

## Interleukin-12 Regulates Chemokine Gene Expression during the Early Immune Response to *Leishmania major*

Colby Zaph and Phillip Scott\*

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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**Following infection with *Leishmania major*, the chemokines XCL1, CXCL10, and CCL2 were preferentially expressed in draining lymph nodes of resistant mice. Neutralization of interleukin 12 (IL-12) or gamma interferon in resistant mice resulted in decreased chemokine expression, while administration of IL-12 to susceptible mice resulted in an increase in the level of chemokine gene expression.**

Experimental infections with *Leishmania major* have proven to be an invaluable tool in examining the factors that contribute to Th subset development in vivo. This system has shown that interleukin 12 (IL-12) and gamma interferon (IFN- $\gamma$ ) are critical to Th1 cell development and resistance to disease, while IL-4 is involved in Th2 development and susceptibility (13). Many studies have focused on the early response to *L. major* infection and have shown that both cytokines and chemokines are part of this response (20, 22). Here we extend these studies to specifically investigate the interplay between cytokines and chemokines during the first 3 days of *L. major* infection.

Specific-pathogen-free 6- to 8-week-old BALB/cByJ and C3HeB/FeJ (Jackson Laboratories, Bar Harbor, Maine) mice were infected in the hind footpads with  $2 \times 10^6$  metacyclic promastigotes (WHO/MHOM/IL/80/Friedlin) selected from stationary parasite cultures using *Arachis hypogea* agglutinin (Sigma Chemical Co., St. Louis, Mo.) as previously described (17). Draining popliteal lymph nodes were removed 4, 16, and 72 h postinfection, and total RNA was extracted using RNeasyStat60 (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's recommendations. Chemokine gene expression was measured using the RiboQuant RNase protection assay (RPA) system (probe mCK5; BD Pharmingen, San Diego, Calif.) following the instructions of the supplier. The quantity of protected RNAs was determined using a phosphorimager and MultiAnalyst software (both from Bio-Rad, Culver City, Calif.). For quantitation, values are expressed as units relative to the L32 rRNA gene.

As early as 16 h postinfection, the chemokines XCL1 (lymphotactin), CXCL10 (IP-10), and CCL2 (MCP-1) were upregulated in the draining lymph nodes of resistant mice (Fig. 1A and B). The genes for these chemokines were not expressed at earlier (4 h) or later (72 h) time points in either mouse strain after infection or in sham-infected animals (data not shown). No change in the expression levels of CCL1, CCL3, CCL4, and CCL5 was evident at 4, 16, or 72 h postinfection (data not shown). To determine if chemokine gene expression required

live parasites, resistant mice were challenged with  $2 \times 10^6$  parasites or an equivalent amount of a freeze-thaw antigen preparation. RPA analysis was carried out on RNA extracted from draining lymph nodes 16 h postchallenge. Dead parasites failed to elicit a chemokine response, implying that active infection is required for the early protective response in resistant mice (Fig. 2).

To better understand the relationship between chemokines and cytokines early after infection with *L. major*, we assessed the ability of lymph node cells from resistant mice to produce chemokines in the absence of IL-12. C3H mice were treated with 1 mg of  $\alpha$ -IL-12 (C17.8, a gift of G. Trinchieri, Schering-Plough Laboratory for Immunological Research, Dardilly, France) 24 h prior to infection with *L. major*. Draining popli-

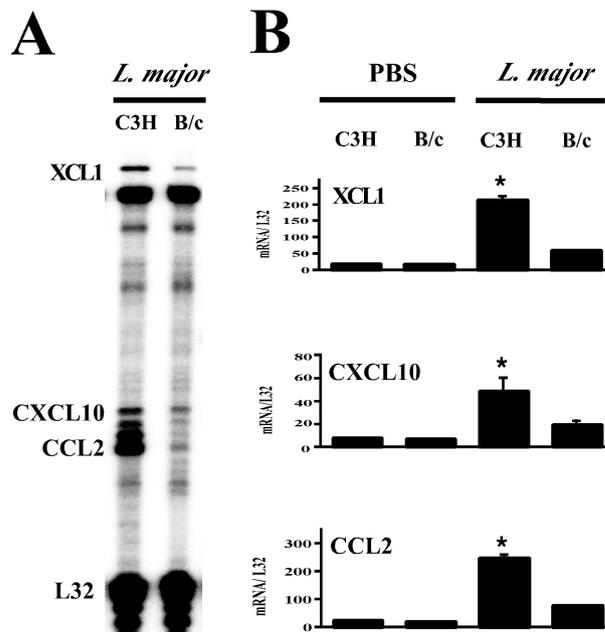


FIG. 1. Chemokines are differentially upregulated in response to *L. major* in resistant and susceptible mice. (A) Representative RPA analysis of RNA isolated from the draining lymph node 16 h postinfection with *L. major*. (B) Graphical representations for averages of five separate experiments. Results represent the means  $\pm$  standard deviations. \*,  $P \leq 0.05$ . PBS, phosphate-buffered saline.

\* Corresponding author. Mailing address: Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104. Phone: (215) 898-1602. Fax: (215) 573-7023. E-mail: pscott@mail.vet.upenn.edu.

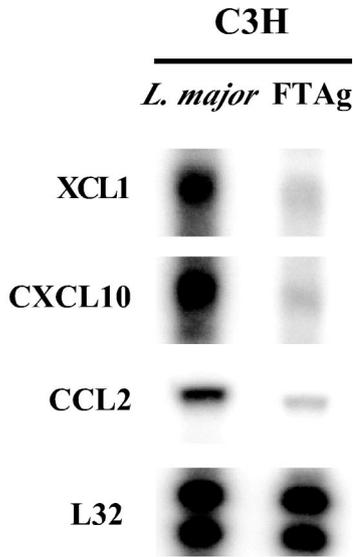


FIG. 2. Chemokine gene expression is dependent upon viable parasites. RPA analysis of RNA isolated from draining lymph nodes 16 h postchallenge with equivalent cell numbers of viable or freeze-thawed *L. major* parasites. Results are representative of two individual experiments.

teal lymph nodes were isolated 16 h postinfection, RNA was extracted, and chemokine gene expression was examined by RPA. Blockade of IL-12 in C3H mice inhibited the infection-induced upregulation of XCL1, CXCL10, and CCL2 (Fig. 3A). Thus, it appears that these chemokines do not initiate IL-12 production, but rather IL-12 is required for the upregulation of specific chemokine genes during the early immune response to

*L. major*. To demonstrate that IL-12 can induce these chemokines, we injected IL-12 into BALB/c mice at the time of infection (0.25  $\mu$ g of recombinant murine IL-12 [S. Wolf and J. Sypek, Genetics Institute, Cambridge, Mass.] per footpad simultaneously with the metacyclic parasites in a volume of 50  $\mu$ l). This treatment resulted in the specific upregulation of XCL1, CXCL10, and CCL2 (Fig. 3B) 16 h postinfection with *L. major*. It is possible that infection-induced IL-4 could actively inhibit chemokine expression in BALB/c mice (3, 10). However infection of BALB/c IL-4-deficient mice did not result in an upregulation of chemokine gene expression (data not shown).

Finally, to determine if the effects of IL-12 were dependent upon increased IFN- $\gamma$  production (20), we examined whether blockade of IFN- $\gamma$  would alter expression of chemokine expression in *L. major*-infected C3H mice. We treated C3H mice with 1 mg of  $\alpha$ -IFN- $\gamma$  (XMG6; DNAX Research Institute, Palo Alto, Calif.) 24 h prior to challenge with *L. major*. Draining popliteal lymph nodes were harvested 16 h postinfection, RNA was extracted, and chemokine gene expression was analyzed by RPA. Figure 3C shows that in the absence of IFN- $\gamma$ , specific chemokine gene expression is not induced.

Several studies have shown that chemokines can promote the production of cytokines, such as IL-12, IFN- $\gamma$ , and IL-4. For example, ligation of CCR5 by CCL4 is reported to induce IL-12 production by CD8 $\alpha^+$  dendritic cells following infection with *Toxoplasma gondii* (1). We saw no preferential expression of CCL3 or CCL4, which suggests that a similar pathway may not operate during leishmaniasis. In contrast, we found that chemokine gene expression was blocked in the absence of IL-12 and IFN- $\gamma$  (Fig. 3). Thus, these data suggest that cytokines precede the expression of chemokines, or at least these

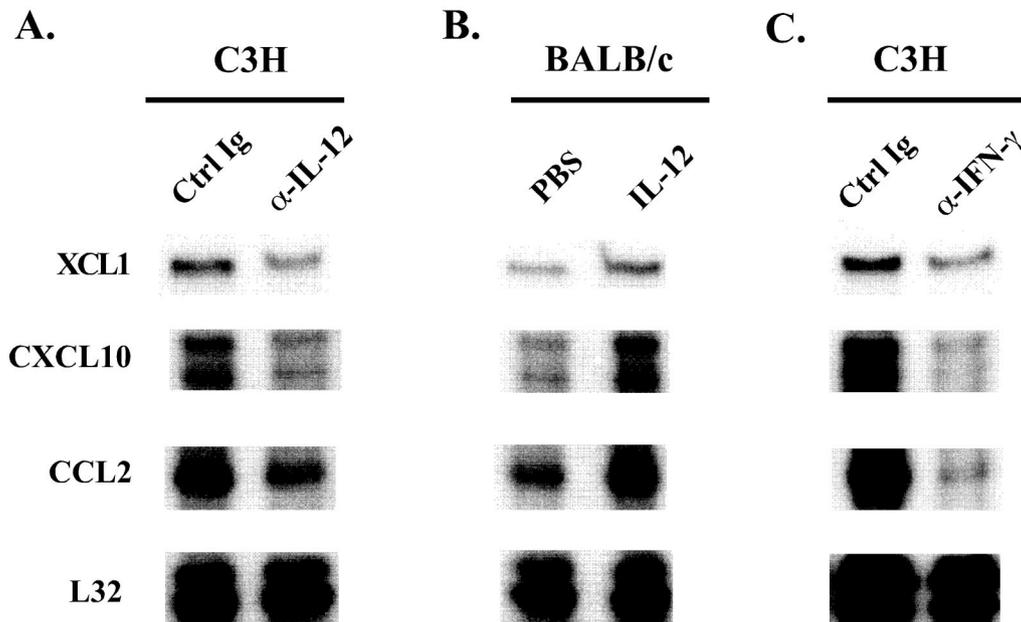


FIG. 3. IL-12 and IFN- $\gamma$  regulate expression of XCL1, CXCL10, and CCL2 in vivo. RPA analysis of RNA isolated from draining lymph nodes from C3H and BALB/c mice at 16 h after infection with *L. major* and treatment with  $\alpha$ -IL-12 (A), IL-12 (B), or  $\alpha$ -IFN- $\gamma$  (C). C3H mice were treated with 1 mg of  $\alpha$ -IL-12 (C17.8) or  $\alpha$ -IFN- $\gamma$  (XMG6) 24 h prior to infection, while BALB/c mice were treated intralesionally with 0.25  $\mu$ g of IL-12 at the time of infection. Results are representative of three separate experiments. PBS, phosphate-buffered saline; Ctrl Ig, control immunoglobulin.

dominant chemokines, in the draining lymph node during infection with *L. major*. These findings agree with studies using other model systems (angiogenesis and *Cryptococcus neoformans* infection), which have also demonstrated a role for IL-12 in chemokine gene expression (8, 21). Our data do not exclude the possibility of a feedback loop, where chemokine expression precedes IL-12 and IFN- $\gamma$  expression and subsequent increased levels of chemokine gene expression are dependent upon IL-12 and IFN- $\gamma$ . However, several reports suggest that chemokines do not upregulate IL-12 and in fact downregulate its expression (2, 7). For example, intravenous treatment with pertussis toxin to block chemokine receptor signaling can render BALB/c mice resistant to infection with *L. major*, and this effect is associated with increased IL-12 production (5).

The functional relevance of the expression of these specific chemokines is not fully understood. XCL1 has been shown to be chemotactic for NK cells and CD8<sup>+</sup> T cells in vitro and in vivo (6, 9). Both cell types are producers of infection-induced IFN- $\gamma$  and may act to enhance resistance to *L. major* (11, 19). CXCL10 has been demonstrated to activate NK cells in vivo, although treatment of *L. major*-infected BALB/c mice with recombinant CXCL10 had no effect on the outcome of disease (22). Recently a role for CXCL10 in the retention of Th1 cells in lymph nodes following injection of *Propionibacterium acnes* has been described (23). It remains to be seen if this phenomenon also contributes to the development of resistance to *L. major*.

There is conflicting evidence on the role of CCL2 during immune responses. On one hand, CCL2 has been shown to inhibit IL-12 production and may be involved in the development of polarized Th2 responses (2, 4, 7). Conversely, CCL2 is induced after infection with *L. major* (12, 22) and has been shown to stimulate the killing of *L. major* by human monocytes (15). Furthermore, CCR2-deficient mice (CCL2 receptor) are susceptible to infection with *L. major*, possibly due to a defect in Langerhans cell migration to the draining lymph node (18). Data from studies of human leishmaniasis have shown a correlation between healing local cutaneous leishmaniasis lesions and expression of CCL2 (14, 16). Thus, CCL2 may be involved in both cell migration and activation.

The development of a protective immune response against *L. major* occurs over several weeks. It is intriguing to hypothesize that events as early as 16 h postinfection may be involved in the development of resistance. The data presented here show a strong correlation between the expression of three specific chemokine genes (XCL1, CXCL10, and CCL2) and mediators of resistance to *L. major*. The functional relationship between chemokines and cytokines will undoubtedly be critical to our understanding of protective immune responses.

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