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Regulatory roles of NKT cells in *Anaplasma phagocytophilum* infection

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Abstract : Human granulocytic anaplasmosis (HGA) is caused by the obligate intracellular bacterium *Anaplasma* (*A.*) *phagocytophilum*. Natural killer T (NKT) cells are key players in host defense against various microbial infections. We investigated the role of NKT cells in immune response to *A. phagocytophilum* infection using NKT-knockout (Jα18-/-) mice. Jα18-/- and wild-type (WT) mice were infected with low-passage *A. phagocytophilum* and assayed for hepatic histopathology and cytokine production during 7 days post-infection. Compared to WT controls, the infected Jα18 -/- mice had much less histopathologic lesions and less apoptosis through day 7, and lower concentrations of IFN-γ and IL-12, but not of IL-10. This result suggests that NKT cells are major components in the pathogenesis of HGA.

Keywords : *Anaplasma phagocytophilum,* human granulocytic anaplasmosis, NKT cells, NKT-knockout mice

Introduction

Human granulocytic anaplasmosis (HGA) is a tick-borne disease caused by *Anaplasma* (*A.*) *phagocytophilum,* an obligate intracellular bacterium that infects and propagates within neutrophil vacuoles [7, 11]. *A. phagocytophilum* not only survives in neutrophils, but also interacts with macrophages, natural killer (NK) cells, and NKT cells to stimulate innate immunity and macrophage activation. Symptomatic infection is variable, and often includes fever, malaise, headache, myalgia, leukopenia, and thrombocytopenia [9, 17]. Several models of HGA exist, including infection of mice, which do not have clinical signs but develop histopathology characteristics of human and equine infections, and infection of horses, which nearly precisely mimics human infection, including the spectrum of clinical severity [3, 15]. Thus, clinical diversity might vary with antigens expressed among circulating natural strains. NKT cells are a unique subset of T lymphocytes that express a semi-invariant T-cell receptor (TCR) and markers of NK cells. NKT cells regulate immune cells, such as T, NK, and dendritic cells because of their capacity to rapidly release large amounts of interferon-γ (IFN-γ) upon activation [6, 12]. NKT-derived IFN-γ plays an important role in both innate and acquired immunity [2, 21, 23] and the cells are important regulators of immune responses in many diseases, including antimicrobial immune responses and Th1/Th2 differentiation [13, 18].

We recently reported that NKT cells, as defined by NK1.1/TCR expression in splenocytes, had a significant reduction among *A. phagocytophilum*-infected animals. This result showed that the early loss of NKT cells are correlated with IFN-γ production and the induction of tissue histopathology in some infection models [12, 13, 22]. In this study, we examined that NKT cells are the major source of IFN-γ that drive innate immune response and proinflammatory histopathology with *A. phagocytophilum* infection.

Materials and Methods

Experimental animals

Female BABL/c (wild-type controls) and NKT knockout (Jα18-/-) mice on the BABL/c background

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that were 6 weeks old were purchased from The Jackson Laboratory (USA). All animals were maintained and used in strict accordance with the guidelines issued by the Johns Hopkins University School of Medicine for animal care.

A. phagocytophilum culture and infection of mice

A. phagocytophilum strain Webster^T was maintained in RPMI 1640 medium supplemented with 5% FBS and 2 mM L-glutamine until >90% of the cells contained morulae. Cultures were coordinated so that low (passage 7-8) materials were available for inoculation. On the day of inoculation, low (p8) passage *A. phagocytophilum*-infected HL-60 cells were centrifuged ($200 \times g$, 10 min) to concentrate the cells, and then each cell pellet was resuspended in sterile PBS for inoculation. Challenge was performed by intraperitoneal injection of 1 ml (10^6 cells/ml) containing either heavily infected (> 90% of cells infected) HL-60 cells.

Histopathologic examination

Three mice of each group (Jα18-/- and wild-type; WT mice) were necropsied on day 0 (1 h after inoculation), 4 and 7. The liver was harvested from each mouse, fixed in zinc fixation solution (BD Pharmingen, USA) and embedded in paraffin for H&E staining. Hepatic histopathologic changes in infected Jα18-/- and WT mice were assessed and ranked for severity, focusing on the size, density of inflammatory lesions, the degree of necrosis and/or apoptosis, and the number of inflammatory foci. All evaluations were performed by investigators blinded to the identity of mouse treatment status and by two microscopists to ensure the fidelity of ranking. Statistical analysis was carried out using one-sided nonparametric statistical tests (Mann-Whitney and Kruskall-Wallis tests) to compare median ranks of groups; p values of < 0.05 were considered significant. To normalize for pathology, the median rank for the mock-infected group at each time was subtracted from the rank of each individual infected mouse. Normalized results were reranked to establish continuous variables and to determine significant differences in hepatic histopathology between infected Jα18-/- and WT mice.

Cytokine analysis

Levels of cytokines in the plasma were determined using mouse Bio-Plex Cytokine Assay kit for Luminex 100 (Bio-Rad Laboratories, USA) according to the manufacturer's recommendations. Briefly, plasma samples and a suspension of capture antibody-conjugated beads were mixed in plate wells and incubated for 30 min. Plates were washed and reacted with biotinylated detection antibodies in the dark for 30 min. After washing, streptavidin-phycoerythrin was added to the wells and the incubation was continued for additional 10 min. Finally, the beads were washed, resuspended with assay buffer, and analyzed on the Luminex 100^{TM} platform. All samples were measured in duplicates. The following analytes were determined: Interleukin-10, 12 and IFN-γ. Statistical significance was determined by application of one-sided Student's t tests and p values of < 0.05 were considered significant.

Results

NKT cell deficiency abrogates hepatic histopathology after _A. phagocytophilum_ infection.

To identify a role for NKT cells in *A. phagocytophilum* infection model, we examined the host defenses and the pathogenesis of anaplasmosis in Jα18-/- mice. The histological analysis of liver showed very less pathological changes in Jα18-/- mice. *A. phagocytophilum*-infected WT mice had more severe hepatic lesions and a larger number of apoptotic cells (Fig. 1). WT mice developed hepatic inflammation on day 4, reducing by day 7. However, at each interval (4.5 h, d4 and d7 post-infection; pi), Jα18-/- mice had less inflammation than WT controls (Fig. 2). When examined over d4 or all d7 pi, the histopathologic severity of infected Jα18-/- was less than in WT mice ($p = 0.048$, d0-5 and $p = 0.031$, d0-7; Mann-Whitney U-test). These data demonstrate that NKT cells are major components of immunopathology in the HGA mouse model.

NKT cells mediate the development of type 1 T cell responses and influence the adaptive immune responses.

The cytokine pattern of NKT cells in the innate phase during *A. phagocytophilum* infection may contribute to the activation of the major T cell lineages, $CD8^+$ and $CD4^+$ T cells, thus influencing the development of type 1 or type 2 immune responses. To further confirm the effect of NKT on *A. phagocytophilum*-driven cytokine production, we examined the cytokine level by plasma in infected mice. WT mice displayed

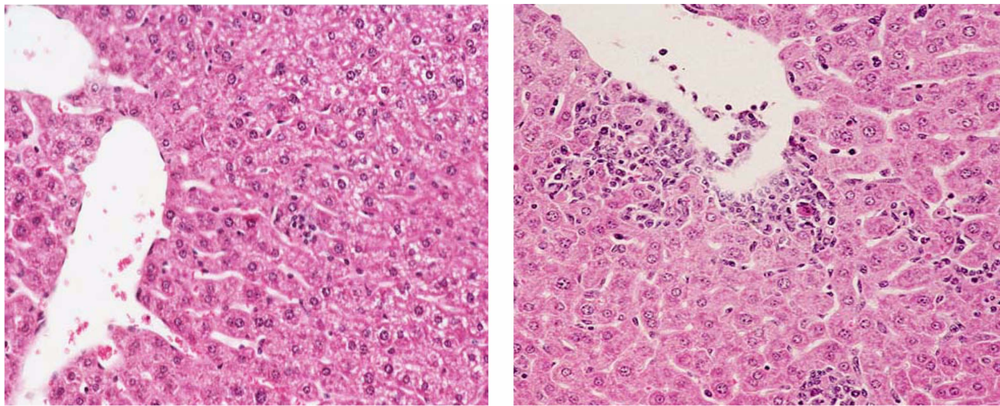

Fig. 1. Hepatic histopathology in *Anaplasma* (*A.*) *phagocytophilum*-infected BABL/c WT (right panel) and Jα18-/- mice (left panel). Note the larger inflammatory lesions and apoptotic cells (right panel). The left panel shows less inflammatory lesions. The preparations were stained with hematoxylin and eosin.

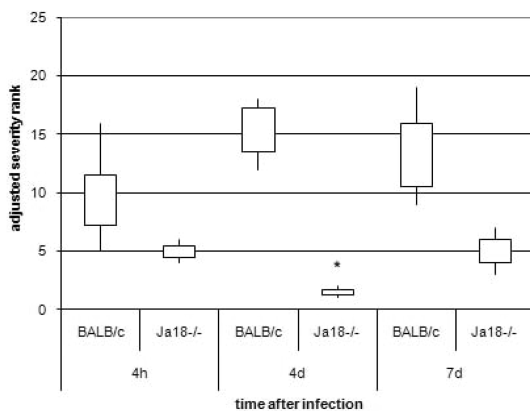

Fig. 2. Comparative hepatic histopathology among *A. phagocytophilum*-infected BABL/c WT and Jα18-/- mice at the time of infection through 7 days post-infection. The data represent the results of three independent experiments with three mice per group. Higher ranks correspond to higher degrees of severity. The bars indicate the means and the error bars indicate the maximum and minimum ranks for replicate mice in each group at each time. An asterisk indicates that the p value is < 0.05, as determined by a Wilcoxon signed-rank test comparing median ranks after reranking at each time.

a type 1 cytokine patterns, characterized by higher levels of IFN-γ and IL-12 compared with the Jα18-/- mice (Figs. 3A and B). NK cells prominently secreted IFN-γ upon *A. phagocytophilum* stimulation, as previously suggested [9]. In addition, they also showed significantly lower level of type 2 cytokine, IL-10. Conversely, Jα18-/- mice exhibited a type 2 cytokine with significantly higher level of IL-10 (Fig. 3C), but lower levels of both IFN-γ and IL-12 on day 7 pi. These results suggest that NKT cells play an important role in regulating the production of Th1 responses.

Discussion

The innate immune system is characterized by rapid responses to pathogens and these are mediated mainly by PMN, MØ, DC, and NK cells [10, 23]. Regarding innate immunity, our previous studies demonstrated the critical role of IFN-γ in induction of severe inflammatory histopathology in a murine model of HGA. *A. phagocytophilum* infection elicit a cell-mediated immune response that results in the production of IFN-γ and up-regulation of other proinflammatory and cytotoxic responses effective at killing intracellular pathogens.

In this study, we demonstrated that *A. phagocytophilum*-infected canonical NKT cell-deficient mice (Jα18-/-) do not develop histopathologic lesions on days 0-7, unlike WT controls. The differences in these parameters between the WT and Jα18-/- mice showed that WT mice exhibit tissue injury and inflammatory histopathology following *A. phagocytophilum* infection. The histopathology in the infected Jα18-/- mice are completely abrogated despite a marked increase in the pathogen load. These observations provide that innate immunity, including that resulting from NK and NKT cells activation, plays an important role in the early histopathology in the murine of HGA.

To determine the role of NKT cells in the pathogenesis of HGA, we examined the levels of the

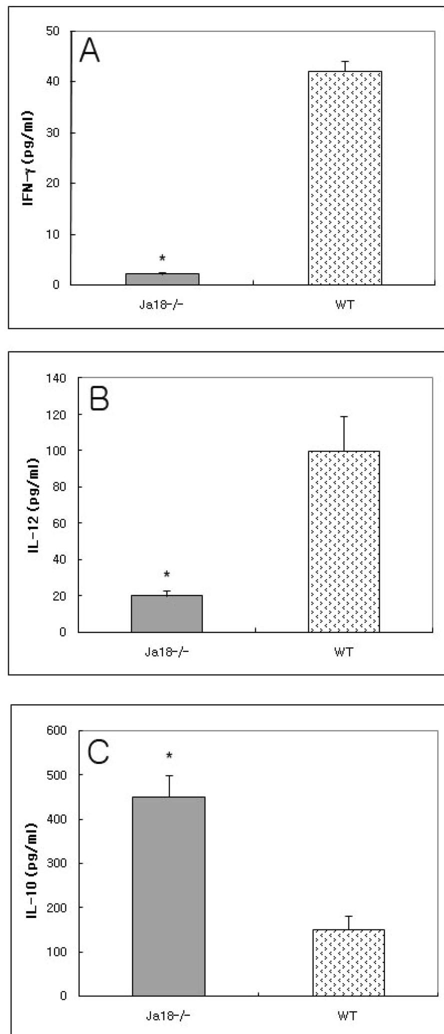

Fig. 3. Cytokines (IFN-γ, IL-12 and IL-10) concentrations in Jα18-/- mice and BABL/c WT controls. At day 7, Jα18-/- mice had significantly lower levels of IFN-γ (A) and IL-12 (B) than infected WT mice. The plasma level of IL-10 (C) was not significantly different for the two groups. Similar results were obtained in three independent experiments. The error bars indicate standard errors of the means. An asterisk indicates that the *p* values less than 0.05 were considered significant.

plasma cytokines in WT and Jα18-/- mice. Early after infection, Jα18-/- mice produced significantly less IFN-γ compared with WT controls. These data are consistent other studies, suggesting that despite rapid induction and the ability of the NK cell to produce IFN-γ, it was a secondary event that depends on IFN-γ release by NKT cells [4]. In addition, lower levels of plasma IL-12 were observed in Jα18-/- mice than in WT mice, indicating that IL-12 has been shown as essential for the activation of NKT cells and their subsequent production of IFN-γ during infection. An interesting finding is that the absence of NKT cells did not influence the high plasma levels of IL-10 (Fig. 3C), which were also observed in infected WT mice. The presence of high levels of IL-10 in Jα18-/- mice suggests that IL-10 is linked to fatal disease. However, the source of IL-10 appears to be independent of NKT cell activation.

Despite the importance of NKT cells, the first cells in the innate immune system to be activated during an infection are DC and other APC, and this is often mediated by Toll receptors that sense bacterial products, leading to activation of the transcription factor NF-κB and the production of proinflammatory cytokines such as IL-12. We previously demonstrated macrophage activation via Toll-like receptor 2 when animals were exposed to *A. phagocytophilum* infection [8], and NK and NKT cells are well known to become activated via this pathogen-associated molecular pattern receptor [5, 19]. Bendelac's group recently described Gram negative bacteria lacking LPS but containing glycolipids reactive with NKT cells that drive IFN-γ release and effect pathogen control [1]. However, this group also studied NKT-deficient mice infected with the non-human pathogen, *Ehrlichia muris*, closely related to *A. phagocytophilum* and also controlled by IFN-γ [16]. Thus, it is likely that NKT cells are activated by certain epitopes specific for pathogens or even strains of the same pathogen species. This antigenic variability may account for differential functional roles of NKT cells in infectious diseases [12-14, 20]. To address this issue microbial pathogens and identification of antigenic effects on NKT may throw more light on immunological activity exerted by NKT cells.

In conclusion, the results of the present study unveiled the important role of NKT cells in host protection against *A. phagocytophilum* infection. Moreover, these findings suggest that NKT cells may be the active early components that bridge innate and adaptive immune responses. Thus, further studies will greatly enhance our understanding on the fact that the different functional NKT subsets may be activated depending on the variability of NKT-mediated immunological outcomes.

Acknowledgments

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