TLR2 and Nod2 Mediate Resistance or Susceptibility to Fatal Intracellular Ehrlichia Infection in Murine Models of Ehrlichiosis

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Abstract

Our murine models of human monocytic ehrlichiosis (HME) have shown that severe and fatal ehrlichiosis is due to generation of pathogenic T cell responses causing immunopathology and multi-organ failure. However, the early events in the liver, the main site of infection, are not well understood. In this study, we examined the liver transcriptome during the course of lethal and nonlethal infections caused by *Ixodes ovatus Ehrlichia* and *Ehrlichia muris*, respectively. On day 3 post-infection (p.i.), although most host genes were down regulated in the two groups of infected mice compared to naïve counterparts, lethal infection induced significantly higher expression of caspase 1, caspase 4, nucleotide binding oligomerization domain-containing proteins (Nod1), tumor necrosis factor-alpha, interleukin 10, and CCL7 compared to nonlethal infection. On day 7 p.i., lethal infection induced highly significant upregulation of type-1 interferon, several inflammatory cytokines and chemokines, which was associated with increased expression levels of Toll-like receptor-2 (TLR2), Nod2, MyD88, nuclear factor-kappa B (NF-κB), Caspase 4, NLRP1, NLRP12, Pycard, and IL-1β, suggesting enhanced TLR signals and inflammasomes activation. We next evaluated the participation of TLR2 and Nod2 in the host response during lethal *Ehrlichia* infection. Although lack of TLR2 impaired bacterial elimination and increased tissue necrosis, Nod2 deficiency attenuated pathology and enhanced bacterial clearance, which correlated with increased interferon-γ and interleukin-10 levels and a decreased frequency of pathogenic CD8+ T cells in response to lethal infection. Thus, these data indicate that Nod2, but not TLR2, contributes to susceptibility to severe *Ehrlichia*-induced shock. Together, our studies provide, for the first time, insight into the diversity of host factors and novel molecular pathogenic mechanisms that may contribute to severe HME.

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Introduction

Human monocytic ehrlichiosis (HME) is an emerging tick-borne disease caused by *Ehrlichia chaffensis*, a Gram-negative obligate intracellular bacterium that lacks lipopolysaccharide (LPS) [1–4]. *E. chaffensis* causes pancytopenia and hepatic dysfunction, which progress to a potentially fatal multiorgan system disorder that mimics toxic shock syndrome despite antibiotic treatment [5–8]. Ehrlichiae infect several myeloid cells, such as macrophages, monocytes, and dendritic cells, and thus cause systemic infection [2,3,9–12]. In mice, innocuous or fatal ehrlichial diseases that mimic different spectra of HME occur following infection with *E. muris* and *Ixodes ovatus Ehrlichia* (IOE), respectively [13–15]. These two *Ehrlichia* species are not only genetically and antigenically related to *E. chaffensis* but also cause human infections [14,15]. Lethal *Ehrlichia* infection is characterized by extensive tissue damage in the absence of overwhelming infection, suggesting an immune-mediated pathology [16–20]. Protective immunity against *Ehrlichia* is mediated by interferon (IFN)-γ production by CD4+ Th1 helper (Th1) cells and natural killer T (NKT) cells [20–22]. However, these cells undergo apoptosis at late stages of severe infection [17,23]. Recently, we demonstrated that cytotoxic and cytokine-producing NK and CD8+ T cells mediate tissue injury and impair anti-*Ehrlichia* protective immunity during lethal *Ehrlichia* infection [20,23].

Innate immune cells express many pattern recognition receptors (PRRs) that are activated upon recognition of pathogen-associated molecular patterns [24–28]. The most characterized PRRs are the TLRs, which are transmembrane proteins localized either at the cell surface or within endosomal membranes. Upon activation, these receptors initiate signaling pathways dependent on adaptor proteins, such as MyD88, that result in activation of nuclear factor-kappa B (NF-κB) [26–30]. Other intracellular PRRs that emerged as sensors for intracellular microbial infection are the nucleotide-binding oligomerization domain (Nod)-like receptor protein (NLR) family, which includes Nod1 and Nod2 [31–34]. Nod1 and Nod2 signal via the adaptor molecule Rip2, a protein kinase required for activation of NF-κB and MAPK cascades, resulting in production of many cytokines and chemokines. Nod1 and Nod2 activation are upstream sensory signals for activation of the inflammasomes in the cytosol, which forms only in response to...
danger signals, including bacterial or viral infection. Activation of the inflammasomes leads to cleavage of caspase 1, which in turn cleaves pro-interleukin (IL)-1β and pro-IL-18, producing biologically active IL-1β and IL-18 [39–42]. These cytokines play different roles in inflammation and host defense against pathogens [39–42]. Currently, there are four defined inflammasomes. NLRP3 and Nalp1 trigger activation in response to extracellular adenosine-5′-triphosphate and pore-forming toxins [43,44]. NLRC4 is able to recognize many bacterial proteins found in the bacterial type III secretion apparatus [39–42]. Absent in melanoma 2 is able to sense cytosolic double-stranded DNA [45].

The first objective of this study was to better understand the pattern of gene expression underlying immune responses against *Ehrlichia* during tissue damage or recovery following lethal or nonlethal infections, respectively. Our results suggest that genes with specific biologic functions, including inflammasomes, TLR2 and Nod2, and several cytokines and chemokines are differentially regulated during mild and severe ehrlichiosis. The second objective is to examine the contribution of Nod2 or TLR2 to host defense against *Ehrlichia* and pathogenesis of HME. Strikingly, we found that TLR2-dependent host responses contribute to protective immunity against *Ehrlichia*. In contrast, Nod2-dependent host responses negatively regulate anti-*Ehrlichia* protective immunity and promote the development of pathogenic immune responses, thus enhancing susceptibility to *Ehrlichia*-induced toxic shock.

**Materials and Methods**

**Ethics Statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Pittsburgh in accordance with the institutional guidelines for animal welfare.

**Mice and *Ehrlichia* infection**

Female C57BL/6J, B6.129-Tlr2^tm1(Km)/J, and B6.129S1-Nod2^tm1Bv/J mice of 8–12 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were housed under specific pathogen-free conditions at the Animal Research Facility in the University of Pittsburgh. Two species of monocytic *Ehrlichia* were used in this study: the highly virulent IOE and the mildly virulent *E. muris* (SPK) buffer (0.5 M K$_2$HPO$_4$, 0.5 M KH$_2$PO$_4$, and 0.38 M NaCl). Ehrlichia were used in this study: the highly virulent IOE and the mildly virulent *E. muris* (SPK) buffer (0.5 M K$_2$HPO$_4$, 0.5 M KH$_2$PO$_4$, and 0.38 M NaCl). All animals were housed under specific pathogen-free conditions at the Animal Research Facility in the University of Pittsburgh. Two species of monocytic *Ehrlichia* were used in this study: the highly virulent IOE and the mildly virulent *E. muris* (SPK) buffer (0.5 M K$_2$HPO$_4$, 0.5 M KH$_2$PO$_4$, and 0.38 M NaCl).

**Reverse transcription and real-time polymerase chain reaction (RT-PCR) arrays**

Quantitative RT-PCR was carried out for groups of genes that are involved with different functions, such as immune regulation, innate and adaptive immune responses, and host cell survival. RNA was isolated from liver tissues using the Ambion RNA isolation kit (Life Technologies, Grand Island, NY), and cDNA was synthesized using the SA Biosciences RT^2^ First Strand Kit (QIAGEN, Valencia, CA) following the manufacturer’s recommendations. The expression levels of ~200 genes were determined using SA Bioscience Pathway Finder RT^2^ Profiler^TM^ PCR arrays for apoptosis, inflammasomes, cytokines, and innate and adaptive immune responses following the manufacturer’s recommendations. Data were collected using an Applied Biosystems 7900 HT Real-Time PCR System. The array plate contained 5 house-keeping genes, including GAPDH and β-actin, and one set for genomic DNA contamination as reference genes and a control. Comparative threshold cycle values were analyzed using SA Biosciences software, and fold regulation values were plotted. Fold regulation values were calculated by dividing the expression fold changes of the candidate genes by the expression fold changes of the reference genes using the comparative threshold cycle method. Upregulation or downregulation of host genes was determined based on comparison with naive mice. Using cut-off criteria, a 5-fold upregulation or downregulation was considered to be significant and of biologic importance.

**Flow cytometry**

Splenocytes were harvested, counted, and resuspended in staining buffer at a concentration of 10^6 cells/tube. FcRs were blocked with a mAb (clone 2.4G2) against mouse CD16 and CD32 for 15 min. The following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PerCP-Cy5.5-, Alexa Fluor-, and allophycocyanin-conjugated monoclonal antibodies (mAb) were purchased from BD Biosciences: anti-CD3 (clone RM4-4), anti-CD8α (clone 53-6.7), and anti-NK1.1 (clone PK136). Appropriate isotype control mAb, including FITC-, PE-, or allophycocyanin-conjugated hamster IgG1 (A19-3), rat IgG1 (R3-34), rat IgG2a (R35-95), mouse IgG1 (X40), and rat IgG2b (A95-1) were purchased from BioLegend (San Diego, CA). Lyt-1, Lyt-2, and granulocyte populations were gated based on forward and side scatter parameters; 20,000–50,000 events were collected using BD LSR or BD FACSScalibur (BD Immunocytometry Systems, San Jose, CA) flow cytometers. Data were analyzed using Flowjo software (TreeStar, Ashland, OR).

**In vitro splenocyte stimulation and cytokine enzyme-linked immunosorbent assay (ELISA)**

Splenocytes were harvested and single-cell suspensions were prepared as described before [19–21]. A total of 2–5×10^6 cells were seeded into a 12-well tissue culture plate in RPMI, supplemented with 10% heat-inactivated fetal bovine serum, 1% HEPEs buffer, and 100 μg/ml penicillin and streptomycin. Splenocytes were cultured with and without IOE antigens. After 48 hours, the culture supernatants were collected and an IFN-γ concentration was determined using the mouse Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations. The minimum detection limit for IFN-γ is 2 pg/ml.

**Bacterial burden determination using real-time PCR**

Total DNA was isolated from liver and spleen tissues using the DNeasy Blood and Tissue kit (QIAGEN). Bacterial burden was determined using a Step One Plus Real-Time PCR machine (Life Technologies, Grand Island, NY) targeting the EM/IOE dsb gene as previously described [16]. The primers and probes used are as follows: EM/IOE dsb-F: 5′-CAG GAT GGT AAA GTA CGT GTG A-3′; EM/IOE dsb-R: 5′- TAG CTA AYG CTG CCT GGA CA-3′; EM/IOE probe: (6FAM)-AGG GAT TTC CCT ATC CTC GGT GAG GC-MGB-BHQ. The eukaryotic housekeeping gene *gapdh* was amplified using the following primers/probes: GAPDH-F: 5′-CAA CTA CAT GGT CTA...
CAT GTT C-3'; GAPDH-R: 5'-TCG CTC TCT GAA GAT G-3'; GAPDH probe: (6FAM)-CGG CAC AGT CAA GGC CGA GAA TGG GAA GC-MGB-BHQ. The comparative cycle threshold method was used to determine the bacterial burden as described previously [19]. The results were normalized to the levels of expression of the gapdh in the same sample and expressed as copy number per 10^4 copies of gapdh [16,30]. PCR analyses were considered negative for ehrlichial DNA if the critical threshold values exceeded 40 cycles.

**Histopathology and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

Tissue sections were fixed in a 10% solution of neutral buffered formalin, dehydrated in graded alcohols, embedded in paraffin wax, and stained with hematoxylin and eosin (H&E). Semi-quantitative analysis of the liver lesions was carried out using three parameters: the number of necrotic cells, the number of apoptotic cells, and the number of inflammatory foci in each high power field (HPF). TUNEL staining was performed on unstained tissue sections, showing apoptotic cell death without focal necrosis, as described previously.

**Statistical analyses**

The two-tailed t test was used for comparisons of mean values for two experimental groups, and one-way analysis of variance was used for comparisons of multiple experimental groups. Data were represented by means ± standard deviations (SDs) or standard errors of the mean. P values P values ≤ 0.001 were considered highly significant (***)/***), P value ≤ 0.01 were considered moderately significant (**), and p values ≤ 0.05 were considered significant (*).

**Results**

**Identification of transcripts altered by lethal *Ehrlicchia* infection**

Previous murine studies indicated that protection against *Ehrlicchia* is mediated by IFN-γ and CD4+ Th1 cells whereas *Ehrlicchia*-induced shock can be attributed to CD4+ Th1 hypersponsiveness and the induction of pathogenic NK and CD8+ T cells mediating host cell apoptosis and necrosis [17–23]. In this study, we examined the expression of several genes that are involved in host cell survival and innate and adaptive immune responses in the livers of murine models of mild and fatal ehrlichiosis caused by *E. muris* and IOE, respectively. We chose to study the liver for two reasons: 1) the liver is the primary site for *Ehrlicchia* infection and pathology in humans and mice [5,7,12,13], and 2) previous studies indicated that the spatial and temporal changes in immune responses in the liver are strong predictors of disease progression in a mouse model of fatal HME [23]. We analyzed gene transcripts relevant to specific pathways, including: apoptosis, inflammasomes, and TLR signaling and innate and adaptive immune responses. Overall, lethal or nonlethal infections induced significant (p ≤ 0.05) downregulation of several genes on day 3 p.i. On the other hand, the majority of gene transcripts were upregulated on day 7 p.i. with both bacterial species but with more dramatic changes in response to lethal than nonlethal infection (Table 1 and 2).

**Lethal ehrlichiosis is associated with pro- and anti-inflammatory cytokines, chemokine storm, and a reduced Th1 response**

We have recently shown that fatal chlirhiosis in humans is associated with higher serum levels of chemokines and pro-inflammatory cytokines than detected in patients with mild ehrlichiosis [17], [23]. Thus, we examined whether similar events exist in murine models of fatal and mild ehrlichiosis. We found that lethally infected mice express significantly elevated levels of several chemokines/chemokine receptors compared to naive and nonlethally infected mice on day 3 p.i., which include CCL3/MIP-1α, CCL4/MIP-1β, CCL6, CCL7/MCP-3, CCL11/Eotaxin 2, CCL9, CCL12, CXCL4, CCR2, CCR3, CXCL5, CXCL9/Mig, CXCL10/IP-10, and CXCL13 (Table 1 and Fig. 1A and 1C). In addition to their roles as chemokine-attractors for macrophages, T cells, NK cells, and granulocytes, most of these chemokines also contribute to the activation of macrophages and T cells. In addition, CXCL10 and CXCL9 chemokines are induced by IFN-γ. On day 7 p.i., in addition to chemokines upregulated on day 3 p.i., lethal infection further induced higher expression of CCL12, CCL6, CCL11, CCL19, CCR7, CXCR1, and CXCL11 compared to nonlethal infection (Table 1 and Fig. 1B and 1D).

Compared to naive or nonlethally infected mice, lethally infected mice have increased expression of *il-1α* at early and late stages of infection, which was associated with upregulation of *tagf1*, but not *tagf2* (Fig. 1E and 1F). TRAF1 and TRAF2 proteins are members of TNF receptors-associated protein family and they mediate signal transduction from various receptors of TNFR superfamily. Expression of the pro-inflammatory *Il-1α* was only elevated in lethally infected mice at early, but not at late, stages of infection (Fig. 1E and 1F). *IL-6* expression was not significantly upregulated in either infection group on day 3 p.i. but was highly significantly upregulated on day 7 p.i. in response to lethal infection than nonlethal infection (Table 1 and Fig. 1E and 1F). Higher expression of *il-1α* in lethally infected mice was also associated with upregulation of anti-inflammatory *il-10* on days 3 and 7 p.i. compared to nonlethally infected and naive mice (Table 1 and Fig 1G and 1H). Notably, differential overexpression of *il-10* during lethal infection did not influence global *ifn-γ* expression, which was comparable in both lethally and nonlethally infected mice on days 3 and 7 p.i. (Table 1 and Fig. 1G and 1H). However, the *ifn-γ/il-10* ratio was lower in lethally compared to nonlethally infected mice on day 3 p.i. (1.35 vs. 5) and on day 7 p.i. (3.5 vs. 22). Since *IL-10* and IFN-γ have suppressor and stimulatory effects on macrophages activation and Th1 responses, respectively, the higher ratio of *ifn-γ/il-10* is a better indication of protective immunity against intracellular pathogens such as *Ehrlicchia* than the level of each cytokine alone. Consistent with our previous studies [17–21], the expression of Th2 or suppressive cytokines, such as *il-4, il-13*, or *TGF-β* was negligible in all groups of mice (data not shown).

Our data also showed that lethal IOE infection induced significantly higher upregulation of *Ifn-β* in the liver than that induced by nonlethal *E. muris* infection (Fig. 1G and 1H). Although IFN-βs known for its anti-viral effect, it promotes induction of chemokines secretion, maturation of dendritic cells and activation of cytotoxic NK cells and is associated with inflammasome activation and regulation of IL-1β secretion [24,25,33]. Further, the expression of IL-1 receptor antagonist (*il-1ra*) was significantly upregulated in nonlethal infection on day 3 p.i., while it was upregulated in lethally infected mice on day 7 p.i. only (Table 1 and Fig. 1G and 1H). IL-1ra is a natural antagonist of IL-1α and IL-1β signaling that prevents uncontrolled immune activation by IL-1α/β through competitive binding to the IL-1 receptor.

**Lethal ehrlichiosis is associated with activation of inflammasomes**

We have previously shown that lethal ehrlichiosis in mice is associated with increased *il-1β* production relative to nonlethal
infection, suggesting inflammasome activation. Lack of IL-18/IL-18R interaction protected mice from lethal Ehrlichia infection, which revealed a detrimental role of IL-18 in this disease process [46]. Our data here show that lethal infection also induced higher expression of \( \text{il-1b} \) on day 7 (Fig. 2B), but not day 3 (Fig. 2A), when compared to nonlethally infected mice. Late \( \text{il-1b} \) expression in lethally infected mice correlated with an early higher expression of \( \text{caspase 1} \) and \( \text{caspase 4} \) (also known as \( \text{caspase 11} \)) on day 3 p.i. compared to nonlethal infection (Fig. 2C). The expression of \( \text{caspase 1} \) was similar in both groups of mice on day 7 p.i., whereas the expression of \( \text{caspase 4} \) remained higher in lethally infected mice than in nonlethally infected mice (Fig. 2D).

Although lethal infection induced higher levels of \( \text{caspases 1 and 4} \) on day 3 p.i., we detected downregulation of several inflammasomes genes (\( \text{nlrp1, nlrp3, nlr12} \)) or slight changes in the expression of several inflammasomes (\( \text{nle4} \) and \( \text{aim2} \)) in both mice groups at that time (Table 2 and Fig. 2E). However, on day 7 p.i., the liver transcriptional profile showed higher levels of \( \text{nlp3} \) and \( \text{nlr12} \) in lethally infected mice, whereas \( \text{nlp3} \) and \( \text{nlr12} \) transcript levels were equally increased in both groups of infected mice compared

### Table 1. Differential gene expression of chemokine and cytokines.

| #  | Gene Symbol | Post infection day 3          | Post infection day 7          |
|----|-------------|-------------------------------|-------------------------------|
|    |             | Non-lethal/ \( E. \) muris   | Lethal/IOE                    | Non-lethal/ \( E. \) muris   | Lethal/IOE |
| 1  | Ccl2        | 1.1                           | -1.3                         | 38.7                         | 978.9      |
| 2  | Ccl3        | 4.6                           | 26.2                         | 5.7                          | 147.7      |
| 3  | Ccl4        | 1.2                           | 13.2                         | 16.6                         | 234.4      |
| 4  | Ccl5        | 1.5                           | 1.0                          | 4.9                          | 78.9       |
| 5  | Ccl6        | 1.4                           | 15.3                         | 2.5                          | 1.9        |
| 6  | Ccl7        | -1.1                          | 641.5                        | 82.4                         | 726.6      |
| 7  | Ccl8        | -1.1                          | 130.3                        | 15.3                         | 281.3      |
| 8  | Ccl9        | 1.7                           | 7.9                          | 3.8                          | 8.2        |
| 9  | Ccl11       | 1.2                           | 2.0                          | 2.7                          | 28.3       |
| 10 | Ccl12       | 2.6                           | 520.1                        | 48.4                         | 452.1      |
| 11 | Ccl19       | -1.5                          | 6.1                          | 13.1                         | 19.5       |
| 12 | Ccl24       | 1.4                           | 16.3                         | 5.5                          | -4.2       |
| 13 | Ccr2        | -1.1                          | 8.9                          | 7.5                          | 3.7        |
| 14 | Ccr3        | 67.0                          | 361.4                        | 3.0                          | 2.4        |
| 15 | Ccr7        | -2.2                          | 2.5                          | 2.2                          | 16.2       |
| 16 | Cxcl1       | 1.5                           | 1.4                          | 23.6                         | 80.4       |
| 17 | Cxcl5       | 1.5                           | 19.9                         | 8.1                          | 14.4       |
| 18 | Cxcl9       | -1.9                          | 221.9                        | 54.9                         | 132.1      |
| 19 | Cxcl10      | -1.2                          | 84.0                         | 27.1                         | 178.0      |
| 20 | Cxcl11      | -27.6                         | 2.8                          | 12.1                         | 130.9      |
| 21 | Cxcl13      | 1.8                           | 12.9                         | 2.8                          | 9.3        |
| 22 | Il1alpha    | 1.6                           | 15.2                         | 4.5                          | 3.7        |
| 23 | Il6         | -1.2                          | 1.1                          | 5.1                          | 24.9       |
| 24 | Tnf         | 1.6                           | 59.3                         | 19.6                         | 52.4       |
| 25 | Tnfsf11     | 1.9                           | 2.6                          | 18.6                         | 2.4        |
| 26 | Tnfsf10b    | 3.0                           | 1.8                          | 2.0                          | 15.0       |
| 27 | Tnfsf10     | -1.3                          | 9.3                          | 4.3                          | 8.2        |
| 28 | Traf1       | 4.0                           | 13.4                         | 6.9                          | 42.9       |
| 29 | Traf2       | -1.3                          | -1.1                         | 125.5                        | 2.4        |
| 30 | Ifnb1       | 1.1                           | 2.2                          | 2.8                          | 275.7      |
| 31 | Ifng        | 4.8                           | 7.1                          | 111.9                        | 154.8      |
| 32 | Il12a       | 1.9                           | 2.6                          | 3.3                          | 11.8       |
| 33 | Il12b       | 1.5                           | 2.1                          | 19.9                         | 32.7       |
| 34 | Il10        | -1.3                          | 35.2                         | 5.3                          | 43.6       |
| 35 | Il1ra       | 2.1                           | 9.4                          | 9.8                          | 181.1      |

1–21 Chemokine and their receptor
22–29 Pro-inflammatory cytokines and signaling genes
30–33 Type-1 interferons and Th1 cytokines
34–35 Anti-inflammatory cytokines
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to naive mice (Table 2 and Fig. 2F). Transcripts for the adaptor molecule, Pycard (PYD and CARD containing domain), were more upregulated in lethal infection on day 7 p.i. than (Fig. 2F). Together, these data suggest that lethal—but not nonlethal— infection is associated with inflammasome activation.

### Table 2. Differential gene expression of TLR, Inflammasomes, and Apoptosis Receptors.

| #  | Gene Symbol | Post infection day 3 | Post infection day 7 |
|----|-------------|----------------------|----------------------|
| 1  | Cd14        | 1.4                  | 1.0                  | 3.5                  | 77.5                  |
| 2  | Cd80        | −2.1                 | 1.0                  | 5.8                  | 8.8                   |
| 3  | Cd40        | −1.4                 | 21.7                 | 17.4                 | 58.3                  |
| 4  | Cd40lg      | −1.6                 | 4.5                  | 3.9                  | 14.2                  |
| 5  | Tlr1        | −3.4                 | 1.9                  | 3.7                  | 7.4                   |
| 6  | Tlr2        | −2.6                 | −5.4                 | 8.0                  | 62.9                  |
| 7  | Tlr3        | −1.9                 | −1.3                 | 1.7                  | 9.7                   |
| 8  | Tlr9        | −2.4                 | 1.2                  | 1.5                  | 16.3                  |
| 9  | Jun         | 1.2                  | 1.4                  | 3.4                  | 20.9                  |
| 10 | Myd88       | −1.6                 | −1.1                 | 2.2                  | 8.8                   |
| 11 | Nf-kb2      | −1.7                 | −2.5                 | 4.4                  | 7.7                   |
| 12 | Nod1        | −1.6                 | 80.8                 | 2.0                  | 1.8                   |
| 13 | Nod2        | 1.8                  | 2.3                  | 43.2                 | 23.0                  |
| 14 | Ripk2       | −1.1                 | 4.4                  | 2.8                  | 6.7                   |
| 15 | Apaf1       | 1.3                  | 1.5                  | 3.0                  | 10.3                  |
| 16 | Birc2       | 6.2                  | 14.1                 | 2.0                  | 6.0                   |
| 17 | Cilta       | 2.1                  | 2.6                  | 122.6                | 16.6                  |
| 18 | Il1b        | 1.7                  | −1.3                 | 2.0                  | 7.8                   |
| 19 | IL-18       | 1.3                  | 7.3                  | −1.7                 | 2.8                   |
| 20 | Casp1       | −1.5                 | 12.5                 | 14.5                 | 15.7                  |
| 21 | Casp12      | −1.9                 | 2.0                  | 6.2                  | 11.3                  |
| 22 | Casp4       | 2.8                  | 32.8                 | 7.1                  | 26.9                  |
| 23 | Nlrc5       | 1.3                  | −1.7                 | 9.0                  | 9.1                   |
| 24 | Nlrp12      | −1.4                 | −1.2                 | −1.2                 | 18.3                  |
| 25 | Nlrp1       | −2.2                 | −3.6                 | 1.1                  | 6.3                   |
| 26 | Nlrp3       | −1.3                 | −2.0                 | −3.0                 | 5.0                   |
| 27 | Bcl2        | 9.9                  | 10.7                 | 6.1                  | 2.8                   |
| 28 | Bcl2I2      | 2.8                  | 1.1                  | 1.8                  | 40.7                  |
| 29 | Bak1        | 2.7                  | 10.2                 | 1.4                  | 1.1                   |
| 30 | Bax         | 3.1                  | 7.3                  | 1.6                  | 2.3                   |
| 31 | Apaf1       | 1.3                  | 1.5                  | 3.0                  | 10.3                  |
| 32 | Aip1        | 2.0                  | 6.6                  | 2.8                  | 1.6                   |
| 33 | Aip2        | 2.1                  | 5.2                  | 2.0                  | 2.1                   |
| 34 | Fasl        | −2.5                 | 10.7                 | 7.2                  | 15.6                  |
| 35 | Fadd        | 5.8                  | −2.6                 | 1.8                  | −10.2                 |
| 36 | Trnsf10 (TRAIL) | −1.3           | 9.3                  | 4.3                  | 8.2                   |

1–4: Accessory and costimulatory molecules  
5–11: Toll-like receptors (TLRs) and TLR signaling  
12–17: Nod1/Nod2 and signaling genes  
18–26: Inflammasomes  
27–36: Apoptosis Receptors  
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Upregulation of TLR2 and Nod2 signaling during late stage of lethal Ehrlichia infection

Host cells express a variety of PRRs that recognize different microbial molecular patterns, among which are extracellular and endosomal TLRs and cytoplasmic Nod1 and Nod2 [24-28]. Our data show that lethal and nonlethal Ehrlichia infection downregulated most Toll-like receptors (tlr2, tlr3, tlr4, and tlr9) on day 3 p.i. (Table 2 and Fig. 3A). However, lethally infected mice had a
significant upregulation of these TLRs on day 7 p.i., mainly \( \text{tlr2} \) (Fig. 3B), which correlated with significant upregulation of \( \text{myd88} \) and \( \text{nf-kb1} \) when compared to nonlethally infected and naïve mice (Table 2 and Fig. 3C and 3D). Levels of transcripts of other adaptor proteins (e.g., \( \text{trif} \), \( \text{tram} \), and \( \text{tirap} \)) in both groups of infected mice were not different from those in naïve mice (data not shown), suggesting that MyD88 is the main protein involved in TLR signaling during ehrlichial infection.

Analysis of intracellular PRRs showed that \( \text{nod1} \) was upregulated 80-fold in lethally infected mice (Fig. 3E), with no significant changes in expression in nonlethally infected mice on day 3 p.i. No change in \( \text{nod2} \) expression was detected in either group of mice on day 3 p.i. (Fig. 3E). However, lethal infection induced higher expression of \( \text{nod2} \) on day 7 p.i. compared to nonlethal infection (Table 2 and Fig. 3F). Activated Nod-2 recruits Ripk2, which activates NF-kB by promoting the ubiquitination of the inhibitor of nuclear factor kappa-B kinase (IKK) subunit of the Ikappa-B kinase complex. Dominant-negative TRAF6 is known to inhibit Ripk2-mediated activation of NF-kB. Our data show that \( \text{ripk2} \) expression was not significantly increased (only 1.8-fold) during nonlethal infection, but was increased approximately 10-fold during lethal infection on day 7 p.i. (Fig. 3F). These data suggest

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that Nod2 ligation can lead to NF-κB activation in lethally—but not in nonlethally—infected mice. Levels of traf6 did not significantly differ in either infected group compared to naïve mice (Fig. 3E and 3F).

Contribution of Tlr2 and Nod2 to Ehrlichia-induced immunopathology and bacterial clearance

Having observed that lethal IOE infection differentially modulates tlr2 and nod2 levels, we decided to elucidate the contributions of TLR2 and Nod2 to the pathogenesis of fatal ehrlichiosis. We infected TLR2-/- and Nod2-/- mice with lethal doses of IOE and compared the outcomes of infection to similarly infected wild type (WT) mice and naïve mice of both strains. Consistent with previous reports [17], WT mice were highly susceptible to lethal IOE challenge where six of six WT mice succumbed to infection on days 9 and 10 p.i. Notably, while TLR2-/- had increased susceptibility to IOE infection with six of six mice succumbed on days 7 and 8 p.i., all Nod2-/- mice survived till days 15 p.i. (Fig. 4A).

Consistent with our previous reports, compared to naïve mice (Fig. 4B and 4F) IOE-infected WT mice developed focal hepatic necrosis and apoptosis on day 7 p.i. (Fig. 4C and 4G). In contrast, IOE-infected Nod2-/- mice had no evidence of necrosis (Fig. 4E) and presented with fewer inflammatory foci in the liver (Fig. 4J). On the other hand, IOE-infected TLR2-/- mice developed extensive necrosis (Fig. 4D) and inflammatory foci (Fig. 4J) compared to infected Nod2-/- and WT mice on day 7 p.i. There was a slight decrease in number of apoptotic cells in Nod2-/- mice compared to WT and TLR2-/- mice on day 7 p.i. (Compare Fig. 4I to Figs. 4G and 4H). Interestingly, lack of Nod2 enhanced bacterial clearance in different organs, and absence of TLR2 increased bacterial burden when compared to infected WT mice on day 7 p.i. (Fig. 4K). These results collectively suggest that TLR2

Figure 3. Differential expression of TLR and NOD genes and downstream signaling molecules during lethal and nonlethal Ehrlichia infection. The expression of TLRs (A and B), transcription factors (C and D), and Nod1 and 2 proteins and their downstream signaling molecules (Ripk2 and TRAF6) (E and F) were examined on days 3 and 7 following lethal (IOE) and nonlethal (E. muris) infection. The expression of TLR2 on day 7 p.i. with IOE was much pronounced than that of other TLRs. The expression of downstream signaling molecules MyD88 and NF-κB was significantly upregulated on day 7 p.i. during IOE infection compared to E. muris infection. Nod1 was differentially upregulated on day 3, and Nod2 was differentially upregulated on day 7 after IOE infection. Data shown represent the mean ± SD of individual liver samples with three mice/group. Data represent two independent experiments (* P = 0.05, * P = 0.01).

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Figure 4. Enhanced resistance of Nod2−/− mice to lethal ehrlichiosis compared to infected wild type and TLR2−/− mice. (A) Survival of WT, TLR2−/− and Nod2−/− mice over 15 days after i.p. infection with high dose of IOE. The data shown represent one of two independent experiments with a total of 6 mice/group. Liver sections from naïve (B and F), IOE-infected WT mice (C and G), IOE-infected TLR2−/− mice (D and H), and IOE-infected Nod2−/− mice (E and I) harvested on day 7 p.i. are stained with H&E. Original magnification for H&E images was 20× and for TUNEL assays was 40×. H&E staining shows that IOE-infected Nod2−/− mice had significant decreases in necrosis compared to infected WT and TLR2−/− mice (arrowheads). TUNEL assay reveals slightly decreased numbers of apoptotic cells (arrows) in Nod2−/− mice with approximately 4–7 apoptotic cells observed per HPF.
and Nod2 play distinct protective and detrimental roles during ehrlichiosis, respectively.

Lack of Tlr2 or Nod2 influence innate immune responses against *Ehrlichia*

We next examined the contribution of Nod2 and TLR2 to protective or pathogenic immune responses mediated by different innate and adaptive immune cells. Absence of Nod2 increased the percentage (Fig. 5A) and absolute number (Fig. 5B) of NKT as well as percentage (Fig. 6 A and 6B) and absolute number of CD4+ T cells (Fig. 6C) in the spleens of infected mice compared to infected WT and TLR2−/− mice. NKT cells and CD4+T cells mediate elimination of intracellular ehrlichiae as shown before (11, 17-23). Although lack of Nod2 did not influence the frequency of pathogenic NK cells, it significantly decreased the percentage and absolute number of pathogenic CD8+ T cells when compared to infected WT and TLR2−/− mice (Fig. 6B and 6C). No significant difference was observed in the total number of NK, CD4+ T cells, and CD8+ T cells between TLR2−/− and WT mice. Interestingly, enhanced resistance and attenuated pathology in IOE-infected Nod2−/− mice correlated with increased antigen-specific production of IFN-γ and IL-10 in the spleen on day 7 p.i. compared to IOE-infected WT and TLR2−/− mice (Fig. 7A and 7B), suggestive of an enhanced Th1 and anti-inflammatory immune responses.

Discussion

In this study, we provide a detailed genome-wide microarray analysis of whole liver during mild and fatal ehrlichiosis, which offers a revealing new perspective on host responses during the course of nonlethal and lethal *Ehrlichia* infection. *Ehrlichia chaffeensis*, the causative agent of HME, leads to disease in Severe Combined Immunodeficiency (SCID) mice but not in immunocompetent mice [17,47]. However, several genes identified in our study are consistent with prior reports that profiled the liver of SCID mice infected with different human isolates of *E. chaffeensis*, each belonging to a different genogroup [12,48]. This suggested that: 1) innate immune responses play a unique role in outcome of *Ehrlichia* infections; and 2) host responses in our murine models of HME using other *Ehrlichia* species (*E. muris* and IOE) mimic the host responses to human *E. chaffeensis* isolates in SCID mice, thus these models are optimal for further analysis of innate and adaptive immune responses.
adaptive immune responses during ehrlichiosis in immunocompetent host.

A notable element within the liver transcriptome profile was broad involvement of multiple pro-inflammatory interleukin and TNF family members and their receptors. However, unlike LPS-positive Gram-negative bacteria, severe infection with LPS-negative *Ehrlichia* induced a concomitant upregulation of both pro-inflammatory cytokine genes and key anti-inflammatory genes, such as *il-10* and *il-1ra*. In experimental models of non-infectious inflammatory diseases, *IL-10* knockout mice develop severe colitis, whereas systemic administration of *rIL-10* can prevent development of colitis [49,50]. *IL-10* mediates an anti-inflammatory function and prevent pathology during infections with intracellular pathogens; however, it also inhibits IFN-γ-mediated activation of phagocytic cells and suppresses the differentiation of protective CD4+Th1 cells, thus inhibit effective elimination of intracellular bacteria [50,51]. The correlation between late, but not early, expression and production of *IL-10* in *E. muris* nonlethally infected mice and IOE-infected *Nod2−/−* mice and survival, protective immunity, minimal pathology and high IFN-γ:IL-10 ratio at early and late stages of infection, suggests that *IL-10* may control excessive inflammatory responses and thus inhibits immunopathology without negatively affecting IFN-γ mediated bacterial elimination. On the other hand, production of *IL-10* and lowered IFN-γ:IL-10 ratio during early and late stages of lethal IOE infection [17–23]. Our data suggest that the development of immunopathology could be attributed to the upregulation of chemokines and their relevant receptors and the recruitment of CD8+ T cells, monocytes, and *NK* cells in the liver of lethally, but not nonlethally, infected mice. Mononuclear and *NK* cells respond to CCL2 (monocyte chemotactic protein [MCP]), CXCL9, CXCL10, and CXCL11 (ligands for the CXCR3) mediate migration of resting human *NK* cells. T cells and neutrophils respond to CCL5 (RANTES) and CXCL8 (IL-8), respectively.

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**Figure 6. Lack of Nod2 increased number of protective CD4+ T cells and decreased frequency of CD8+ T cells during severe *Ehrlichia* infection.** Spleen cells were harvested from IOE-infected *Nod2−/−*, TLR2−/−, and WT mice on day 7 p.i., and cells were analyzed directly *ex vivo* to determine the frequency of CD4+ and CD8+ T cells. (A) The dot plot shows the percentage of CD3+CD4+ T cells in naive mice and IOE-infected WT, TLR2−/−, and *Nod2−/−* mice. (B) CD3+ cells were gated and further analyzed for expression of CD4 and NK1.1. NK1.1+ cells are thus divided into CD4+ T cells (upper quadrant) and CD8+ T cells (lower quadrant). *Nod2−/−* mice have higher percentages of CD4+ T cells but lower percentages of CD8+ T cells compared to other groups of mice. (C) The absolute numbers of CD4+ and CD8+ T cells in the four groups of mice. Dot plot data are from a representative mouse from each group. The absolute number of cells represents the means ± SD with three mice/group and is representative of three independent experiments.

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**Figure 7. Enhanced resistance to *Ehrlichia* infection in *Nod2−/−* mice is associated with increased IFN-γ and IL-10 production.** WT C57BL/6, TLR2−/−, and *Nod2−/−* mice were infected with IOE. Splenocytes were harvested on day 7 p.i. and stimulated *in vitro* with IOE antigens. At 48 hours after *in vitro* antigen stimulation, the supernatant was collected and examined for IFN-γ (A) and IL-10 (B) by enzyme-linked immunosorbent assay. The data are expressed as the mean ± SD for three mice in each group. The data shown are from one experiment that is representative of three independent experiments.

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Enhanced resistance in Nod2 -/- could be also due to effective cells occur via cross-presentation during lethal ehrlichiosis [46]. Other studies have shown that Nod1, acting in ligation. Thus, Nod1 and TLR2 both function as costimulatory protective NKT and antigen-specific CD4+T cells (Fig. 4E). Enhanced Nod2 attenuated immunopathology (Fig. 4A and B) and enhanced induction of pathogenic cytotoxic CD8+ T cells during Ehrlichia-induced shock. Indeed, our data demonstrate that the absence of Nod2 attenuated immunopathology (Fig. 4A and B) and enhanced protective immunity and bacterial clearance (Fig. 4E). Enhanced resistance of Nod2 -/- mice could contribute to the induction of pathogenic cytotoxic CD8+ T cells by CD8+ dendritic cells [60]. Thus, it is possible that an early upregulation of Nod1 followed by upregulation of Nod2 could contribute to the induction of pathogenic cytotoxic CD8+ T cells during Ehrlichia infection. In conclusion, our study indicates Nod2 mediates the dysregulated inflammatory responses and immunopathology during lethal ehrlichiosis and TLR2 mediates effective clearance of ehrlichiae in the absence of Nod2 signals. Our data thus define for the first time unique molecular pathogenic mechanisms that may account for the development of Ehrlichia-induced shock. Targeting these pathways could represent a novel immunotherapeutic strategy to combat these important infections and the associated pathology.

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Author Contributions

Conceived and designed the experiments: NI PC QY. Performed the experiments: PC QY AK OA. Analyzed the data: NI PC QY AK OA. Contributed reagents/materials/analysis tools: NI PC QY AK OA. Wrote the paper: NI PC QY.
References

1. Lin M, Rikihisa Y (2003) Ehrlichia chaffeensis and Anaplasma phagocytophilum lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. Infect Immun 71: 3324–31.

2. Bryant CE, Spring DR, Gangloff M, Gay NJ (2006) Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Microbiol 6: 351–357.

3. Huang H, Lin M, Wang X, Käck T, Mottaz H, et al. (2008) Proteinomic analysis of and immune responses to Ehrlichia chaffeensis lipoproteins. Infect Immun 76: 1345–1352.

4. Ismail N, Stevenson HL, Walker DH (2007) Relative importance of the early treatment with doxycycline in human ehrlichiosis. Medicine (Baltimore) 86: 57–60.

5. Paddock CD, Childs JE (2003) Histologic, serologic, and molecular analysis of persistent ehrlichiosis in a murine model. Am J Pathol 158:757–69.

6. Ratnasamy N, Everett ED, Roland WE, McDonald G, Caldwell CW (1996) The interaction between Ehrlichia phagocytophilum and Acinetobacter baumannii. J Comp Path 123: 163–171.

7. Szymczak WA, Deepe GS Jr (2009) The CCL7-CCL2-CCR2 axis regulates IL-4 production in human infection and is differentially regulated by GATA-1. J Immunol 182: 3405–14.

8. Hara H, Oyama H, Akiyama J, Watanabe N, Takeuchi O, et al. (2010) IL-18 receptor chain expression on CD4 T cells, but not B cells, mediates colitis in interleukin-10-deficient mice. J Exp Med 180: 1207–1216.

9. Tsuchiya K, Hara H, Nomura T, Yamamoto T, Daim S, et al. (2010) Differentiation: Critical Down regulatory Role of IL-4. J Exp Med 194: 143–156.

10. Tsui CC, Tsai SH, Lu CC, Hu ST, Wu TS, et al. (2012) Activation of an NLRP3 inflammasome restricts Mycobacterium kansasii infection. PLoS One 7: e36292.

11. Sheng Y, Nishioka K, Nakajima T, Sato H (2010) Infection of mice with the intracellular pathogen Ehrlichia chaffeensis. Infect Immun 78: 6160–20.

12. Stevenson HL, Crossley EC, Walker DH (2007) Relative importance of T-cell subsets in monocytotropic Ehrlichia infection. Infect Immun 75: 3604–13.

13. Asseman C, Maurer S, Lech J, Coffman RL, Powrie F (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 190: 995–1004.

14. Anand PK, Malireddi RK, Lukens JR, Vogel P, Bertin J, et al. (2012) NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. Nature 486: 389–393.

15. Tsuchiya K, Hara H, Nomura T, Yamamoto T, Daim S, et al. (2010) Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. Cell Host Microbe 8: 471–483.

16. Brou P, Miotte J, Jones JW, Vance RE, Monack DM (2010) Differential cytokine production by IL-4 and contact with infected dendritic cells. J Immunol 184: 5172–8.

17. Haring JS, Hart JT (2009) Interleukin-18-related genes are induced during the contraction phase but do not play major roles in regulating the dynamics or function of the T-cell response to Listeria monocytogenes infection. Infect Immun 77: 1094–903.

18. Humann J, Lenz LL (2010) Activation of naive NK cells in response to Listeria monocytogenes requires IL-18 and contact with infected dendritic cells. J Immunol 184: 5172–8.

19. Wajnberg GM, Yager E, Shilo K, Collins DM, Chu FK (1998) Infection of the laboratory mouse with the intracellular pathogen Ehrlichia chaffeensis. Infect Immun 66: 3892–9.

20. Okada H, Rajima T, Kawahara M, Rikihisa Y (2001) Ehrlichia proliferation and acute hepatocellular necrosis in immunocompetent mice experimentally infected with the HE strain of Ehrlichia, closely related to Ehrlichia chaffeensis. Comp Path 123: 163–171.

21. Sotomayor EA, Popov VI, Feng HM, Walker DH, Olano JP (2001) Animal model of fatal human monocytotropic Ehrlichiosis. Am J Pathol 156:757–69.

22. Olano JP, Wen G, Feng HM, McBride Jr, Walker DH (2004) Histologic, serologic, and molecular analysis of persistent ehrlichiosis in a murine model. Am J Pathol 165:997–1006.

23. Hirose H, Kawasaki H, Kawasaki Y, Nakagawa J, Hara H, et al. (2003) Intradermal environment promotes a protective type-1 response against lethal infection of T-cell subsets in monocytotropic E. chaffeensis infection. Infect. Immun 71: 3324–31.

24. Medzhitov R, Janeway C Jr (2000) Innate immune recognition: mechanisms and responses during fatal T-cell subsets in monocytotropic E. chaffeensis infection. Infect. Immun 71: 3324–31.
55. Jia T, Serbina NV, Brandl K, Zhong MX, Leinet IM, et al. (2008) Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection. J Immunol. 180:6846–53.

56. Crane MJ, Hokeness-Antonelli KL, Salazar-Mather TP (2009) Regulation of inflammatory monocyte/macrophage recruitment from the bone marrow during murine cytomegalovirus infection: role for type I interferons in localized induction of CCR2 ligands. J Immunol 183: 2810–2817.

57. Hokeness-Antonelli KL, Crane MJ, Salazar-Mather TP (2007) IFN-alpha beta-mediated inflammatory responses and antiviral defense in liver is TLR9-independent but MyD88-dependent during murine cytomegalovirus infection. J Immunol 179: 6176–6183.

58. Delbridge LM, O’Riordan MX (2007) Innate recognition of intracellular bacteria. Curr Opin Immunol 19: 10–16.

59. Lamkanfi M (2011) Emerging inflammasome effector mechanisms. Nat Rev Immunol 11: 213–220.

60. Mariathasan S, Monack DM (2007) Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Rev Immunol 7: 31–40.

61. Rahman MA, Cheng Z, Matsuo J, Rikihisa Y (2012) Penicillin-binding protein of Ehrlichia chaffeensis: cytokine induction through MyD88-dependent pathway. J Infect Dis 206: 110–116.

62. Mercier BC, Vezzé E, Fogeron ML, Debaud AL, Tomkowiak M, et al. (2012) NOD1 cooperates with TLR2 to enhance T cell receptor-mediated activation in CD8 T cells. PLoS One 7: e42170.

63. Abdul-Sater AA, Koo E, Hacker G, Ojcius DM (2009) Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen Chlamydia trachomatis. J Biol Chem 284: 26789–96.