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c-Jun NH₂-Terminal Kinase 2 Inhibits Gamma Interferon Production during *Anaplasma phagocytophilum* Infection[∇]

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Gamma interferon (IFN- γ) plays a critical role in the early eradication of *Anaplasma phagocytophilum*. However, the mechanisms that regulate IFN- γ production upon infection remain poorly understood. Here we show that c-Jun NH₂-terminal kinase 2 (JNK2) inhibits IFN- γ production during *A. phagocytophilum* infection. *jnk2*-null mice were more refractory to infection with *A. phagocytophilum* and produced increased levels of IFN- γ after challenge with the pathogen. The resistance of *jnk2*-null mice to *A. phagocytophilum* infection was due to elevated levels of IFN- γ secreted by conventional and natural killer (NK) T cells. The administration of α -galactosylceramide, a strong NK T-cell agonist, increased IFN- γ release and protected mice from *A. phagocytophilum*, further demonstrating the inhibitory effect of JNK2 on IFN- γ production. Collectively, these findings provide strong evidence that JNK2 is an important regulatory protein for IFN- γ secretion upon challenge with *A. phagocytophilum*.

Anaplasma phagocytophilum infection causes human granulocytic anaplasmosis (HGA), and the organism can infect neutrophils, neutrophil precursors, and endothelial cells (13, 26). HGA is often asymptomatic or mild, with fevers, headache, myalgia, thrombocytopenia, or neutropenia. Severe complications may include septic shock, respiratory distress syndrome, rhabdomyolysis, myocarditis, acute renal failure, hemorrhage, neuropathy, hepatitis, and opportunistic infections (2, 12, 13).

The cytokine gamma interferon (IFN- γ) contributes to the eradication of *A. phagocytophilum* (1, 21, 24, 30, 31, 38). IFN- γ gene-null mice have a markedly elevated *A. phagocytophilum* load compared to that of wild-type mice during the early phase of infection. The mechanisms, however, that regulate IFN- γ production upon infection remain poorly understood. We hypothesized that the c-Jun NH₂-terminal kinase (JNK) pathway could be important for the regulation of IFN- γ production because the JNK pathway is important for T helper 1 differentiation (11, 33, 40). We found that c-Jun NH₂-terminal kinase 2 (JNK2) inhibited IFN- γ production during *A. phagocytophilum* infection. This study represents the first step toward identifying inhibitory proteins that modulate IFN- γ secretion upon *A. phagocytophilum* infection.

MATERIALS AND METHODS

Bacterial strain, lipid antigens, and mouse strains. The NCH-1 isolate of *A. phagocytophilum*, recovered from a patient with HGA, was used throughout these studies (36). *Sphingomonas* sp. lipids PBS 57 and PBS 74 were described previously (23). α -Galactosylceramide (α -GalCer) and globotrihexosylceramide (GB3) were purchased from Alexis Biochemicals (Switzerland). C57BL/6, BALB/c, *cd1d*-null, and *rag1*-null mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Cd1d*-null mice were backcrossed onto the BALB/c background for at least 10 generations. *jnk2*-null mice, described previously (40), were backcrossed onto the C57BL/6 background for at least six generations. All mice used for in vivo infection were 4 to 12 weeks of age, sex matched, and maintained under specific pathogen-free conditions at Yale University School of Medicine. The experiments were done in accordance with the Yale University institutional animal care and university committee guidelines.

***A. phagocytophilum* infection and detection methods.** *A. phagocytophilum* was cultured in HL-60 cells (catalog no. 240-CCL; American Type Culture Collection, Manassas, VA) grown in Iscove's modified Dulbecco's medium (Gibco BRL/Life Technologies, Grand Island, NY) supplemented with 20% fetal calf serum at 37°C with 5% CO₂ (19). Cells were sustained with medium replacement once a week. *A. phagocytophilum*-infected cells were maintained as described previously with an equal volume of uninfected cells added to infected cells and diluted 1:5 with fresh medium (37). Cell-free *A. phagocytophilum* was collected from 95% *A. phagocytophilum*-infected HL-60 cells. Infected cells were centrifuged for 10 min at 4,000 \times g. The cell pellet was resuspended in culture medium and lysed by six passages through a 25-gauge needle, followed by six passages through a 27-gauge needle. The cell lysate was centrifuged at 1,200 \times g for 3 min, and the supernatant was used for infection. *A. phagocytophilum* was separated from host cells, heat killed for 45 min at 65°C, and used for stimulation assays.

Blood from *rag1*-null mice chronically infected with the *A. phagocytophilum* NCH-1 strain (20 to 25% neutrophil infection) was used to inoculate inbred immunocompetent and gene-deficient mice. *A. phagocytophilum* infection in *rag1*-null mice was determined by using the percentage of morulae-containing granulocytes (*A. phagocytophilum* aggregates) examined in a peripheral blood smear. Slides were stained with Diff-Quick (Baxter Healthcare Corporation, Miami, FL) and examined for morulae by using light microscopy (1).

Cytokines and antibodies. Recombinant murine interleukin-12 (IL-12)p40/p70 and IFN- γ were purchased from BD Pharmingen (San Diego, CA). IL-18 and anti-IL-18 antibodies for enzyme-linked immunosorbent assay (ELISA) were purchased from MBL (Woburn, MA). Anti-mouse monoclonal antibodies for

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cytokine and surface labeling were purchased from BD Pharmingen, as follows: purified anti-IL-12p40/p70 (C15.6, rat immunoglobulin G1 [IgG1]); purified anti-IFN- γ (R4-6A2, rat IgG1); fluorescein isothiocyanate-conjugated anti-CD69 (HL2F3, Armenian hamster IgG1); phycoerythrin-Cy5 (PE-Cy5)-conjugated anti-CD4 (H129.19, rat IgG2a), PE-Cy7-conjugated anti-CD8 (SK1, mouse IgG1); PE-conjugated anti-IFN- γ (XMG1.2, rat IgG1); PE-Cy7-conjugated anti-IFN- γ (XMG1.2, rat IgG1); and fluorescein isothiocyanate-conjugated anti-CD8 (SK1, mouse IgG1).

T-cell hybridoma assay. The NK T hybridoma cell line V α 14-J α 18 DN32.D3 was described previously (25). DN32.D3 cells (2.5×10^5 cells) were cocultivated with 2.5×10^5 cells from either wild-type or *jnk2*-null bone marrow-derived dendritic cells (BMDC) as previously described (25) in the presence of stimulants for 18 h. IL-2 release in cultured supernatants was measured using CTLL-2 indicator cells (3). BMDC were prepared from bone marrow cells that were collected by removing the femur bones of mice, cutting off the ends of the bones, and flushing out the bone marrow with RPMI 1640 medium injected with a syringe. The pooled cells were harvested by centrifugation at $1,600 \times g$ for 10 min and resuspended in 1 ml of red blood cell lysis buffer (Sigma, St Louis, MO) for 5 min at room temperature. These cells were washed in RPMI 1640 medium and cultured in RPMI 1640 medium containing 500 U of murine recombinant granulocyte-macrophage colony-stimulating factor (mGM-CSF)/ml for 6 days prior to analysis. BMDC were collected in ice-cold phosphate-buffered saline and used for T-cell hybridoma assays.

***A. phagocytophilum* restimulation assays and flow cytometry analysis.** Wild-type and *jnk2*-null mice were euthanized, and spleens and livers were removed 6 days post-*A. phagocytophilum* infection. Following spleen removal, the splenocytes from wild-type and *jnk2*-null mice were restimulated with *A. phagocytophilum* for 18 h and surface stained with anti-CD4 and anti-CD8. For the intracellular staining, brefeldin A was added 3 h before harvesting. For liver cell isolation, age- and sex-matched C57BL/6 and *jnk2*-null mouse livers were perfused, and the differential centrifugation method using colloidal silica particles coated with polyvinylpyrrolidone (Percoll) was used to isolate mononuclear cells (7). CD1d- α -GalCer tetramers were generated and used as described previously (5). Flow cytometry was performed as described previously (25) using a FACScalibur flow cytometer (BD Biosciences). Data were analyzed using Cell Quest Pro software (BD Biosciences).

Determination of cytokine levels in infected mice. Retroorbital bleeding of the wild-type and the gene-deficient mice was performed at indicated time points. Sera from animals in each group were pooled, and cytokine levels were assessed using sandwich ELISA.

Quantification of *A. phagocytophilum* in the peripheral blood of mice. To quantify the *A. phagocytophilum* load in the peripheral blood, 100 μ l of anticoagulated peripheral blood from gene-deficient mice and from wild-type mice was incubated with 900 μ l of erythrocyte lysis buffer (Sigma, St. Louis, MO) at room temperature for 20 min. DNA was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA), according to the manufacturer's recommendation. DNA samples were mixed with an iQ SYBR green I supermix (Bio-Rad, Hercules, CA) in an iCycler thermal cycler (Bio-Rad, Hercules, CA). Quantitative reverse transcription (qRT)-PCR conditions were performed as previously described (29). DNA levels were normalized to that of the mouse β -actin gene (GenBank accession number X03672). The *A. phagocytophilum* 16S rRNA (GenBank accession number M73224) gene was then quantified.

Statistical analysis. *P* values were calculated by using experimental and control data, using the nonpaired Student *t* test. Statistical significance was set at a *P* value of <0.05 .

RESULTS

JNK2 inhibits IFN- γ production and promotes resistance to *A. phagocytophilum* infection. To define the precise role of JNK2 in IFN- γ production during *A. phagocytophilum* infection, we stimulated wild-type and *jnk2*-null spleen cells with heat-killed *A. phagocytophilum*, a potent IFN- γ inducer. We noticed increased IFN- γ release from *jnk2*-null cells upon *A. phagocytophilum* stimulation compared to that from control cells (Fig. 1A). IL-4 secretion was not evident upon *A. phagocytophilum* stimulation in both the wild-type and the *jnk2*-null cells, suggesting that *A. phagocytophilum* is a strict Th1 inducer (Fig. 1B). We then infected *jnk2*-null mice with *A. phagocytophilum* to determine whether JNK2 plays an inhibitory role in

IFN- γ production in vivo. The levels of IFN- γ in murine peripheral blood increased dramatically after 3 to 4 days following infection and returned to baseline levels at day 8 (Fig. 1C). IFN- γ secretion was twofold higher in *jnk2*-null mice at days 3 and 4 than in wild-type mice (Fig. 1C).

We then investigated whether IL-12 and IL-18 contribute to IFN- γ secretion upon *A. phagocytophilum* infection. IL-12 and IL-18 have a synergistic effect on IFN- γ secretion (10, 18). Furthermore, both cytokines have been shown to be important for the eradication of *A. phagocytophilum* from the host (30, 31). IL-18 secretion was significantly higher in *jnk2*-null mice, suggesting that there is an association with IFN- γ production in vivo (Fig. 1D). IL-18, however, may also play a role in *A. phagocytophilum* infection that is IFN- γ independent, because IL-18 was also produced after IFN- γ production and returned to baseline levels (between days 8 and 12). On the other hand, IL-12 levels in the peripheral blood of *jnk2*-null mice were slightly reduced compared to that of wild-type mice (Fig. 1E).

IFN- γ plays an important role in the host's defense against *A. phagocytophilum* infection (1, 21, 24, 30, 31, 38). Therefore, we determined the pathogen's burden in wild-type and *jnk2*-null mice after infection. *A. phagocytophilum* infection in the peripheral blood of *jnk2*-null mice was decreased 10- to 16-fold at days 4 and 8 postinfection (Fig. 1F). We did not observe any differences between the bacterial loads of wild-type and *jnk2*-null mice at day 10 when both mouse groups were clearing the infection. Taken together, our results suggest that JNK2 has an inhibitory role in IFN- γ production upon *A. phagocytophilum* stimulation.

JNK2 ablation results in greater IFN- γ production by CD4⁺ and CD8⁺ T cells upon *A. phagocytophilum* stimulation. Increased levels of IFN- γ in the sera of *jnk2*-null mice may be due to elevated IFN- γ production by T cells. To determine whether JNK2 regulates the production of IFN- γ in CD4⁺ and CD8⁺ T cells, intracellular staining was used. We performed our analysis with CD4⁺ and CD8⁺ T cells at day 6 postinfection because the IFN- γ response was more likely to be triggered by CD4⁺ and CD8⁺ T cells at this stage. Overall, CD4⁺ and CD8⁺ T cells produced low levels of IFN- γ upon infection, and nonrestimulated CD4⁺ and CD8⁺ T cells from wild-type and *jnk2*-null mice had only minimal IFN- γ production (Fig. 2A and B). However, IFN- γ levels were twofold higher in restimulated CD4⁺ and CD8⁺ T cells from *jnk2*-null mice than in those from wild-type animals. Taken together, these results strongly suggest that JNK2 inhibits IFN- γ production by CD4⁺ and CD8⁺ T cells upon *A. phagocytophilum* stimulation.

JNK2 inhibits IFN- γ production by NK T cells. We examined the role of natural killer (NK) T cells in immunity to *A. phagocytophilum* infection because previous studies have demonstrated that CD1d-restricted NK T cells contribute to the defense against *Ehrlichia muris* (25), an organism closely related to *A. phagocytophilum*. Choi et al. (8) have also suggested that NK T cells are activated during infection. We first assessed the expression of JNK2 in tetramer-sorted CD1d-restricted NK T cells from heterozygotes *jnk2* and *jnk2*-null mice cocultivated with wild-type BMDC stimulated with heat-killed *A. phagocytophilum*. The analysis revealed that activated CD1d-restricted NK T cells from heterozygous *jnk2* mice were able to phosphorylate JNK (Fig. 3A). On the other hand, *jnk2*-null mice did not show any JNK phosphorylation when NK T cells

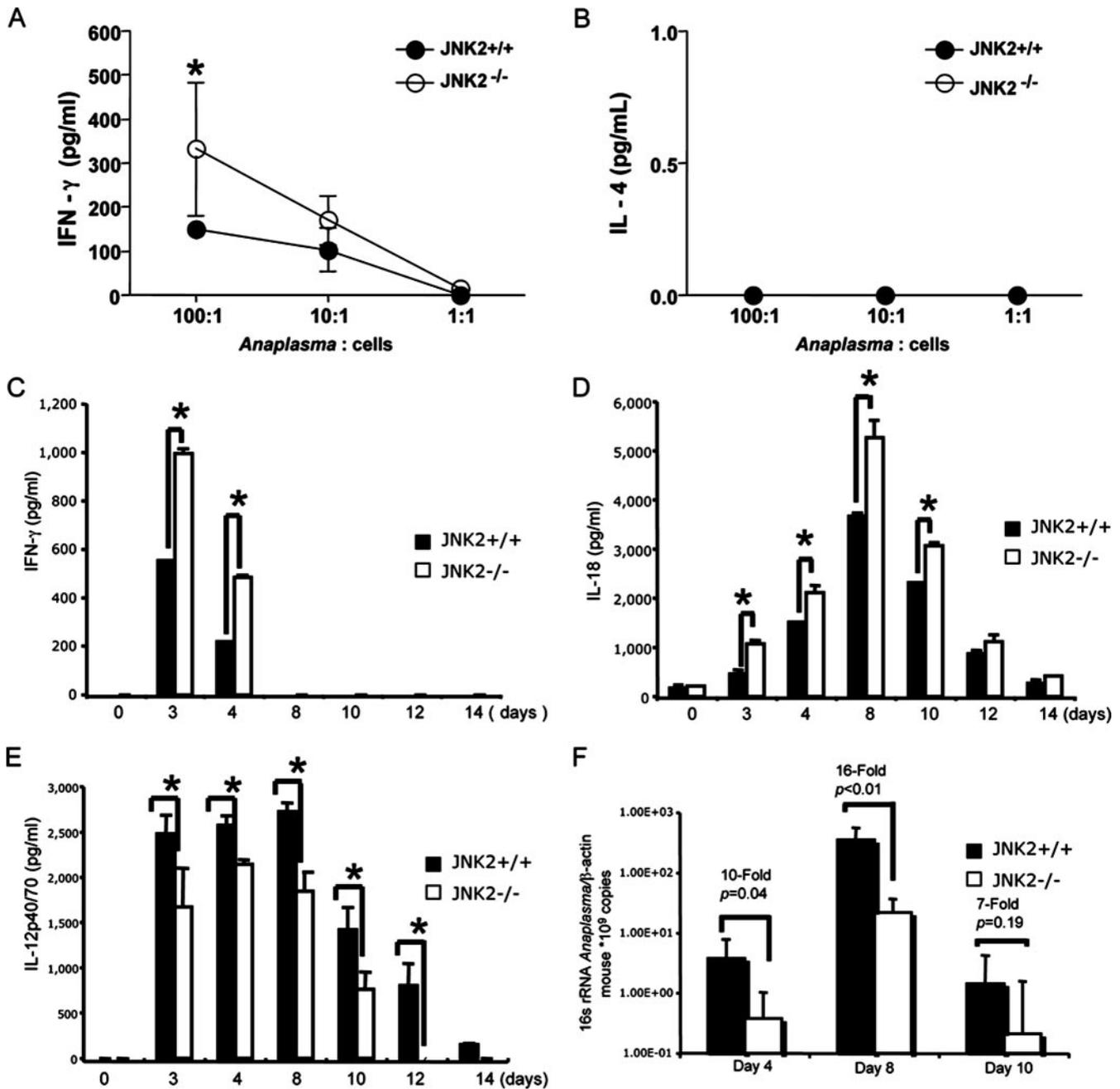


FIG. 1. JNK2 deficiency increases IFN- γ levels in peripheral blood and decreases murine susceptibility to *A. phagocytophilum* infection. Wild-type and *jnk2*-null mice were euthanized, and spleens were removed 6 days post-*A. phagocytophilum* infection. Wild-type ($n = 5$) and *jnk2*-null ($n = 5$) mouse spleen cells (10^6 cells) were restimulated with heat-killed *A. phagocytophilum* (at multiplicities of infection of 100, 10, and 1 PFU/cell) for 18 h, and levels of IFN- γ (A) and IL-4 (B) were measured by ELISA. Serum concentrations of (C) IFN- γ , (D) IL-18, and (E) IL-12p40/p70 were measured by ELISA in wild-type and *jnk2*-null mice infected with *A. phagocytophilum* ($n = 6$ per group). (F) Wild-type ($n = 10$) and *jnk2*-null ($n = 11$) mice were infected with *A. phagocytophilum*, and the bacterial load was measured in peripheral blood using qRT-PCR. Experiments were repeated twice, and the results for one representative experiment are shown. Data represent the means \pm standard deviations. The asterisks denote statistical significance. Statistical significance was calculated using the unpaired Student's *t* test ($P < 0.05$).

were activated. Nonphosphorylated levels of JNK proteins were expressed at levels that were comparable in both groups of mice. These results strongly suggest that JNK proteins play a critical role in signaling processes during NK T-cell activation.

We then evaluated the NK T-cell response to *A. phagocyto-*

philum infection by using mice that do not carry functional CD1d-restricted NK T cells. *Cd1d*-null mice had a higher pathogen load at day 2 than the wild-type animals (Fig. 3B), which is consistent with an early role of NK T cells in *A. phagocytophilum* immunity. The reduced capacity to eradicate *A. phagocytophilum* correlated with lower levels of IFN- γ in the

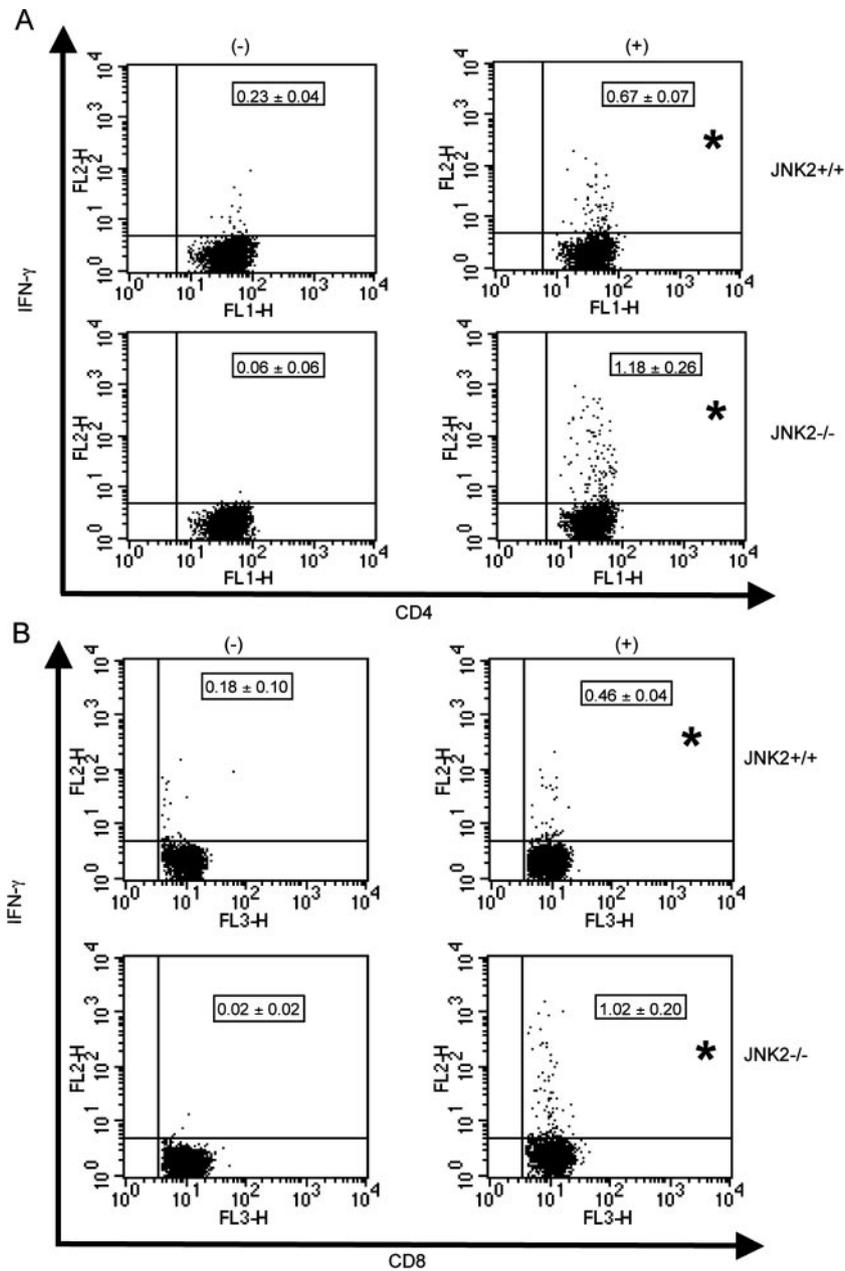


FIG. 2. JNK2 negatively regulates IFN- γ production in CD4⁺ and CD8⁺ T cells after *A. phagocytophilum* restimulation. Wild-type and *jnk2*-null mice ($n = 6$ per group) were euthanized, and spleens were removed 6 days post-*A. phagocytophilum* infection. Splenocytes (5×10^6 cells) from wild-type and *jnk2*-null mice were cultured ex vivo for 18 h in the presence or absence ($n = 3$ per group) of heat-killed *A. phagocytophilum* (10^7 bacterial cells), and CD4⁺ and CD8⁺ T-cell production of intracellular IFN- γ was determined. Numbers in the upper-right quadrants are the percentages of IFN- γ -producing cells in (A) CD4⁺ and (B) CD8⁺ T cells. The signs (-) and (+) indicate the presence or absence of heat-killed *A. phagocytophilum*. Data represent the means \pm standard deviations. The asterisks denote statistical significance. Statistical significance was calculated using the unpaired Student's *t* test ($P < 0.05$).

peripheral blood of *cd1d*-null mice (Fig. 3C). We did not detect differences in IFN- γ secretion between the wild-type and the *cd1d*-null mice at days 5 and 8, which was consistent with similar *A. phagocytophilum* loads in the peripheral blood samples of both mouse groups (Fig. 3B).

To further support the role of NK T cells in *A. phagocytophilum* clearance, the NK T hybridoma cell line DN32.D3 was cocultivated with BMDC from heterozygote and *cd1d*-null mice in the presence of heat-killed *A. phagocytophilum*. The

cd1d-null cell culture showed reduced levels of IL-2 after *A. phagocytophilum* stimulation (Fig. 3D). Similar results were obtained after the culture was stimulated with the NK T-cell agonist α -GalCer. These results obtained with BALB/c *cd1d*-null mice show that NK T cells play a direct role in the early control of the *A. phagocytophilum* burden.

We then examined whether in vivo stimulation with α -GalCer would promote enhanced IFN- γ production in *jnk2*-null mice. α -GalCer has a potent stimulatory effect on NK T cells

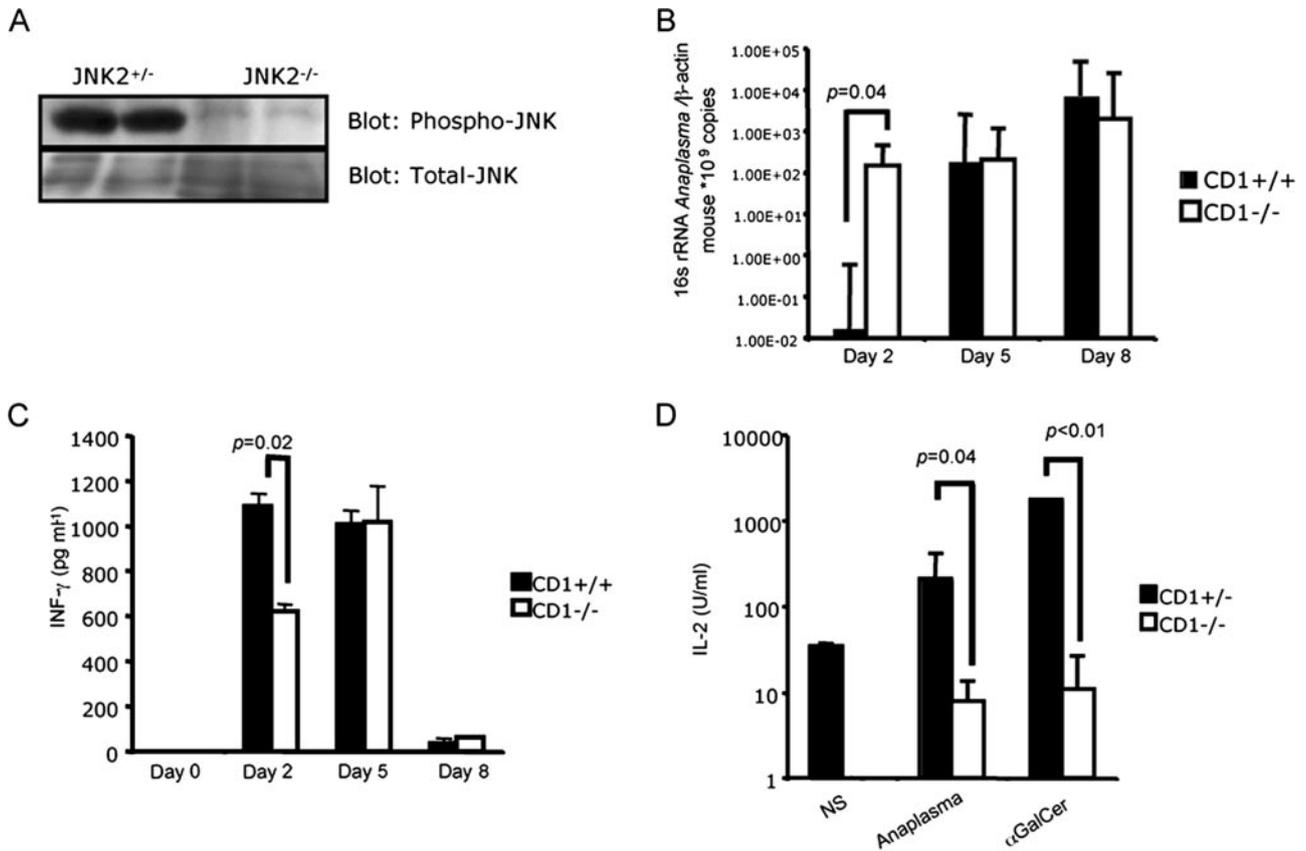


FIG. 3. NK T cells play an important role in early *A. phagocytophilum* immunity. (A) Tetramer-sorted CD1d-restricted NK T cells (2.5×10^5 cells) from heterozygous *jnk2* ($n = 4$) and *jnk2*-null ($n = 4$) mice were cocultivated with wild-type BMDC (2.5×10^5 cells) in the presence of heat-killed *A. phagocytophilum* (at a multiplicity of infection of 100 PFU/cell) for 20 min. Cells were then lysed, and proteins (20 μ g) were normalized and subjected to immunoblotting using the phospho (Thr-183/Tyr-185)-JNK and total JNK antibodies as indicated. (B) Wild-type and *cd1d*-null mice were infected with *A. phagocytophilum*, and the bacterial load was measured in peripheral blood using qRT-PCR. (C) Serum concentrations of IFN- γ in 6- to 12-week-old wild-type ($n = 5$) and *cd1d*-null ($n = 5$) mice were measured by ELISA at the indicated time points. (D) The IL-2 response of the V α 14 NK T hybridoma DN32.D3 (2.5×10^5 cells) cell line cocultivated with heterozygous *cd1d* or *cd1d*-null dendritic cells (2.5×10^5 cells) in the presence of 10^7 heat-killed *A. phagocytophilum* or α -GalCer (100 ng/ml) cells for 18 h is indicated; NS, no *A. phagocytophilum*. Data represent the means \pm standard deviations. Statistical significance was calculated using the unpaired Student's *t* test ($P < 0.05$).

and has been used extensively as an NK T-cell stimulant in vivo (15, 27, 35). α -GalCer stimulation increased IFN- γ in the peripheral blood samples of *jnk2*-null mice at 24 h postinjection (Fig. 4A). On the other hand, IL-4 production peaked at 2 h postinjection in the wild-type genotype and was increased com-

pared to that in the *jnk2*-null mice (Fig. 4B). The molecule GB3 was used as a negative control and did not show any stimulatory effect postinjection. Thus, our results suggest that JNK2 inhibits IFN- γ production by NK T cells.

Next, we prepared BMDM from wild-type and *jnk2*-null

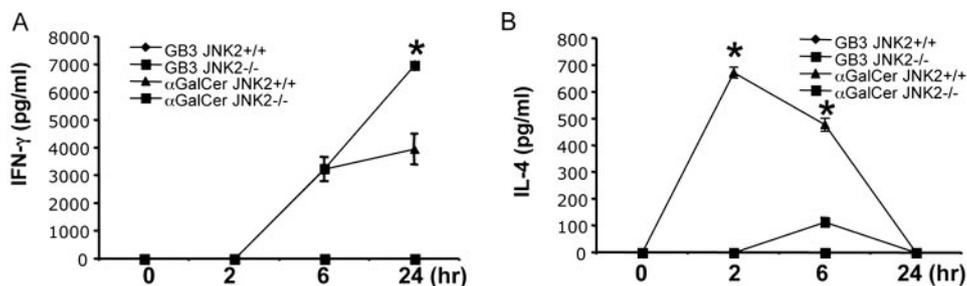


FIG. 4. IFN- γ response in *jnk2*-null mice is increased upon glycolipid stimulation. Wild-type ($n = 10$) and *jnk2*-null ($n = 10$) mice were stimulated with an intraperitoneal injection of α -GalCer (250 ng/mouse) or GB3 (250 ng/mouse), and levels of (A) IFN- γ and (B) IL-4 were determined at the indicated time points, using ELISA. Data represent the means \pm standard deviations. Statistical significance was calculated using the unpaired Student's *t* test ($P < 0.05$).

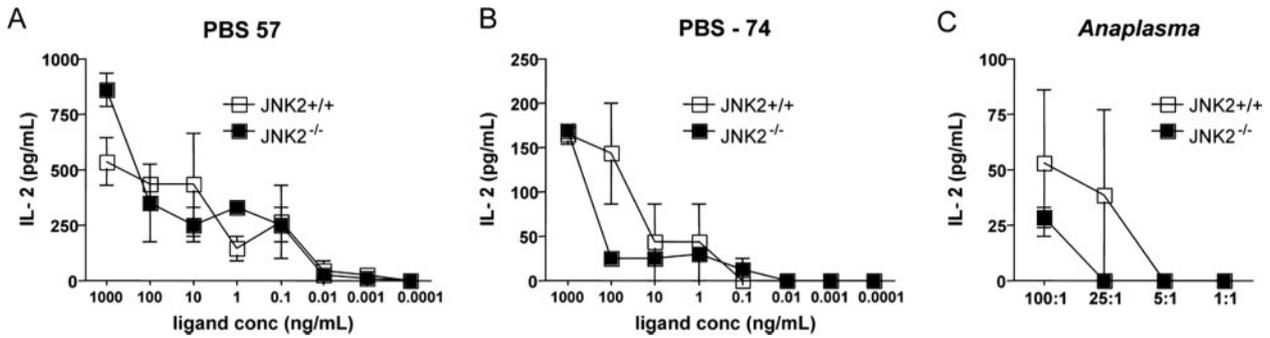


FIG. 5. JNK2 does not alter glycolipid presentation upon stimulation. BMDM (2.5×10^5) from wild-type ($n = 5$) and *jnk2*-null ($n = 5$) mice were cocultivated with the V α 14 NK T hybridoma DN32.D3 cell line (2.5×10^5 cells) in the presence of different concentrations of the NK T-cell agonists (A) PBS 57 and (B) PBS 74 and (C) heat-killed *A. phagocytophilum* (at multiplicities of infection from 100 to 1 PFU/cell) for 18 h, and the levels of the cytokine IL-2 were measured. The results of one representative experiment are shown. Data represent the means \pm standard deviations.

mice to determine whether the increased IFN- γ production by NK T cells observed for *jnk2*-null cells was due to enhanced lipid presentation. To test this hypothesis, we cocultivated the NK T hybridoma cell line DN32.D3 with BMDM from either the wild-type or the *jnk2*-null mice for 18 h in the presence of the NK T agonists PBS 57 and PBS 74 and measured IL-2

poststimulation. We did not detect differences between levels of IL-2 secretion by the NK T hybridoma cell line from either the wild-type or the *jnk2*-null cells, suggesting that JNK2 does not play a role in glycolipid presentation upon stimulation by BDMC (Fig. 5A and B). Similar results were obtained after stimulation with *A. phagocytophilum* (Fig. 5C). Taking these

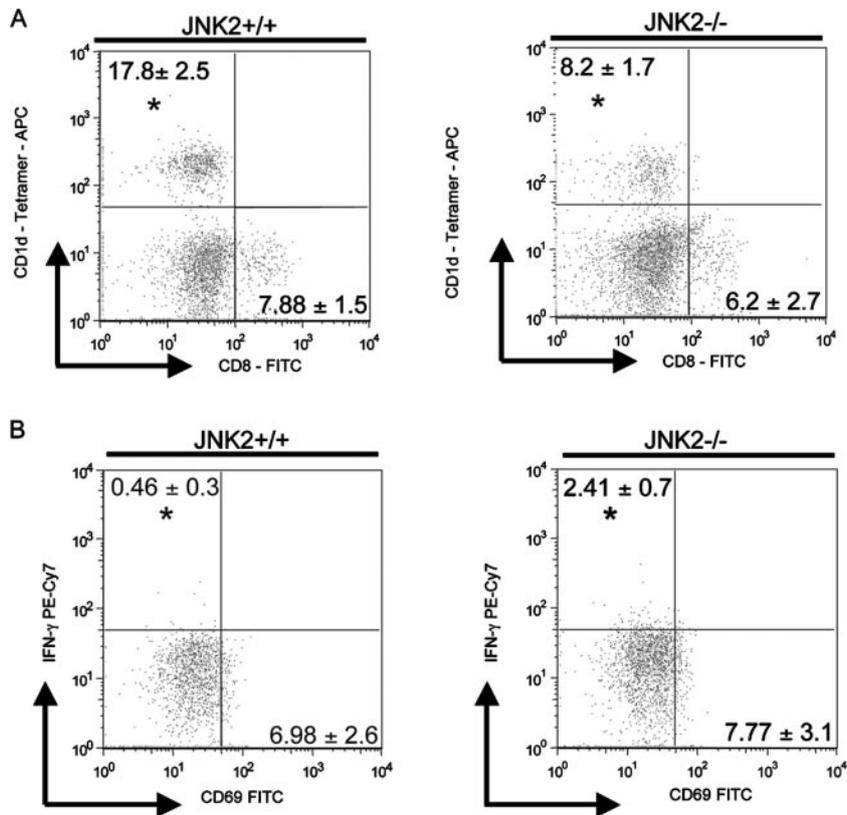


FIG. 6. JNK2 ablation increases NK T-cell-derived IFN- γ production in *jnk2*-null mice after *A. phagocytophilum* restimulation. (A) Wild-type and *jnk2*-null mice were euthanized, and liver cells were removed 6 days post-*A. phagocytophilum* infection. Liver cells (10^6 cells) from wild-type ($n = 16$) and *jnk2*-null ($n = 16$) mice were restimulated with 10^7 heat-killed *A. phagocytophilum* for 18 h, and mouse mononuclear liver cells were stained (tetramer $^+$ and CD8 $^+$). The percentage of cells in the V α 14 NK T-cell gate is indicated. APC, antigen-presenting cells. (B) V α 14 NK T restimulated liver cells from wild-type and *jnk2*-null mice were stained (tetramer $^+$ CD69 $^+$), and the levels of IFN- γ were determined. The results of one representative experiment are shown. Data represent the means \pm standard deviations. Statistical significance was calculated using the unpaired Student's *t* test ($P < 0.05$). FITC, fluorescein isothiocyanate.

findings together, we conclude that JNK2 directly controls IFN- γ production mediated by NK T cells, since lipid presentation is not affected.

***A. phagocytophilum* stimulation promotes increased IFN- γ production by CD1d-restricted NK T cells from *jnk2*-null mice.** We used *A. phagocytophilum* to investigate whether JNK2 has a repressive role in IFN- γ production mediated by CD1d-restricted NK T cells. Wild-type and *jnk2*-null mice were infected with *A. phagocytophilum* for 6 days, and mononuclear liver cells were harvested and restimulated for 18 h.

It has been recently demonstrated that activated NK T cells down-regulate the V α 14 NK T receptor after antigen stimulation and that the effector functions of activated V α 14 NK T cells, such as cytokine production, remain intact (16, 39). We tested *A. phagocytophilum* reactivity to the V α 14 invariant NK T-cell receptor using α -GalCer-CD1d tetramers. We noticed a twofold down-regulation of the V α 14 NK T receptor from *jnk2*-null cells after *A. phagocytophilum* restimulation, which suggested enhanced NK T-cell activation from these cells (Fig. 6A). Indeed, increased activation of NK T cells from *jnk2*-null mice was directly correlated with higher IFN- γ production (Fig. 6B). Collectively, our data demonstrate that JNK2 has an inhibitory role in IFN- γ production mediated by CD1d-restricted NK T cells.

Enhanced clearance of *A. phagocytophilum* by stimulated NK T cells. We reasoned that the activation of CD1d-restricted NK T cells could facilitate *A. phagocytophilum* clearance from the host. This hypothesis was supported by two main findings: (i) the clearance of the bacterium from *cd1d*-null mice was decreased at day 2 postinfection, and (ii) strong evidence indicated that NK T cells participate in *A. phagocytophilum* immunity. We therefore determined whether the activation of NK T cells by the CD1d-restricted lipid α -GalCer could result in the faster elimination of *A. phagocytophilum*. Wild-type and *jnk2*-null mice were given an intraperitoneal injection of α -GalCer and GB3, followed by infection with *A. phagocytophilum* 24 h later. GB3 was used as a negative control. We chose to infect mice with *A. phagocytophilum* 24 h postinjection because IFN- γ release peaks at 1 day poststimulation, as demonstrated previously (Fig. 4).

We compared the bacterial load and the level of IFN- γ release as a measure of protection against *A. phagocytophilum* infection. Overall, both the wild-type and the *jnk2*-null mice injected with GB3 showed more-than 1,000-fold more bacteria in the peripheral blood compared to mice that received the α -GalCer injection treatments at day 2 postinfection (Fig. 7A). The protective effect of α -GalCer during *A. phagocytophilum* infection decreased considerably at days 5 and 7. Similar bacterial loads among all treatment groups were observed at day 9 postinfection. The effect of α -GalCer injection in *jnk2*-null mice was more robust than in wild-type mice. Wild-type mice injected with α -GalCer carried 28- and 5.7-fold fewer bacteria at days 5 and 7, respectively, than those receiving the GB3 treatment. On the other hand, *jnk2*-null mice injected with α -GalCer carried 1,000- and 18-fold fewer bacteria at days 5 and 7, respectively, than the GB3-treated mice. Decreased levels of bacteria in the peripheral blood of wild-type and *jnk2*-null mice injected with α -GalCer were correlated with higher levels of IFN- γ release (Fig. 7B). As expected, *jnk2*-null mice injected with α -GalCer presented a robust IFN- γ re-

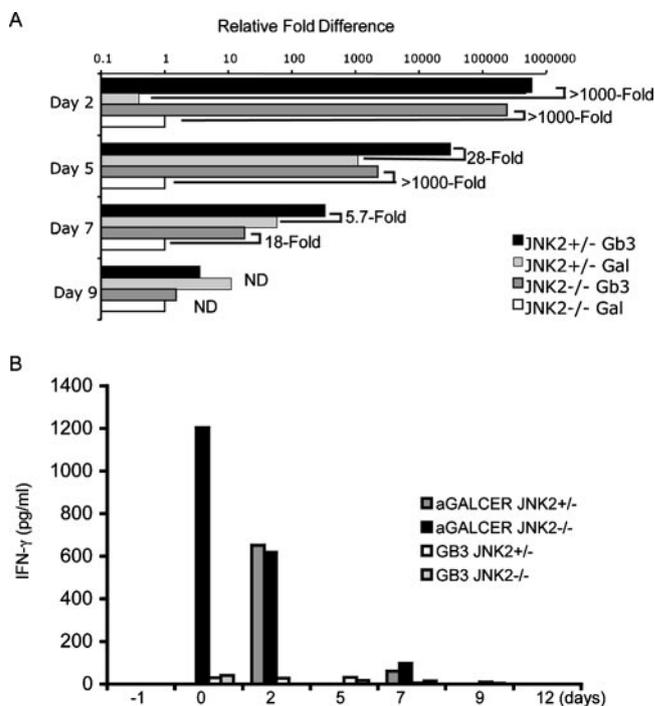


FIG. 7. α -GalCer-injected mice are protected against *A. phagocytophilum* infection. Heterozygous *jnk2* ($n = 5$) and *jnk2*-null ($n = 7$) mice were challenged with live *A. phagocytophilum* 24 h after receiving intraperitoneal injections of α -GalCer (200 ng per mouse). Heterozygous *jnk2* ($n = 4$) and *jnk2*-null ($n = 5$) mice were injected with GB3 (200 ng per mouse) and used as a negative control. (A) The relative bacterial load differences between the blood of gene-sufficient mice and that of gene-deficient mice were calculated by normalizing the bacterial copy numbers of *jnk2*-null (α -GalCer injected) mice to 1, compared to those of mice receiving other treatments. (B) Pooled serum concentrations of IFN- γ were measured by ELISA at the indicated times. Data represent the means \pm standard deviations. ND, no relative bacterial load difference in the blood of infected mice.

sponse at 24 h postinjection. Surprisingly, the kinetics of IFN- γ release was more delayed in wild-type mice injected with α -GalCer. Wild-type mice injected with α -GalCer only showed a great IFN- γ response 2 days postinfection when they encountered *A. phagocytophilum*. Taken together, these observations suggest that JNK2 inhibits NK T-mediated IFN- γ production upon *A. phagocytophilum* infection.

DISCUSSION

We describe evidence showing that JNK2 directly inhibits IFN- γ production, mediated by conventional and NK T cells upon *A. phagocytophilum* stimulation. These results are in disagreement with a previous observation (40), which indicated that the differentiation of precursor CD4⁺ T cells into effector Th1 cells was impaired in *jnk2*-null mice. We suggest that opposite phenotypes may occur when different stimulants are used to study the IFN- γ response in *jnk2*-null mice. Yang et al. (40) used the concanavalin A model to understand the role of JNK2 in Th1 differentiation, while we used *A. phagocytophilum* to decipher how JNK2 regulates IFN- γ response upon infection.

A. phagocytophilum, *Ehrlichia*, and *Sphingomonas* are gram-

negative bacteria that belong to the *Alphaproteobacteria* and constitute one of the most ubiquitous classes of bacteria on earth (4). Therefore, we decided to investigate whether *A. phagocytophilum* has the capacity to induce IFN- γ production mediated by CD1d-restricted NK T cells. Our results show that *cd1d*-null mice have an impaired capacity to clear *A. phagocytophilum* during the early phase of infection, which correlated with lower levels of IFN- γ in the peripheral blood of *cd1d*-null mice.

α -GalCer tetramers demonstrated that *A. phagocytophilum* has the capacity to stimulate CD1d-restricted NK T cells. Indeed, our report of experiments with livers from wild-type and *jnk2*-null mice shows that NK T cells from *jnk2*-null mice down-regulate the T-cell receptor after *A. phagocytophilum* restimulation. The decreased number of CD1d-tetramer-reactive T cells may likely correspond to an enhanced NK T-cell response from *jnk2*-null mice to *A. phagocytophilum*. Decreased numbers of tetramer-positive CD1d-restricted NK T cells had an inverse correlation with IFN- γ production. NK T-cell receptor down-regulation as a feature of NK T-cell activation has been suggested in several models, including *A. phagocytophilum*, *Listeria* spp., *Salmonella* spp., and *Toxoplasma gondii* (6, 8, 9, 32, 34). However, it is also possible that the NK T-cell receptor down-regulation in our studies could be a consequence of T-cell receptor stimulation via activated cell death (14, 17, 22). Further experimentation needs to be done to determine the cause of NK T-cell receptor down-regulation upon *A. phagocytophilum* infection.

We applied three different approaches to determine the inhibitory role of JNK2 in NK T-cell function upon *A. phagocytophilum* infection. First, we found that IFN- γ production is increased in *jnk2*-null mice upon stimulation with NK T-cell agonists. Second, we determined that the enhanced NK T-cell response from *jnk2*-null mice upon *A. phagocytophilum* infection is not due to increased glycolipid presentation by dendritic cells. Third, we injected wild-type and *jnk2*-null mice with α -GalCer and observed a strong protective effect at day 2 postinfection. The injection of NK T agonists (e.g., α -GalCer) therefore could theoretically be used as a vaccine for therapy against *A. phagocytophilum* infection. However, the design of NK T-cell therapies against HGA must be applied with caution. NK T cells may function as a double-edged sword: activated NK T cells may aggravate the symptoms of human anaplasmosis as opposed to ameliorating the disease (20, 28). To summarize, our data clearly indicate that JNK2 has a repressive role in IFN- γ production mediated by conventional and NK T cells upon *A. phagocytophilum* infection. The knowledge provided herein should permit a better understanding of *A. phagocytophilum* immunity and contribute to the development of prophylactic measures that thwart the spread of HGA.

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