

Infection with the Helminth *Nippostrongylus brasiliensis* Does Not Interfere with Efficient Elimination of *Mycobacterium bovis* BCG from the Lungs of Mice

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Received 25 July 2001/Returned for modification 13 September 2001/Accepted 5 March 2002

Infection with *Mycobacterium tuberculosis* continues to be one of the major global health threats. Strong mycobacterium-specific Th1 immune responses correlate with protection, and decreased Th1 responses correlate with disease progression. In contrast, the impact of Th2 responses on the development of protective immune responses to mycobacteria remains unclear. To analyze whether ongoing Th2 responses present in the lung influence the development of a protective Th1 immune response to mycobacteria, we coinfectd mice with the helminth *Nippostrongylus brasiliensis* and *Mycobacterium bovis* BCG. We found that the T cells from the lymph nodes of coinfectd mice secreted significantly less gamma interferon than did the T cells from mice infectd with *M. bovis* BCG after in vitro stimulation with purified protein from *M. tuberculosis* when 10⁸ CFU of *M. bovis* BCG were used for the infection. This result indicates that the helminth infection reduced the Th1 immune response to the mycobacteria in the lung. However, mycobacterial clearance was not delayed in the coinfectd animals. Importantly, the infection with BCG after the helminth infection did not reduce the helminth-induced Th2 response in the lung, ruling out the possibility that the lack of a reduction in bacterial clearance in the coinfectd mice was due to a downmodulation of the helminth-induced Th2 response. Taken together, our results suggest that ongoing Th2 responses in the lung do not necessarily lead to increased susceptibility to mycobacterial infection.

Mycobacterium tuberculosis and *Mycobacterium bovis* are facultative intracellular parasites which tend to reside within macrophages. Infections with these bacteria cause tuberculosis in humans and livestock. Macrophages infectd with mycobacteria interact with both CD4⁺ and CD8⁺ T cells, inducing the release of cytokines by both macrophages and T cells, which in turn activate antimicrobial macrophage functions, usually leading to the control of the mycobacterial infection (5, 24). This effect is mostly mediated by gamma interferon (IFN- γ) and tumor necrosis factor alpha (5, 6, 14, 15, 18, 24). However, the cause of individual variations in susceptibility to mycobacterial disease is only partly understood. It is believed that protection is associated with strong Th1 cell-mediated immune responses, whereas Th2 immune responses with high interleukin-4 (IL-4), IL-5, and IL-10 levels promote disease (5, 24). Supporting this view are the findings that both IL-4 and IL-10 can downmodulate macrophage functions in vitro, which explains how the presence of IL-4 and/or IL-10 could interfere with the effective elimination of mycobacteria in vivo (2, 8, 9, 25). Furthermore, there is also evidence suggesting that the presence of IL-10 delays mycobacterial clearance (1, 7, 16, 17, 21, 22). However, other studies have failed to observe this effect (11, 23). Production of IL-4 and IL-5 during mycobacterial infection does not seem to play a major role in the efficient elimination of mycobacteria in immunocompetent mice (11, 23). However, eosinophils (dependent upon the production of IL-5 [4, 20])

may at least potentially play both positive and negative roles in the elimination of mycobacteria, since it has been reported that eosinophils can take up and kill mycobacteria but also contribute to a more rapid growth of the bacteria in IFN- γ receptor-deficient mice (3, 19). Taken together, the impact of Th2 responses on the efficient elimination of mycobacteria in vivo is unclear. This may also be an important issue in the design of new vaccines or therapies that aim to protect humans from tuberculosis. Furthermore, humans exposed to mycobacteria or harboring a dormant mycobacterial infection may develop allergen- or helminth-induced Th2 responses in their lifetime, possibly leading to increased susceptibility to a primary infection with mycobacteria or to a reactivation of a previously controlled infection. In particular, infections with helminths may play a role in the pathogenesis of tuberculosis in the developing countries of the world, since exposure to mycobacteria and simultaneous infection with helminths are very common.

To address the question of whether the Th2 response initiated by a helminth infection influences susceptibility to mycobacteria, C57BL/6 mice were infectd intraperitoneally (i.p.) with 1,000 L3 larvae of the helminth *Nippostrongylus brasiliensis* and intranasally (i.n.) 4 days later with either 2 \times 10⁴ or 1 \times 10⁸ CFU of *M. bovis* bacillus Calmette-Guérin (BCG) (strain Copenhagen; generously provided by Jürgen Hess) as described previously (10). Helminths initiate a strong Th2 response characterized by eosinophilia and the secretion of IL-4, IL-5, and IL-10 by T cells, first in the lung and then in the gut (12). The experimental protocol we used was aimed at ensuring that the Th1 immune response to the mycobacteria occurred at the same time and site as the Th2 response induced

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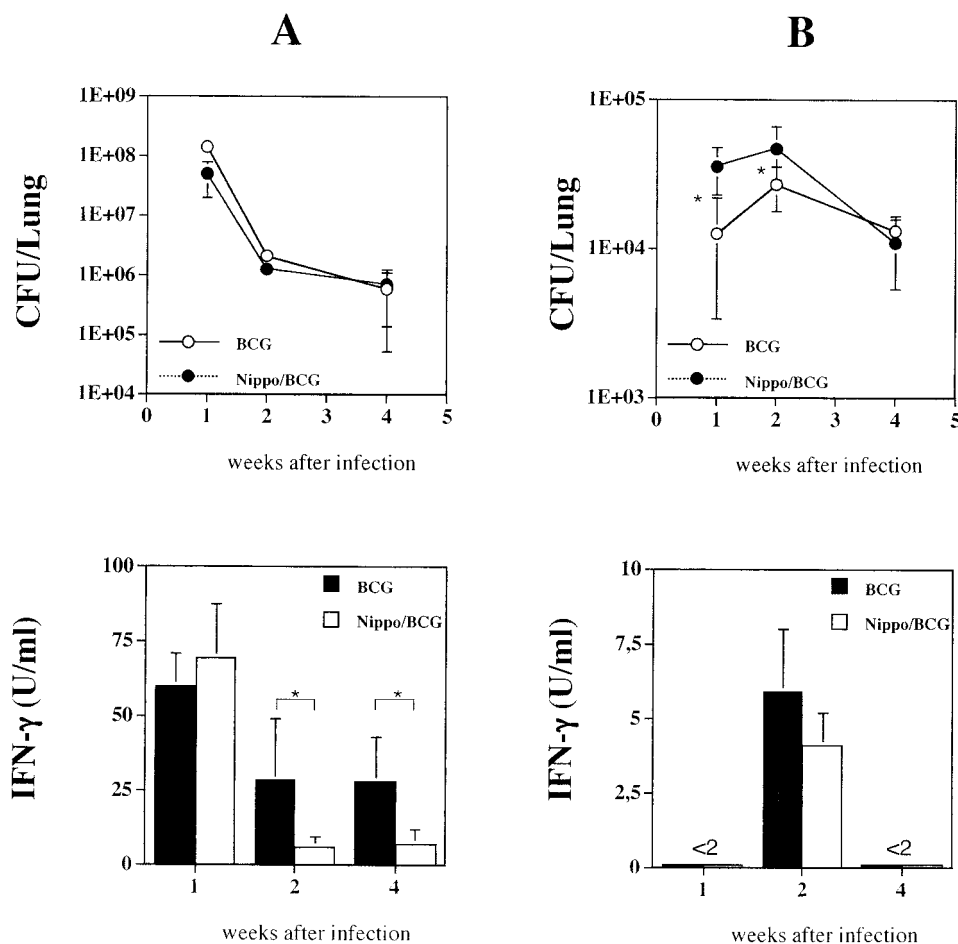


FIG. 1. Mice coinfecting with the helminth *N. brasiliensis* (Nippo) and *M. bovis* BCG show no delay in bacterial clearance in comparison to mice infected only with *M. bovis* BCG. Mice were coinfecting with 1,000 L3 larvae of the helminth *N. brasiliensis* (i.p., day 0) and then with 1×10^8 or 2×10^4 CFU of *M. bovis* BCG (i.n., day 4). In parallel, mice were also infected with similar amounts of *M. bovis* BCG only. One, two, and four weeks after the mycobacterial infection, the numbers of CFU per lung were determined for the different groups of mice. In parallel to the preparation of the lungs, single-cell suspensions (2×10^5 /well) of total MLN cells from mice at 1, 2, and 4 weeks postinfection were stimulated in vitro for 48 h with PPD (20 μ g/ml). The level of IFN- γ present in the supernatants was determined by ELISA. Shown are the mean values with standard deviations (error bars) obtained when 1×10^8 CFU (A) or 2×10^4 CFU (B) of *M. bovis* BCG were used for the i.n. infection of five to eight mice per group. Statistical analysis was performed by using the unpaired Student *t* test. Asterisks indicate *P* values of <0.05 .

by the helminths migrating through the lung on their way to the gut. We then analyzed whether this Th2 response reduced the development of a protective Th1 immune response to *M. bovis* BCG. One, two, or four weeks after infection, the mice were sacrificed, their lungs were removed, and the number of bacteria present in the lungs was determined as described previously (10, 11). The number of *M. bovis* BCG bacteria present in the lungs of coinfecting mice was compared with the number detected in the lungs of mice infected only with *M. bovis* BCG at the same time points. Furthermore, the antimycobacterial Th1 response induced in the lungs was analyzed for the different groups of mice. For this purpose, mediastinal lymph node (MLN) cells from the different groups of mice were stimulated in vitro with purified protein derivative from *M. tuberculosis* (PPD) (20 μ g/ml; Statens Serum Institute, Copenhagen, Denmark), and the amount of IFN- γ present in the culture supernatant was determined as described previously (10, 11). Since it has previously been reported that an infection with *M. bovis* BCG prior to an infection with *N. brasiliensis* decreased the

Th2 response to helminths in the lung (10), we also analyzed whether the *M. bovis* BCG infection 4 days after the helminth infection could produce a similar effect. For this purpose, mice were infected with *N. brasiliensis* only or with *N. brasiliensis* and, 4 days later, 10^8 CFU of *M. bovis* BCG (see above). Eleven days later, the mice were sacrificed, bronchoalveolar lavages (BAL) were performed, and single-cell suspensions of MLN cells were prepared. BAL fluid cells were counted and stained with hematoxylin and eosin, and the number of eosinophils was identified microscopically. The MLN cells were stimulated in vitro for 48 h on anti-CD3-bound plates in the presence of IL-2. The levels of IL-4, IL-5, and IL-10 present in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (11).

Our results showed that bacterial clearance was not delayed in the coinfecting mice compared to that in animals infected only with 10^8 CFU of *M. bovis* BCG (Fig. 1A). A repetition of the experiment also showed that the helminth infection did not result in a delay of bacterial clearance (data not shown). In

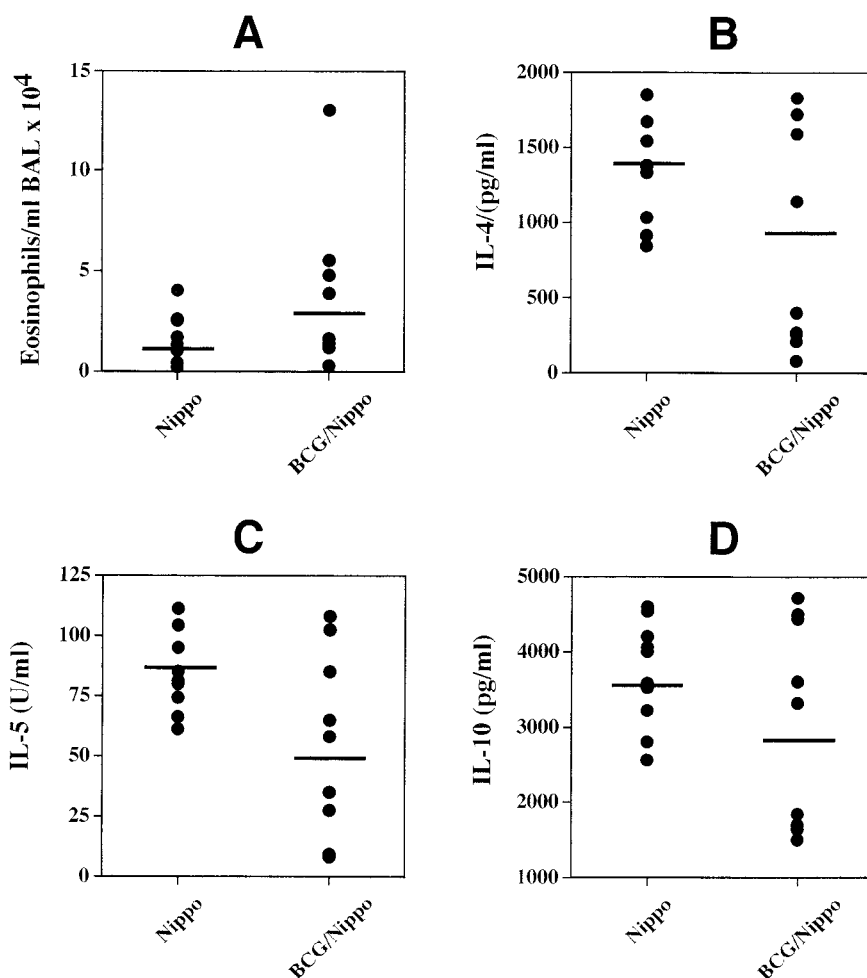


FIG. 2. Infection with *M. bovis* BCG does not inhibit the development of a Th2 response in the lungs of mice previously infected with *N. brasiliensis* (Nippo). In an experiment separate from the one described in the legend to Fig. 1, mice were either infected with 1,000 L3 larvae of the helminth *N. brasiliensis* only (i.p., day 0) or coinfecting with *N. brasiliensis* (i.p., day 0) and 10⁸ CFU of *M. bovis* BCG (i.n., day 4). Eleven days after the initial infection with *N. brasiliensis*, BAL were performed and single-cell suspensions of MLN cells were prepared. BAL fluid cells were counted and stained with hematoxylin and eosin, and the different cell types were identified microscopically. The MLN cells were stimulated in vitro for 48 h on anti-CD3-bound plates in the presence of IL-2. The amounts of IL-4, IL-5, and IL-10 in the supernatants were determined by ELISA. Shown are the numbers of eosinophils present in the BAL fluid (A) and the amounts of IL-4 (B), IL-5 (C), and IL-10 (D) secreted by the MLN cells after in vitro stimulation of individual mice in each group. The mean numbers of eosinophils and levels of cytokines detected in the BAL fluid and supernatants from the MLN cells of the different groups of mice are indicated. The experiment was repeated once with similar results.

contrast, mice coinfecting with the helminths and 2×10^4 CFU of *M. bovis* BCG eliminated the mycobacteria significantly more slowly at 1 and 2 weeks but not 4 weeks after infection than mice infected only with the mycobacteria (Fig. 1B). However, a repetition of this experiment showed no delay in bacterial clearance in mice coinfecting with *N. brasiliensis* and *M. bovis* BCG (1 week, $30.9 \times 10^3 \pm 14.0 \times 10^3$ CFU in BCG-only mice versus $32.9 \times 10^3 \pm 14.2 \times 10^3$ CFU in coinfecting mice; 2 weeks, $84.4 \times 10^3 \pm 32.3 \times 10^3$ CFU in BCG-only mice versus $58.5 \times 10^3 \pm 16.6 \times 10^3$ CFU in coinfecting mice; 4 weeks, $27.5 \times 10^3 \pm 12.2 \times 10^3$ CFU in BCG-only mice versus $13.6 \times 10^3 \pm 8.0 \times 10^3$ CFU in coinfecting mice [mean values with standard deviations of seven mice per group]). Although the results of one of the two experiments suggest that an infection with *N. brasiliensis* leads to a delay in bacterial clearance when 2×10^4 CFU of *M. bovis* BCG are used for the

infections, a repetition of the experiment (with 5×10^4 CFU of *M. bovis* BCG) revealed no difference in the rates of bacterial clearance between coinfecting mice and mice infected only with *M. bovis* BCG (data not shown). Taken together, these data suggest that infection with helminths does not significantly interfere with the efficient elimination of *M. bovis* BCG from the lungs of mice. One week after infection with 10⁸ CFU of *M. bovis* BCG, the T cells from the draining lymph nodes of the lungs of coinfecting mice secreted similar amounts of IFN- γ after mycobacterium-specific restimulation in vitro. However, 2 and 4 weeks after infection, significantly less IFN- γ was produced by the T cells from the MLN of the coinfecting mice after restimulation with PPD (Fig. 1A). When 2×10^4 CFU of *M. bovis* BCG were used for the i.n. infection, PPD-induced IFN- γ secretion could be observed only at 2 weeks after infection. At this time point, the T cells from the MLN of coinfecting

mice produced amounts of IFN- γ similar to those produced by the T cells from the MLN of mice infected only with *M. bovis* BCG (Fig. 1B).

Taken together, these results suggest that the development of a Th1 immune response after infection with *M. bovis* BCG is reduced by the helminth infection when 1×10^8 CFU (at 2 and 4 weeks but not 1 week after mycobacterial infection) but not 2×10^4 CFU of *M. bovis* BCG are used. However, the elimination of *M. bovis* BCG from the lungs of mice was not delayed by the helminth infection. This result was surprising since it is well established that infections with the helminth *N. brasiliensis* induce strong Th2 responses in the lung, leading to the secretion of IL-4 and IL-10, which in turn have been associated with the deactivation of macrophages and the inefficient elimination of intracellular parasites and bacteria (2, 5, 24). A possible explanation for this finding is that the Th1 immune response that was initiated after the development of the helminth-induced Th2 response shut down the Th2 response, so no effect on bacterial clearance could be detected in the helminth-infected mice. This effect was reported to occur when mice infected with *N. brasiliensis* were treated with recombinant IL-12 a few days after the infection (13). However, the levels of Th2-associated cytokines and the numbers of eosinophils in the lungs of coinfecting mice at 1 week after infection with 10^8 CFU of *M. bovis* BCG were similar to those in mice infected only with *N. brasiliensis* (Fig. 2). This clearly indicates that the *M. bovis* BCG infection 4 days after the helminth infection did not inhibit the generation of an *N. brasiliensis*-induced Th2 response in the lung.

In conclusion, our results demonstrate that the Th1 response mounted against *M. bovis* BCG leads to the efficient elimination of the bacteria from the lungs of mice, irrespective of an ongoing Th2 response at the same time and in the same organ. However, it remains to be elucidated if the same effect will be observed when more-virulent mycobacteria such as *M. avium* or *M. tuberculosis* are used for the coinfection studies. It is also possible that the Th2 response induced by *N. brasiliensis* has a stronger inhibitory effect on bacterial clearance when a poorly replicating strain of, for example, *M. avium* is used. The rationale behind this view is that the weak Th1 response induced by slow-growing relatively avirulent mycobacteria may not be sufficient to overcome the potential negative effects of IL-4 and/or IL-10 on macrophage activation. Furthermore, due to the short-lived Th2 response induced in the lungs by infection with *N. brasiliensis*, the use of multiple infections with *N. brasiliensis* or *Schistosoma* eggs for coinfection studies may yield different results.

This work was supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung, and the Bayerisches Staatsministerium für Wissenschaft, Forschung und Kunst.

We thank Susanne Stumpf and Ursula Tatsch for their excellent technical assistance.

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