

Reconstitution of *Mycobacterium leprae* Immunity in Severe Combined Immunodeficient Mice Using a T-Cell Line¹

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Unlike human lepromatous leprosy, the murine infection with *Mycobacterium leprae* is localized and self limiting. It is possible, however, to protect mice against foot pad infection with leprosy bacilli by vaccination with killed armadillo-derived *M. leprae*⁽²³⁾ or its subunits^(8, 9). In the murine model of leprosy, the restriction or prevention of mycobacterial growth in mouse foot pads is the manifestation of protective immunity.

By using adoptive transfer of cells, several laboratories have assessed the role of immune responses in promoting protection against leprosy bacilli. These studies have indicated that murine resistance to infection with this mycobacterium depends on T-cell-mediated immune responses^(10, 14). However, interpretation of data derived from studies in which cells were transferred to nonirradiated⁽¹⁰⁾ or sublethally irradiated recipients^(10, 14) may be misleading due to the difficulty in discriminating host from donor lymphocytes.

We used severe combined immunodeficient (SCID) mice to examine the ability of adoptively transferred BALB/c-derived *M. leprae*-responsive T cells to confer protection against infection with leprosy bacilli. SCID mice have an autosomal recessive

mutation that prevents the assembly of functional antigen receptor V region genes⁽²¹⁾. As a result, these mice lack immunocompetent B and T cells in their peripheral lymphoid organs⁽³⁾. The H-2d SCID mice are congenic to the immunocompetent C.B-17 mouse strain which is allotype coisogenic to BALB/c mice⁽¹⁾. This study shows that adoptively transferred T cells engraft effectively, maintain their functional activity, and control *M. leprae* infection.

MATERIALS AND METHODS

Mice. Female C.B-17 SCID mice were bred and housed in the University of California San Francisco (UCSF) pathogen-free animal care facility. Female, 6 to 8-week-old BALB/c mice were purchased from Jackson Laboratory, Bar Harbor, Maine, U.S.A.

Leprosy bacilli. A well-characterized strain of *M. leprae*, initially isolated from a lepromatous patient and maintained in the laboratory by passage through mouse foot pads, was used to infect the mice. After 22 years of passage in mouse foot pads, this isolate synthesizes the *M. leprae*-specific phenolic glycolipid⁽⁵⁾. Armadillo-derived, irradiation-killed, whole *M. leprae* were provided by Dr. P. J. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A., through contract AI-05074.

Generation of *M. leprae*-responsive T cells. BALB/c mice were injected intradermally in the right flank with 100 μ l of 10⁷ whole irradiation-killed *M. leprae* emulsified in Freund's incomplete adjuvant as described by Shepard, *et al.*⁽²³⁾. Eight days later the spleens were removed and the spleen cells were freed from the red blood cells by a brief incubation in 0.84% am-

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monium chloride. T cells were enriched by the passage of dispersed splenic cells through nylon wool columns after a 1-hr incubation at 37°C. To activate T cells *in vitro*, 1×10^6 *M. leprae*-primed, nylon-wool-nonadherent cells and 1×10^6 irradiated (2500 rad from a cesium source) unseparated spleen cells were cultured in 16-mm wells (Costar, Cambridge, Massachusetts, U.S.A.) with 5 µg/ml sonicated *M. leprae* in a final volume of 1 ml of Iscove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, New York, U.S.A.) supplemented with 5% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 µg of streptomycin/ml—hereafter referred to as complete medium (CM). Cultures were maintained for 7 days at 37°C in a humidified 10% CO₂ atmosphere. After 7 days, lymphoblasts were isolated by Ficoll-Hypaque gradient centrifugation. These cells were propagated in CM supplemented with 20 U recombinant IL-2/ml (a gift from Cetus Corporation, Emeryville, California, U.S.A.) in the presence of an equal number of *M. leprae*-pulsed antigen presenting cells (APC). APC were derived from *M. leprae*-nonimmune BALB/c mice. Dispersed spleen cells from naive mice were incubated with 5 µg/ml sonicated leprosy bacilli for 16 hr at 37°C before irradiation (2500 rad) and used as APC/feeder cells. Seven days after the last restimulation, lymphoblasts (referred to as immune T cells) were again isolated from the feeder cells by using the procedures described above. These cells were then used for flow cytometric and functional analysis, as well as reconstitution of the SCID mice.

Adoptive cell transfer. SCID mice, six per group, were reconstituted with either immune T cells or freshly isolated splenic T lymphocytes (nonimmune T cells) by intraperitoneal injection. Immune T cells were isolated from the irradiated APC by Ficoll-Hypaque gradient centrifugation at the end of the growth cycle and prior to transfer. The nonimmune T cells were enriched by passage of dispersed spleen cells derived from naive BALB/c mice through nylon wool columns. Ten million viable cells, determined by trypan blue exclusion, were resuspended in 500 µl phosphate buffered saline (PBS) for injection into each mouse.

Twenty-four hr later reconstituted recipients, as well as nonreconstituted controls, were infected with leprosy bacilli as described.

***M. leprae* infection.** SCID, as well as BALB/c mice, were infected in both hind-foot pads with 5000 live *M. leprae* derived from the foot pads of mice near the peak of logarithmic multiplication. Protection was evaluated at the peak of mycobacterial multiplication, when the numbers of acid-fast bacilli (AFB) from each of three naive BALB/c mice infected with the same *M. leprae* inoculum reached 5×10^5 per foot pad.

Assessment of protective immunity. At the peak of mycobacterial multiplication in the nonimmune BALB/c mice (7 to 9 months postinfection), blood was collected from the tail vein of each animal and peripheral blood mononuclear cells were freed from red blood cells by a brief incubation with 0.84% ammonium chloride for flow cytometric analysis. The mice were then sacrificed and their spleens and foot pads were harvested. The spleen cells were dispersed into single cell suspensions and used for a) flow cytometric analysis, b) generation of lymphokine, and c) measurement of proliferative activity. The number of AFB in both hindfoot pads were enumerated by a standard microscopic technique⁽²²⁾. The differences between groups were determined by comparing the geometric mean of the number of AFB in the right and left hindfoot pads by Fisher's exact median test⁽¹²⁾.

Immunofluorescence analysis. Phycoerythrin (PE)-conjugated anti-CD4 (L3T4) monoclonal antibodies (mAb), FITC-conjugated anti-CD8 (Ly-2) mAb (Becton Dickinson, Mountain View, California, U.S.A.), and FITC-conjugated anti-CD3 mAb (PharMingen, San Diego, California, U.S.A.) were used for surface marker analysis. FITC- or PE-conjugated rat IgG2a (PharMingen) were used as negative controls. The spleen cells as well as the peripheral blood mononuclear cells were suspended in PBS containing 2% FCS and 0.01% sodium azide (staining buffer) and were incubated at 4°C for 45 min with the FITC- and/or PE-conjugated mAb. After extensive washing, the cells were fixed with 1.0% paraformaldehyde in the staining buffer and ex-

aminated on a FACScan analytical flow cytometer (Becton-Dickinson) (13). Only cells of lymphocyte size and complexity were analyzed based on forward and 90° scatter gates. Photomultiplier tube voltage and color compensation between the FITC and PE channels were determined using Becton-Dickinson CaliBRITE particles. Percent specific staining is defined as the percent staining obtained in the positive sample minus the percent staining in the negative control.

Generation of lymphokine-containing supernatants. One ml of spleen cells suspended in CM (1×10^6 cells/ml) was dispensed into 16-mm diameter wells (Costar). After 48 hr of culture in either the absence or the presence of 2.5 μ g/ml Concanavalin A (ConA; Sigma Chemical Co., St. Louis, Missouri, U.S.A.), the supernatant from each culture was separated from the cells by centrifugation and stored at -70°C until assayed.

Proliferation assay. Freshly isolated spleen cells (5×10^4 /well) were stimulated with a previously determined optimal dose of ConA or *M. leprae*. All proliferation assays were performed in round-bottom microtiter wells in a final volume of 200 μ l of CM. The cultures were maintained for 4 to 5 days at 37°C in a 10% CO₂-humidified atmosphere. [³H]Thymidine (1 μ Ci) was added to each well 16 hr before the cells were harvested. Immune T cells (10×10^4 /well) were cultured with 25×10^4 of irradiated (2500 rad), freshly isolated spleen cells in 200 μ l of IL-2-free CM per well in the presence or absence of stimuli as described above. Following 3 days of culture, these cultures were pulsed with [³H]thymidine for 6 hr. The results of these assays are expressed as a) mean counts per minute (cpm) of quadruplicate cultures; b) Δ cpm, which represents cpm in cells cultured with the stimulus minus cpm in cells cultured without the stimulus; or c) stimulation index (SI) determined by the following equation: SI = cpm in cells cultured with the stimulus/cpm in cells cultured without the stimulus.

Lymphokine assay. The levels of interferon-gamma (IFN- γ) in the spleen cell culture supernatant were measured by ELISA (Genzyme, Cambridge, Massachusetts, U.S.A.) according to the manufacturer's protocol. The sensitivity of this assay is 125

pg IFN- γ /ml according to the manufacturer.

RESULTS AND DISCUSSION

A T-cell line was established from pooled splenic T lymphocytes of BALB/c mice immunized with whole irradiation-killed leprosy bacilli. These cells, the "immune T cells," underwent DNA synthesis ($56,887 \pm 6531$ cpm/ 10^5 cells, SI = 35) and released IFN- γ (53 ng/ 10^6 cells) in response to sonicated *M. leprae*.

The spleen and blood cells from the recipient SCID mice were analyzed for the expression of surface CD4 and CD8 molecules by flow cytometry to assure that both immune T cells as well as the BALB/c-derived nonimmune T cells had engrafted successfully. Although the cellularity of spleens from SCID mice reconstituted with either the immune T cells or the nonimmune T cells was lower than that of the immunocompetent BALB/c mice, the data shown in Figure 1 demonstrate that the adoptively transferred T cells have populated the recipients. An average of 12×10^6 and 5×10^6 cells were obtained from spleens of SCID mice reconstituted with the immune or nonimmune T cells, respectively. Spleens from the BALB/c mice contained about 10^8 cells. In individual SCID mice, a variable fraction of splenic lymphocytes expressed the CD4 or CD8 surface molecules. In five SCID mice engrafted with the immune T cells, CD4+ T cells and CD8+ T cells comprised $16.7 \pm 11.6\%$ and $5 \pm 3.5\%$ of the spleen cells, respectively. The frequency of cells expressing these surface markers in the blood of these animals was comparable to that found in the spleens (Fig. 1). A higher percentage of lymphocytes in the spleen and blood of SCID mice reconstituted with nonimmune T cells displayed CD4 and CD8 surface molecules. Although SCID mice tend to become "leaky" with age (i.e., express CD4+ and CD8+ α/β TCR+ T cells) (2), only $4.9 \pm 0.9\%$ and $1.5 \pm 1.1\%$ of splenic lymphocytes were, respectively, CD4+ and CD8+ T cells in nonreconstituted control SCID mice. The distribution of CD4+ and CD8+ T cells in the *M. leprae*-infected BALB/c mice was comparable to that of the age-matched normal BALB/c mice and was, as expected, significantly higher than that ob-

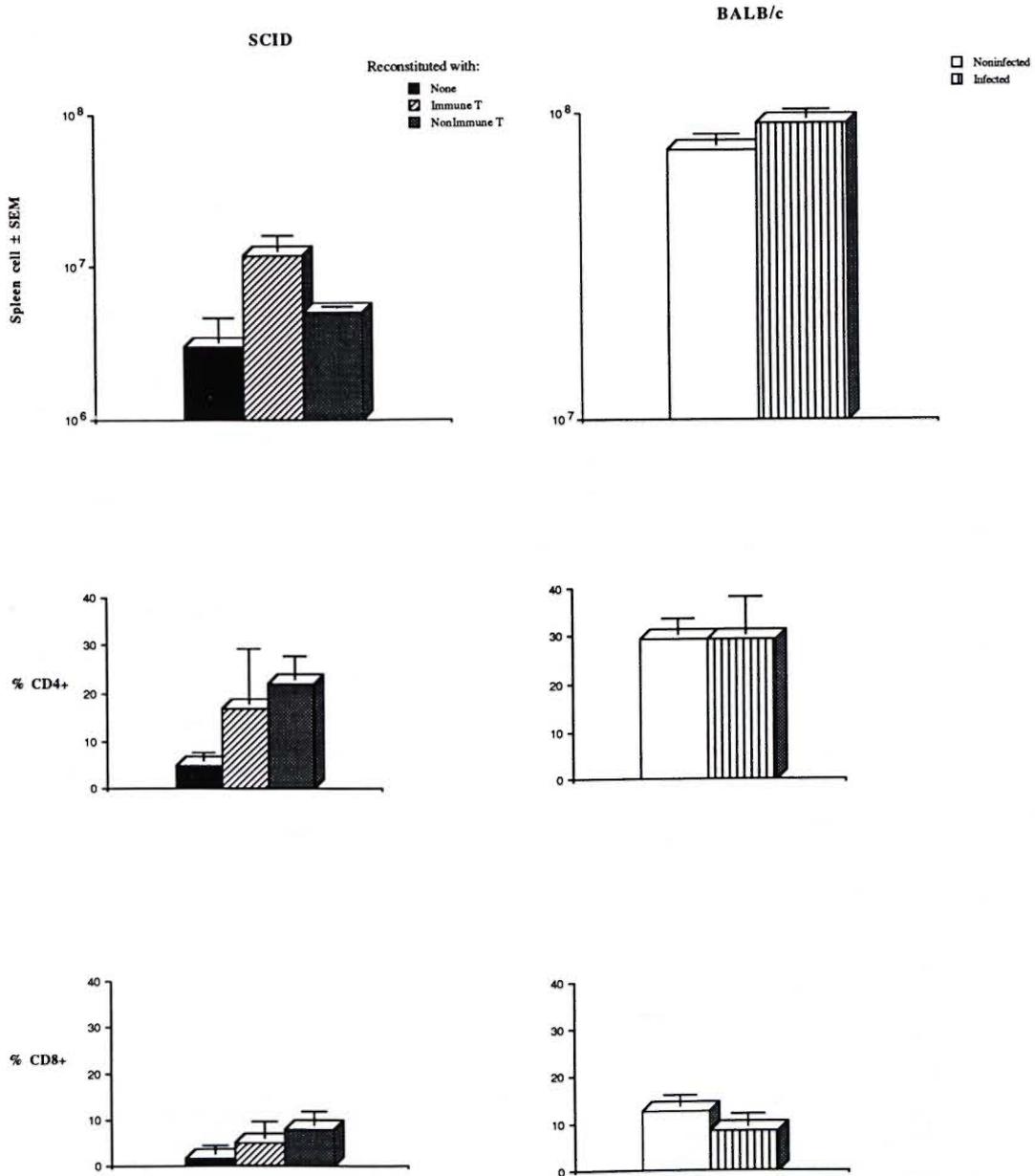


FIG. 1. CD4+ and CD8+ lymphocytes in the spleens of reconstituted SCID mice.

served in the reconstituted SCID mice (Fig. 1).

To determine if the donor cells had maintained their functional activity, the ability of the spleen cells from each mouse to produce IFN- γ and proliferate upon *in vitro* stimulation was assessed at the termination of the study. Spleen cells taken from non-reconstituted SCID mice failed to undergo proliferation when stimulated with ConA

(Δ cpm = 84) (Fig. 2). These data complement the findings generated by flow cytometry and rule out the possibility that the nonreconstituted SCID mice became "leaky" during the course of these studies. In contrast, spleen cells taken from animals reconstituted with the immune, as well as the nonimmune, T cells produced readily detectable levels of IFN- γ (Fig. 2). Splenic lymphocytes from animals recon-

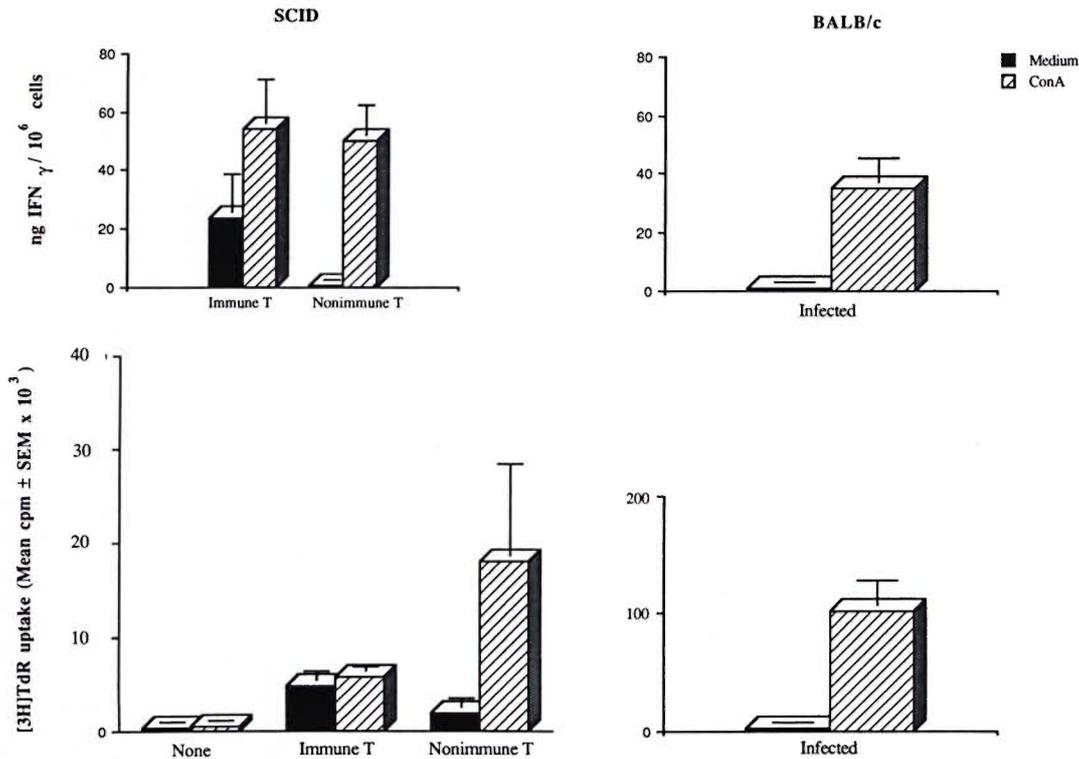


FIG. 2. ConA stimulations of splenic lymphocytes *in vitro* from reconstituted SCID mice.

stituted with nonimmune T cells demonstrated a marked increase in the uptake of [3 H]thymidine when stimulated with ConA (Δ cpm = 16,140, SI = 9.6) (Fig. 2). Spleen cells from mice reconstituted with the immune T cells displayed a high level of DNA synthesis even in the absence of ConA, reflecting a state of activation (Fig. 2). As expected, spleen cells taken from the *M. leprae*-infected, control BALB/c mice responded to ConA by lymphokine production and DNA synthesis (Fig. 2).

Depending on the cell availability, the response of spleen cells from animals in each group to sonicated leprosy bacilli was also assessed. The profile of response provoked by *M. leprae* was similar to that induced by ConA. Uptake of [3 H]thymidine (mean cpm \pm S.E.M. from three animals per group) by spleen cells taken from mice reconstituted with sonicated *M. leprae* was $25,161 \pm 990$ cpm compared to 1109 ± 528 cpm in control cultures kept in medium alone (SI = 2.3). Spleen cells from animals reconstituted with the immune T cells showed com-

parable levels of DNA synthesis in the presence or absence of *M. leprae* (5490 ± 1509 and 5326 ± 960 cpm, respectively).

Only SCID mice reconstituted with the immune T cells 24 hr before infection with 5000 bacilli exhibited significant protection against infection with leprosy bacilli. The number of AFB per foot pad in these animals ranged from 5.49×10^4 to 8.33×10^4 (mean number of $6.9 \pm 1.6 \times 10^4$ per foot pad), as compared to an average of $18.92 \pm 11.0 \times 10^5$ AFB per foot pad in nonreconstituted SCID mice ($p < 0.03$) (Fig. 3). The yield of *M. leprae* in the foot pads of SCID mice populated with immune T cells was also significantly lower than that found in naive BALB/c mice (mean number of $1.78 \pm 0.76 \times 10^5$ AFB per foot pad, $p < 0.05$), and at levels previously found only in BALB/c mice that had been effectively immunized against *M. leprae* (8). In agreement with this observation, SCID mice populated with the immune T cells restricted the growth of *M. leprae* more effectively than those reconstituted with nonimmune T cells ($6.9 \pm 1.6 \times 10^4$ versus 2.77 ± 0.88

$\times 10^5$ AFB per foot pad, $p < 0.03$) (Fig. 3). Although the number of *M. leprae* in the foot pads of SCID mice populated with non-immune T cells was less than that found in nonreconstituted SCID mice (Fig. 3), the difference was not statistically significant.

These findings confirm earlier observations implicating the central role of T cells in providing protection in the murine model of leprosy (10,14). We used SCID mice because data generated in this leprosy model are more convincing than those derived from sublethally irradiated recipients (14). Considering the long duration of this infection (6 to 9 months), the possibility that endogenous cells may restore the host's immunity in the irradiated recipients cannot be ruled out. We elected to use an unseparated T-cell line in these initial studies because CD8+ T cells appear to play a pivotal role in the control of mycobacterial infections (4,6,11,20) and because highly purified CD8+ T cells do not engraft successfully into SCID mice (19). These studies in SCID mice demonstrate that after the initial selection *in vitro*, immune T cells homed effectively (Fig. 1) and were able to exert their anti-*M. leprae* activity (Fig. 3). The ability of CD4+ T cells to confer protection against leprosy bacilli in the absence of CD8+ remains to be studied. A role for CD4+ (L3T4+), but not CD8+ (Lyt-2+), T cells in the protective immune responses against *M. bovis* has been suggested by adoptive transfer studies in thymectomized mice treated with either anti-L3T4 or anti-Lyt-2 monoclonal antibody (16). The ability of immune T cells to control the growth of leprosy bacilli in the recipient SCID mice may be caused, in part, by the activation of infected macrophages by elaboration of IFN- γ . Such an antimycobacterial function has been ascribed to IFN- γ (15,17,18). Additional studies on the effect of treating reconstituted SCID mice with anti-IFN- γ neutralizing antibody should provide support for this possibility. Alternatively, immune T cells were able to reduce the *M. leprae* load by direct interaction with the infected macrophages (4). Consistent with this possibility is our unpublished observation that these immune T cells were able to lyse BALB/c-derived peritoneal macrophages pulsed with killed *M. leprae*, as revealed by the release of [51 Cr]sodium chromate (data not shown).

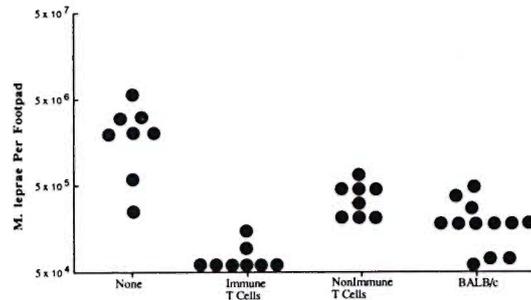


FIG. 3. Growth of *M. leprae* in the foot pads of reconstituted SCID mice.

This study demonstrates that transferred immune T cells populated SCID mice and maintained their functional activities for the duration of this study (275 days). This study further suggests that these cells are able to migrate to the sites of infection and upon reactivation *in vivo* to reduce the mycobacterial burden of macrophages.

SUMMARY

To test whether *Mycobacterium leprae*-immune T cells can confer protection against infection with leprosy bacilli, severe combined immunodeficient (SCID) mice were reconstituted with a BALB/c-derived, *M. leprae*-responsive, T-cell line. Flow cytometric analysis of spleen and peripheral blood cells confirmed reconstitution with T cells. *In vitro* lymphokine production and the proliferation of spleen cells from the reconstituted animals established that the donor cells had maintained their functional activity for the duration of the study (275 days). The transfer of immune T cells 24 hr before foot pad infection with leprosy bacilli resulted in a profound reduction in *M. leprae* multiplication, as compared to the nonreconstituted SCID mice. The yield of acid-fast bacilli in the foot pads of SCID mice reconstituted with the *M. leprae*-immune T cells also was significantly lower than that found in naive BALB/c mice, and at levels previously found only in BALB/c mice that had been immunized effectively. These experiments demonstrate that *M. leprae*-immune T cells home effectively and control *M. leprae* infection in SCID mice.

RESUMEN

Para investigar si las células T inmunes al *Mycobacterium leprae* pueden conferir protección contra la

infección por el bacilo de la lepra, se reconstituyeron ratones con inmunodeficiencia combinada severa (SCID) con células T respondedoras al *M. leprae* derivadas de ratones BALB/c. Los análisis por citometría de flujo de las células de bazo y de sangre periférica, confirmaron el éxito de la reconstitución. La producción *in vitro* de linfocinas y la proliferación de las células de bazo de los animales reconstituídos indicaron que las células del donador mantuvieron su actividad funcional durante todo el tiempo del estudio (275 días). En comparación con los ratones SCID no reconstituídos, la transferencia de las células inmunes 24 horas antes de la infección de las almohadillas plantares con los bacilos de la lepra, ocasionó una profunda reducción en la multiplicación del *M. leprae*. La recuperación de bacilos ácido-ratones SCID reconstituídos con las células T inmunes al *M. leprae* también fue significativamente menor que la recuperación de bacilos a partir de los ratones BALB/c intáctos, y los niveles de recuperación fueron solo similares a los encontrados en ratones BALB/c inmunizados contra el bacilo de la lepra. Estos experimentos demuestran que las células T inmunes al *M. leprae* se establecen de manera efectiva y controlan la infección por el *M. leprae* en los ratones SCID.

RÉSUMÉ

Dans le but de tester si des cellules T immunisées vis-à-vis de *Mycobacterium leprae* peuvent conférer une protection contre une infection par des bacilles de la lèpre, on a administré à des souris sévèrement immunodéficientes une lignée de cellules T dérivées de BALB/c et réagissant à *M. leprae*. Une analyse cytométrique de flux au niveau de la rate et des cellules du sang périphérique a confirmé la reconstitution des cellules T. La production de lymphokine *in vitro* et la prolifération des cellules spléniques chez les animaux traités ont confirmé que les cellules administrées avaient maintenu leur activité fonctionnelle pour la durée de l'étude (275 jours). Le transfert de cellules T immunes 24 h avant l'infection du coussinet plantaire par des bacilles lépreux a résulté en une diminution importante de la multiplication de *M. leprae*, par comparaison avec les souris non traitées. La charge en bacilles acidorésistants des coussinets plantaires des souris traitées par les cellules T immunisées vis-à-vis de *M. leprae* était également significativement plus faible que celle trouvée chez les souris BALB/c de souche, et seulement comparable à celle trouvée chez des souris BALB/c effectivement immunisées. Ces expériences démontrent que les cellules T immunisées vis-à-vis de *M. leprae* abritent effectivement et contrôlent l'infection par *M. leprae* chez les souris sévèrement immunodéficientes.

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