ThermoFisher SCIENTIFIC
Immunology at Work
Resource Center
Learn more
invitrogen

The Journal of Immunology

# JNK1 Is Required for T Cell-Mediated Immunity Against *Leishmania major* Infection

This information is current as of August 9, 2022.

Stephanie L. Constant, Chen Dong, Derek D. Yang, Mark Wysk, Roger J. Davis and Richard A. Flavell

*J Immunol* 2000; 165:2671-2676; ;
doi: 10.4049/jimmunol.165.5.2671
http://www.jimmunol.org/content/165/5/2671

**References** This article **cites 28 articles**, 10 of which you can access for free at:
http://www.jimmunol.org/content/165/5/2671.full#ref-list-1

**Why *The JI*?** Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2000 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

# JNK1 Is Required for T Cell-Mediated Immunity Against *Leishmania major* Infection[1]

**Stephanie L. Constant,[2]* Chen Dong,[2]*† Derek D. Yang,‡ Mark Wysk,§ Roger J. Davis,§ and Richard A. Flavell[3]*†**

**c-Jun N-terminal kinase (JNK) is a mitogen-activated protein kinase that plays important regulatory roles in helper T cell differentiation. In the current study, we used *Jnk1*-deficient mice to examine the function of JNK during an in vivo pathogenic infection, leishmaniasis, which is strongly influenced by Th1/Th2 effector mechanisms. The data show that *Jnk1*-deficient mice, despite their usually genetically resistant background, were unable to resolve *Leishmania* infections. *Jnk1*$^{-/-}$ mice displayed reduced delayed-type hypersensitivity in response to the pathogen, which was associated with a T cell defect. We found that, although these mice can direct an apparent Th1-response, there is also simultaneous generation of *Leishmania*-specific Th2 responses, which possibly down-modulate protective Th1-mediated immune function. These findings demonstrate that the negative regulation of Th2 cytokine production by the JNK1 signaling pathway is essential for generating Th1-polarized immunity against intracellular pathogens, such as *Leishmania major*. *The Journal of Immunology*, 2000, 165: 2671–2676.**

During immune responses, naive CD4$^+$ helper T cells differentiate into two effector cell subsets, Th1 and Th2, which are characterized on the basis of the profile of cytokines they produce and the type of immune regulation they perform (1). Th1 CD4$^+$ T cells are potent initiators of inflammatory-type responses, due to their ability to secrete high levels of cytokines, such as IFN-$\gamma$ and TNF-$\beta$. In contrast, Th2 CD4$^+$ T cells are characterized by the production of IL-4, IL-5, IL-9, IL-10, and IL-13, which promote some forms of humoral immunity (notably the generation of IgG1 and IgE isotypes) and allergic-type responses. There is also strong cross-regulation between the two Th subsets, which, under some circumstances, can result in the dominance of one of the two subsets and therefore a bias toward one type of immune response. This has best been demonstrated in a variety of pathogenic infections, including leishmaniasis, schistosomiasis and leprosy (reviewed in Ref. 2).

Infection using the protozoal parasite *Leishmania major* provides an ideal model for studying the induction of Th1 vs Th2 responses because resistance to infection depends on a strong Th1 response (3). Most laboratory strains, such as C57BL/6 (B6)[4] and C3H, mount the strong Th1-type responses that are associated with clearance of the parasites and resolution of infection. In contrast, strains such as BALB/c are biased toward Th2-type responses, making them susceptible to the infection. Several studies suggest that this susceptibility is likely due to a burst of IL-4 production by activated Ag-specific CD4$^+$ T cells early in the course of infection, promoting Th2 differentiation (3). Cytokines produced by Th2 cells have been shown to abrogate the initiation of macrophage activation, required for clearance of *L. major* infections (4). Moreover, when overexpressed in vivo, Th2 cytokines render mice of a resistant genetic background susceptible (5).

Polarized differentiation of CD4$^+$ T cells toward one Th subset over the other can be influenced by a number of factors, including the cytokine environment, priming Ag dose, Ag/TCR interaction affinity, and costimulatory signals (reviewed in Refs. 6 and 7). The molecular mechanisms by which these factors orchestrate Th cell differentiation is still unclear, but one can postulate that they must converge at intracellular signaling molecules, which in turn modulate the activity of transcription factors responsible for cytokine gene expression (7, 8). c-Jun N-terminal kinase (JNK), which phosphorylates c-Jun and enhances AP-1 transcriptional activity, is a candidate for such signal integration (9). JNK and AP-1 activation in T cells has been originally shown to require both TCR/CD3 and the costimulatory molecule CD28 (10, 11). In addition, AP-1 has been reported to be important for the regulation of Th1 and Th2 cytokine genes (12). To address the function of JNK in helper T cell differentiation, we have recently generated and analyzed *Jnk1*-deficient mice. Quite surprisingly, in vitro-activated *Jnk1*$^{-/-}$ naive Th cells preferentially differentiated into Th2 cells, whereas wild-type cells became mostly Th1 cells (13). This phenomenon was likely caused by increased IL-4 production in response to anti-CD3 stimulation. An enhancement in Th2 responses was also seen in *Jnk1*$^{-/-}$ effector cells primed either in vitro by polyclonal activation or in vivo by immunization with keyhole limpet hemocyanin in alum (13). Interestingly, we found that *Jnk1*$^{-/-}$ Th cells remained capable of Th1 differentiation and IFN-$\gamma$ production in the presence of an environment polarizing toward Th1. However, the in vitro-differentiated *Jnk1*$^{-/-}$ Th1 populations also made detectable amounts of Th2 cytokines, indicating a failure to

*Section of Immunobiology, Yale University School of Medicine, and †Howard Hughes Medical Institute New Haven, CT 06520; ‡Lilly Research Laboratory, Eli Lilly and Company, Indianapolis, IN 46285; and §Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605

Received for publication March 20, 2000. Accepted for publication June 16, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded by National Institutes of Health Grant AI-39158 awarded to S.L.C. and the Howard Hughes Medical Institute. C.D. and D.D.Y. were Associates and R.A.F. and R.J.D. are Investigators of the Howard Hughes Medical Institute. C.D. is currently supported by a postdoctoral fellowship from the Arthritis Foundation.

[2] S.L.C. and C.D. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Richard A. Flavell, Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, 310 Cedar Street, FMB 412, P.O. Box 208011, New Haven, CT 06520-8011. E-mail address: fran.manzo@yale.edu

[4] Abbreviations used in this paper: B6, C57BL/6; JNK, c-Jun N-terminal kinase; DTH, delayed-type hypersensitivity.

Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

down-regulate Th2 responses. Thus, JNK1 may serve as a negative regulator of Th2 cytokine expression (13).

To assess the role of JNK1 in regulation of in vivo immune responses, in the current study, we examined the physiological consequences of an *L. major* infection in *Jnk1*$^{-/-}$ mice on a Th1-biased 129 × B6 MHC$^{b}$ background. We found that, unlike wild-type 129 × B6 or B6 mice, *Jnk1*$^{-/-}$ mice were unable to resolve the infection. Although these mice could initiate anti-*Leishmania* Th1 responses to some extent, they also displayed an enhanced Th2 response. These results indicate that inhibition of Th2 cytokine production by JNK1 is an essential regulatory mechanism that ensures Th1-polarized reaction and immunity against intracellular pathogens, such as *L. major*.

## Materials and Methods

### *Mice*

The *Jnk1*-deficient and wild-type control mice were generated as described previously (13) and were maintained as homozygotes on a 129 × B6 $F_2$ genetic background. C57BL/6 and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD).

### *Parasites and infection protocol*

*L. major* promastigotes of the WR309 substrain were maintained at 23°C in Schneider's Drosophila Medium (Life Technologies, Grand Island, NY) supplemented with 20% FCS and 50 μg/ml gentamicin. Inoculates of the parasites were passaged through BALB/c mice every fifth culture split to maintain infectivity. For experiments, mice were infected in the right hind foot with $2 \times 10^6$ stationary phase promastigotes in 10 μl PBS. The course of infection in each group of mice was monitored by weekly measurements of footpad thickness of the infected right vs uninfected left foot using spring-loaded calipers. Feet were also monitored for lesion formation and ulceration.

### *Induction and measurement of delayed-type hypersensitivity (DTH) responses*

*L. major* lysate was prepared by subjecting stationary phase promastigotes in PBS to two rounds of 1-min sonication, followed by filter sterilization. Protein content of lysate batches was determined by 280:260 nm spectrophotometry. Mice were challenged at 6 wk postinfection in the uninfected left foot with 40 μg of the lysate in 10 μl PBS, and footpad thickness was measured after 48 h using spring-loaded calipers.

### *Assaying for* Leishmania*-specific serum Ig*

At 7–8 wk postinfection, mice were sacrificed and blood isolated by cardiac puncture. Serum was separated by centrifugation of blood samples at 2000 rpm for 5 min and then frozen at −20°C until used. For assays, ELISA plates were coated overnight at 4°C with 10 μg/ml *L. major* lysate (prepared as described above) in borate saline buffer, pH 8.4. Serum samples, starting at a 1:50 dilution, were diluted down in two-fold increments in borate saline buffer containing 1% BSA and 0.1% Tween 20 and incubated for 1 h at 37°C. All subsequent ELISA steps were conducted for 1 h at 37°C in borate saline buffer containing 1% BSA. Detection of IgG2a, IgG1, or IgE isotypes was conducted using biotinylated anti-Ig purchased from PharMingen (San Diego, CA) for IgG2a and IgG1, and from Biosource (Camarillo, CA) for IgE. Allotypic differences between B6 mice vs 129 × B6 and BALB/c mice was taken into account, with anti-Ig reagents of the appropriate specificity for the different strains. ELISAs were developed using HRP-streptavidin (Zymed, San Francisco, CA) followed by a tetramethylbenzidine one-step substrate system (Dako, Carpinteria, CA). Color development was stopped using 6 M HCl and plates then read at 450 nm using an EL340 automated microplate reader (Bio-Tek Instruments, Winooski, VT).

### *In vitro restimulation of lymph node cells*

At 4 wk postinfection, mice were sacrificed and their popliteal and inguinal lymph nodes isolated and pooled. Cell suspensions were prepared and set up at $8 \times 10^6$/ml in 24-well tissue culture plates in the presence of different concentrations of *L. major* lysate (prepared as described above). All cultures were set up in Click's Medium (Life Technologies) supplemented with 5% FCS. Supernatants were collected for cytokine analysis after 4 days of culture.

### *Stimulation of IL-12 production by macrophages and dendritic cells*

For macrophage experiments, mice were injected i. p. with 1.5 ml thioglycolate (3% in PBS) and 3 days later peritoneal macrophages were harvested and cultured overnight in tissue culture plates at $4 \times 10^6$/ml in Click's Medium plus 5% FCS. The following day, nonadherent cells were removed and the remaining adherent macrophages incubated in the presence or absence of recombinant IFN-γ (10 U/ml) plus LPS (10 ng/ml). For dendritic cell experiments, spleen cells were incubated at 37°C at $10^7$/ml in medium plus 5% FCS in 100-mm tissue culture petri dishes and 2 h later the nonadherent cells were removed by washing the plates with warm medium. The remaining adherent cells were cultured overnight and the following day the loosely adherent dendritic cells were collected by gentle pipetting. This typically yields populations of dendritic cells enriched to 80–85%. Suspensions of dendritic cells were plated in 96-well microtiter plates at $1 \times 10^5$/well in the presence of anti-CD40L (provided by Dr. Marilyn Kehry at Boehringer Ingelheim) at 1:100 dilution or medium alone. Supernatants from both macrophage and dendritic cell cultures were collected 48 h later and assayed for the presence of IL-12 p40 (see below).

### *Stimulation of $NO_2^-$ production by macrophages*

Adherent peritoneal macrophages (prepared as above) were stimulated with either IFN-γ (10 U/ml) plus LPS (10 ng/ml) or plus *L. major* lysate (50 μg/ml), or medium alone. Culture supernatants were collected 48 h later and assayed for the presence of $NO_2^-$ using a nitrate/nitrite colorimetric kit (Alexis Biochemicals, San Diego, CA).

### *Assaying for cytokines in culture supernatants*

Supernatants were assayed for the presence of IFN-γ, IL-4, IL-5, IL-12 p40, and IL-13 cytokines using kits purchased from Endogen (Woburn, MA) for IFN-γ, IL-4, and IL-5, PharMingen for IL-12, and R&D Systems (Minneapolis, MN) for IL-13. All assays were conducted according to manufacturers' specifications. Concentrations of each cytokine were calculated based on standard curves generated from recombinant cytokines provided with the kits.

## Results and Discussion

Our earlier studies of CD4$^+$ differentiation in *Jnk1*$^{-/-}$ mice suggested that JNK1 may serve as a negative regulator of Th2 cytokine expression (13). To understand the function of JNK1 in immunity against a pathogenic infection, leishmaniasis, we infected *Jnk1*$^{-/-}$ mice with *L. major*. Immunity against *L. major* has been shown to exhibit a strong Th1/Th2 dichotomy and is typically characterized as a Th1 response in B6, 129, or 129 × B6 mixed genetic backgrounds and a Th2 response in BALB/c mice (3). We first examined whether the course of a *L. major* infection would be altered in *Jnk1*$^{-/-}$, as compared with wild-type, mice. *Jnk1*$^{-/-}$, as well as 129 × B6, B6, and BALB/c mice were infected in the right hind foot with *L. major* promastigotes, and footpad thickness was measured using calipers over the course of several weeks to establish susceptibility vs resistance to the infection. As shown in Fig. 1, the expected patterns of footpad swelling were observed in susceptible BALB/c mice (a gradual increase in lesion formation, leading to ulceration of the lesions by 7 wk postinfection) and resistant B6 and 129 × B6 mice (an initial small increase in lesion formation followed by a plateau in response). Strikingly, however, the pattern of lesion formation in *Jnk1*$^{-/-}$ mice was almost identical with that observed in the BALB/c strain, with lesions becoming ulcerated by 7 wk postinfection. These data clearly show that, despite their resistant genetic background, *Jnk1*$^{-/-}$ mice are impaired in their ability to resolve a *L. major* infection. This in turn suggested that JNK1 is required to make an appropriate immunological response to clear the infection.

In leishmaniasis, the clearance of the *L. major* parasite species is known to be mediated by DTH responses, characterized by the influx and activation of *Leishmania*-specific Th1 CD4$^+$ T cells at the site of infection (3). To test the ability of *Jnk1*$^{-/-}$ mice to mount a *Leishmania*-specific DTH response, mice were challenged 6 wk postinfection with *L. major* lysate in the uninfected left foot

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

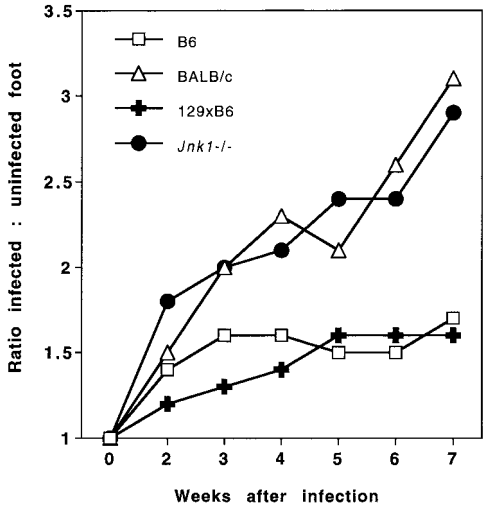


**FIGURE 1.** Course of *L. major* infection is altered in *Jnk1*$^{-/-}$ mice. Mice were infected in the right foot with $2 \times 10^6$ stationary phase *L. major* promastigotes in 10 $\mu$l PBS. At weekly intervals, footpad thickness of right and left feet was measured using spring-loaded calipers. The mean footpad thickness of infected and uninfected feet was determined for each group of mice, and the ratio of these means calculated and plotted. The plot shows data from two combined experiments, with $n = 5$ mice for each group.

and DTH-induced swelling measured with calipers after 48 h. Fig. 2 shows the DTH induced by antigenic challenge in *Jnk1*$^{-/-}$ mice as compared with similarly treated 129 × B6, B6, and BALB/c mice. Both 129 × B6 and B6 groups show strong levels of DTH, associated with their ability to resolve *L. major* infections. In comparison, both BALB/c and *Jnk1*$^{-/-}$ mice show a markedly reduced level of DTH, although the deficiency was more pronounced in the susceptible BALB/c. These data suggest that *Jnk1*$^{-/-}$ mice have an intermediate phenotype with regards to their ability to mount efficient Th1-mediated DTH responses. Despite this, based on the results observed in Fig. 1, it seems likely that such reduced DTH responses are not sufficient to completely resolve *L. major* infections.

The results depicted in Figs. 1 and 2 suggested that *Leishmania*-infected *Jnk1*$^{-/-}$ mice were impaired in type I immune functions.

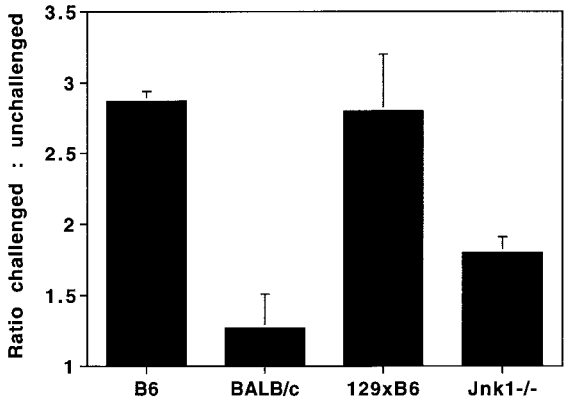


**FIGURE 2.** *L. major*-specific DTH responses are impaired in *Jnk1*$^{-/-}$ mice. Mice were infected in the right foot with $2 \times 10^6$ stationary phase *L. major* promastigotes in 10 $\mu$l PBS. Six weeks after infection, the uninfected left feet were challenged with 40 $\mu$g *L. major* lysate in 10 $\mu$l PBS. Footpad thickness of the left feet before and 48 h after lysate challenge was measured using spring-loaded calipers and the ratio of footpad thickness for each mouse before and after challenge calculated. For each group of mice, plots show the mean ratio ± SE, with $n = 3$.

Control and clearance of *L. major* infections requires effective macrophage activation, with the production of nitric oxide ($NO_2^-$) mediating parasite destruction. To assess whether macrophages from *Jnk1*$^{-/-}$ mice might have some primary defect in their ability to produce $NO_2^-$, which could account for their failure to resolve an ongoing infection, we measured levels of $NO_2^-$ release following different types of stimulation. As shown in Fig. 3, macrophages from *Jnk1*$^{-/-}$ were as competent as macrophages isolated from wild-type control mice to produce $NO_2^-$, suggesting no primary defect in their function.

We then investigated whether *Jnk1*$^{-/-}$ mice have a defect in the activation of the innate immune system that renders them susceptible to infection, a subject which had not been addressed in our previous analysis. Because IL-12 produced by activated APCs has been shown to be required to establish Th1-mediated immune responses against infections with *L. major* (14, 15), we also examined whether macrophages and dendritic cells from *Jnk1*$^{-/-}$ mice might be impaired in IL-12 production. Thioglycolate-elicited peritoneal macrophages from wild-type or knockout animals were treated with or without LPS plus IFN-$\gamma$. As shown in Fig. 4, the macrophages from *Jnk1*$^{-/-}$ mice did not have any impairment in their capacity to produce IL-12 following stimulation. In fact, they produced higher amounts of IL-12 than the wild type. Dendritic cells are the other major source of IL-12 in vivo. It has been suggested that, during *Leishmania* infection, these cells make IL-12 in response to CD40 ligation by T cells. Therefore, we isolated splenic dendritic cells from control or knockout animals and activated them with CD40 ligand. Similar to our results with macrophages, *Jnk1*$^{-/-}$ dendritic cells were not defective in producing IL-12 (Fig. 4). These results indicate that the JNK1 signaling pathway is not required for the activation of IL-12 expression. Interestingly, we have previously shown that the p38 pathway, acting through MKK3 MAP kinase kinase, plays a major role in IL-12 induction in both macrophages and dendritic cells (16). Therefore, it seems unlikely that the failure of *Jnk1*$^{-/-}$ mice to resolve their infection is in the APC compartment at the level of either IL-12 production or effector function.

In view of the above data and our previous finding that *Jnk1*-deficient T cells are defective in their functional differentiation, we focused our analysis on helper T cells. To analyze whether the impairment in Th1-mediated functions might be due to deficient Th1 differentiation, we examined adaptive immune components in response to the *Leishmania* infection. First, serum was isolated

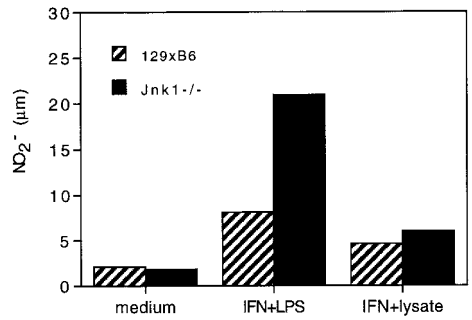


**FIGURE 3.** Macrophage function is normal in *Jnk1*$^{-/-}$ mice. Peritoneal exudate cells were collected and pooled from thioglycolate-treated *Jnk1*$^{-/-}$ and wild-type mice ($n = 2$) and plated overnight at $2 \times 10^6$/ml to enrich for adherent cells. Adherent macrophages were then cultured for 2 days in the presence of IFN-$\gamma$ (10 U/ml) + LPS (10 ng/ml) or IFN-$\gamma$ (10 U/ml) + *L. major* lysate (50 $\mu$g/ml) or medium alone. Culture supernatants were tested for the presence of $NO_2^-$ using a nitrate/nitrite colorometric kit. Bar graphs show the mean concentration from duplicate wells.

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

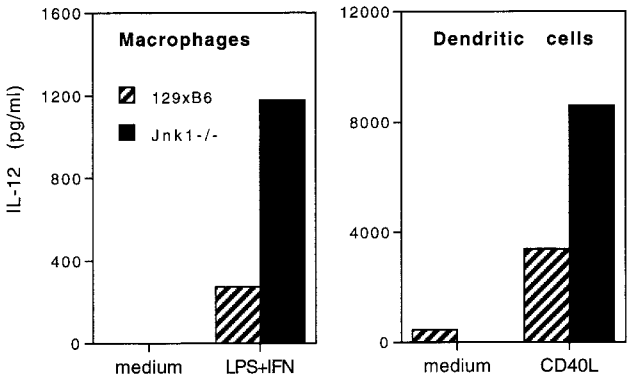


**FIGURE 4.** Macrophages and dendritic cells from *Jnk1*$^{-/-}$ mice show no defect in IL-12 production. Peritoneal exudate cells were collected from thioglycolate-treated *Jnk1*$^{-/-}$ and wild-type mice ($n = 2$) and plated overnight at $2 \times 10^6$/ml to enrich for adherent cells. Adherent macrophages were then cultured for 2 days in the presence of IFN-$\gamma$ (10 U/ml) + LPS (10 ng/ml) or medium alone. Dendritic cells were enriched from the spleen of *Jnk1*$^{-/-}$ and wild-type mice using a 2-h adherence step followed by overnight culture. Enriched dendritic cells were then cultured for two days at $5 \times 10^5$/ml in the presence of CD40L (1:100) or medium alone. Supernatants collected after 2 days of culture were tested for the presence of IL-12 p40 using an ELISA kit. Bar graphs show the mean concentration from duplicate wells.

from each group of mice at 7 wk postinfection and assayed for the presence of *Leishmania*-specific Ab of different isotypes (IgG2a, IgG1, and IgE). No *Leishmania*-specific IgE was detected in any of the mice (data not shown); however, significant levels of both IgG2a and IgG1 isotypes were detected (Fig. 5). Whereas all groups of mice produced *Leishmania*-specific IgG2a, the Th2-dependent IgG1 isotype was detected in only BALB/c and *Jnk1*$^{-/-}$ mice, albeit at a reduced titer in the latter. This suggests that the *Jnk1*$^{-/-}$ mice have enhanced Th2-type responses relative to their 129 × B6 wild-type counterparts.

To further distinguish whether the impairment of immunity against *L. major* infection in *Jnk1*$^{-/-}$ mice was the result of a reduction in Th1 differentiation or enhanced Th2 responses in vivo, we examined the profile of cytokines generated by the various groups of mice (Fig. 6). Lymph node cells draining the site of a 4-wk infection in *Jnk1*$^{-/-}$, 129×B6, and BALB/c mice were restimulated in vitro with *L. major* lysate and 4 days later culture supernatants were assayed for the presence of various cytokines. *Jnk1*$^{-/-}$ cells produced IFN-$\gamma$ at the same level as wild-type cells, whereas only half the amount was generated by BALB/c cells. This, together with normal IgG2a titers, suggests that Th1 effector cell development in vivo is normal in *Jnk1*$^{-/-}$ mice. When we measured cytokines usually associated with Th2-type responses, we found IL-5 and IL-13 were selectively produced by BALB/c and *Jnk1*$^{-/-}$ cells (Fig. 6). This is consistent with our previous report that in vitro-differentiated *Jnk1*$^{-/-}$ Th1 populations also produced detectable levels of Th2 cytokines (13). This suggests that strong Th2 responses were induced in *Jnk1*$^{-/-}$ mice following in vivo infection, supporting our earlier hypothesis that JNK1 is not required for Th1 differentiation, but negatively regulates Th2 cytokine production. Interestingly, whereas BALB/c mice produced high levels of IL-4, *Jnk1*$^{-/-}$ cells produced only very small amounts of the cytokine. These findings correlate with our previous observations following in vivo immunization in *Jnk1*$^{-/-}$ mice using keyhole limpet hemocyanin in alum, where T cells made five-fold more IL-5, but only 30% more IL-4, than wild-type cells after the in vitro restimulation of draining lymph node cells (13). Although our current and previous observations agree well with regard to patterns of cytokine production, the virtual absence of an enhancement in IL-4 production in the cultures derived from *Jnk1*$^{-/-}$ mice is difficult to explain in the light of Ag-specific IgG1 being detected in their serum (Fig. 5). It is possible that IL-4 is being produced initially at higher levels during in vitro restimulation but that the cytokine is being reused over time. In other words, *Jnk1*$^{-/-}$ cells may not be able to sustain IL-4 expression during restimulation. In support of this, we were able to detect higher levels of the cytokine when culture supernatants were sampled after only 3, instead of 4, days of culture (S.C. and C.D., unpublished observations). However, it should also be emphasized that the virtual absence of IL-4 in cultures of restimulated lymph node cells does not necessarily correlate with an absence of the cytokine during an ongoing infection in vivo. Even if only low levels of IL-4 are produced in vivo, these may be sufficient to induce class switching to the IgG1 isotype (17). Interestingly, it has been shown that IL-13, although not capable of inducing class switching in B cells directly, can act on B cells to increase their survival and hence Ab production (18). When we examined our culture supernatants for the presence of IL-13, we were able to detect high levels both in the BALB/c and *Jnk1*$^{-/-}$ but not the wild-type

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

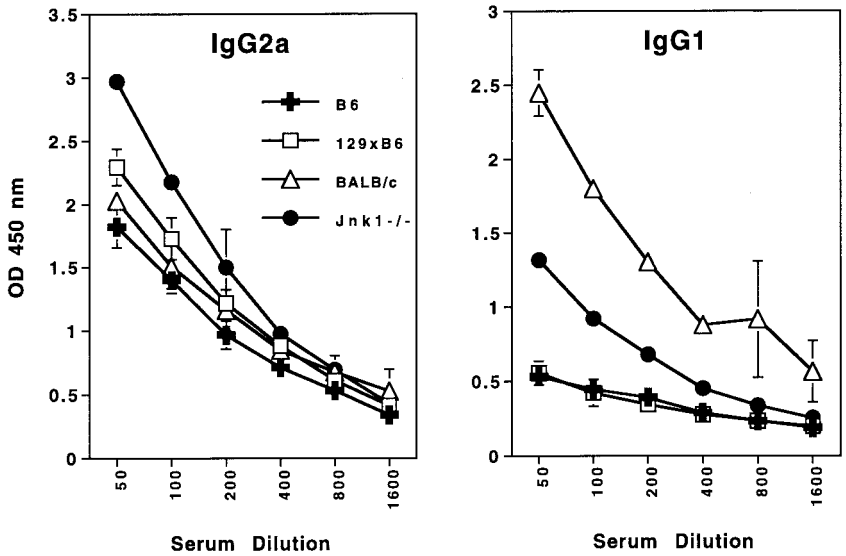


**FIGURE 5.** Ig isotypes in *Jnk1*$^{-/-}$ mice following *L. major* infection. Serum was obtained from mice 7 wk after infection in the right foot with $2 \times 10^6$ stationary phase *L. major* promastigotes. *L. major*-specific Ig isotypes were measured in each serum sample using isotype-specific ELISAs as described in *Materials and Methods*. Plots show the OD 450 nm readings obtained for *Leishmania*-specific IgG2a and IgG1 isotype ELISAs at different dilutions of serum. For each group of mice, plots show the mean OD ± SE, with $n = 4$.

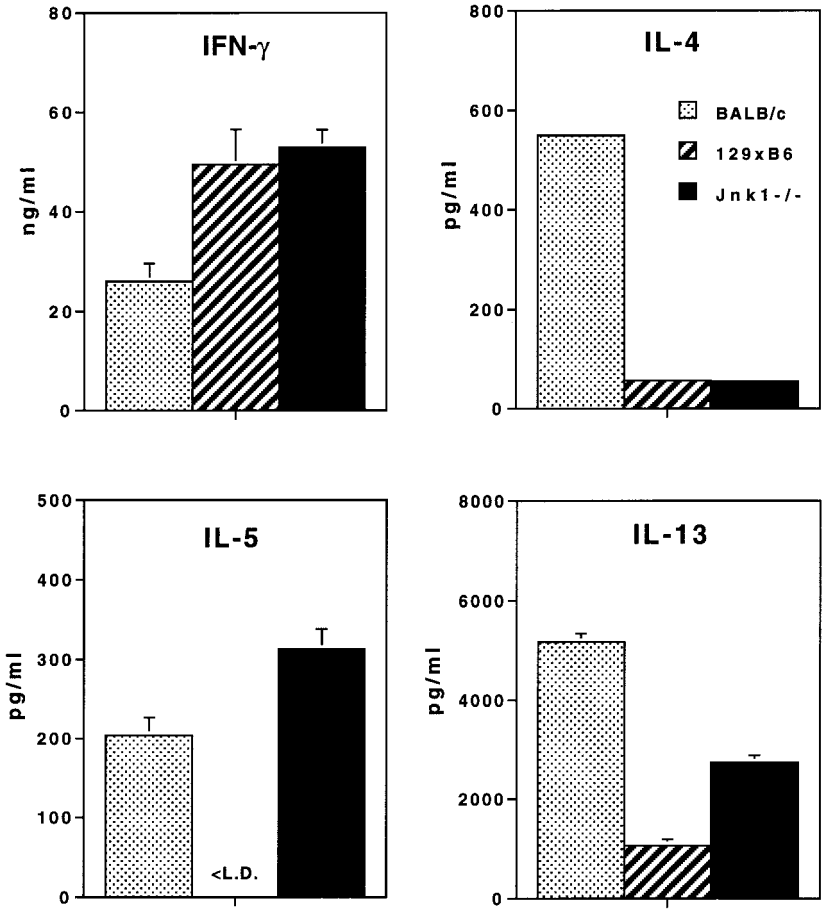


**FIGURE 6.** *L. major*-infected *Jnk1*$^{-/-}$ mice produce Th2-type cytokines. Mice were infected in both feet with $2 \times 10^6$ stationary phase *L. major* promastigotes and 4 wk later popliteal and inguinal lymph nodes were isolated and pooled for each group of mice ($n = 2$). Lymph node cell suspensions were set up in 24-well tissue culture wells at $8 \times 10^6$/ml in the presence of different concentrations of *L. major* lysate and 4 days later culture supernatant was collected for cytokine analysis. ELISAs for the detection of IFN-γ, IL-4, IL-5, and IL-13 were performed. Plots show the mean of duplicate wells ± SD obtained after stimulation with 50 μg/ml lysate. No cytokines were detected in any of the groups in the absence of lysate. <L.D., Below the limit of detection of the ELISA.

groups, so it is also possible that in vivo this cytokine would help amplify any small induction of *Leishmania*-specific IgG1 in *Jnk1*$^{-/-}$ mice. In support of this hypothesis, IL-13 transgenic mice on a B6 genetic background were reported recently to be defective in mounting an effective immunity against *L. major* (19). Moreover, several groups have now shown that IL-4Rα-deficient BALB/c mice are able to resist *L. major* infections (20, 21).

Another cytokine that has been associated with promoting the differentiation of Th2-type cells is IL-10. Although we observed some difference in the production of IL-10 after 2 wk of infection between *Jnk1*$^{-/-}$ and wild-type control mice, this was small (1.7-fold). Although such a difference might contribute to the observed down-regulation in early Th1 differentiation in *Jnk1*$^{-/-}$ mice, it is unlikely that this alone could be the mechanism. Furthermore, the major role of IL-10 during leishmaniasis is thought to be in the inhibition of Th1-mediated effector responses rather than altering initial priming events (22).

Taken together, the data suggest that *Jnk1*$^{-/-}$ mice exhibit a strikingly enhanced Th2 response following infection by *L. major*, although this phenotype is not as extreme as that induced in infected BALB/c mice. Because Th1-associated responses can also be detected during the course of infection, this argues against a failure of Th1 cells to develop into *Jnk1*$^{-/-}$ mice, in agreement with our earlier published observations (13). Instead, it is likely that any ongoing Th1-type immune responses are being down-modulated by the simultaneous production of Th2 cytokines, most notably IL-13, and potentially, IL-10. This study strongly supports the hypothesis that JNK1 is involved in the negative regulation of Th2 cytokine production, and further demonstrates that the negative regulation of Th2 responses by JNK1 plays an indispensable role in generation of a Th1-polarized reaction during *L. major* infection in mice of a resistant (Th1-dominant) genetic background.

We have previously shown that there is an enhancement of NF-ATc nuclear accumulation in *Jnk1*$^{-/-}$ cells in response to anti-CD3 stimulation, independent of IL-4 (13). Recently, we have identified the biochemical mechanism by which JNK1 regulates NF-ATc nuclear localization (23). JNK1 can bind to and phosphorylate NF-ATc, which then inhibits targeting of calcineurin phosphatase. Mutation of two serine residues that are phosphorylated by JNK to alanine rendered constitutive nuclear localization of NF-ATc molecules. NF-ATc has been shown to bind the IL-4 promoter and to be essential for Th2 development and cytokine production (24, 25). Interestingly, NF-ATc mutant molecules that are constitutively nuclear could activate a minimal IL-4 promoter reporter (23), suggesting that greatly enhanced nuclear accumulation of NF-ATc in activated JNK1-deficient T cells might account for the exacerbated production of Th2 cytokines by these cells. Knockout mice for NF-ATp, another member of the NF-AT family, show a strikingly similar phenotype to *Jnk1*$^{-/-}$ mice (26, 27). These mice, also on a 129 × B6 background, were reported to be susceptible to *L. major* infection (26). Thus, NF-ATc and NF-ATp, both of which can bind to the IL-4 promoter (28), may antagonize each other in regulating Th2 cytokine production. JNK1, by regulating NF-ATc nuclear localization, may modulate the balance of this Yin-Yang pair and therefore the outcome of immunity. The results presented here clearly demonstrate that JNK1 is essential for protective in vivo Th1 responses and immunity against *L. major* through the inhibition of Th2 responses.

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022

## Acknowledgments

We thank K. Lee and J. Stein for excellent technical assistance, F. Manzo for assistance with manuscript preparation, Dr. K. Bottomly for helpful discussion, and Dr. D. Leitenberg for critical reading of the manuscript.

## References

1. Bottomly, K. 1988. A functional dichotomy in CD4$^+$ T lymphocytes. *Immunol. Today 9:268.*
2. Bevan, M. J., and A. Sher. 1993. Immunity to infection: lessons on the immune response from the war on bugs. *Curr. Opin. Immunol. 5:477.*
3. Reiner, S. L., and R. M. Locksley. 1995. The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol. 13:151.*
4. Liew, F. Y., S. Millott, Y. Li, R. Lelchuk, W. L. Chan, and H. Ziltener. 1989. Macrophage activation by interferon-$\gamma$ from host-protective T cells is inhibited by interleukin (IL) 3 and IL 4 produced by disease-promoting T cells in leishmaniasis. *Eur. J. Immunol. 19:1227.*
5. Leal, L. M. C. C., D. W. Moss, R. Kuhn, W. Muller, and F. Y. Liew. 1993. Interleukin-4 transgenic mice of resistant background are susceptible to *Leishmania major* infection. *Eur. J. Immunol. 23:566.*
6. Constant, S. L., and K. Bottomly. 1997. The induction of Th1 and Th2 CD4$^+$ T cell responses: the alternative approaches. *Annu. Rev. Immunol. 15:297.*
7. Dong, C., and R. A. Flavell. 2000. Cell fate decision: Th1 and Th2 subsets in immune responses. *Arthritis Res. 2:179.*
8. Leitenberg, D., and K. Bottomly. 1999. Regulation of naive T cell differentiation by varying the potency of TCR signal transduction. *Semin. Immunol. 11:283.*
9. Ip, Y. T., and R. J. Davis. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. *Curr. Opin. Cell. Biol. 10:205.*
10. Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell 77:727.*
11. Rincón, M., and R. A. Flavell. 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J. 13:4370.*
12. Foletta, V. C., D. H. Segal, and D. R. Cohen. 1998. Transcriptional regulation in the immune system: all roads lead to AP-1. *J. Leukocyte Biol. 63:139.*
13. Dong, C., D. D. Yang, M. Wysk, A. J. Whitmarsh, R. J. Davis, and R. A. Flavell. 1998. Defective T cell differentiation in the absence of *Jnk1*. *Science 282:2092.*
14. Scharton-Kersten, T., L. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott. 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *J. Immunol. 154:5320.*
15. Hondowicz, B. D., T. M. Scharton-Kersten, D. E. Jones, and P. Scott. 1997. *Leishmania major*-infected C3H mice treated with anti-IL-12 mAb develop but do not maintain a Th2 response. *J. Immunol. 159:5024.*
16. Lu, H.-T., D. D. Yang, M. Wysk, E. Gatti, I. Mellman, R. J. Davis, and R. A. Flavell. 1999. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (*Mkk3*)-deficient mice. *EMBO J. 18:1845.*
17. Noben-Trauth, N., P. Kropf, and I. Muller. 1996. Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science 271:987.*
18. Lai, Y. H., and T. R. Mosmann. 1999. Mouse IL-13 enhances antibody production in vivo and acts directly on B cells in vitro to increase survival and hence antibody production. *J. Immunol. 162:78.*
19. Matthews, D. J., C. L. Emson, G. J. McKenzie, H. E. Jollin, J. M. Blackwell, and A. N. J. McKenzie. 2000. IL-13 is a susceptibility factor for *Leishmania major* infection. *J. Immunol. 154:1458.*
20. Noben-Trauth, N., W. E. Paul, and D. L. Sacks. 1999. IL-4- and IL-4 receptor-deficient BALB/c mice reveal differences in susceptibility to *Leishmania major* parasite substrains. *J. Immunol. 162:6132.*
21. Mohrs, M., B. Ledermann, G. Kohler, D. A., A. Gessner, and F. Brombacher. 1999. Differences between IL-4 and IL-4 receptor $\alpha$-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. *J. Immunol. 162:7302.*
22. Powrie, F., S. Menon, and R. L. Coffman. 1993. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *Eur. J. Immunol. 23:3043.*
23. Chow, C.-W., C. Dong, R. A. Flavell, and R. J. Davis. 2000. JNK inhibits substrate targeting by protein phosphatase calcineurin to NFATc1. *Mol. Cell. Biol. 20:5227.*
24. Yoshida, H., H. Nishina, H. Takimoto, L. E. M. Marengere, A. C. Wakeham, D. Bouchard, Y. Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription factor NFATc1 regulates lymphocyte proliferation and Th2 development. *Immunity 9:627.*
25. Ranger, A. M., M. R. Hodge, E. M. Gravellese, M. Oukka, L. Davidson, F. W. Alt, F. C. de la Brousse, T. Hoey, M. Grusby, and L. H. Glimcher. 1998. Delayed lymphoid repopulation with defects in IL-4 driven responses produced by inactivation of NFATc. *Immunity 8:125.*
26. Kiani, A., J. P. Viola, A. H. Lichtman, and A. Rao. 1997. Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity 7:849.*
27. Hodge, M. R., A. M. Ranger, C. F. de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity 4:397.*
28. Timmerman, L. A., J. I. Healy, S. N. Ho, L. Chen, C. C. Goodnow, and G. R. Crabtree. 1997. Redundant expression but selective utilization of nuclear factor of activated T cells family members. *J. Immunol. 159:2735.*

Downloaded from http://www.jimmunol.org/ by guest on August 9, 2022