Received: 14 December 2020 | Revised: 1 February 2021 | Accepted: 2 February 2021

DOI: 10.1111/imr.12960

Immunological Reviews WILEY

INVITED REVIEW

INVITED REVIEW THEMED ISSUE

Susceptibility and resistance in leprosy: Studies in the mouse model

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Funding information
Leprosy Research Initiative and Turing Foundation, Grant/Award Number: 703.15.43; National Institute of Allergy and Infectious Diseases, Grant/Award Number: AAI 15006 and AI50027

Abstract

Leprosy is a chronic granulomatous infectious disease caused by the pathogen, *Mycobacterium leprae*, and the more recently discovered, *M. lepromatosis*. Described in 1873, *M. leprae* was among the first microorganisms to be proposed as a cause of a human infectious disease. As an obligate intracellular bacterium, it has still not thus far been reproducibly cultivated in axenic medium or cell cultures. Shepard's mouse footpad assay, therefore, was truly a breakthrough in leprosy research. The generation of immunosuppressed and genetically engineered mice, along with advances in molecular and cellular techniques, has since offered more tools for the study of the *M. leprae*–induced granuloma. While far from perfect, these new mouse models have provided insights into the immunoregulatory mechanisms responsible for the spectrum of this complex disease.

KEYWORDS
clofazimine, interferon-gamma, interleukin-17, *M. leprae*, *M. lepromatosis*, NOS2

1 | ANIMAL MODELS FOR LEPROSY

For nearly 150 years, there have been numerous attempts by scientists to propagate *Mycobacteriu M. lepromatosis* and find a suitable animal model representative of the spectrum of leprosy. Johnstone[1] reviewed the early endeavors to cultivate *M. leprae* in various animals, which included a vast array of mammals, birds, and cold-blooded animals (Table 1). Most of these attempts yielded disappointing results, with either no response by the host or only a mild inflammation at the site of inoculation. This may have been due simply to the natural resistance of these hosts to *M. leprae* infection. However, in many of the early efforts the *M. leprae* inoculum that was used was likely of poor quality as it was crude material of unknown viability and concentration derived from patient biopsies. Furthermore, *M. leprae* is a rather fastidious organism and little was known at the time regarding its long doubling time of 12-14 days, its requirement for cool temperatures, or an appropriate infection route.

Based on the knowledge of the preference of *M. leprae* for the cooler areas of the body, Charles Shepard[2] inoculated *M. leprae* into the footpads of mice (*Mus musculus*) and demonstrated limited growth of the organisms over several months. Importantly, he was able to reproducibly passage and propagate the bacilli into subsequent mice. This mouse footpad assay has permitted the culture of multiple isolates of *M. leprae* and *M. lepromatosis*[3] from human lesions, as well as the evaluation of new leprosy drugs and regimens, the documentation of drug resistant strains, and fundamental immunological studies including testing the efficacy of vaccines.[4] While this review will focus primarily on the mouse model, a brief overview of other useful animal models is provided here.

The nine-banded armadillo (*Dasypus novemcinctus*) was demonstrated in 1971[5] to develop leprosy after inoculation with *M. leprae*. Their low body temperature of 32-35°C underlies the fact that infection disseminates to all of the tissues, resulting in massive numbers of organisms in the spleen, liver, and lymph nodes. Histopathological

This article is part of a series of reviews covering Immunity to Mycobacteria appearing in Volume 301 of Immunological Reviews.

TABLE 1 Early attempts to develop animal models for leprosy

Animal	First known attempt
Mammals	
Rabbits	Niesser (1881)[a]
Dogs	Niesser (1881)
Guinea pigs	Kobner (1882)
Rats	Kobner (1882)
Cats	Damsch (1883)
Pigs	Arning (1885)
Monkeys	Tedeschi (1893)
Java	Kobner (1882)
Macaque	Nicolle (1906)
Rhesus	Reenstierna (1926)
Wedge-capped capuchin	McKinley (1932)
Patas	Roffo (1927)
Japanese	Saito (1949)
Mice	Sugai (1909)
Chimpanzees	Marchoux and Bourret (1907)
Hamsters	Adler (1937)
Gerbils	Saito (1949)
Korean Chipmunks	Lew et al (1973)
Slender Lorises	Narayanan (1976)
Armadillos	Kirchheimer and Storrs (1971)
Hedgehogs	Klingmuller 1979)
Chinchillas	Binford (1987)
Fruit bats	Binford (1987)
Lemmings	Binford (1987)
Meadow voles	Binford (1987)
Opossums	Binford (1987)
Birds	
Pigeons	Kobner (1882)
"Fowls"	Ota (1939)
Chickens	Nonaka (1940)
Paddy birds	Saito (1949)
Canaries	Saito (1949)
Parrots	Saito (1949)
Love birds	Saito (1949)
Chick embryos	Nakagawa and Nakamura (1954)
Fish	
Eels	Kobner (1882)
Loaches	Kobner (1882)
Goldfish	Couret (1911)
Saltwater fish	Couret (1911)
Rainbow Perch	Chaussinand and Besse (1951)
Amphibians	
Frogs	Kobner (1882)
Tadpoles	Couret (1911)
Toads	Saito (1949)
Reptiles	
Turtles	Couret (1911)
Snakes	Couret (1911)
Lizards	Fite et al (1964)
Alligators	Fite et al (1964)

[a]Complete citations can be found in ref. 1.

elements across the spectrum of leprosy have been described,[6] although most armadillos are susceptible to *M. leprae* infection and only 15%-20% of the animals are resistant. For years, the armadillo has been used to cultivate large numbers of *M. leprae* for the purification of leprosy research reagents,[7] which can be obtained through the Biodefense and Emerging Infections Research Resources Repository (https://www.beiresources.org/). More recently, the armadillo is advancing as a model for leprosy-specific neuropathy.[8,9] As an experimental animal, the armadillo remains an exotic species requiring capture in the wild. However, gravid females will successfully give birth in captivity, and their genetically identical quadruplicate offspring are excellent models for the study of host genetics.[10,11] Since the sequencing of the armadillo genome in 2011,[12] some immunological reagents, including recombinant interleukin-2 (IL-2)[13] and recombinant interferon-gamma (IFN-γ),[14] have been generated. Numerous RT-PCR reagents[15] necessary to probe the model for clues to leprosy pathogenesis have been developed as well. Armadillos naturally infected with *M. leprae* are found in the Gulf Coast area of the United States and in other parts of the Americas,[16,17] and their role as reservoir hosts for transmission of leprosy to humans has been documented.[18-20]

Among non-human primates, sooty mangabey monkeys (*Cercocebus atys*) have been shown to harbor natural infection with *M. leprae*.[21] Natural infection in chimpanzees (*Pan troglodytes*) has also been documented.[22-24] In these animals, however, it was not certain whether they had been infected while feral, or if infection occurred upon exposure to *M. leprae*–infected humans while being temporarily housed for shipment after their capture. Serological[25] and molecular[26] studies have now provided evidence that *M. leprae* can be transmitted from humans to non-human primates, and between non-human primates. Recently, leprosy in wild chimpanzees has been observed (https://www.biorxiv.org/content/10.1101/2020.11.10.374371v1). Experimentally, mangabey monkeys inoculated with *M. leprae* developed a disseminated disease which resembles human leprosy, both clinically and histologically.[27] Experimental leprosy has also been established in African green monkeys (*Cercopithecus aethiops*),[28,29] the white-handed gibbon (*Hylobatus lar*),[30] rhesus monkeys (*Macaca mulatta*),[28] and a chimpanzee.[31] Immunological reagents that react in primates[32] are becoming increasingly available, but acquisition and long-term maintenance of non-human primates require specialized facilities and can be prohibitively expensive.

Recently, adult zebrafish (*Danio rerio*) were used to study *M. leprae*–induced granulomas. Upon infection, histopathological

examination revealed well-formed, non-caseating granulomas, which developed rapidly and controlled *M. leprae* growth.[33] Early nerve damage was examined in zebrafish larvae, which have the added advantages of being optically transparent and lacking in adaptive immunity at this stage.[34] In this model, PGL-1–induced nitric oxide synthase, produced by *M. leprae*–infected macrophages, generated reactive nitrogen species which caused demyelination of adjacent nerves. While the study of peripheral nerves is difficult in this non-mammalian model, zebrafish are relatively inexpensive, have a low body temperature, and many genetic mutants are available.[35]

Red squirrels (*Sciurus vulgaris*) in Great Britain having lesions resembling those of leprosy have been reported.[36-39] Red squirrels in Brownsea Island, England, were found to harbor a natural infection with *M. leprae*, whereas *M. lepromatosis* infection was found in red squirrels in England, Scotland, and Ireland. Leprosy infection has not been detected in squirrels in other parts of Europe.[40,41] Therefore, red squirrels appear to be a reservoir of infection in the British Isles, although current inter- and intra-species transmission patterns are unknown. Red squirrels are not currently being used as leprosy research models, primarily because of their protected species status.[42]

2 | LEPROSY SPECTRUM

Leprosy is predominantly a disease of skin, mucous membranes of the upper respiratory tract, and peripheral nerves, the cooler parts of the body.[43] There is a considerable range in the manifestations of clinical leprosy as the course of the disease is largely a result of the cell-mediated immune response, or lack thereof, of the host toward the antigens of *M. leprae*. The World Health Organization (WHO) developed a disease classification scheme which, based on the number of lesions and the presence of bacilli in skin smears, describes patients as either paucibacillary or multibacillary to aid in the recommendation of the course of therapy. A more detailed five-stage clinical-immunopathological spectrum was developed by Ridley and Jopling.[44] The Ridley-Jopling classification of leprosy considers the appearance of skin lesions, the presence of acid-fast bacilli in the skin and nerves, motor and sensory nerve changes, and histopathological findings. This scheme describes a comprehensive spectrum consisting of indeterminate, tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL), and lepromatous (LL) phases.

The earliest recognizable stage of leprosy is indeterminate leprosy. It is important to understand that indeterminate does not mean that one is unsure of the diagnosis of leprosy; rather, it means that it is not yet known where in the spectrum the disease should be classified. Indeterminate leprosy often goes unrecognized as it presents with a single or a few hypopigmented lesions, perhaps with some sensory loss, minimal histological changes, and rare acid-fast bacilli. Spontaneous healing may occur. Some individuals, however, progress into one of the established polar forms of leprosy (TT or LL) or to an unstable borderline stage.

TT leprosy is a localized disease with one, or at most, a few well-circumscribed skin lesions with extreme anesthesia. Peripheral nerves in the vicinity of the skin lesion(s) may be enlarged. Histologically, there are very few demonstrable acid-fast bacilli. Well-organized granulomas consisting of epithelioid cells, multinucleated giant cells, and a distinct organization of CD4+ and CD8+ T cells are observed.[45] Individuals with TT leprosy manifest a strong cell-mediated immunity to *M. leprae* antigens but produce relatively low levels of antibody. Much of the clinical picture in TT leprosy is due to the cell-mediated immune responses of the host to the relatively few bacilli in sites adjacent to nerves.

LL leprosy is the widespread, anergic form of the disease. Lepromatous granulomas, or lepromas, in advanced LL leprosy may contain 10^{10} *M. leprae* per gram of tissue. Histologically, they are characterized by an enormous infiltration of macrophages filled with large numbers of acid-fast bacilli. A foamy appearance of the cells is due to copious amounts of lipids. CD8+ cells predominate among the few T cells that are found in the LL lesions. Nerve destruction is characterized by the massive numbers of bacilli seen in surrounding macrophages, perineural cells, and Schwann cells. There is a strong antibody response. Although LL leprosy patients are not afflicted with a general immunosuppression, there is a striking and clearly identified specific T cell anergy for the antigens of *M. leprae*.

Borderline leprosy includes those presentations of disease between LL leprosy and TT leprosy. The majority of persons with leprosy fall into this area of the spectrum. The signs and symptoms of borderline leprosy are due to a combination of bacterial multiplication and the host's cell-mediated immune response to *M. leprae*. BT leprosy exhibits more skin lesions than TT leprosy. Satellite lesions develop near the edges of the larger lesions, and individual lesions are larger but still with well-defined edges. Peripheral nerve damage is generally more prevalent and more severe in BT leprosy than in TT leprosy. Histopathologically, BT leprosy skin lesions are similar to those of TT leprosy except that the granulomas are less organized and do not extend up to involve the basal layer of the epidermis. The numbers of *M. leprae* in BT lesions vary from undetectable to a few bacilli. In contrast, BL leprosy is closer to the LL end of the disease spectrum. Skin lesions of BL leprosy contain predominantly macrophages but with far more lymphocytes than in LL leprosy. The numbers of acid-fast bacilli are greater than in BT leprosy but less than in LL leprosy. BB leprosy is rare because it is the most unstable. A patient with BB leprosy can develop clinical, bacteriological, and histopathologic features of more tuberculoid disease, and this shift is termed "upgrading." A shift from the BB leprosy region to more lepromatous disease is called "downgrading."

3 | MOUSE MODELS

3.1 | Conventional and athymic nude mice

Immunocompetent mice, such as C57Bl/6 or BALB/c, are quite resistant to infection with *M. leprae*. Using Shepard's infection model, a low-dose suspension of a few thousand bacilli is inoculated into the hind footpads where it grows locally and plateaus at approximately

10^5-10^6 organisms by 4-6 months postinfection. It is postulated that infection during the first few months is governed primarily by innate immunity. The growth peak, or plateau phase, is indicative of the onset of adaptive cell-mediated immunity that results in killing of the bacilli.[46] It is important to note that if >10^6 *M. leprae* are inoculated into the footpad, there is no subsequent growth as this dose immunizes the mouse. In our studies, we inoculate 6 × 10^3 freshly harvested, viable *M. leprae* into each hind footpad and monitor bacterial growth (Figure 1A) and granuloma development, both histopathologically and by immunohistochemical staining, over a 12- to 18-month infection period. Immunocompetent mice develop a modest granulomatous response, consisting of a mild lymphocytic infiltrate, few epithelioid cells, and small focal collections of macrophages, located immediately underneath the epidermis with minimal invasion of deeper tissue (Figure 1B). These properties of limited growth and mild pathology led researchers to regard immunocompetent mice as resembling TT leprosy, but it is actually more of a self-healing type of presentation.

In contrast, athymic nude mice cannot mount a cell-mediated immune response and *M. leprae* grow ostensibly without constraint (Figure 1A). Bacterial numbers can reach 1 × 10^{10} or more acid-fast bacilli per footpad. The granuloma in athymic nude mice resemble LL. In the absence of mature T cells, infected footpad tissue becomes a massive leproma (Figure 1B), where the macrophages are packed with bacilli. Local fibroblasts, striated muscle cells, and nerves are heavily infected as well. The athymic nude mouse model has been used for years to culture *M. leprae*, and along with studies in thymectomized-irradiated mice and rats, nude rats, and severe combined immunodeficiency (SCID) mice,[47-49] this strain defined the role of the T cell in cell-mediated immunity to leprosy. Furthermore, it enabled detailed evaluation of drug regimens, and allowed for detection of persisting drug sensitive *M. leprae* from treated patients. We currently use these mice to routinely propagate *M. leprae* and *M. lepromatosis*.[3]

In the course of our studies using *M. leprae* infection of mice, we also developed a companion strategy to the Shepard growth method that would allow us to better assess the immune responsiveness to *M. leprae* at the site of infection and permit a more in-depth exploration of the cellular composition of the footpad lesion. This protocol utilized a higher dose of *M. leprae* and was loosely based on the lepromin test. The lepromin test[43] is a skin test previously used to classify clinical disease in humans. In general, a large dose of killed, whole bacilli were injected intradermally, and the ensuing granulomatous response was examined grossly for induration and histologically for cellular composition. A variety of *M. leprae* preparations, obtained from human or armadillo tissues and subjected to different purification protocols, were used over the years for the lepromin test. The lepromin test is not a diagnostic test for leprosy and bears no similarity to the tuberculin skin test for tuberculosis. The lepromin test is read at ~4 weeks, not 48 h, and it is not a delayed-type hypersensitivity response. Rather, a positive lepromin test is prognostic and determines whether or not one can mount a successful granulomatous response to *M. leprae*. Persons who have never been exposed to *M. leprae* can mount a positive lepromin test response. A negative response means one cannot mount a granulomatous response to the organism. If the individual is a patient, a positive lepromin test classifies the patient in the TT-BT range of the spectrum. A negative test suggests BL-LL.

In our footpad induration protocol, mice are injected in the footpad with a high dose of *M. leprae*, and induration is measured

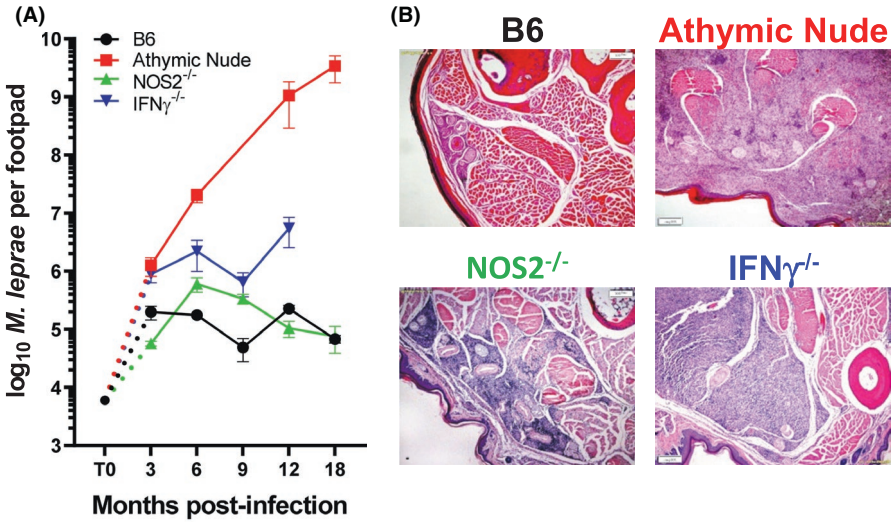

FIGURE 1 Growth of *M. leprae* and granulomatous response in infected footpads. A, B6, athymic nude, NOS2$^{-/-}$, and IFN-$\gamma^{-/-}$ mice were infected in the hind footpads with 6 × 10^3 *M. leprae*, and growth was monitored for 18 mo. B, Histological examination (hematoxylin and eosin stains) of *M. leprae*–infected footpads at 9 mo postinfection. B6 develop a small granuloma comprised of lymphocytes, epithelioid cells, and macrophages. Athymic nude mouse footpads become engorged with *M. leprae* filled macrophages. In NOS2$^{-/-}$ mouse footpads, the large granulomatous response infiltrated the perineurium and muscle bundles, whereas in IFN-$\gamma^{-/-}$ footpads, the large unorganized infiltration was composed of epithelioid macrophages with randomly interspersed lymphocytes

weekly as an external determination of cellular infiltration. As shown in Figure 2A, immunocompetent B6 mice develop significant footpad induration over the first few weeks which peaks at ~4 weeks, similarly to a positive lepromin test. In contrast, there is virtually no early induration in athymic nude mice, and the footpad only slowly increases in size as the macrophages infiltrate the tissue to accommodate the growing *M. leprae*. Using this strategy, we developed procedures to isolate the cells from the granulomas and culture them in vitro. Immunocompetent mouse footpad granulomas contained mostly macrophages and T cells, while cells isolated from *M. leprae*–infected athymic nude mouse footpads consisted primarily of engorged macrophages (Figure 2B). Furthermore, these footpad granuloma cells could be analyzed for cytokine and chemokine expression and production by RT-PCR and ELISA, respectively. These cells can also be characterized via extracellular and intracellular flow cytometry.

3.2 | Knockout mice

The extremes provided by the immunocompetent B6 and BALB/c strains and the immunosuppressed athymic nude strain have been productively employed in various chemotherapeutic and immunotherapeutic leprosy studies. However, we wanted to explore the possibility of mouse models that may represent borderline disease. Immunologically, the borderline area of the leprosy spectrum is the most interesting, as BT, BB, and BL leprosy can be clinically unstable and prone to inadequately understood immunological complications.[43] In order to modify the immune response in immunocompetent mice, we tried several chemical, for example, aminoguanidine, L-N^6-(1-iminoethyl)-lysine, and immunological, for example, antibodies, cytokines, and manipulations with some success, but the protracted nature of *M. leprae* infection made these studies difficult to carry out long term. The widespread availability of genetically engineered mice, therefore, aided our studies immensely.

There are now literally hundreds of commercially available genetically engineered mouse strains, including targeted gene knockouts, conditional knockouts, tissue-specific knockouts, knock-in mutations, and humanized mice. Using such mice afforded us more options for analyzing the effects of infection compared with the extremes of the immunocompetent and athymic nude mouse strains. Of course, the mechanisms that regulate inflammation and immunity are complex and changeable. They can be influenced not only by genetic factors but also by a number of intrinsic qualities, such as age, hormones, or microbiome, as well as external burdens such as poor nutrition, co-infections with immunomodulating parasites, viruses, or worms, or exposure to other mycobacteria. Any of these conditions can affect both short- and long-term immune status.[50] Yet, single-gene knockout mice with deficiencies at specific points of the immune cascade provided a good starting point for dissecting responses to infection with *M. leprae*. Furthermore, these strains could suggest targets for prevention, early diagnosis, and improved treatments. Therefore, we chose to first examine mice with knockouts in genes important for cell-mediated immunity.

3.2.1 | IFN-γ knockout (IFN-$\gamma^{-/-}$) mice

One of the most interesting strains has been the IFN-$\gamma^{-/-}$ mice.[51,52] IFN-γ production by CD4+ T cells plays a key role in immunity to

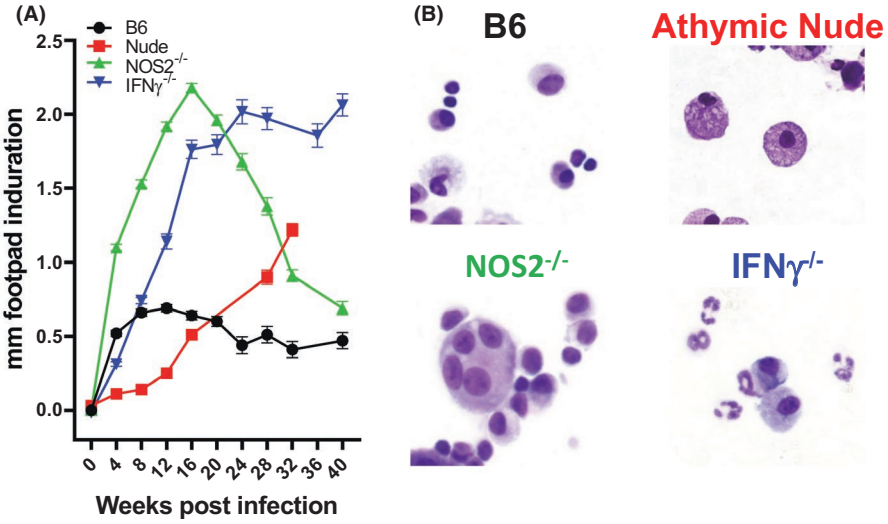

FIGURE 2 Footpad induration and isolated granuloma cells. A, B6, athymic nude, NOS2$^{-/-}$, and IFN-$\gamma^{-/-}$ mice were infected in the hind footpads with 30 × 10^6 *M. leprae*, and induration was measured with a Vernier caliper. B, Cells were isolated from the footpad granulomas at 16 wk postinfection, and cytospin preparations were stained with a Diff-Quik staining kit. B6 footpad granulomas contained macrophages and T cells. Athymic nude footpads are comprised of heavily infected macrophages (photograph originally published in ref. 103). T cells, macrophages, and multinucleated giant cells were recovered from NOS2$^{-/-}$ footpads. IFN-$\gamma^{-/-}$ footpads contained large numbers of neutrophils

mycobacterial infections. Studies with *M. tuberculosis* were done with mice having disruptions in the IFN-γ gene (IFN-$\gamma^{-/-}$) or the IFN-γ receptor[53-55] gene. Infection of IFN-$\gamma^{-/-}$ mice with *M. tuberculosis* resulted in decreased survival time, unrestricted growth of the bacilli, and necrotic granulomas. Adoptive transfer experiments from IFN-$\gamma^{-/-}$ mice to TB-infected nude mice showed that protection afforded by CD8+ T cells required IFN-$\gamma^{-/-}$ production.[56]

When IFN-$\gamma^{-/-}$ mice were infected with *M. leprae*, the bacilli multiplied beyond the peak reached in wild type mice to reach a plateau approximately one log higher (Figure 1A); however, the bacilli did not grow unchecked as with the athymic nude mice. By 9 months postinfection, the footpads of the *M. leprae*–infected IFN-$\gamma^{-/-}$ mice were visibly enlarged. Histopathologically, the footpads of IFN-$\gamma^{-/-}$ mice exhibited large aggregates of mononuclear cells replacing 40%-75% of the soft tissue (Figure 1B). The infiltrates were not organized into well-formed granulomas but were composed primarily of epithelioid macrophages with small aggregates of lymphocytes randomly interspersed.

We also examined the host response of the IFN-$\gamma^{-/-}$ mouse to *M. leprae* infection using the high dose footpad induration protocol. Early in our studies, we intended to harvest the tissue only at four weeks postinoculation, as is done in the human lepromin test. However, as studies with our various knockout mice progressed, we discovered that the granulomatous response to the bacilli evolved quite strikingly over the course of several months and was distinct among the various strains. Therefore, we extended these studies to evaluate the evolution of the granulomatous response at early (4 weeks), mid (16-20 weeks)-, and late (>32 weeks) time points. While the induration plateau was steadily maintained in control mice, induration of the IFN-$\gamma^{-/-}$ footpads increased steadily over a 16-week period (Figure 2A). At this time, it reached a plateau and maintained this level of induration for 40 weeks. IFN-$\gamma^{-/-}$ footpad cells harvested at 4 months postinfection were analyzed by RT-PCR, ELISA, and flow cytometry. Interestingly, while few Ly6G+ neutrophils were found in the B6 footpads, there was a substantial increase in both the number and percentage of neutrophils in the IFN-$\gamma^{-/-}$ footpads (Figure 2B). While similar levels of IL-10 were expressed, significantly higher levels of IL-17A and CXCL-5 were expressed in IFN-$\gamma^{-/-}$ granuloma footpads (Figure 3A). Flow cytometric analyses showed a higher percentage of both CD4+ IL-17+ and CD8+ IL-17+ cells in the IFN-$\gamma^{-/-}$ mice (Figure 3B) compared with B6 mice. Furthermore, high amounts of IL-17 were generated by IFN-$\gamma^{-/-}$ footpad cells upon stimulation with *M. leprae* membrane antigen (MLMA) (Figure 3C). These data indicate a predominantly Th17 response to *M. leprae* in these mice. The properties of augmented but not uncontrolled growth of *M. leprae*, enhanced granulomatous infiltration with only scattered lymphocytes present, and reduced Th1-type cytokine production are attributes present in BL leprosy. Moreover, the infiltration of neutrophils suggests the possibility that this strain may be manipulated for leprosy reactions (see below).

3.2.2 | NOS2$^{-/-}$ mice

Another mouse strain which showed some features of borderline leprosy was the inducible nitric oxide synthase knockout (NOS2$^{-/-}$). Cytokine (primarily IFN-γ)-activated macrophages are the primary

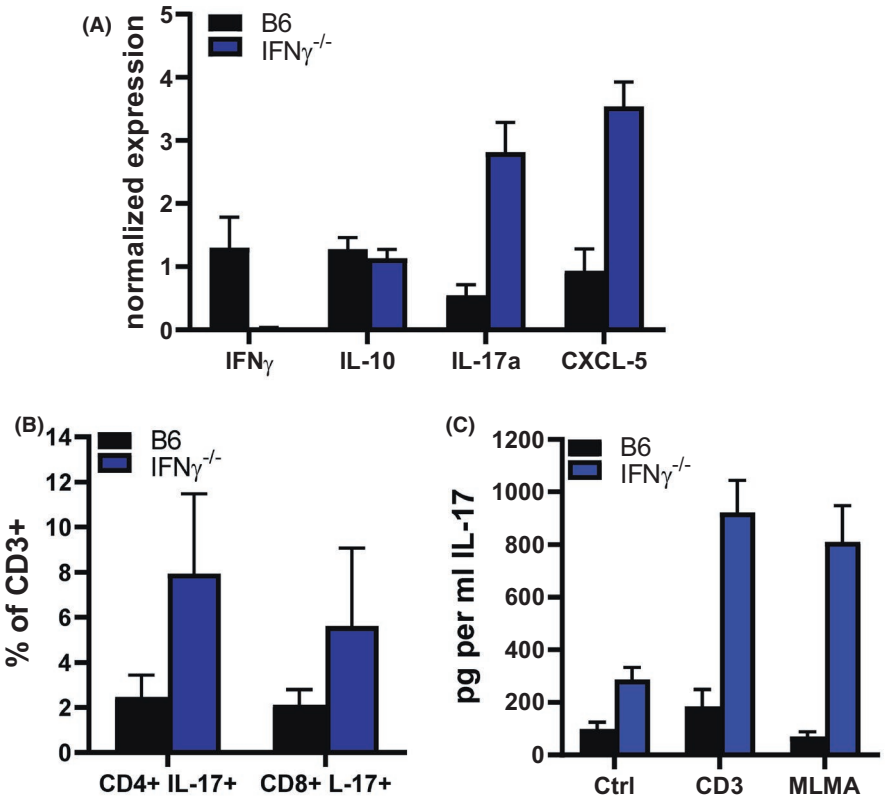

FIGURE 3 IL-17 generation in *M. leprae*–infected INF-$\gamma^{-/-}$ footpads. B6 and IFN-$\gamma^{-/-}$ mice were infected in the hind footpads with 30 × 10^6 *M. leprae*. A, IFN-γ, IL-10, IL-17, and CXCL-5 expression determined by RT-PCR on footpad tissue. Granuloma cells were isolated and IL-17 production was determined by B. flow cytometry and C. ELISA on supernatants from isolated cells cultured in vitro with MLMA

effector cells in the control of intracellular pathogens, including *M. leprae*.[57] Two powerful products produced by activated macrophages are toxic radicals of nitrogen and oxygen.

Reactive nitrogen intermediates (RNI) generated from L-arginine[58] by a high-output, cytokine-inducible isoform of nitric oxide synthase (NOS2) are principal components of the antimicrobial effector mechanisms of activated macrophages. Competitive inhibitors of L-arginine, such as N^G-monomethyl-L-arginine (L-NMA), aminoguanidine (AG), and L-N^6-(1-iminoethyl)-lysine (L-NIL) have been employed to demonstrate the role of RNI in macrophage-mediated resistance to infection with *M. leprae* and *M. tuberculosis* in vitro,[59,60] and their in vivo administration has confirmed the importance of this pathway in resistance to *M. tuberculosis*.[61-63] The generation of knockout mouse strains with targeted disruptions in the NOS2 gene has enabled long-term *M. leprae* infection studies without the possible side effects of chemical inhibitors. As shown in our own studies[64] and those of Nathan's group,[63] growth of *M. tuberculosis* and the granulomatous response in the tissues was markedly enhanced in NOS2$^{-/-}$ mice, while time-to-death following infection was decreased. In contrast, NOS2-deficient mice could control *M avium* growth in vivo.[65]

We had previously shown the importance of RNI in IFN-γ–induced macrophage-mediated resistance to *M. leprae* in vitro using both L-NMA–treated control macrophages[59] and macrophages from NOS2$^{-/-}$ mice.[66] Therefore, *M. leprae* growth in vivo was assessed in an effort to determine whether, as with *M. tuberculosis* infection, macrophage incapacity in vitro would correlate with exacerbated growth in vivo. NOS2$^{-/-}$ mice were infected with *M. leprae* using the low-dose growth protocol and bacterial multiplication was monitored over several months.[67] There was a slightly enhanced growth of *M. leprae* at 6-9 months postinfection, but the number of *M. leprae* in the footpads then declined yielding a marked reduction in the number of bacilli at 15-17 months postinfection (Figure 1A). Interestingly, NOS2$^{-/-}$ mice exhibited a 10-fold larger, organized granuloma, composed of numerous epithelioid cells, a few macrophages, and dense collections of lymphocytes. In areas, the granuloma infiltrated the perineurium and muscle bundles (Figure 1B). Using the footpad induration protocol, there was a rapid enlargement of the footpads in NOS2$^{-/-}$ mice (Figure 2A) which peaked at 16 weeks but then began to decline. The majority of the lymphocytes which infiltrated the footpads of both the B6 and NOS2$^{-/-}$ strains were CD3+ T cells; few B220+ B cells were present. However, both the number and percentage of CD3+ cells were augmented in NOS2$^{-/-}$ footpads. Furthermore, in the NOS2$^{-/-}$ footpads, a higher percentage of these cells displayed activation markers. Cytokine gene expression in the developing granuloma of the footpad tissues of NOS2$^{-/-}$ mice was examined by quantitative RT-PCR. While present in control mice, cytokines crucial to an effective Th1 response, such as IFN-γ, IL-2, and tumor necrosis factor (TNF) and chemokines important in granuloma formation, including CCL-3 and CCL4, were expressed at significantly higher levels in NOS2$^{-/-}$ footpads. Interestingly, higher levels of IL-10 were also generated in NOS2$^{-/-}$ footpads. The CD4+ CD44^{hi} cells from NOS2$^{-/-}$ footpads also produced higher levels of IFN-γ when stimulated in vitro with *M. leprae* membrane antigen. NOS2$^{-/-}$ mice, therefore, develop more rapid, extensive chronic inflammation with the presence of multinucleated giant cells (Figure 2B). These attributes, along with the controlled growth of *M. leprae*, led us to categorize this strain as BT leprosy.

3.2.3 | *phox91*$^{-/-}$ mice

In contrast, we saw little effect on *M. leprae* infection in the absence of reactive oxygen intermediates (ROI). Early studies by Sharp[68] showed that *M. leprae* was susceptible to killing by hydrogen peroxide in a cell-free system. More recently, Cole's group[69,70] determined that the genes for KatG, a catalase-peroxidase enzyme, and AhpD, part of a peroxidase and peroxynitrite reductase system, are pseudogenes in *M. leprae*, verifying the reason that neither a catalase nor a peroxidase is generated. Yet, *M. leprae* is able to remain viable and proliferate inside the macrophage, the primary host cell. How *M. leprae* survives inside the macrophage, especially with regard to defense against ROI, has been attributed to a number of factors. First, *M. leprae* is only a weak stimulus of the macrophage oxidative burst. This may be due its entry via the complement (C) 3 receptor[71] or a scavenging of ROI by cell wall glycolipids such as PGL-1[72] and LAM.[73] *M. leprae* also expresses both *sodC* and *sodA*[74] and produces a superoxide dismutase. Thus, the toxic effects of superoxide should be effectively suppressed by *M. leprae* and the production of the subsequent ROI should be greatly reduced. Taken together, these in vitro studies suggest that *M. leprae* should be competent in dealing with the ROI host defense mechanism.

We examined the role of ROI for the effective host response to experimental leprosy using mice which have a disruption in the 91 kD subunit of the NAPDH oxidase cytochrome b (*phox91*$^{-/-}$).[75] Multiplication of *M. leprae* in *phox91*$^{-/-}$ footpads was elevated early in infection compared with B6 mice, but growth was subsequently controlled as counts similar to those observed in B6 mice were obtained later in infection. Histopathologically, a mild granulomatous response developed in both strains with no tissue destruction. Footpad induration was similar in B6 and *phox91*$^{-/-}$ with the lymphocyte infiltration into the granuloma consisting primarily of activated effector cells. Th1 cytokines, chemokines, and inducible nitric oxide synthase were expressed at in *phox91*$^{-/-}$ mice footpads at levels comparable to the immunocompetent strains. When infected with *M. leprae* in vitro, normal macrophages from B6 and *phox91*$^{-/-}$ mice supported bacterial viability, whereas IFN-γ–activated macrophage killed *M. leprae* in a RNI-dependent manner, confirming that production of RNI is the more important effector mechanism utilized by activated murine macrophages to inhibit *M. leprae*.

3.2.4 | p40$^{-/-}$ mice

The cytokines, IL-12, produced primarily by macrophages and monocytes, and IL-23, produced by macrophages and dendritic cells, are important in the regulation of innate and adaptive immunity. IL-12

induces the production of IFN-γ by T and NK cells early in infections and promotes the development of a Th1-type cell–mediated immune response, whereas IL-23 stimulates the production of IL-17 by Th17 cells and is important for the development of chronic inflammation and granuloma formation.[76] IL-12 and IL-23 are heterodimeric cytokines which share a common p40 subunit, and p40 has been shown to be important in host defense against mycobacterial disease.[77,78]

To study the roles of IL-12/23 in experimental leprosy, *M. leprae* infection was evaluated in p40$^{-/-}$ mice.[79] p40$^{-/-}$ mice infected using the growth protocol exhibited significantly enhanced growth of *M. leprae*, reaching 10^5 by 3 months postinfection and continuing to multiply to reach 10^6 by 12 months postinfection. However, p40$^{-/-}$ mice developed only a mild inflammation. Likewise, footpad induration was markedly decreased in p40$^{-/-}$ compared with that of B6 mice. Similarly to B6 footpads, leukocyte accumulation into p40$^{-/-}$ footpads consisted primarily of CD4+ T cells and CD11b+ macrophages; however, the percent CD4$^+$ T cell infiltration was reduced and the percent CD8$^+$ cell infiltration was augmented in p40$^{-/-}$ mice. Expression of cytokines crucial to an effective Th1 response (IFN-γ and TNF) and chemokines important in granuloma formation (CCL2, CCL3, CCL4) were significantly lower in p40$^{-/-}$ footpads compared with B6 footpads. In summary, compared with B6 mice, p40$^{-/-}$ mice exhibited a decreased ability to control *M. leprae* growth and evidenced reduced footpad induration with altered CD4$^+$ and CD8$^+$ T cell composition, presumably due to the lack of protective IL-12 and proinflammatory IL-23, respectively.

3.2.5 | IL-10$^{-/-}$ mice

IL-10, generated primarily by T cells and macrophages, exerts immunosuppressive and anti-inflammatory actions. IL-10$^{-/-}$ mice experimentally infected with various pathogens tend to succumb rapidly from a severe pathology associated with an intense production of inflammatory mediators. Studies in several leprosy endemic populations have revealed IL-10 polymorphisms associated with both susceptibility and resistance to leprosy.[80] IL-10 has been detected in lesions of multibacillary patients[81] and likely contributes to the leprosy-specific unresponsiveness seen in LL leprosy.[82]

Upon infection, *M. leprae* growth in IL-10$^{-/-}$ mice showed similar growth kinetics as control mice.[67] However, this deficiency in IL-10 did not further restrict growth or augment inflammation in the already highly resistant B6 mice. Histopathologically, IL-10$^{-/-}$ mice developed a slight increase in the infiltration of lymphocytes, macrophages, and especially epithelioid cells to the site of infection. Mild, yet significant, increases in both footpad induration and in the numbers of cells isolated from the footpad were observed in IL-10$^{-/-}$ mice.

3.2.6 | TNF$^{-/-}$ and TNFR1$^{-/-}$ mice

TNF plays a pivotal role in inflammatory phenomena that culminate in either pathogenesis or resistance in mycobacterial disease. The classic studies by Kindler et al[83] showed that TNF is produced by many cell types and demonstrated a role in regulating granuloma structural organization and maintenance through the recruitment and migration of leukocytes into lesions. In addition, TNF is important for triggering activation of IFN-γ–primed macrophages for antimicrobial activity, inducing apoptosis or necrosis, and inducing cellular differentiation. In leprosy, TNF expression has been detected in leprosy lesions.[84] In general, the amount of TNF has correlated with cell-mediated immunity, with higher levels expressed in TT leprosy as compared to LL leprosy. In addition, studies have associated TNF gene alleles as relevant to increased clinical susceptibility to *M. leprae* infection,[80] although the actual role of TNF in cellular dynamics within that microenvironment of long-term infection remains experimentally undefined.

To study the role of TNF in experimental leprosy, *M. leprae* footpad infection was evaluated in TNF$^{-/-}$ and TNFR1$^{-/-}$ mice.[85] *M. leprae* growth was augmented 10-fold throughout the 9-12 months infection period compared with control mice. Histopathologically, TNF$^{-/-}$ and TNFR1$^{-/-}$ mice developed an extensive but diffuse lymphocytic infiltration. Footpad induration was initially delayed in both knockout strains but did develop by 28 days postinfection. Flow cytometric analyses demonstrated an increased activated CD4+ T cells and I-Ab+ macrophage accumulation in TNF$^{-/-}$ and TNFR1$^{-/-}$ footpads. Expression of inflammatory cytokines and chemokines was elevated in TNF$^{-/-}$ and TNFR1$^{-/-}$ footpads compared with control mice. These data indicate that TNF plays a role in the development of an organized and protective granuloma in experimental leprosy.

3.2.7 | Lymphotoxin-alpha (LTα$^{-/-}$) mice

An absence of the related cytokine, LTα, had quite a different effect.[85] LTα is implicated in the development of secondary lymphoid organs and in cell-mediated immunity against intracellular pathogens. Previous studies had shown that although LTα-deficient mice can amass similar accumulations of T cells at the site of *M. tuberculosis* lung infection, T cells fail to properly co-localize with granuloma macrophages, resulting in abnormal granulomas with elevated bacterial load.[86] LTα deficiency does not impair TNF production, and conversely, LTα production is independent of TNF. A polymorphism, LTAα+ 80, which has been found within the promoter region of the gene encoding LTα, is an important risk factor for developing leprosy per se.[87] LTα has been detected in TT leprosy lesions and in type 1 reactions, which supports its purported role as a proinflammatory cytokine.

Mycobacteriu M. lepromatosis footpad infection was evaluated in LTα-deficient chimeric (cLTα$^{-/-}$) mice.[85] Targeted disruption of LTα results in the loss of secondary lymphoid tissue; therefore, in order to have mice with peripheral lymph nodes populated with LT$\alpha^{-/-}$ lymphocytes, chimeric knockout mice were generated by reconstitution of irradiated RAG mice with LT$\alpha^{-/-}$ bone marrow (cLT$\alpha^{-/-}$). Irradiated RAG mice reconstituted with B6 bone marrow (cB6) served as controls. cLTα$^{-/-}$ mice were deficient in both the

soluble and membrane-bound forms of LTα. Multiplication of *M. leprae* in the footpad of cB6 mice reached on the order of 10^5 bacilli by three months postinfection and remained at this level up to nine months postinfection. There was no difference between cB6 and cLTα$^{-/-}$ mice in the number of acid-fast bacilli recovered from footpads at three and six months postinfection. However, acid-fast bacilli counts were slightly but significantly higher in the cLTα$^{-/-}$ mice at nine months postinfection. These data show that LTα is not essential for controlling the growth of *M. leprae* during early infection, but may influence survival of *M. leprae* late infection. Histopathologically, few lymphocytes accumulated in the footpads of cLTα$^{-/-}$ mice and footpad induration could not be sustained. Flow cytometric analyses demonstrated that few lymphocytes could be recovered from cLTα$^{-/-}$ footpads, but the popliteal lymph nodes contained more CD3+ cells compared with cB6 mice suggesting an aberration in cell trafficking. In addition, there was a five- to 50-fold lower level of expression of inflammatory cytokines and chemokines in *M. leprae*–infected cLTα$^{-/-}$ footpads. These data indicate that LTα regulates the induction and maintenance of the granulomatous response during chronic *M. leprae* infection, and this may underlie the association of genetic variants in the LTα gene and human leprosy.

4 | LEPROSY REACTIONS

Perhaps the most difficult and challenging aspect in the treatment of leprosy is the care and management of reactional episodes. Up to 40% of leprosy patients will undergo these painful and debilitating immunological events. Both type 1 and type 2 reactions can cause severe inflammation, require prolonged and recurrent specialized inpatient care, and can exacerbate and lead to irreversible nerve damage, paralysis, and deformity.[43] Leprosy reactions typically occur in the borderline and LL leprosy areas of the disease spectrum. They can occur during leprosy drug treatment and even after completion of the treatment regimen when patients are technically cured of leprosy. Little is known about what induces reactions. Dead bacteria can remain in skin and delicate nerve tissues for years following treatment completion, representing a hoard of persistent antigens. It has also been observed that certain "stresses," for example, puberty, menstruation, pregnancy, postpartum, vaccination, illness, and psychological strain that may alter complex immunological balances can precipitate a reactional episode.[88] The mechanisms that underlie and modulate the onset of leprosy reactions are not well understood but are likely manifestations of immunoregulatory changes, both systemically and in the microenvironment of the leprosy granulomas. Predicting and preventing reactional episodes would be of great benefit[89] as existing treatments often have side effects which limit application and effectiveness, especially when they must be administered long term.

Type 1 (reversal) reactions appear as the result of an increase in cell-mediated immunity and affect patients classified in the BT to BL areas of the spectrum. They are characterized by their localized and gradual onset. Histologically, type 1 reactions consist of an influx of mononuclear cells, edema, and erythema in pre-existing lesions. If a Type 1 reaction upgrades, lymphocyte numbers may increase. In intense type 1 reactions, necrosis accompanied by severe nerve damage may occur.

Type 2 (erythema nodosum leprosum [ENL]) reactions are characterized by the abrupt development of groups of tender, erythematous skin nodules and fever. Because type 2 reactions can involve any tissue containing *M. leprae* antigens, ENL lesions are not confined to the skin or pre-existing lesions, but can involve the eye, joints, nasal mucosa, and other tissues. Histologically, type 2 reactions often present an influx of neutrophils on a BL or LL background. A vasculitis involving arterioles or venules may be demonstrable. Immunologically, type 2 reactions have long been considered manifestations of an Arthus type of hypersensitivity reaction.

Global transcriptional profiles of peripheral blood mononuclear cells from leprosy patients in reaction revealed immunity-related pathways. The "complement and coagulation" pathway was common in both type 1 and type 2 reactions, as was IFN-γ as an upstream regulator.[90] An increase in CXCL-10 expression was uniquely associated with type 1 reactions.[91,92] Decreased C4 and increased IgM, IgG1, and C3d-associated immune complexes in patients newly diagnosed with leprosy were associated with subsequent development of type 2 reaction.[93] Decreased C4 at diagnosis was also found in those who developed type 1 reaction. Another study examining gene expression profiles in leprosy lesions found "cell movement" pathways associated with neutrophil recruitment as a major factor in Type 2 reactions.[94] Recently, studies using longitudinal analyses of leprosy patients before, during, and after type 1 reaction revealed an up-regulation of IFN-γ downstream genes[95] and identified a five-gene transcriptomic signature for onset type 1 reactions.[89]

4.1 | Immunotherapy in leprosy patients

A number of studies attempted to modify the anergic responses of LL patients to *M. leprae* and augment cell-mediated immunity by means of local administration of purified recombinant human cytokines. Multiple intradermal injections of IFN-γ into the lesions of LL and BL patients[96] evoked a notable migration of T cells, predominantly CD4+, and monocytes into the dermis. The bacillary load was reported to have also decreased. A persistent granulomatous response showing characteristic epithelioid and multinucleated giant cells was observed. In addition, repeated intramuscular injections of IFN-γ induced a generalized, intradermal mononuclear cell infiltration of CD3+, CD4+, CD8+, and CD1a+ T cells and Leu-M5+ mononuclear phagocytes. Unfortunately, IFN-γ administration induced an ENL reaction in 60% of the LL patients.[97] Upon intradermal injection of IL-2 into LL lesions,[98] both CD4+ and CD8+ cells migrated into the lesion, and epidermal keratinocytes expressed activation markers. There was also evidence of the destruction of *M. leprae*–parasitized granuloma macrophages and a reduction in the bacterial index in the lesions. More recently, as the use of biologics for conditions such as arthritis has increased, incidences of leprosy reactions have

occurred.[99] For example, Scollard et al[100] reported two patients who developed clinical borderline leprosy while being treated with Infliximab, an anti-TNF antibody therapy they had been prescribed for arthritis. The development of leprosy under these circumstances highlighted the protective aspects of TNF which had likely kept a subclinical infection with *M. leprae* under control. More intriguing, however, was that when the infliximab therapy was discontinued, both patients developed type 1 reactions within one month. Thus, fluctuations in TNF can profoundly affect the development, progression, and stability of borderline leprosy. These immunotherapy studies in humans undeniably demonstrated that the position on the leprosy spectrum could be purposely upgraded to augment cell-mediated immunity, but at the risk of harmful consequences such as the induction of reactions.

5 | MOUSE MANIPULATIONS

We have attempted several manipulations, in both in vitro models and in vivo *models*, to disrupt the status quo of the mouse response to *M. leprae* infection.

5.1 | Reconstitution with IFN-γ–activated macrophages in an in vitro model of LL leprosy

Krahenbuhl's group[101,102] showed that macrophages heavily infected with *M. leprae*, either derived from the athymic nude mouse footpad granuloma or infected in vitro, were refractory to activation with IFN-γ. From these data, he proposed that any killing of the bacilli in LL leprosy lesions would have to be accomplished by newly arriving, previously activated macrophages infiltrating the granuloma. To further test this hypothesis, we developed an in vitro model of the LL granuloma and used it to study the fate of *M. leprae* in a LL leprosy lesion with and without immunotherapeutic intervention.[103] Target cells, consisting of granuloma macrophages harvested from the footpad of *M. leprae*–infected athymic nude mice, were co-cultured with normal or IFN-γ–activated effector macrophages. The bacilli were recovered and assessed for viability. *M. leprae* recovered from target macrophages, either cultured alone or in the presence of normal effector macrophages, exhibited high viability. In contrast, bacilli recovered from target macrophages co-cultured with IFN-γ–activated macrophages were killed. An effector:target ratio of at least 5:1, a co-culture incubation period of 3-5 days, and production of RNI, but not ROI, were required. Neither IFN-γ nor TNF was required during the co-cultivation period for killing of the bacilli, but cell-to-cell contact was necessary for both augmentation of bacterial metabolism by normal macrophages and inhibition of *M. leprae* by activated effector macrophages. Conventional and confocal fluorescent microscopy revealed that the bacilli from target macrophages were acquired by the effector macrophages. These data suggest that, in the absence of cell-mediated immunity, the influx of fresh macrophages into a LL leprosy lesion sustains growth and viability. However, if the new effector macrophages are activated beforehand, they have an opportunity to kill the *M. leprae* they acquire from the infected macrophages. These studies were performed in the absence of cytotoxic T cells. Exactly how the new macrophages acquire *M. leprae* from the infected granuloma macrophages is currently being studied.

5.2 | Overcoming anergy in an in vivo model of LL leprosy

Reasoning that the overwhelming growth of *M. leprae* in the footpads of athymic nude mice could be an intriguing model on which to exert an abrupt induction of cell-mediated immunity to *M. leprae* antigens in a granuloma that resembled LL, we performed adoptive transfer of *M. leprae*–sensitized T cells. However, we were disappointed to observe remarkably little effect in the recipient athymic nude mice with regard to inflammation or killing of *M. leprae*. Previous studies had shown prominent secretion of prostaglandin (PG) E_2 by ex vivo cultured granuloma cells from *M. leprae*–infected athymic nude footpads as well as from human LL leprosy biopsies.[101] Based on this experience and on reports that essential fatty acid deficiency enhances cell-mediated immunity by reducing production of prostaglandins with immunosuppressive actions, these adoptive transfer experiments were redesigned to suppress PGE_2 production in *M. leprae*–infected recipient athymic nude mice prior to adoptive transfer of activated T cells. *M. leprae*–infected mice were fed either a control or linoleic acid-free diet for 3 months prior to adoptive transfer of *M. leprae*–primed, T-enriched lymphocytes.[104] In brief, activated T cells adoptively transferred into recipient mice fed control diets induced little reduction in *M. leprae* viability. In contrast, *M. leprae* from recipient mice fed the essential fatty acid–deficient diet exhibited a markedly reduced viability upon adoptive transfer of activated T cells. These data showed that induction of PGE_2 production by *M. leprae* or its constituents could negatively modulate the function of the T cells in the microenvironment of the lepromatous granuloma and result in a localized immune deficiency in the vicinity of the bacilli.

5.3 | Modification of neutrophil infiltration in *M. leprae*–infected IFN-$\gamma^{-/-}$ footpads

Upon infection of IFN-$\gamma^{-/-}$ mice with *M. leprae*, there was a persistent and long-term infiltration of Ly6G+ neutrophils into the footpads, a response seen in neither B6 nor athymic nude mouse footpads, nor any other knockout mouse strain that we had examined. This response was especially interesting since the presence of neutrophils is a hallmark of type 2 reactions. In our initial attempts to modify this cellular response, we treated IFN-$\gamma^{-/-}$ mice with intra-footpad inoculations of recombinant IFN-γ. We observed a reduction in the size of the footpads, emphasizing the dynamic nature of this granulomatous response. However, we know that macrophages from IFN-$\gamma^{-/-}$ mice are functionally normal and can be activated by IFN-γ to kill *M. leprae*,[51] so this decrease in the induration of IFN-$\gamma^{-/-}$ footpads may simply have been due to the killing of the bacilli in these mice. Therefore, we tried an alternate approach. The

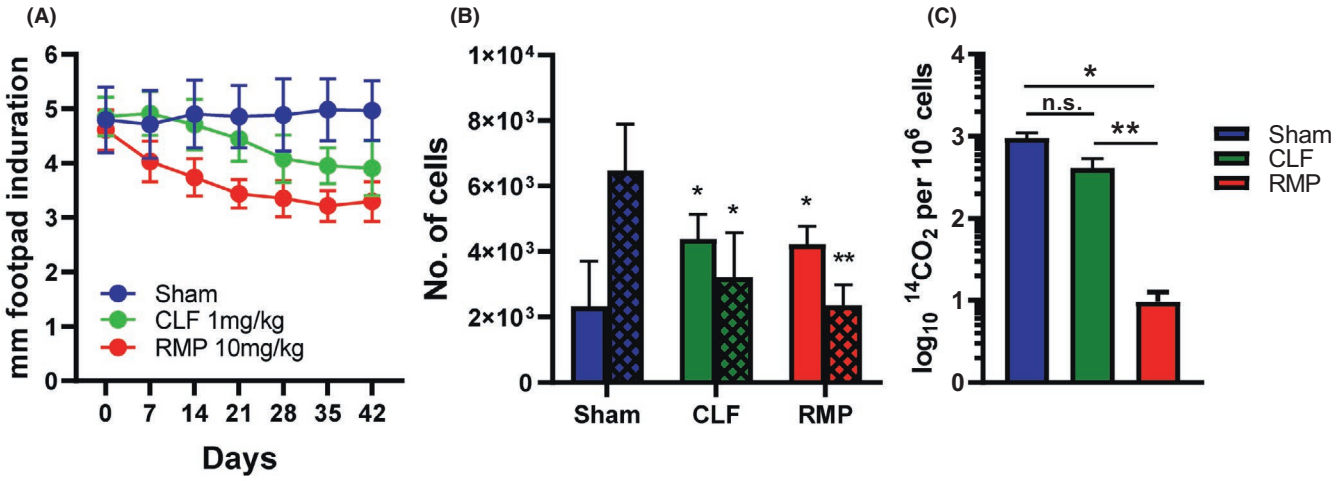

FIGURE 4 Modulation of *M. leprae*–infected INF-$\gamma^{-/-}$ footpad induration and cellular composition by Clofazimine and rifampin. IFN-$\gamma^{-/-}$ mice were infected in the hind footpads with 30×10^6 *M. leprae*. At 24 wk postinfection, mice were treated daily for 10 d with vehicle (sham), 1 mg/mL clofazimine (CLF), or 10 mg/kg rifampin (RMP). A, Footpad induration was measured with a vernier caliper. B, At 42 d, footpads cells were isolated and the number of macrophages (open bars) and neutrophils (hatched bars) were determined by flow cytometry. C, *M. leprae* viability was assessed by radiorespirometry

incidence of type 2 reactions has been reported to be lower in leprosy patients treated with clofazimine.[105] Therefore, we treated mice via gastric gavage with 10 daily doses of a very low, non-bactericidal dose of 1 mg/kg Clofazimine or the standard bactericidal dose of 10 mg/mL Rifampin. Footpad induration was monitored over six weeks. As shown in Figure 4A, significant reductions in footpad induration were observed with both drug treatments. Flow cytometric analyses of granuloma footpad cells (Figure 4B) showed that the high infiltration of neutrophils seen in sham-treated IFN-$\gamma^{-/-}$ mice were significantly reduced upon both clofazimine and rifampin treatment. Interestingly, this reduction in neutrophils in clofazimine-treated mice occurred without bacterial killing (Figure 4C). These data suggest that clofazimine may subdue type 2 reactions by directly regulating neutrophil trafficking. The mechanisms of this process are currently being studied.

5.4 | Generation of double knockout mouse strains via cross-breeding

As noted earlier, the NOS2$^{-/-}$ mouse strain responded to *M. leprae* infection in a manner that resembled BT leprosy. They developed a large granulomatous response, generated high levels of TH1 cytokines and chemokines, and restricted bacterial growth; however, elevated levels of IL-10 were also detected at the site of infection. While IL-10 is an immunosuppressive cytokine that has been associated with multibacillary leprosy, variations in IL-10 expression have been observed upon the occurrence or treatment of reactional episodes.[92,106-108] It has been proposed[109] that the presence of IL-10 in reaction lesions is an attempt to activate regulatory pathways which can suppress proinflammatory pathways and temper tissue damage from excessive inflammation. Therefore, we questioned whether disruption of IL-10 in the NOS2-/- strain would exacerbate the model further toward a more inflammatory or even "reactional" state. To study this, we cross-bred the NOS2$^{-/-}$ and IL-10$^{-/-}$ strains to create a double knockout strain, 10NOS2$^{-/-}$.

Like the control and parent strains, 10NOS2$^{-/-}$ mice controlled growth of *M. leprae*.[67] Similar to the NOS2$^{-/-}$ strain, inflammatory infiltrates replaced muscle bundles in the footpad granuloma, replacing nearly 50% of the muscle mass. Induration and the number of cells isolated from the footpad were also significantly higher in 10NOS2$^{-/-}$ mice than in either B6 or IL10$^{-/-}$, although similar to NOS2$^{-/-}$. In addition, 10NOS2$^{-/-}$ footpads yielded increased numbers of CD4$^+$ and CD8$^+$ lymphocytes. Upon examination of T cells isolated from the granulomas of *M. leprae*–infected mice and re-stimulated in vitro with various *M. leprae* native or recombinant antigen preparations, 10NOS2$^{-/-}$ CD4+ and CD8+ T cells produced elevated levels of IFN-γ compared with the other three strains. Furthermore, CD4+ T cells were present inside fragmented nerves. It is important to point out that the numbers of *M. leprae* present in the footpads of these different strains were the same. In fact, all four strains killed the bacilli. The difference in pathology and nerve damage was due to the host response. While the magnitude of T cell infiltration into the granuloma was primarily regulated by NOS2, a concurrent lack of IL-10 resulted in an intensified *M. leprae* antigen responsiveness and T cell invasion of nerves.

6 | CONTRIBUTIONS OF THE MOUSE MODEL

6.1 | Molecular enumeration and viability of *M. leprae*

Mycobacteriu M. lepromatosis is uncultivable in axenic media and must be enumerated by direct microscopic counting of the bacilli. This procedure is complex, labor-intensive, and suffers from

limited sensitivity and specificity. Using primers to amplify a shared region of the multicopy repeat sequence (RLEP) specific to *M. leprae*, we have developed real-time PCR assays for quantifying *M. leprae* DNA in biological samples.[110] Because of the intermediate level of growth of *M. leprae* in the TNFR1$^{-/-}$ footpad, this knockout strain, along with immunocompetent mice, athymic nude mice, and armadillo tissue, was used to assess the validity, accuracy, and reproducibility of this molecular enumeration assay. The RLEP assay was sensitive and showed excellent correlation with microscopic counting. We have also developed a molecular enumeration assay for *M. lepromatosis*.[3] Molecular enumeration is a rapid and objective means to estimate the numbers of *M. leprae* in tissues, and application of the technique can facilitate work with this agent in many laboratories.

We have also spent a considerable amount of time over the years to develop a number of surrogate viability assays for *M. leprae*. One of our most widely used assays is a radiorespirometry assay, which measures bacterial metabolism of palmitic acid. With a sensitivity of approximately one million organisms, this assay can determine viability from bacilli harvested from in vitro cultures and was one of the first assays used to show that activated macrophages can kill *M. leprae*.[57] Another technique is a live-dead fluorescent staining procedure based on Syto9 and propidium iodide.[111] Perhaps the most exciting development is a molecular viability assay based on the expression of *hsp18* and *esxA* transcripts from a defined number of *M. leprae*.[112] An advantage of this assay is that it can be performed on fixed tissues, lending it well to mouse footpad tissues or human biopsies. Furthermore, since it utilizes a standard curve, it determines absolute viability at time of harvest and does not rely on a paired "pretreatment" sample. The outcome of the molecular viability assay correlates with both radiorespirometry and the mouse footpad assay. Lastly, the molecular viability assay can detect *M. leprae* killing by either immunological, that is, activated macrophages, or chemotherapeutic means. In drug studies, it can differentiate between bactericidal and bacteriostatic drug activities, enabling even short-term efficacy studies without the need for sub-passage of *M. leprae* in mice. Development and optimization of the molecular enumeration and viability assays depended on the mouse model, and with these advances, we have developed a drug evaluation pipeline[113] which evaluates a new drug candidate against non-dividing *M. leprae* in a cell-free axenic analysis, non-dividing *M. leprae* in intracellular macrophage cultures, and multiplying *M. leprae* in the mouse footpad assay.

6.2 | Antigen responsiveness and diagnostic tests

A top priority in leprosy research is the implementation of a field-friendly diagnostic test for detection of early leprosy,[114] and the development and assessment of such a test is an area of intense research.[115-117] To aid in this endeavor, animal models have been used to screen immune responsiveness to various *M. leprae* antigens with a goal of providing an interface between the production of these test antigens and their evaluation in human field trials. Lahiri et al[118] tested the sensitivity for antigen-induced IFN-γ production in a carefully defined mouse model of early leprosy infection. Since there may be no lesion present in subclinical leprosy, they examined splenic T cells for a systemic response to footpad infection and found that several *M. leprae* recombinant proteins induced significant levels of IFN-γ secretion. Hagge et al[67] investigated the CD4+ and CD8+ T cell responses at the site of infection in an inflammatory footpad model of infection. They found upregulated IFN-γ responses in the granuloma by CD4+, and especially CD8+, T cells to ML0380 (GroES), ML2038 (bacterioferritin), and ML1877 (EF-Tu) in lesions associated with nerve damage. Antigen responsiveness to a variety of recombinant *M. leprae* proteins has also been evaluated in peripheral blood mononuclear cells from *M. leprae*–infected armadillos.[15] *M. leprae* antigens used in this study were specifically chosen based on their recognition by T cells derived from *M. leprae* exposed humans.[119,120] They found that armadillos expressed a strong IFN-γ response to these same antigens. Armadillos have also been recently used to test new skin test reagents.[121] Collectively, these studies support the utility of these defined animal models of *M. leprae* infection for examining antigens currently under consideration for improved T cell–based diagnostic assays for leprosy in humans.

6.3 | Vaccines

There is ample evidence that cell-mediated immunity, effected by both CD4+ and CD8+ T cells, is instrumental in host defense against mycobacterial diseases. It is commonly stated that 95% of people are resistant to developing leprosy upon exposure to *M. leprae*. This rather anecdotal estimate is largely based on subjective observed incidences of disease in close contacts of leprosy patients in highly endemic areas. A review of epidemiological studies in the pre-antibiotic era supports this conclusion[122]; however, this phenomenon remains difficult to investigate and quantify as leprosy is often unrecognized and undiagnosed for years. Indeterminate leprosy has been known to resolve or progress into the leprosy spectrum. Furthermore, TT leprosy is considered the highly resistant form of the disease, but it is still leprosy. Viable organisms are found in the lesions and nerve damage often accompanies this resistance. So, what actually constitutes protection in leprosy?

Initial attempts to define protective immunity to *M. leprae* with the goal of producing a vaccine were studied in the mouse footpad by Shepard[123] who determined that heat-killed *M. leprae* (HKML) stimulated the protection against subsequent footpad challenge with live *M. leprae*. Walker et al[124] demonstrated that HKML induced a Th1-type cytokine response. In a study utilizing CD4+ T cell knockout (CD4$^{-/-}$) mice and CD8+ T cell knockout (CD8$^{-/-}$) mice (manuscript in preparation), vaccination with HKML primed spleen cells from both the CD4$^{-/-}$ and CD8$^{-/-}$ strains as well as control mice for substantial IFN-γ production upon in vitro stimulation with *M. leprae* antigen. Moreover, vaccination with HKML

induced a substantial inhibition of *M. leprae* growth in control and $CD8^{-/-}$ footpads. In contrast, however, growth of *M. leprae* in HKML-vaccinated $CD4^{-/-}$ footpads was not inhibited but similar to non-vaccinated mice.

Live, but not dead, BCG can confer protective immunity against *M. leprae* challenge and induce immunotherapeutic protection when administered after footpad infection. However, field trials of BCG as a vaccine for leprosy have yielded widely variable results (reviewed in ref. 125,126 Other whole cell vaccine candidates for leprosy include *M habana*, *M vaccae*, *M indicus pranii*, and ICRC bacilli.[127]

New defined vaccines are also being developed and tested in animal models. The LepVax vaccine is composed of a hybrid recombinant protein made of four *M. leprae* antigens, ML2531, ML2380, ML2055, and ML2028 (LEP-F1) which are prepared as a stable emulsion with a synthetic, TLR4 agonist (GLA-SE) as adjuvant.[128] LepVax reduced growth of *M. leprae* in mouse footpads; furthermore, LepVax, administered as postexposure immunoprophylaxis to armadillos infected with high doses of *M. leprae*, reduced sensory nerve damage and both delayed and alleviated motor nerve damage. A phase 1 trial with LepVax was recently completed and reported to be well-tolerated.[129] Another attractive option in vaccine development is a vaccine that is cross-protective for both leprosy and tuberculosis. Vaccination with *M. tuberculosis* Ag85B-ESAT6 or *M. leprae* Ag85B-ESAT6, both formulated in GLA-SE, reduced growth of both organisms in mouse footpads.[130] In fact, *M. leprae* infection was controlled better by the *M. tuberculosis* recombinant vaccine than by the *M. leprae* recombinant vaccine.

6.4 | Chemotherapeutics

Currently, treatment for leprosy in most parts of the world entails a WHO-recommended multidrug therapy comprised of three drugs: dapsone, a weakly bactericidal sulfone drug which interferes with dihydrofolic acid synthesis by blocking dihydroteroate synthetase; rifampicin, a bactericidal drug which binds to the β-subunit of the bacterial RNA polymerase and interferes with RNA synthesis; and clofazimine, a riminophenazine dye with immunosuppressive properties. While quite effective, the leprosy treatment regimens are quite long, at least six months for paucibacillary leprosy and 12 months for multibacillary leprosy. This lengthy course of therapy can exacerbate adverse drug side effects and make compliance difficult. This in turn can lead to relapse and drug resistance. Hence, better drugs with shorter regimens would be beneficial for control measures.

Testing new drugs for anti–*M. leprae* activity is not an easy undertaking. One must have a ready supply of viable leprosy bacilli against which to assay the test compounds. Through an interagency agreement with National Institutes of Allergy and Infectious Diseases, the NHDP maintains four strains of *M. leprae* and two strains of *M. lepromatosis*. As *M. leprae* only multiplies in living hosts, for example, humans, armadillos, and mice, these strains are carefully maintained through continuous passage in athymic nude mice. Aliquots of leprosy bacilli with a viability of >90% are routinely harvested and supplied to the leprosy research community for a wide variety of studies (https://www.hrsa.gov/hansens-disease/research/index.html). In addition, because of the slow growth and long generation time drug studies in the mouse footpad can take months to complete.

Early drug studies were performed using immunocompetent mice. It is important to note that drug therapy for any infection does not likely kill every single pathogenic organism, but instead decreases the number of bacteria to a less detrimental level so that a developing immune response can complete the job. Therefore, we and others use the athymic nude mouse for drug efficacy studies because it increases the sensitivity of the assay by allowing evaluation solely of the drug's antimicrobial properties without contribution from an acquired cell-mediated immune response. We also employ a variation of Shepard's kinetic mouse footpad model. With this method, footpads of athymic nude mice are infected with *M. leprae* and treatment begins one to two months postinfection, when the bacteria are in exponential growth. Footpads are harvested at an early and later time point post-treatment completion, and *M. leprae* growth and viability are measured using molecular techniques. One measures the delay of growth in the treated group compared with control mice, and thereby bactericidal versus bacteriostatic outcomes can be differentiated.[131]

However, could other immunological aspects benefit drug efficacy studies? One such contribution could lie in the development of chemoprophylaxis regimens for contacts of those diagnosed with leprosy. Ideally, postexposure prophylaxis should be safe, effective, easy to administer, and not costly.[114] It should also be a brief and simple regimen so as not to promote any untoward anxiety or stigma that may ensue from a confusion between prophylaxis and treatment for leprosy. With this in mind, a variety of proposed treatment regimens could be carried out in mice with differing levels of immunity, for example, immunocompetent, athymic nude,[132] or even knockout strains, to determine the least intrusive, yet effective, chemoprophylaxis regimen for different levels of immunity. Then, as immunodiagnostic assays for leprosy become more refined and predictive of susceptibility to *M. leprae* infection,[133] a more personalized chemoprophylaxis regimen could be recommended based on a multibacillary or paucibacillary disease prediction.

7 | CONCLUSIONS AND OUTLOOK

An essential feature in the successful containment of chronic intracellular infections is the ability to develop and maintain an effective granulomatous response. While examination of patient biopsies has implicated several cytokines as responsible for disease susceptibility and pathology, it is difficult to characterize the longitudinal development of the chronic granuloma microenvironment generated in response to *M. leprae* infection when such infection may have existed sub-clinically for years prior to diagnosis. The advantage of animal models is that we know the dose, time, and site of infection and can, for the most part, plan accordingly.

TABLE 2 Attributes for classification of mouse strains in the leprosy spectrum

Characteristic	B6	10NOS2$^{-/-}$	NOS2$^{-/-}$	IFN-γ$^{-/-}$	Athymic nude
ML growth	+	+	+	++	+++
Granuloma	Minimal	Destructive	Destructive	Unorganized	Leproma
FP induration	+	+++	+++	++	–
Lymphoid cells	CD4 > CD8	↑CD4↑CD8	↑CD4	↑CD4	–
Myeloid cells	Epithelioid	Epithelioid	Epithelioid	↓MΦ/↑PMN	Foamy
Cytokine response	TH1	↑TH1	↑TH1	TH2/TH17	TH2
Classification	Self-healing	TT	BT	BL	LL

Over the years, we have evaluated, in vivo and in vitro, the host cell–mediated immune response to mouse footpad infection with *M. leprae* in more than 10 different strains of mice, many of which are presented here. These studies focused on the *M. leprae*–induced granuloma, its histopathology, cellular composition, the immunological products the cells generate, and their ability to kill or, conversely, provide a niche, for *M. leprae*. Interestingly, comparing our findings in the knockout mouse strains with intact immunocompetent control mice at one end of the leprosy spectrum and athymic nude mice on the other, no knockout of a single cytokine, T cell type, or antimicrobial mechanism transformed them into an immunosuppressed model like the athymic nude mouse. Nevertheless, based on their unique characteristic response, some mouse strains could be placed in the leprosy spectrum (Table 2). These findings underscore the presence of alternative mechanisms of host resistance in these animals, the very mechanisms which may be functioning in borderline leprosy.

An ideal animal model of leprosy would recapitulate the clinical and histopathological spectrum of disease, the peripheral neuritis, and the reactional episodes. The current animal models each contribute to some of these aspects. There is as of yet no really good animal model, mouse or otherwise, for leprosy reactions, although ENL has been described in a sooty mangabey monkey.[27] The closest we have come so far to a reaction in a mouse is with our double knockout 10NOS2$^{-/-}$ strain. However, with the ever-increasing numbers of genetically altered animals and the sophisticated methods used to generate such strains, the potential for reaction models and better representatives of the leprosy spectrum is definitely possible.

ACKNOWLEDGEMENTS

This work was supported by the NIH National Institute of Allergy and Infectious Diseases (AI50027 and AAI15006), and the Leprosy Research Initiative (LRI) and the Turing Foundation under LRI Grant number 703.15.43.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLAIMER

The views expressed in this publication are solely the opinions of the author and do not necessarily reflect the official policies of the U.S. Department of Health and Human Services, the Health Resources and Services Administration, or the Agency for Healthcare Research and Quality, nor does mention of the department or agency names imply endorsement by the U.S. Government.

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How to cite this article: Adams LB. Susceptibility and resistance in leprosy: Studies in the mouse model. *Immunol Rev*. 2021;301:157–174. https://doi.org/10.1111/imr.12960