NOTES

Innate Immune Response to *Anaplasma phagocytophilum* Contributes to Hepatic Injury

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In mice, *Anaplasma phagocytophilum* control is independent of phagocyte oxidase (phox), inducible NO synthase (NOS2), tumor necrosis factor (TNF), and MyD88 Toll-like receptor signaling. We show that despite evasion of these host responses, phox, NOS2, TNF, and MyD88 are activated and contribute to inflammation and hepatic injury more than *A. phagocytophilum* itself.

Anaplasma phagocytophilum is an obligate intraneutrophilic tick-borne bacterium that causes human and animal granulocytic anaplasmosis (HGA). In the murine model of HGA, host immune response is more important for histopathologic lesions than is pathogen load (24, 29). Deficiency in inducible nitric oxide synthase (NOS2), tumor necrosis factor (TNF), or NADPH phagocyte oxidase (phox) has little impact on *A. phagocytophilum* control (5, 32). Likewise, infected Toll-like receptor 2 (TLR2)-deficient (*Tlr2*−/−), TLR4-deficient, and *Myd88*−/− mice attain pathogen load levels similar to those of controls (32), despite *A. phagocytophilum* vs triggering NF-κB activation through TLR2 in vitro (17). *A. phagocytophilum*-infected mice lacking gamma interferon have fewer histopathologic changes and increased pathogen loads, yet gamma interferon is dispensable for pathogen control (2, 24). Whether induced immune effectors cause the inflammatory tissue damage underlying disease is unknown. We hypothesized that *A. phagocytophilum*, although able to avoid innate immunity, nevertheless induces innate immune effectors, resulting in inflammatory hepatic lesions, which are elicited independently of bacterial load.

A. phagocytophilum (strain MRK) was grown in HL-60 cells, and C.B17 SCID mice were infected as described previously (32). For experimental infections, mice were injected intraperitoneally with 100 μl of blood that was pooled from infected or uninfected (HL-60 cells only) SCID mice and diluted 1:5 in phosphate-buffered saline. When tested retrospectively, this dilution corresponded to 10^4 to 10^6 *A. phagocytophilum* genome equivalents; both the method and the inoculum size were within established ranges for mouse models of *A. phagocytophilum* infection (2, 3, 20). For each paired wild-type C5JBL/6

(B6)/knockout mouse experiment, the same inoculum size was used.

Mouse strains used for this study were obtained as previously described (32) and were as follows: *Cybb*−/− (gp91phox−/−) and *Nos2*−/− mice (C B6 background), *Tnf*−/− mice (B6 background), B6 *Myd88*−/− mice backcrossed for three generations onto a C3H background, B6 *Tlr2*−/− mice backcrossed for eight generations onto a C3H/HeJ background, B6 and C3H/HeN control mice, and C.B17 SCID mice. All mice were housed under barrier conditions and were between 4 and 16 weeks of age. For pilot studies, three infected and three mock-infected *Cybb*−/− mice, *Nos2*−/− mice, and B6 mice were examined at days 16, 25, and 43 postinfection, and three infected *Tnf*−/− mice and B6 mice were examined at days 3, 7, and 14. For confirmation, six *Tnf*−/−, nine *Cybb*−/−, nine *Nos2*−/−, nine C3H/HeJ *Tlr2*−/−, and nine *Myd88*−/− mice (infected and mock infected) as well as B6 or C3H control mice were used; three mice were examined at each time point. *Cybb*−/− and *Nos2*−/− mice were examined at days 7, 21, and 42; *Tnf*−/− mice were tested at days 3 and 7 postinfection (p.i), and C3H/HeJ *Tlr2*−/− and *Myd88*−/− mice were examined at days 3 and 7 and at 2 to 3 weeks p.i.

Animals were sacrificed and tissues fixed, embedded, and cut into 5-μm sections for hematoxylin and eosin staining. Two independent reviewers ranked hepatic inflammatory lesions for severity after normalizing for uninfected controls. Severity was assessed so that high rank numbers reflected higher degrees of tissue inflammation and injury severity. Severity was determined by examining (i) the extents of liver injuries (hepatocyte apoptosis, parenchymal necrosis), (ii) the numbers and relative areas of inflammatory lesions (small multifocal infiltrates to extensive focal infiltration), and (iii) the densities and types of inflammatory infiltrates (predominant or mixed granulocytic, mononuclear, or histiocytic). For infected *Cybb*−/−, *Nos2*−/−, and paired B6 mice, the median rank for uninfected controls was subtracted from that for infected animals to adjust for background, and then samples were reranked to generate

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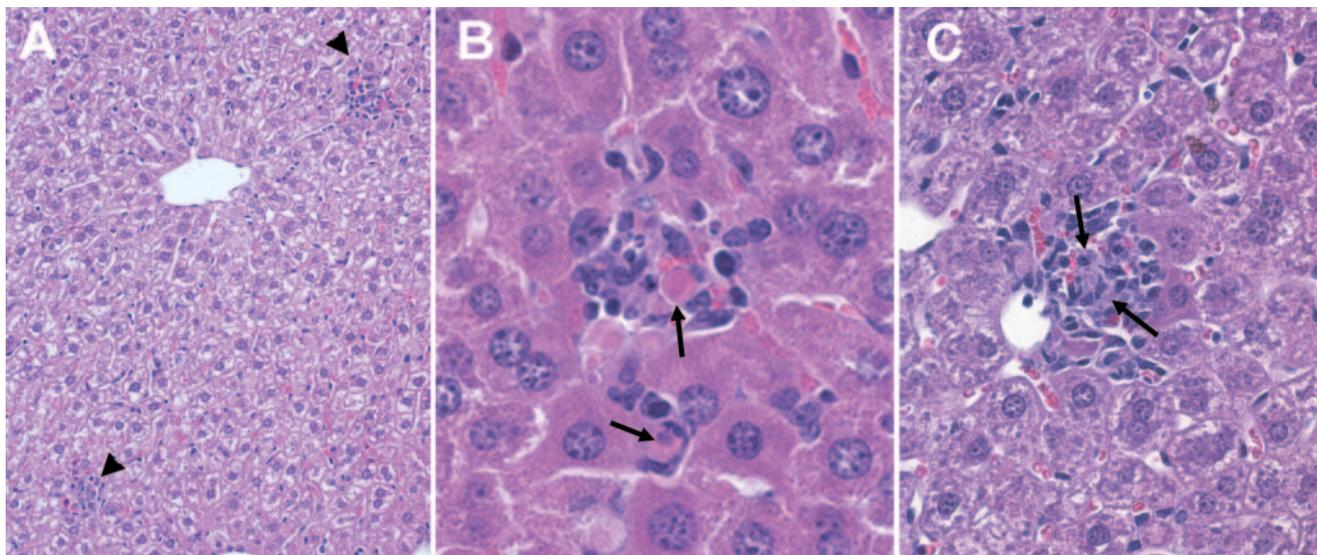
FIG. 1. Hepatic histopathology with *A. phagocytophilum* infection in mice. B6 and knockout mice displayed inflammation, which included (A) scattered parenchymal lesions (arrowheads) and lobular hepatitis (B6 mice, day 7 p.i. [original magnification, ×64]), (B) focal lesions with mixed inflammatory infiltrates and apoptotic hepatocytes (arrows) (B6 mice, day 3 p.i. [original magnification, ×160]), and (C) granulomas and granulomatous infiltrates characterized by the presence of epithelioid macrophages (arrows) surrounded by lymphocytes ($Cybb^{-/-}$ mice, day 7 p.i. [original magnification, ×160]).

group medians. For other knockout mice, histopathologic results were compared to those for uninfected wild-type mice to assure the absence of concurrent infections or aberrant pathological lesions, but no adjustments for genetic background were made. Because differences between knockout and wild-type mice were potentially not normally distributed, we utilized nonparametric Mann-Whitney statistical analysis; P values of <0.05 were considered significant.

We previously reported that no significant differences in *A. phagocytophilum* tissue or blood loads between wild-type and knockout mice were detected at any interval (32). In pilot studies (not shown), histopathologic data pooled from multiple time points showed more-severe hepatic injury in infected $Cybb^{-/-}$ mice than in $Nos2^{-/-}$ or B6 mice ($P < 0.05$), especially at day 43; no differences were noted between infected $Nos2^{-/-}$ and B6 mice. No significant differences were detected between infected $Tnf^{-/-}$ mice and B6 controls at any time point, although there was a trend toward more-severe injury in B6 mice at day 7 ($P = 0.06$).

In confirmatory studies, all B6 and knockout mice had some hepatic parenchymal and perivascular inflammatory lesions (Fig. 1). Histopathologic ranks are shown in Fig. 2. At the earliest time point, B6 mice had more-severe histopathologic changes ($P < 0.05$) than $Tnf^{-/-}$ mice, a trend also noted at day 7 ($P = 0.06$). Likewise, B6 mice had higher severity rankings than $Nos2^{-/-}$ or $Cybb^{-/-}$ mice on day 7 ($P < 0.05$). By day 42, when hepatic granulomatous lesions were observed in infected $Cybb^{-/-}$ mice, severity exceeded the baseline observed for uninfected $Cybb^{-/-}$ mice ($P < 0.05$) and for infected B6 mice ($P = 0.14$), although not by a statistically significant degree. This increase in hepatic histopathologic severity was noted for $Cybb^{-/-}$ mice only at day 42. No differences in histopathology were identified for other tissues (spleen, lymph node, lung, and bone marrow).

Among mice with disrupted TLR signaling, no hepatic pathology differences were noted between infected C3H/HeJ $Tlr2^{-/-}$ (TLR2/4-defective) mice and B6 controls at any time. No differences between $Myd88^{-/-}$ mice and infected C3H/HeN controls were detected, whereas $Myd88^{-/-}$ mice had less-severe histopathologic changes than infected B6 controls at all times ($P < 0.05$).

These data show that inflammatory and innate immune pathways are activated during *A. phagocytophilum* infection, even though *A. phagocytophilum* has evolved mechanisms to avoid killing by activated effectors (14). We previously showed that innate and adaptive immune responses related to TLR2, TLR4, MyD88, phox, NOS2, and TNF are dispensable for the control of *A. phagocytophilum* (32). This discrepancy raises the question of whether *A. phagocytophilum* remains unaffected because it downregulates innate immune activation in vivo or whether it is neither susceptible nor accessible to innate immune effectors.

Histopathologic injury in the HGA murine model does not correlate with bacterial load, and the pathogen itself may not directly cause tissue injury (23, 24, 29). The reduced histopathologic changes in phox- or NOS2-deficient mice suggest that early innate immune responses elicit inflammatory lesions related to reactive oxygen intermediates (ROI) and reactive nitrogen species (11). Some differences between pilot and confirmatory studies reflect the use of pooled data, the lack of early time intervals, and bias caused by the higher level of histopathologic injury severity in $Cybb^{-/-}$ mice at day 43 in the pilot study. TNF appears to have only a limited role in the hepatic injury that accompanies *A. phagocytophilum* infection, since a significant reduction in severity was observed in only one experiment at a single time point. These kinetics imply that inflammation is largely the result of innate and not adaptive immunity, a finding also supported by the severe histopatho-
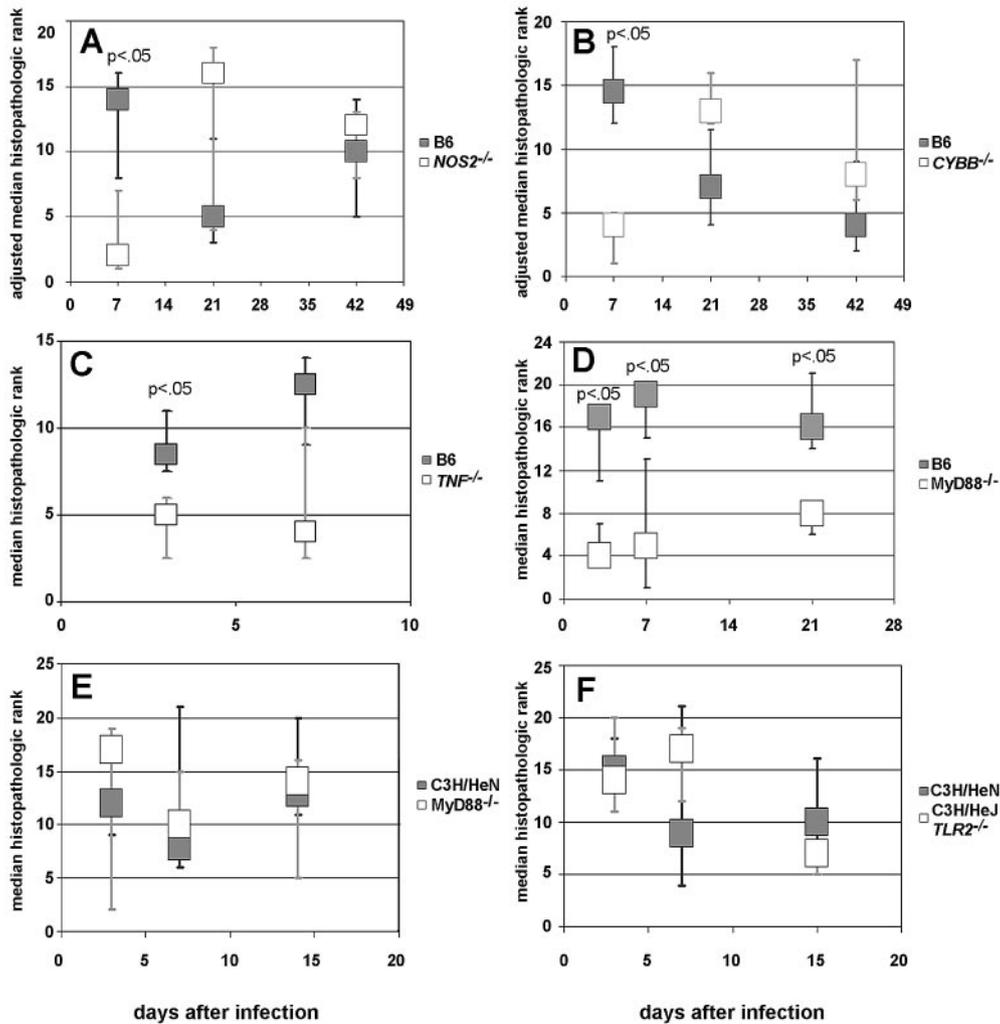
FIG. 2. Median histopathologic ranks in hepatic tissues from *A. phagocytophilum*-infected knockout and control mice. High histopathologic rank numbers correspond to higher degrees of tissue inflammation and severity of injuries. On day 7 of infection, B6 mice had significantly higher ($P < 0.05$) severity ranks than did matched (A) $Nos2^{-/-}$ and (B) $Cybb^{-/-}$ mice. (C) On day 3, wild-type B6 mice had histopathologic lesions significantly more severe ($P < 0.05$) than those of matched $Tnf^{-/-}$ mice, a trend also noted at day 7 ($P = 0.06$). (D) Comparison to the B6 background revealed that $Myd88^{-/-}$ mice had histopathologic lesions less severe ($P < 0.05$) than those of infected B6 controls at all time points. (E) When an examination on the C3H/HeN background was conducted, no significant differences between $Myd88^{-/-}$ mice and infected C3H/HeN controls were detected at any time point. (F) Additionally, no differences were noted between infected C3H/HeJ $Tlr2^{-/-}$ mice (TLR2/4 defective) and infected C3H/HeN controls at any time point.

logic changes among infected SCID mice (12). A role for innate immunity via TLRs is also suggested by the inhibition of inflammation in MyD88-deficient B6 mice, because MyD88 is critical for signaling by most TLRs (6, 31), and *A. phagocytophilum* is able to induce NF-κB activation via TLR2 but not TLR4 (17). However, this hypothesis is contradicted by the similar levels of inflammation for infected MyD88-deficient C3H mice and for TLR2- and TLR4-deficient C3H mice. These data suggest that with *A. phagocytophilum* infection, inflammatory signaling in C3H mice (i) proceeds via a MyD88-independent pathway and (ii) involves alternative TLRs or unidentified ligands differentially expressed between C3H and B6 mice.

Inflammatory lesions among $Nos2^{-/-}$, $Tnf^{-/-}$, and B6 mice resolved with time. More-severe lesions in $Cybb^{-/-}$ mice at day 42 were characterized by granulomatous infiltrates similar to

those seen in cases of chronic granulomatous disease (28). The prolonged inflammation illustrates that phox contributes to termination of the inflammatory response in accordance with the immunoregulatory properties of ROI (8). Interestingly, *A. phagocytophilum* downregulates phox expression and activity in HL-60 cells (4, 13, 15, 26) and induces neutrophil interleukin-8 expression, thereby promoting recruitment of new host cells (3, 29). Thus, infection of neutrophils by *A. phagocytophilum* likely delays inflammation resolution, which is aggravated by the complete absence of phox, as seen for $Cybb^{-/-}$ mice (8, 25, 30).

In early *A. phagocytophilum* infection, NOS2 promotes hepatic inflammation, consistent with our experiments in which NOS activity was inhibited pharmacologically (11). In contrast, $Nos2^{-/-}$ mice infected by intracellular pathogens such as viruses, *Francisella tularensis*, or *Coxiella burnetii* often demonstrate more-severe inflammation (1, 10, 21, 22), which can be

attributed to the lack of NO-dependent feedback inhibition or to the high pathogen load levels after loss of reactive nitrogen species effector function (7, 8). We conclude that *A. phagocytophilum* survival mechanisms for intraneutrophilic growth, i.e., modulations of NOS2, ROI, TNF, and interleukin-8, paradoxically enhance inflammation and tissue injury (9, 29). This situation is distinct from that for many other intracellular pathogens that impede innate and adaptive immune responses to evade killing (16, 18, 19, 27). Thus, *A. phagocytophilum* may have evolved novel adaptations for propagation and pathogenesis not utilized by other intracellular pathogens.

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AUTHOR'S CORRECTION

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Volume 13, no. 7, p. 806–809, 2006. Page 806, column 1, line 22: "*A. phagocytophilum* (strain MRK) . . . ." should read "*A. phagocytophilum* (strain Webster) . . . ."