BCL-6-Deficient Mice Reveal an IL-4-Independent, STAT6-Dependent Pathway That Controls Susceptibility to Infection by *Leishmania major*

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BCL-6-Deficient Mice Reveal an IL-4-Independent, STAT6-Dependent Pathway That Controls Susceptibility to Infection by Leishmania major

Alexander L. Dent,* T. Mark Doherty,† William E. Paul,‡ Alan Sher,† and Louis M. Staudt†*

The BCL-6 gene negatively regulates Th2 responses as shown by the finding that BCL-6-deficient (BCL-6−/−) mice develop a lethal Th2-type inflammatory disease. The response of inbred mouse strains to infection with Leishmania major is under genetic control; BALB/c mice are susceptible and develop a progressive parasite burden, whereas most other common laboratory strains of mice are resistant to infection. We found that BCL-6−/− mice on a resistant genetic background (C57BL/6 × 129 intercrossed mice) were highly susceptible to L. major infection; they resembled BALB/c mice in terms of lesion size, parasite load, and the production of Th2 cytokines. BCL-6−/−IL-4−/− double-mutant mice were also susceptible to L. major infection and produced 10-fold higher levels of the Th2 cytokine IL-13 than IL-4−/− littermate controls. By contrast, BCL-6−/−STAT6−/− double-mutant mice were resistant to L. major infection despite also producing elevated levels of IL-13. These results show that STAT6 is required for susceptibility to L. major infection and suggest that IL-13 signaling through STAT6 may contribute to a nonhealing, exacerbated L. major infection. The Journal of Immunology, 1999, 163: 2098–2103.

Infection of mice with the parasite Leishmania major has been a useful model system to study the development of Th cell subsets during the course of a natural immune response (reviewed in Ref. 1). Infection of mouse strains such as C57BL/6 and 129/SvEv with L. major results in a healing immune response and in the development of Th1 cells, whereas infection of BALB/c mice with L. major results in a nonhealing, progressive disease and in the development of Th2 cells. An explanation for these different outcomes of L. major infection in mice is that IFN-γ produced by Th1 cells is required for optimal activation of macrophage microbicidal function (2, 3), whereas the Th2 cytokines IL-4, IL-10, and IL-13 suppress macrophage activation and permit intracellular parasite growth (4–6). The factors regulating the development of Th1 vs Th2 responses during L. major infection in different strains of mice has been an area of vigorous investigation. Most mouse strains produce Th1 responses after L. major infection. However, infected BALB/c mice produce an early burst of IL-4, which appears to skew the developing T cell response toward the Th2 subset (7). BALB/c mice have a subset of CD4 T cells bearing specific TCRs that appear to have been preprogrammed to secrete IL-4 after activation by L. major Ags (8). Interference with this IL-4 production in BALB/c mice by anti-IL-4 Abs or by gene disruption converts the BALB/c anti-L. major response to a healing response in most, but not all, cases (9–11). Conversely, disruption of the genes for IFN-γ or IL-12 can switch an L. major-resistant mouse strain to an L. major–susceptible strain (12, 13). Thus, manipulation of the Th cell response has been shown to be critical for the outcome of L. major infection in mice.

Studies of mutant BCL-6−/− mice have shown BCL-6 to be an important regulator of Th2 cell responses (14–16). BCL-6−/− mice characteristically develop a spontaneous and fatal Th2-type inflammation of the heart and lungs. Moreover, immunization of BCL-6−/− mice with a protein Ag in adjuvant results in the generation of Th2 cells and Th2-type inflammation. Interestingly, the Th2 inflammatory response proceeds even in BCL-6−/−IL-4−/− mice and BCL-6−/−STAT6−/− double-mutant mice, suggesting that BCL-6 controls Th2 differentiation by a pathway independent of signaling through the IL-4R (16). BCL-6 is a transcriptional repressor protein that binds DNA motifs that closely resemble the IFN-γ activation site motifs recognized by the STAT transcription factors (14). Furthermore, BCL-6 can repress IL-4–induced transcription mediated by STAT6 (14). Thus, one hypothesis is that BCL-6 may regulate Th2 differentiation by modulating transcription induced by cytokine signaling through STAT proteins.

To further characterize the role of BCL-6 in the development of Th2 cells and Th2 responses, we infected BCL-6−/− mice (on a C57BL/6 × 129 intercrossed background) with L. major. We found that lack of functional BCL-6 resulted in a nonhealing L. major response that correlated with the development of Th2 cells. BCL-6−/−IL-4−/− mice were still susceptible to L. major infection, but BCL-6−/−STAT6−/− mice were resistant. These results indicate that susceptibility to L. major infection in this model depends on signaling through STAT6. IL-4 and IL-13 are both products of Th2 cells, and both signal through STAT6 (17–19). The fact that disruption of IL-4 alone did not make BCL-6−/− mice resistant to L. major suggests that IL-13 plays a major role in mediating nonhealing L. major responses in the absence of BCL-6.

Materials and Methods
Mice
BCL-6−/−, BCL-6−/−IL-4−/−, and BCL-6−/−STAT6−/− mice were generated as previously described (14, 16). The IL-4−/− mouse line was described by Kopf et al. (20). The STAT6−/− mouse line was described by Shimoda et al. (21). Animals were housed in an American Association for the Advancement of Science laboratory.
Murine IL-13 was measured by ELISA with reagents obtained from R&D Systems (Minneapolis, MN), and the sensitivity of the IL-13 ELISA was 8 pg.

**Results**

*BCL-6−/− mice are susceptible to *L. major* infection*

Because BCL-6 negatively regulates the development of Th2 cells, we decided to test whether BCL-6−/− mice would be susceptible to infection with the parasite *L. major*. The genetic background for the BCL-6−/− mice is a mix of C57BL/6 (B6) and 129Sv (129) strains, and both the C57BL/6 and 129 mouse strains are reported to be resistant to *L. major* infection (1, 13). Therefore, BCL-6−/− mice, littermate B6-129 wild-type mice, and BALB/c mice were infected with *L. major* by s.c. injection of 10⁶ parasites in one rear footpad. The infections were allowed to proceed only 5 wk due to the high frequency of early death of the BCL-6−/− mice. Nevertheless, at this time, differences between resistant and susceptible strains of mice usually become apparent. Indeed, at this time point, the swelling of both the BCL-6−/− and BALB/c footpads was significantly greater than that of wild-type B6-129 footpads (Fig. 1). Moreover, the footpads of both BCL-6−/− and BALB/c mice had characteristic skin ulcers and tissue necrosis (Table I). Next, the number of viable parasites in the lymph node draining the infection site was assayed. The parasite loads of BCL-6−/− and BALB/c mice were comparable, and both types of mice had 2 orders of magnitude more parasites than wild-type B6-129 mice (Fig. 2). T cell responses to *L. major* infection were assayed by testing cytokine production after polyclonal stimulation of the T cells from the draining lymph nodes. Both BCL-6−/− and BALB/c mice produced significantly higher levels of the Th2 cytokines IL-4 and IL-13 than did wild-type B6-129 mice (Fig. 3). The BCL-6−/− mice actually made significantly greater IL-4 and IL-13 than did wild-type B6-129 mice (Fig. 3).

FIGURE 1. Footpad swelling from B6–129, BCL-6−/−, BALB/c, IL-4−/−, BCL-6−/− IL-4−/−, STAT6−/−, and BCL-6−/− STAT6−/− mice 5 wk postinfection. Footpad swelling is calculated as the size difference in millimeters between infected and uninfected footpads from the same mouse. Average swelling is shown ±SE. Asterisks indicate statistically significant results as evaluated by Student’s *t* test, and the accompanying *p* values are shown. BCL-6−/− and BALB/c values were evaluated with respect to wild-type values. The BCL-6−/− IL-4−/− values were evaluated with respect to IL-4−/− values. The BCL-6−/− STAT6−/− values were evaluated with respect to STAT6−/− values. The BCL-6−/− IL-4−/− *p* value with respect to the wild-type value was <0.001. The BCL-6−/− STAT6−/− *p* value with respect to the wild-type value was 0.134.
of Th2 cytokines in both BCL-6−/- mice and BALB/c mice correlated with their nonhealing responses to the parasite infection. These data, taken together, show that BCL-6 controls both the production of Th2 cytokines and the susceptibility to *L. major* infection.

**Susceptibility to *L. major* infection in BCL-6−/− mice does not require IL-4**

In BALB/c mice, disruption of IL-4 function by anti-IL-4 Abs or IL-4 gene targeting can block susceptibility to *L. major* infection (9–11), although there are exceptions to these findings (23, 24). We therefore studied the role of IL-4 in the susceptibility of BCL-6−/− mice to *L. major* infection by mating the BCL-6−/− mice to mice genetically incapable of making IL-4 (IL-4−/− mice) (20). BCL-6−/−IL-4−/− double-mutant mice and IL-4−/− littermates were infected with *L. major* and analyzed 5 wk after infection.

BCL-6−/−IL-4−/− mice developed footpad swelling and parasite counts comparable in magnitude to those observed in BCL-6−/− mice and significantly greater than those observed in either IL-4−/− or wild-type mice (Figs. 1 and 2). In fact, the footpad pathology in BCL-6−/−IL-4−/− mice was worse than that in BCL-6−/− mice in terms of both skin ulceration and tissue necrosis (Table I). Another indication of *L. major* disease progression is the dissemination of parasites to the spleen, typically observed in *L. major*-susceptible mice. The spleens of wild-type, BCL-6−/−, IL-4−/−, and BCL-6−/−IL-4−/− mice were therefore assayed for the presence of parasites (Table II). Although the spleens of only one of 10 wild-type mice and one of five IL-4−/− mice were positive for parasites, every spleen examined from BCL-6−/− and BCL-6−/−IL-4−/− mice contained parasites. Thus, in terms of footpad pathology and parasite load, the disruption of IL-4 gene expression did not affect the progression of *L. major* infection in BCL-6−/− mice. Because IL-4−/− mice do not produce IL-4, we assessed the generation of a Th2 response by measuring IL-5 and IL-13 secretion by T cells from the draining lymph nodes of infected mice. Although T cells from infected BCL-6−/−IL-4−/− mice produced only modestly elevated (2×) levels of IL-5 compared with T cells from infected IL-4−/− mice (data not shown), the BCL-6−/−IL-4−/− T cells produced 10-fold more IL-13 than the IL-4−/− T cells (Fig. 3). T cells from infected BCL-6−/−IL-4−/− mice produced 3-fold less IFN-γ than T cells from infected IL-4−/− mice (Fig. 3). Thus, as judged by the elevated secretion of IL-13 and reduced secretion of IFN-γ, BCL-6−/−IL-4−/− mice developed a Th2-like response during *L. major* infection, although the levels of IL-13 produced were lower than those observed in infected BCL-6−/− mice (Fig. 3).

**Susceptibility to *L. major* infection in BCL-6−/− mice is STAT6 dependent**

Because STAT6 is a critical transducer of signaling by IL-4 and IL-13, we tested whether disruption of STAT6 would affect the susceptibility of BCL-6−/− mice to *L. major* infection. BCL-6−/−STAT6−/− mice and STAT6−/− littermates were infected with *L. major* and analyzed 5 wk after infection. STAT6−/− mice displayed parasite counts in the range of wild-type mice (Fig. 2). BCL-6−/−STAT6−/− mice, in marked contrast to BCL-6−/− and BCL-6−/−IL-4−/− mice, displayed parasite counts that were not significantly greater than those observed in littermate STAT6−/− mice (Fig. 2). Consistent with the lower parasite counts, the footpad pathology of BCL-6−/−STAT6−/− mice was

### Table I. Gross pathology of Leishmania-infected footpads

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Skin Ulceration</th>
<th>Tissue Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0/28</td>
<td>0/28</td>
</tr>
<tr>
<td>BCL-6−/−</td>
<td>4/6</td>
<td>1/6</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>IL-4−/−BCL-6−/−</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>STAT6−/−</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>STAT6−/−BCL-6−/−</td>
<td>2/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Data in table for 5 wk postinfection.
* Positive footpad/total examined.
* Statistical significance with respect to wild type (*p < 0.05*).

### Table II. Frequencies of Leishmania infiltration into the spleens of infected mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Paraise Positive</th>
<th>Ave. No. Parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1/10</td>
<td>3.2</td>
</tr>
<tr>
<td>BCL-6−/−</td>
<td>3/3</td>
<td>200</td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>1/5</td>
<td>0.2</td>
</tr>
<tr>
<td>IL-4−/−BCL-6−/−</td>
<td>5/5</td>
<td>18</td>
</tr>
<tr>
<td>STAT6−/−</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>STAT6−/−BCL-6−/−</td>
<td>0/4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data in table for 5 wk postinfection.
* Positive spleens/total examined.
* Statistical significance with respect to wild type (*p < 0.05*).
also less severe than that of BCL-6−/− mice and BCL-6−/− IL-4−/− mice (Fig. 1 and Table I). Most strikingly, none of the BCL-6−/− STAT6−/− mice displayed dissemination of parasites to the spleen. Taken together, these results show that the loss of STAT6 greatly decreases the susceptibility BCL-6−/− mice to infection with L. major.

T cells from infected BCL-6−/− STAT6−/− mice produced more IL-13 than those from STAT6−/− littermate controls, but the IL-13 response was less vigorous than that in BCL-6−/− or BCL-6−/− IL-4−/− mice (Fig. 3). The BCL-6−/− STAT6−/− T cells did not secrete detectable IL-4 and did secrete somewhat more IFN-γ than STAT6−/− controls. Thus, the strong Th2-like response observed in infected BCL-6−/− mice was attenuated by the absence of STAT6, although T cells from BCL-6−/− STAT6−/− mice still produced elevated levels of IL-13.

FIGURE 3. Cytokine measurements after Con A stimulation of the draining LN cells of B6-129, BCL-6−/−, BALB/c, IL-4−/−, BCL-6−/− IL-4−/−, STAT6−/−, and BCL-6−/− STAT6−/− mice 5 wk postinfection. Average amounts of IFN-γ, IL-4, and IL-13 are shown ± SE. Asterisks indicate statistically significant results as evaluated by Student’s t test, and the accompanying p values are shown. All values were evaluated with respect to wild-type values. Other statistically different differences are: for IFN-γ, the p value for BCL-6−/− IL-4−/− with respect to IL-4−/− is 0.019; for IL-13, the p value for BCL-6−/− IL-4−/− with respect to IL-4−/− is 0.004; and the p value for BCL-6−/− STAT6−/− with respect to STAT6−/− is 0.018.

Discussion

We have found that the BCL-6 oncogene plays an important role in regulating responses to L. major in mice; disruption of the BCL-6 gene is sufficient to turn a resistant mouse strain into a susceptible strain. Additionally, we have found a novel IL-4-independent, STAT6-dependent pathway that regulates susceptibility to L. major in BCL-6−/− mice. This IL-4-independent, STAT6-dependent pathway implicates IL-13 as a mediator of susceptibility to L. major infection.

BCL-6−/− mice display two prominent immune deficits: an abnormal development of Th2 cells and an inability of B cells to generate a germinal center response (14). The B cell defect in these mice is unlikely to play a major role in their response to L. major infection; a recent study showed that the presence or the absence of B cells does not affect the course of L. major infection in either C57BL/6 or BALB/c mice (25). Moreover, both BCL-6−/− and BCL-6−/− STAT6−/− mice have similar B cell defects (A. L. Dent, unpublished observations), but differ in their resistance L. major infection. On the other hand, the uncontrolled Th2 cell differentiation in BCL-6−/− mice could well contribute to their susceptibility to L. major infection. Previous studies of BALB/c mice (with a wild-type BCL-6 gene) have suggested that IL-4 is required for the generation of a Th2-dominated immune response to L. major (9–11). Unlike other models of Th2 differentiation, however, the production of Th2 cytokines in BCL-6−/− mice is independent of functional IL-4 and STAT6 genes (16). Indeed, we observed that BCL-6−/− STAT6−/− mice and BCL-6−/− IL-4−/− mice developed Th2-type heart and lung inflammation at the same rate as BCL-6−/− mice. This novel pathway to Th2 development in BCL-6−/− mice presumably explains why BCL-6−/− IL-4−/− mice were able to develop a nonhealing, Th2-like response to L. major. The fact that BCL-6−/− STAT6−/− mice develop Th2-type inflammatory disease at the same rate as BCL-6−/− mice contrasts with the finding that these two strains differ in their susceptibility to L. major infection. Thus, the inflammatory phenotype characteristic of BCL-6−/− mice is genetically separable from the L. major susceptibility phenotype. This is noteworthy because it demonstrates that the L. major susceptibility of BCL-6−/− mice is not merely secondary to a general immunodeficiency associated with their inflammatory disease.

In this study we found that BCL-6−/− IL-4−/− mice developed severe L. major infections, while BCL-6−/− STAT6−/− mice were resistant to L. major infection. IL-4 and IL-13 are homologous proteins and are the only two cytokines that have been shown to physiologically activate the STAT6 transcription factor (17–19). Because the absence of IL-4 did not alter the susceptibility of BCL-6−/− mice to L. major, the present results suggest that IL-13 signaling through STAT6 contributes to the pathologic, nonhealing L. major response in BCL-6−/− mice. A role for IL-13 in L. major infection is supported by Noben-Trauth et al., who observed significant differences in susceptibility of IL-4−/− BALB/c mice vs IL-4R−/− BALB/c mice when infected with the L. major substrain IR173 (23). Moreover, other recent studies have found that IL-13 is an important factor for controlling intestinal parasite infection (26–28). In studies with IL-4−/− mice, IL-4Rα−/− mice, and STAT6−/− mice, IL-13 was a more potent factor than IL-4 in the expulsion of N. brasiliensis parasites from the intestine (27, 28). In addition, a recent study using IL-13-deficient mice has shown IL-13 to be critical for the management of infection with the intestinal worm Trichuris muris (26). Thus, the accumulated evidence suggests that IL-4 and IL-13 have both unique and overlapping functions in immune responses to parasites.
It is not clear at present what cell type would be critically affected by IL-13 during L. major infection. Naïve T cells have IL-4R but not IL-13 receptors, and consequently, IL-13 cannot drive Th2 differentiation in vitro (29). However, a recent report demonstrated that T cells from IL-13−/− mice are deficient in Th2 generation in vitro (30). This defect did not reflect a role for IL-13 during the in vitro culture, because addition of exogenous IL-13 did not reverse the defect in Th2 differentiation of IL-13−/− T cells. Rather, it appears that IL-13 exerted an effect on T cells in vivo that impaired their ability to respond to IL-4 and differentiate to Th2 cells both in vivo and in vitro. Thus, it is possible that IL-13 might augment Th2 responses in vivo and thereby influence the magnitude of the Th2 response of BCL-6−/− mice to during L. major infection. This possibility is compatible with the observation that T cells from BCL-6−/− STAT6−/− mice produced lower levels of Th2 cytokines at 5 wk postinfection than did T cells from BCL-6−/− mice and BCL-6−/− IL-4−/− mice at 5 wk postinfection. Although BCL-6−/− STAT6−/− T cells can produce high levels of Th2 cytokines 2 wk after immunization with a protein Ag plus adjuvant (16), STAT6-mediated IL-13 signaling may be required to maintain Th2 responses in BCL-6−/− STAT6−/− mice at longer time points.

Alternatively, IL-13 may regulate the response of non-T cells during L. major infection. IL-13 may play a critical role in L. major infection due to its ability to inhibit macrophage activation (6, 31). Activated macrophages are important for the elimination of the L. major parasite, and IL-13 may modulate the L. major response by inhibiting macrophage function during the infection. Recently, it was demonstrated that IL-13 can inhibit the production of nitric oxide by activated wild-type macrophages but not activated STAT6−/− macrophages (32). Thus, the healing response of BCL-6−/− STAT6−/− mice to L. major infection might be due to the lack of an inhibitory action by IL-13 on STAT6-deficient macrophages, thereby allowing these macrophages to effectively control the growth of the parasite. BCL-6−/− STAT6−/− mice may therefore have lower Th2 responses in response to L. major infection because the macrophages have controlled the infection, which would result in less T cell stimulation and differentiation.

STAT6 has also been shown to be an important factor in the murine immune response to L. mexicana (33). In contrast to infection of mice with L. major, L. mexicana forms chronic infections in most strains of mice, including C57BL/6 and 129/Sv. Although not as well characterized as the murine response to L. major, the nonresolving response of mice in response to L. mexicana infection correlates with the induction of a Th2-type response. Disruption of IL-4 signaling by mutation of the STAT6 gene therefore prevents the formation of a nonhealing response to L. mexicana infection, resulting in the formation of a healing Th1 type of response (33). Although the study by Stamm et al. (33) reveals a critical role for STAT6 in regulating the murine immune response to Leishmania infection, no mention was made of the role of IL-13.

The response of BCL-6−/− mice to L. major infection again highlights the critical role of BCL-6 in regulating T cell differentiation to Th2 cells in vivo. The genetic absence of BCL-6 was sufficient to convert a Th1 immune response to L. major to a response in which Th2 cytokines predominated. Indeed, T cells from infected BCL-6−/− mice produced higher levels of Th2 cytokines than did T cells from infected BALB/c mice. The mechanisms by which BCL-6 regulates Th cell differentiation are not known at present. BCL-6 is not constitutively expressed by T cells, and BCL-6 is not expressed after mitogenic activation of T cells in vitro (A. L. Dent, unpublished observations). However, BCL-6 is highly expressed in vivo in germinal center T cells as well as in a subset of CD30-positive T cells scattered throughout the T cells zones of secondary lymphoid organs (34, 35). The in vivo activation signals that lead to this high level of BCL-6 expression in T cells are not known. Conceivably, though, the expression of BCL-6 in this T cell subset may be necessary to block a pathway to Th2 differentiation in vivo (reviewed in Ref. 36). This pathway is distinct from the previously described Th2 differentiation pathways in that it can proceed in the absence of IL-4 and STAT6. Future work will address whether BCL-6 induction is required in all T cells to prevent Th2 differentiation or whether this novel, BCL-6-dependent, regulatory pathway is engaged in only a subset of T cells responding to certain antigenic challenges.

References


