Stat1 Negatively Regulates Immune-Mediated Injury with *Anaplasma phagocytophilum* Infection

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Stat1 Negatively Regulates Immune-Mediated Injury with Anaplasma phagocytophilum Infection

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Human granulocytic anaplasmosis (HGA) is caused by the obligate intracellular bacterium Anaplasma phagocytophilum. Our data previously demonstrated that A. phagocytophilum induces an immunopathologic response by activating IFN-α production through the Stat1 signaling pathway. In this study, we investigated the broader role of Stat1 signaling in the host response to infection with A. phagocytophilum. In Stat1 knockout (KO) compared with wild-type mice, A. phagocytophilum infection was more highly pathogenic as characterized by the unanticipated development of clinical signs in mice including markedly increased splenomegaly, more severe inflammatory splenic and hepatic histopathology, >100-fold higher blood and splenic bacterial loads, and more elevated proinflammatory cytokine/chemokine responses in serum. CD4+ and CD8+ T lymphocyte populations were significantly expanded in spleens of A. phagocytophilum–infected Stat1 KO mice compared with wild-type mice. The leukocyte infiltrates in the livers and spleens of A. phagocytophilum–infected Stat1 KO mice also contained expansions in neutrophil and monocyte/macrophage populations. Importantly, A. phagocytophilum–infected Stat1 KO mice did not demonstrate induction of inducible NO synthase in splenocytes. These results show that Stat1 plays an important role in controlling bacterial loads but also by unexpectedly providing an undefined mechanism for dampening of the immunopathologic response observed with A. phagocytophilum infection. The Journal of Immunology, 2014, 193: 000–000.

Anaplasma phagocytophilum is the obligate intracellular bacterium that causes human granulocytic anaplasmosis (HGA) (1). Human infection ranges from a mild to severe febrile disease with inflammatory complications such as septic and toxic shock–like syndromes and acute respiratory distress syndrome (2–4). The majority of human infections caused by A. phagocytophilum do not result in severe disease, but when severe, the underlying pathogenesis relates to induction of a macrophage activation syndrome (5). Although clinical signs with A. phagocytophilum infection in murine models are not observed, the inflammatory disease process can be tracked by histopathologic studies (6). In the murine model, inflammatory lesion severity is also closely linked to macrophage activation by IFN-γ, leading to increased production of NO, proteases, cytokines, and chemokines, among other inflammatory mediators of tissue injury (7–10). Paradoxically, A. phagocytophilum–induced increases in plasma IFN-γ in mice that lead to severe inflammatory histopathology do so in the absence of significant bacterial loads (7).

In fact, A. phagocytophilum infection in IFN-γ− or IFN-γ− receptor knockout (KO) mice significantly reduces histopathologic inflammation, unrelated to pathogen burden (7, 11, 12).

STAT1 mediates most of the biological functions of both type I IFN (IFN-α/β) and type II IFN (IFN-γ) and is important in host innate and adaptive immune responses to viruses and other intracellular pathogens, especially including intracellular bacteria (13, 14). Although IFN-α/β depends on the presence of the canonical signaling molecules STAT1, STAT2, and IFN regulatory factor 9, IFN-γ signaling is mostly dependent on STAT1 (13, 15). While IFN-α/β–dependent mechanisms interfere with virus replication, in contrast, IFN-γ mediates resistance against intracellular bacteria and protozoa, primarily through its ability to activate macrophages. The loss of STAT1 function in humans or mice results in a dramatically increased susceptibility to viral, bacterial, and protozoan infections (13, 14, 16). A. phagocytophilum infection–induced IFN-γ signaling leads to phosphorylation of Stat1 in mice (17), and dexamethasone suppression of STAT1 expression reduces inflammatory signaling and disease severity in A. phagocytophilum–infected horses (18). However, the direct effects and impact of STAT1-mediated signaling on host immunity during A. phagocytophilum infection have not been otherwise studied. Therefore, the aim of this study was to determine what if any role STAT1 and related signaling play in inflammatory lesions and disease with A. phagocytophilum infection.

Materials and Methods

Mice and A. phagocytophilum infection

Six-week-old 129Sv wild-type (WT) mice and Stat1 KO mice (129Sv/SvEv-Stat1<sup>−/−</sup>) were purchased from Taconic Farms (Germantown, NY). All animals were maintained under specific pathogen-free conditions and used in strict accordance with the guidelines and protocols approved by The Johns Hopkins University School of Medicine Animal Care and Use Committee. Experiments were repeated three times; experiment 1 used 20 WT and 25 Stat1 KO mice; experiment 2 used 30 WT and 30 Stat1 KO mice; and experiment 3 used 19 WT and 19 Stat1 KO mice.

Low passage (p4) A. phagocytophilum Webster strain was used. On the day of inoculation, 10<sup>7</sup> heavily infected (>90%) HL-60 cells were
centrifuged (2000 × g, 10 min) to concentrate the cells. Cell-free *A. phagocytophilum* was isolated by syringe lysis, cellular debris removed by centrifugation at 3000 × g for 10 min, and the host cell-free *A. phagocytophilum* were then harvested from the supernatant by centrifugation at 12,000 × g for 30 min. Bacteria were resuspended in PBS for experimental infections and injected i.p. into mice. PBS alone was used for mock infections. Four to five mice of each strain were assigned to *A. phagocytophilum*–infected and mock-infected cohorts for evaluation at time intervals as follows: 4 h, 2, 4, 7, 10, and, for some experiments, 14 d postinfection (p.i.). All experiments were repeated at least twice to confirm reproducibility.

**Histopathology and immunohistochemistry**

At each interval p.i., four to five *A. phagocytophilum*–infected WT (129Sv) mice and four to five *A. phagocytophilum*–infected Stat1 KO mice were euthanized by CO₂ asphyxiation and necropsied for harvest of plasma, liver, and spleen. Plasma was collected by centrifugation of EDTA-anticoagulated blood obtained after cardiac puncture; after centrifugation, the top 50% plasma fraction was used for analysis of cytokine concentrations, and the bottom 50% cellular fraction was used for analysis of *A. phagocytophilum* bacteremia by quantitative PCR (qPCR). Liver and spleen removed at necropsy were fixed in 10% buffered formalin. The weights of the harvested spleens were measured on days 4, 7, 10, and 14. The tissues were processed and embedded in paraffin, prepared as 5-μm sections; one section for each tissue was used for H&E staining. Histopathologic changes in WT and Stat1 KO mice were investigated for severity as described previously (10, 19).

For splenic immunohistochemistry, 5-μm-thick paraffin-embedded tissue sections were deparaffinized and hydrated through a graded alcohol series before Ag retrieval in heat-induced sodium citrate buffer (pH 6) for 30 min. The primary Abs used were against CD3 (all T lymphocytes; Abcam, Cambridge, U.K.), CD4 (Abcam), CD8 (BD Pharmingen, San Jose, CA), CD45R/B220 (B lymphocytes; BD Pharmingen), and Gr-1/Ly6G and Ly6C (neutrophils and granulocytes; BD Pharmingen) according to the manufacturer’s instructions. After primary Ab reactions, tissue sections were stained with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) or biotinylated anti-rat IgG (Vector Laboratories) for 1 h at room temperature, washed, and incubated with VECTASTAIN ABC Reagent (Vector Laboratories) for 30 min. After washing, tissue sections were reacted with peroxidase substrate solution (Vector Laboratories), rinsed, counterstained, mounted, examined by light microscopy, and photographed.

**Table I.** Comparison of spleen weights between *A. phagocytophilum*–infected Stat1 KO and WT mice (mean weight in mg ± SD)

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>WT</th>
<th>Stat1 KO</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>75 ± 6</td>
<td>78 ± 5</td>
</tr>
<tr>
<td>4</td>
<td>113 ± 16</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>7</td>
<td>175 ± 14</td>
<td>283 ± 45</td>
</tr>
<tr>
<td>10</td>
<td>240 ± 71</td>
<td>573 ± 73</td>
</tr>
<tr>
<td>14</td>
<td>255 ± 17</td>
<td>698 ± 117</td>
</tr>
</tbody>
</table>

Data are representative of three independent experiments.

*p value*: 0.537; 0.030; 0.881; 0.003; 0.015.
FIGURE 2. Gross morphology of spleens from *A. phagocytophilum*–infected WT and *Stat1* KO mice at day 10 p.i. Note the marked enlargement of the spleen and the pale color of the spleen in *Stat1* KO mice that results from the massive infiltration by leukocytes.

qPCR for *A. phagocytophilum*

* *A. phagocytophilum* blood loads were determined at 4 h, 2, 4, 7, 10, and 14 d p.i. All bacterial blood concentrations were calculated based on blood volume without normalization to leukocyte numbers. In brief, after centrifugation, blood samples were divided into cellular and plasma fractions, each comprising 50% of the volume of the sample. DNA in 100 μl cellular fraction was extracted after adding 100 μl PBS in accordance with the manufacturer’s instructions (Qiagen DNA blood kit; Qiagen, Valencia, CA), reconstituting the sample to the original whole blood volume. The final elution was prepared into 200 μl buffer so that the proportion of blood DNA volume to extracted blood volume was maintained. Real-time qPCR was performed as previously described targeting multicopy *msp2*. Bacteria were quantified comparing triplicate threshold cycle values to a standard curve using cloned single-copy *msp2* and normalizing to a single genome equivalent by dividing the calculated copy numbers by 100 to account for the 100 copies of *msp2* per *A. phagocytophilum* genome (19). The analytical sensitivity of this assay is regularly as low as 1 infected cell/ml blood, approximately equivalent to 10 bacteria/ml blood.

Bacterial loads in the spleen were determined in WT and *Stat1* KO mice following *A. phagocytophilum* infection as described (17). Briefly, spleens were soaked in RNA later (Qiagen) and stored at 4°C. RNA was extracted from 30 mg spleen tissue using recommendations per the RNAeasy mini kit (Qiagen). RNA was reverse transcribed to cDNA and then tested as for blood DNA. For quantitative purposes, each sample was normalized to *ActB* transcription to control for RNA variation and adjusted to compensate for an average number of 350 *msp2* transcripts per bacterium to establish bacterial load per microgram of splenic tissue (20).

Serologic testing by indirect fluorescent Ab method

The indirect immunofluorescence assay for *A. phagocytophilum* Abs was performed to assess humoral immune response to infection in WT and *Stat1* KO animals. Briefly, plasma samples were screened at a 1:80 dilution, using *A. phagocytophilum*–infected HL-60 cells fixed to wells of 12-well Ag slides, and incubated for 1 h. Diluted anti-mouse IgG (γ-chain specific) FITC-conjugated secondary Ab (Invitrogen) was added to each well, and the slides were incubated for 1 h. The slides were washed in PBS, soaked for 5 min in 0.005% Evans blue, and washed again in PBS to remove residual stain. Slides were then mounted and examined with a fluorescent microscope. Mouse plasma samples that reacted at the screening dilution were titrated to end point.

Flow cytometry for immunophenotyping and quantification of cell subsets

Spleens from each *A. phagocytophilum*–infected mouse were removed at necropsy, and splenocytes were isolated and purified as previously described (21). Briefly, the spleens were minced, erythrocytes lysed in RBC lysis buffer (Sigma-Aldrich), cell debris removed, and cells were resuspended in PBS. Splenocytes were incubated with allophycocyanin-labeled anti-mouse CD3ε (BD), FITC-labeled anti-mouse CD4 (BD Biosciences), PerCP-labeled anti-mouse CD8 (BD Biosciences), and PE-labeled anti-mouse CD49b (DX5α; BD Biosciences) in one tube and allophycocyanin-labeled anti-mouse Gr-1/Ly6G and Ly6C (BD) in the other tube, respectively, at 4°C for 15 min. Stained cells were washed twice with PBS containing 0.5% BSA (Sigma-Aldrich), fixed with 1% paraformaldehyde (Sigma-Aldrich), and examined by flow cytometry using an FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR), and gates were set based on isotype-matched control Abs. Data were examined as proportion of all gated cells (not shown) and as absolute numbers per milligram of splenic tissue. Because 129Sv mice do not express NK1.1, a well-recognized marker for NK and NKT cells, we used CD49b (DX5α) that is expressed on the surfaces of these cells in 129Sv mice. Thus,
NK cells were defined as CD3ε^2CD49b^+ whereas NKT cells were defined as CD3ε^+CD49b^+.

Plasma cytokine analysis

Cytokines and chemokines in the plasma at each time point from each *A. phagocytophilum*–infected WT or *Stat1* KO mouse were detected separately using a mouse CBA in flammation kit (BD Biosciences) according to the manufacturer’s instructions. Acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences). A total of 300 bead events for each cytokine was collected. Analysis of cytometric bead array data were performed using the FCAP Array 3.0 software (Soft Flow, St. Louis, MO). Keratinocyte chemoattractant (KC; CXCL1), monocyte chemotactic protein (MCP)-3 (CCL7), and MCP-5 (CCL12) quantifications were determined using an ELISA kit (Instant ELISA; eBioscience, San Diego, CA).

Western blotting for inducible NO synthase, total Stat1, and p-Stat1

Spleens harvested on days 7 and 10 from *A. phagocytophilum*–infected WT or *Stat1* KO mice were minced and then dispersed into a single-cell suspension. Splenocytes from infected and mock-infected mice were separately pooled, and all pools were stimulated with and without LPS at 0, 1, and 5 ng/ml for 1 h, separately suspended in lysis buffer (Pierce, Rockford, IL), and assayed for protein content (bicinchoninic acid protein assay; Pierce). Cell lysates were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes (iBlot Gel transfer Device; Invitrogen). After blocking, the membranes were incubated with rabbit Abs to p-Stat1, total Stat1, inducible NO synthase (iNOS; Cell Signaling Technology, Danvers, MA), and actin (Millipore, Billerica, MA). The nitrocellulose blots were then washed, incubated with HRP-conjugated goat anti-rabbit Ab (HRP; Cell Signaling Technology) and HRP Substrate (Millipore) in SNAP i.d.

FIGURE 4. Comparison of H&E splenic histopathology (A and B) and immunohistochemical detection of CD3^+ T lymphocytes (C and D), CD45R/B220 B lymphocytes (E and F), F4/80 macrophages (G and H), and Gr-1/Ly6G and Ly6C neutrophils/granulocytes (I and J) between *A. phagocytophilum*–infected Stat1 KO (left panels) and WT (right panels) mice at day 10 p.i. Whereas white pulp with germinal centers and expanded paracortical and medullary regions were observed among infected WT mice (A), in Stat1 KO mice, the white pulp was almost completely effaced and replaced by a marked expansion of the paracortical and medullary regions with mononuclear cells and numerous neutrophils (B). In *A. phagocytophilum*–infected KO mice, there was a marked expansion of the paracortex and medulla by T lymphocytes (C), macrophages (G), and neutrophils/granulocytes (I); B lymphocytes were clustered in a few residual follicles (E). In contrast, among infected WT mice, the distribution of T lymphocytes [predominantly in paracortex and germinal centers (D)], B lymphocytes [almost exclusively in follicles, (F)], and macrophages [in paracortex and medulla, (H)] was as anticipated. Neutrophils and granulocytes were nearly absent from all areas of the spleen of infected WT mice (B and J). Original magnification ×100 (A–J). Original magnification ×400 (K and L). Data shown are representative from individual animals out of three repeated experiments with between 36 and 60 animals in each repeat.
Protein Detection System (Millipore), and then visualized using a Kodak Gel Imaging System (Raytest USA, Wilmington, NC). In each case, the blots were stripped with Restore Western blot Stripping Buffer (Pierce) and reprobed. Actin was used to allow normalization of protein loading.

Statistical analyses

Data were evaluated for normality before statistical analysis. Quantitative data that was normally distributed was analyzed by two-tailed, two-sample unequal variance Student t tests, in which variation was presented as SD or SEM for small sample sizes. For nonnormally distributed data, statistical analysis was conducted by two-sided nonparametric Mann-Whitney U tests, with values displayed in box and whisker plots as median, first, and third quartiles (box) and minimum and maximum (whiskers). The p values <0.05 were considered significant.

Results

The effect of Stat1 KO on A. phagocytophilum bacterial load

To assess how Stat1 KO mice respond to A. phagocytophilum infection, bacterial loads in the blood and spleen were compared between infected and mock-infected animals by qPCR. Following A. phagocytophilum infection in WT mice, approximately similar quantities of bacteria were present per microliter of blood and per milligram of spleen from days 4–10 (Fig. 1). In contrast, in Stat1 KO mice compared with WT mice, bacteria loads were significantly increased >400- and >750-fold in the blood at days 7 and 10, respectively (Fig. 1, top panel). Similarly, bacterial loads in spleens of Stat1 KO mice at days 7 and 10 p.i. were >700- and 48-fold higher than in WT mice, respectively (Fig. 1, bottom panel).

A. phagocytophilum infection causes severe clinical signs in Stat1 KO mice

As anticipated, no clinical signs were detected in WT mice. Surprisingly, Stat1 KO mice infected with A. phagocytophilum developed severe clinical signs between days 7 and 14 p.i., including reduced activity, piloerection, crouching, dehydration, and weight loss. Weight loss in Stat1 KO mice started after day 7 p.i. and peaked by day 14. Infected Stat1 KO mice recovered slowly after day 21 p.i., and none died except when humanely euthanized at the completion of experiments. Splenic weights of Stat1 KO mice infected with A. phagocytophilum increased 7.4- to 8.0-fold between days 4 and 14 versus 1.8- to 2.3-fold compared with infected WT mice (Table I). Splenomegaly was especially evident between days 7 and 14, most enlarged after day 10 (Fig. 2), and persisted to at least day 22, when all animals were euthanized.

A. phagocytophilum–infected Stat1 KO mice develop severe inflammatory histopathology

To determine the severity of tissue injury in A. phagocytophilum–infected Stat1 KO mice, histopathologic examination was performed on liver and spleen from all mice. As previously demonstrated, the livers from A. phagocytophilum–infected WT mice showed small localized accumulations of lymphocytes, macrophages, and occasionally neutrophils, with or without apoptotic cells in hepatic lobules and perivenular portal regions. In A. phagocytophilum–infected WT mice, changes in hepatic histopathology were first evident on day 2, peaked at days 4–7, and gradually decreased by day 10, returning to baseline by day 14 (Fig. 3). In contrast, the livers of A. phagocytophilum–infected Stat1 KO mice revealed more severe inflammatory pathology and tissue injury, including intense lymphohistiocytic inflammatory cell infiltrates throughout hepatic lobules, in portal regions and central zones, with some infiltrates bridging these zones. In addition, greater degrees of focal necrosis and increased numbers of apoptotic cells were observed as compared with WT mice. The dramatic hepatic histopathology demonstrated significant perivascular and perportal infiltrates, including many neutrophils found first at day 7 and most pronounced at day 10 (Fig. 3), and this was sustained through day 14 when the experiments were terminated. The splenomegaly observed in infected Stat1 KO mice was primarily the result of marked expansion of the red pulp by inflammatory cell infiltrates. The spleens from A. phagocytophilum–infected Stat1 KO mice revealed almost completely effaced white pulp (splenic lymphoid follicles) replaced by mononuclear and neutrophil inflammatory cell infiltrates compared with A. phagocytophilum–infected WT mice after 10 d (Fig. 4). The more severe splenic pathology observed in Stat1 KO mice positively correlates with the markedly higher bacterial tissue loads.

To examine the infiltrating cell populations responsible for these significant histopathologic changes, immunohistochemical phenotyping was performed. In the spleens of A. phagocytophilum–infected Stat1 KO mice at day 10 p.i., the accumulation of B lymphocytes into follicles was significantly decreased compared with infected WT mice, and although most B cells were largely still confined to small germinal centers, more were distributed into the splenic sinuses in Stat1 KO versus WT mice (Fig. 4). In contrast, the numbers of T lymphocytes, macrophages, and neutrophils (Fig. 4), and including CD4+ and CD8+ T lymphocytes (Fig. 5), were all markedly increased in the spleens of A. phago-

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**FIGURE 5.** Comparison of splenic histopathology (top panel) and immunohistochemical detection of CD4 and CD8 T lymphocytes between A. phagocytophilum–infected Stat1 KO (left panels) and WT (right panels) mice at day 10 p.i. Splenic histopathology is as described in Fig. 4. As anticipated among infected WT mice, CD4 and CD8 T lymphocytes were largely restricted to follicular germinal centers and paracortex (CD4) and paracortex and medulla (CD8). In contrast, infected Stat1 KO mice demonstrated a marked increase in numbers and a highly homogeneous distribution throughout the splenic parenchyma that was largely devoid of cortex and follicles. Data shown are representative from individual animals out of three repeated experiments with between 36 and 60 animals in each repeat. Original magnification ×100.
FIGURE 6. Selected splenic cell populations in *A. phagocytophilum*–infected Stat1 KO mice analyzed by flow cytometry for immunophenotyping. (A) Compared with infected WT mice, significantly more CD4+ and CD8+ T lymphocytes, B lymphocytes, neutrophils, NKT (CD3ε+CD49b+) lymphocytes, and NK (CD3ε−CD49b+) lymphocytes infiltrated the spleen in *A. phagocytophilum*–infected Stat1 KO mice at days 10 and/or 14 p.i. Data are shown as means and SEM. (B) Cumulative area graphs that show kinetic and quantitative comparisons of immune and inflammatory cell components in spleens of WT and Stat1 KO mice infected by *A. phagocytophilum*. Note the marked difference in total cellular infiltrates and particularly marked increase in Gr1+ neutrophils and granulocytes at days 10 and 14 p.i. with infection of Stat1 KO mice. Note that no data were available for Gr1+ neutrophils/granulocytes on days 0 and 4. Data shown are representative from individual animals out of three repeated experiments with between 36 and 60 animals in each repeat. The *p* values are shown for each comparison and were calculated using two-sample unequal variance, two-sided Student *t* tests. The *p* values < 0.05 were considered significant.
cytophilum–infected Stat1 KO mice at day 10 compared with WT mice. These findings demonstrate that loss of Stat1 leads to a dysregulated immune response to A. phagocytophilum as compared with WT mice. Moreover, this response is associated with ineffective control of bacterial infection, with extensive tissue inflammation and severe clinical disease not previously observed in murine models of HGA.

Quantitative changes in infiltrating immune and inflammatory cells among splenocytes

To determine changes among infiltrating cell populations associated with the loss of white pulp in the spleen of Stat1 KO mice infected with A. phagocytophilum, flow cytometry was performed comparing splenocytes from A. phagocytophilum–infected WT and Stat1 KO mice. Flow cytometric analyses showed that CD3, CD4, and CD8 T lymphocyte populations and neutrophils (Gr-1) were increased in A. phagocytophilum–infected Stat1 KO mice (Fig. 6A). From days 0 to 7, there were minor differences in T cell population numbers or proportions between A. phagocytophilum–infected Stat1 KO and WT mice. The number of CD3+CD4+ T lymphocytes was significantly increased in A. phagocytophilum–infected Stat1 KO mice compared with WT mice at day 10 and also for CD3+CD8+ T lymphocytes at day 14 (Fig. 6). In contrast to the immunohistologic studies, the numbers of CD19+ B lymphocytes increased only in infected Stat1 KO mice at day 10, but unlike the CD3+CD4+ (p = 0.009), CD3+CD8+ (p = 0.014), Gr1+ (p < 0.001), and CD3+CD49b+ (p = 0.005) cells, the overall proportion of B cells among all immunophenotyped splenocytes did not significantly differ between WT and Stat1 KO mice (p = 0.237). Compared to WT mice, the absolute numbers (Fig. 6A) and proportions (not shown) of Gr1+ neutrophils in A. phagocytophilum–infected Stat1 KO mice was increased 3.3- (p = 0.007) and 9.8-fold (p = 0.005), respectively, at day 10, and 2.7- (p = 0.003) and 8.0-fold (p = 0.005), respectively, at day 14. As expected, the numbers of CD3−CD49b−NKT and CD3−CD49b+ NK cells were significantly increased at almost each interval p.i. in both WT and Stat1 KO mice (p < 0.046, except for WT day 10; data not shown). However, infection in Stat1 KO mice yielded an expansion of CD3−CD49b+ NKT cells that was markedly and significantly greater than in WT mice at days 10 p.i. (3.5-fold; p = 0.008) and 14 (5.7-fold; p = 0.007) (Fig. 6A). In contrast, the overall numbers of CD3+CD49b+ NK cells/mg spleen did not change with infection in Stat1 KO mice versus WT at any time point p.i., except for day 4, and this change was likely not biologically relevant. Overall, cellular infiltrates occurred with the same kinetics but vastly different quantities in the WT and Stat1 KO mice (Fig. 6B). The total number of immunophenotyped immune and inflammatory cells in the spleens of infected Stat1 KO mice peaked on day 14 at 21,595 cells/mg tissue or 1.6 × 10^7 cells/spleen, 10-fold more than infected WT animals (Fig. 6B), although the greatest difference was 13-fold on day 10 with 6.7 × 10^7 total cells/spleen in infected Stat1 KO mice.

**Indirect immunofluorescence assay for A. phagocytophilum IgG Abs**

A. phagocytophilum IgG Ab titers are shown in Table II. Plasma obtained from each A. phagocytophilum–infected WT and Stat1 KO mouse was reactive with titers in excess of 640 at day 14 p.i. When geometric mean titers were compared between infected cohorts on days 10 and 14 p.i., those in plasma from Stat1 KO mice were always higher (p < 0.001) than in plasmas from A. phagocytophilum–infected WT mice (Table II).

| Table II. IFA Ab Titers between WT and Stat1 KO Mice after A. phagocytophilum Infection at Days 10 and 14 p.i. |
|-------------|-------------|-------------|-------------|
| Host Mouse | Day 10 | Day 14 |
| WT | 640 | 640 |
| Stat1 KO | 5,120 | 10,240 |
| Geometric mean Stat1 KO | 761 | 761 |
| Geometric mean Stat1 KO | 4,305 | 8,611 * |

Results are representative of two experiments.

* p < 0.001, Student t test.

IFA, indirect immunofluorescence assay.

A. phagocytophilum–infected Stat1 KO mice develop more robust inflammatory plasma cytokine responses

To characterize whether Stat1 KO mice generate significantly higher proinflammatory cytokine concentrations than WT mice after A. phagocytophilum infection, we examined the levels of several plasma cytokines (Fig. 7A) and chemokines (Fig. 7A) for which genes are known to be transcriptionally regulated by NF-κB and/or Stat1. Among A. phagocytophilum–infected Stat1 KO mice, levels of cytokines driven by binding of NF-κB to κB sequences in their gene promoters, including IFN-γ, IL-12p70, IL-6, TNF-α, CCL2 MCP-1, CCL7 MCP-3, and CCL5 RANTES, increased to significantly higher levels compared with WT mice at days 7 and 10 p.i.; CXCL1 (KC) increased only at day 7, and IL-10, IL-17, CCL12 MCP-5, and CCL3 macrophage inflammatory protein-1α were either not different or of questionable biological difference between the two groups at any time p.i. Of the two cytokines/chemokines analyzed for which gene promoters also have Stat1 IFN-γ–activated sites, changes in CCL2 levels were significantly higher and potentially biologically relevant, whereas IL-17 levels were not. Overall, there was a marked increase in proinflammatory cytokines and chemokines with infection in Stat1 KO mice compared with WT (Fig. 7B) that was particularly accentuated at days 10 and 14 and included the unique increase in TNF-α and a dramatic increase in KC (CXCL1).

**iNOS and Stat1 expression by Western blot**

We previously showed that phosphorylated Stat1 is not expressed or weakly expressed in splenocytes of uninfected mice, but peaks in splenocytes from infected mice at day 7 post–A. phagocytophilum infection (17). To further determine whether Stat1 and phosphorylated Stat1 are required for the induction of iNOS, we examined iNOS protein expression when pStat1 expression peaked after infection at days 10 and 14 p.i. (CXCL1).

To establish a link between iNOS expression and Stat1 expression was not detected in Stat1 KO splenocytes. However, splenocytes from A. phagocytophilum–infected WT mice showed Stat1 and p-Stat1 expression at day 7, independent of LPS stimulation (Fig. 8B).
FIGURE 7. Analysis of serum cytokines and chemokines in Stat1 KO (black bars) and WT (gray bars) mice p.i. with *A. phagocytophilum*. (A) Most cytokines including IFN-γ, IL-6, IL-12, and TNF-α, were significantly increased in the serum of infected Stat1 KO. WT mice exhibited smaller changes at day 10 p.i. In contrast, although IL-10 concentrations were higher in Stat1 KO mice, the increases were much lower than compared with the counterbalancing changes in IFN-γ and IL-12. TNF-α levels did not change in WT mice over the course of the infection, but increased markedly by day 10 in Stat1 KO animals. IL-17 concentrations were elevated in Stat1 KO mice compared with WT, but the levels were low in both and of questionable biological relevance. Serum levels of CXCL1, CCL2, CCL3, and CCL5 were significantly elevated at either day 7 or 10 in *A. phagocytophilum*-infected Stat1 KO mice compared with WT mice. The levels of CCL3 and CCL12 were generally low in both WT and Stat1 KO mice and of questionable biological relevance. Although the levels of CCL7 were elevated at days 7 and 10, the differences between WT and Stat1 KO mice was not significant. (B) Kinetic and quantitative comparison of the plasma cytokines and chemokines in WT and Stat1 KO mice infected by *A. phagocytophilum*. (Figure legend continues)
suppressed by dampening or eliminating Stat1 signaling (17, 18). A critical question is whether features of disease severity could be to its role in counterbalancing IFN-γ effects (7). This would also necessarily impact signaling by type I and type III IFNs (IFN-α, IFN-β, and IFN-λ) that generate IFN-stimulated gene factor 3 complex (or Stat1/Stat2/IFN regulatory factor 9) to bind IFN-stimulated response elements in genes and activate inflammatory antiviral responses as well (13, 15).

Paradoxically, the studies in this paper demonstrate that the absence of Stat1, the major proinflammatory signaling pathway for IFN-γ, converts the subclinical infection phenotype universally observed with A. phagocytophilum mouse models to one with severe clinical signs (reduced activity, piloerection, crouching, dehydration, and weight loss), dense infiltration of inflammatory cells, and robust proinflammatory cytokine/chemokine responses compared with WT controls. Although this study also demonstrates that Stat1 is not essential to eventual resolution of the infection, it implies that STAT1 plays an important role in innate immune protection and provides evidence of an unexplained and potentially novel STAT1-dependent homeostatic mechanism that dampens host inflammatory and immune organ/tissue damage with A. phagocytophilum infection.

It is perhaps not surprising that significantly more inflammation occurs owing to the increased bacterial loads observed in the A. phagocytophilum–infected Stat1 KO mice, as this phenotype has been observed with both bacterial, protozoan, and viral infections (14, 16, 23, 24). This observation also underscores the necessity of macrophage activation mediated by IFN-γ signaling via Stat1 for control of pathogen loads, as previously observed with Toxoplasma gondii (23). Inflammatory and innate immune signaling with A. phagocytophilum infection is thought to be initiated in one of several ways. The first recognized pathway is via TLR2, but not TLR4, that drives NF-kB and proinflammatory gene transcription (9, 10). In fact, the data in this study demonstrate that most of the chemokines and cytokines upregulated in the Stat1 KO mice p.i. are driven by NF-kB that binds to the kB sites in promoter sequences at gene loci that could easily be explained by TLR activation. Similarly, loss of the MyD88 adapter for TLR2 such as with infection in KO mice also diminishes inflammatory phenotype without reducing bacterial loads (8).

Alternatively, inflammasome activation could occur via Rip1, IPAF/NLR4C, ASC/caspase-1 and possibly nucleotide-binding oligomerization domain–like receptors that in part regulate the IL-1β and the IFN-γ/IL-18 axis, although neither IL-1β nor IL-18 are regularly detected among infected horses or humans (5, 18, 25–27). A role for this pathway is also suggested by the reduction in histopathologic tissue damage in ASC KO mice infected by A. phagocytophilum (J.H. Pedra, D.G. Scorpio, and J.S. Dumler, unpublished observations). Finally, it is well established that p38 MAPK plays a role in A. phagocytophilum pathogenesis and could also be a determinant of proinflammatory response via AP-1 as well (28–31). Regardless, it is relatively easy to understand how inflammatory signaling and tissue injury can occur with A. phagocytophilum infection in mice, horses, or humans.

The biological consequences of proinflammatory signaling via NF-kB, AP-1, and IFN-γ/Stat1 have many redundancies, but the latter allows predominantly for a process by which macrophages become activated for effector responses such as increased microbial killing (16, 22, 23, 32). Similarly, type I IFNs can also promote inflammatory response and antiviral effects (13, 15).

Note the marked differences between WT and Stat1 KO mice at days 7 and 10 in particular: 1) the lack of TNF-α in WT compared with its marked increase at days 7 and 10 p.i. with infection of Stat1 KO mice; 2) the marked difference in KC (CXCL1) at days 7 and 10 between WT and Stat1 KO mice; and 3) the relative levels of IFN-γ and IL-12 versus IL-10 in WT versus Stat1 KO mice. Data shown are representative from individual animals out of three repeated experiments with between 36 and 60 animals in each repeat. The p values are shown for each comparison and were calculated using two-sample unequal variance, two-sided Student t tests. The p values < 0.05 were considered significant.
However, the loss of Stat1 signaling would lead to reduced signal capacity owing to the fact that both type I (IFN-α and IFN-β) and type II (IFN-γ) IFNs use Stat1, either as hetero- or homodimers, for activation of downstream transcription. The increased severity of inflammatory disease in A. phagocytophilum–infected Stat1 KO mice compared with WT mice is driven by a more pronounced Th1 response reflected in the marked increase in serum IFN-γ, IL-6, IL-12, TNF-α, MCP-1, and RANTES. This occurred in the absence of iNOS generation and presumably other macrophage activation-related effector mechanisms, was associated with the greater degree of tissue inflammatory cell infiltrates, and was associated with a significant defect in control of bacteria load. An important target of both type I and type II IFNs is upregulation of iNOS that generates NO, an important antimicrobial effector with macrophage activation (23, 33, 34). Maximal expression of iNOS from Nos2 requires cumulative signals that interact with κB, IFN-γ–activated sites, and IFN-stimulated response element sites on its promoter. In this study, we confirm that Stat1 KO mice were unable to produce detectable iNOS, probably as a result of the loss of two of the three major transcriptional activators. In turn, its absence confirms the loss of macrophage activation despite a more severe inflammatory process that, despite greater tissue severity compared with WT animals, lacked the degrees of necrosis and pyroptosis observed with infection in the absence of IL-10 and extreme macrophage activation (7).

Two observations focus attention toward the importance of Stat1 signaling with A. phagocytophilum infection. First, the lack of Stat1, despite log orders higher concentrations of IFN-γ, results in worsened bacterial loads and increased proinflammatory response, observed previously in other intracellular pathogen infection models (16, 23, 32, 35). This is remarkable because it delineates the independence of Stat1 for inflammatory response and the dependence on Stat1 for expression of antimicrobial control effector mechanisms. The second observation derives from the concept that infection in IFN-γ KO mice results in high bacterial loads as anticipated, but loss of proinflammatory response, in the presence of intact Stat1 signaling. The paradox in these two observations is that the loss of the predominant signaling pathway for IFN-γ, Stat1, converts the infection into a unique highly inflammatory phenotype associated with significant clinical signs where none had been detected before. This likely indicates that Stat1 signaling also promotes a homeostatic counterweight to inflammation, the nature of which is not clear. Although various autoimmune processes can mimic the inflammatory phenotype observed in this study (36, 37), to the best of our knowledge, the phenotype of reduced inflammation with IFN-γ deficiency that is reversed and worsened by loss of its chief transcriptional activator has not been previously reported.

Several mechanisms exist that could be involved in this homeostatic balance. First, the only well-demonstrated anti-inflammatory mechanism for A. phagocytophilum is the production of IL-10, as infection in IL10 KO mice results in a marked increase in inflammation, tissue necrosis, and presumably pyroptosis (7), and severity of HGA in humans and horses is related to the balance between IL-10 and IFN-γ serum concentrations (5, 18).

The well-recognized control that IL-10 exerts against IFN-γ–mediated gene expression and macrophage activation is a likely explanation for this observation (22). In this study, the plasma levels of IL-10 were not significantly higher in the Stat1 KO mice than in WT; yet, the ratio between IFN-γ and IL-10 was significantly skewed, potentially leading to loss of this feedback-inhibitory process. As IL-10 signaling is not dependent on Stat1 to a great degree, this scenario seems likely (38). In fact, a critical difference between the histopathologic inflammatory phenotype observed with IL10 KO versus Stat1 KO animals infected with A. phagocytophilum is the relative lack of necrosis and pyroptosis observed in the latter model, presumably owing to the lack of macrophage activation and generation of macrophage antimicrobial effector mechanisms. Other potential regulatory processes lost with Stat1 KO could be suggested with loss of suppressor of cytokine signaling expression or reduced production of TGF-β (39, 40). Although neither was investigated in this study, suppressor of cytokine signaling expression has been implicated in intracellular survival of A. phagocytophilum in neutrophils where expression of IFN-γ receptor α-chain CD119 is diminished, leading to reduced Stat1 dimerization and signaling (41). In contrast, no study of the role that TGF-β plays with A. phagocytophilum infection has been conducted.

Finally, given the lack of pathogen control in the presence of high plasma IFN-γ levels raises questions about the precise mechanisms that control A. phagocytophilum. Although it is likely that a combination of innate immune and inflammation-related factors contribute to disease phenotype, they also likely work to control the pathogen. However, as previously demonstrated, specific Ab alone can exert control over A. phagocytophilum and other Anaplasmataceae infections (42, 43), and Stat-1 deficiency does not diminish Ab response. Although the significantly higher levels of Ab generated in the Stat1 KO mice could simply reflect the increased bacterial load, it is possible that Ab in this context could exert an increased effect on pathogen control. Additional experimental work, including a determination of whether Ab can directly neutralize A. phagocytophilum, will be needed.

Overall, our findings show that Stat1 KO mice develop more severe disease and Th1-skewed acute inflammation in response to A. phagocytophilum infection compared with WT mice. We demonstrate that Stat1 is fundamentally important for host defenses against A. phagocytophilum infection, leading to macrophage activation and, inevitably, control of infection. We have shown that absence of Stat1 results in an enhanced immune response triggered by A. phagocytophilum infection. These observations further suggest that severity of A. phagocytophilum infection could be in part linked to polymorphisms of STAT1 in humans as well (44). Further corroboration of these observations and extended studies of immune control mechanisms will expand our understanding of the complex immune processes that lead to disease with A. phagocytophilum infection.

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References


