-} mice were stimulated for 30 min with the WT *A. phagocytophilum* (MOI, 100), mitochondrial oxidants rotenone (40 μ M) and antimycin (40 μ g/ml), or the NOX-dependent respiratory burst chemical PMA (5 μ M). ROS generation in the mitochondria was measured by flow cytometry using the fluorescent probe MitoSOX (2.5 μ M). (I) BMDMs (2×10^5) from wild-type mice were stimulated with the WT or the *lpda1* *A. phagocytophilum* strains (MOI, 10) at the indicated time points. Comparisons were performed by two-way ANOVA—Bonferroni (*lpda1*, WT); data are presented as means $\pm$ SEM. Experiments were repeated at least twice. *, $P < 0.05$; NS—not significant. (-), nonstimulated cells.

expansion of lymphoid follicles and marked extramedullary hematopoiesis in the red pulp (26, 27). Hematopoietic changes were characterized by increased numbers of Ter-119⁺ erythroid precursors, B lymphocytes, and Gr-1⁺ granulocytic cells postinfection. Marked extramedullary hematopoiesis characterized by substantially increased granulopoiesis and erythropoiesis was also found in *E. muris* infection of mice (33). Altered CXCL12/CXCR4 signaling—a critical pathway in hematopoietic and progenitor cell mobilization—appears to be involved in infection by both *A. phagocytophilum* and *E. muris* (26, 33).

One intriguing result from our study is that signaling in neutrophils and signaling in macrophages during *A. phagocytophilum* infection were not alike. Neutrophils were refractory to *lpda1::TnHimar1* mutant infection and triggered a much lower NF- κ B response compared to the wild-type strain results. In contrast, macrophages stimulated with the *A. phago-*

cytophilum lpda1::TnHimar1 mutant had higher levels of NF- κ B signaling and were fully capable of binding and internalizing the *lpda1::TnHimar1* mutant. These results suggest that LPDA1 may play a functional role in phagocytosis of *A. phagocytophilum*. Independent groups have determined that tetrasaccharide sialyl Lewis (sLe^x) present on the protein P-selectin glycoprotein ligand-1 (PSGL-1) by *A. phagocytophilum* is required for human neutrophil infection (22, 25). On the other hand, *A. phagocytophilum* uses α -1,3-fucosylation but not PSGL-1 for infection of murine neutrophils (8) and it is known that some *A. phagocytophilum* strains may use PSGL-1-dependent and -independent routes of cell invasion (46, 49). Future experiments comparing and contrasting the NF- κ B pathway in neutrophils and macrophages during *A. phagocytophilum* infection should uncover signaling differences between these cell types. The synergistic effect of neutrophil-macro-

phage interaction and the contribution of *A. phagocytophilum* LPDA1 in the immunological synapse should be emphasized.

In summary, the results provided here demonstrate a role of *A. phagocytophilum* LPDA1 in infection-induced immunopathology. Further investigation examining how LPDA1 contributes to *A. phagocytophilum* pathogenesis and immunobiology will be critical for understanding HGA etiology.

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