CD1-Restricted T Cell Recognition of Microbial Lipoglycan Antigens

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It has long been the paradigm that T cells recognize peptide antigens presented by major histocompatibility complex (MHC) molecules. However, nonpeptide antigens can be presented to T cells by human CD1b molecules, which are not encoded by the MHC. A major class of microbial antigens associated with pathogenicity are lipoglycans. It is shown here that human CD1b presents the defined mycobacterial lipoglycan lipoarabinomannan (LAM) to αβ T cell receptor–bearing lymphocytes. Presentation of these lipoglycan antigens required internalization and endosomal acidification. The T cell recognition required mannosides with α(1→2) linkages and a phosphatidylinositol unit. T cells activated by LAM produced interferon γ and were cytolytic. Thus, an important class of microbial molecules, the lipoglycans, is a part of the universe of foreign antigens recognized by human T cells.

The family of CD1 molecules is expressed in the thymus, on antigen-presenting dendritic cells in various tissues, on mantle zone B cells, and on cytokine-activated monocytes (*1*, *2*). CD1 proteins are remotely homologous to MHC in their α1 and α2 domains (*3*). Unlike MHC class I and class II proteins, human CD1 molecules are encoded on chromosome 1 and are nonpolymorphic. Recent data indicate that human CD1b molecules present nonpeptide components of mycobacteria to $CD4^-8^-$ (double negative, DN) αβ T cell receptor (TCR)–bearing T cells (*4*). In addition, CD1b does not require the TAP1 and TAP2 transporters or the genes encoding DMA and DMB for their expression and antigen-presenting function (*2*, *5*). Thus, CD1 molecules may mediate a MHC-independent antigen-presenting pathway (*4*).

To characterize antigens presented by CD1 molecules to T cells, we derived mycobacteria-reactive DN $\alpha\beta^+$ T cell lines from the skin lesion of a leprosy patient (line LDN4) and from the peripheral blood mononuclear cells (PBMCs) of a normal donor (line BDN2) (*6*). These T cells were examined for their ability to respond to subcellular fractions of mycobacteria in the presence of CD1-expressing antigen-presenting cells (APCs) (Fig. 1A). Substantial T cell antigen activity for both cell lines was associated with a soluble fraction obtained after repeated extraction of cell walls with SDS. This fraction contains several cell wall–associated proteins as well as the glycosylphosphatidylinositols (GPIs), lipoarabinomannan (LAM), lipomannan (LM), and phosphatidylinositol mannosides (PIMs). We found that the LAM-depleted soluble cell wall fraction [prepared by Triton X-114 partitioning (*7*)] did not induce detectable T cell proliferation. To confirm that the antigenic activity was attributable to a lipoglycan, we tested highly purified LAM and showed it to maintain T cell stimulatory activity. Recognition of purified LAM from *Mycobacterium leprae* was restricted by CD1b for both cell lines, as only CD1b-specific monoclonal antibodies (mAbs) blocked T cell proliferation (Fig. 1B). Furthermore, DN T cells lysed LAM-pulsed monocytes in a CD1b-restricted manner (Fig. 1C) (*8*). LAM induced these DN T cells to secrete the type 1 cytokine pattern, with large amounts of interferon γ (IFN-γ) and small amounts of interleukin-4 (IL-4) (Fig. 1D) (*9*). These

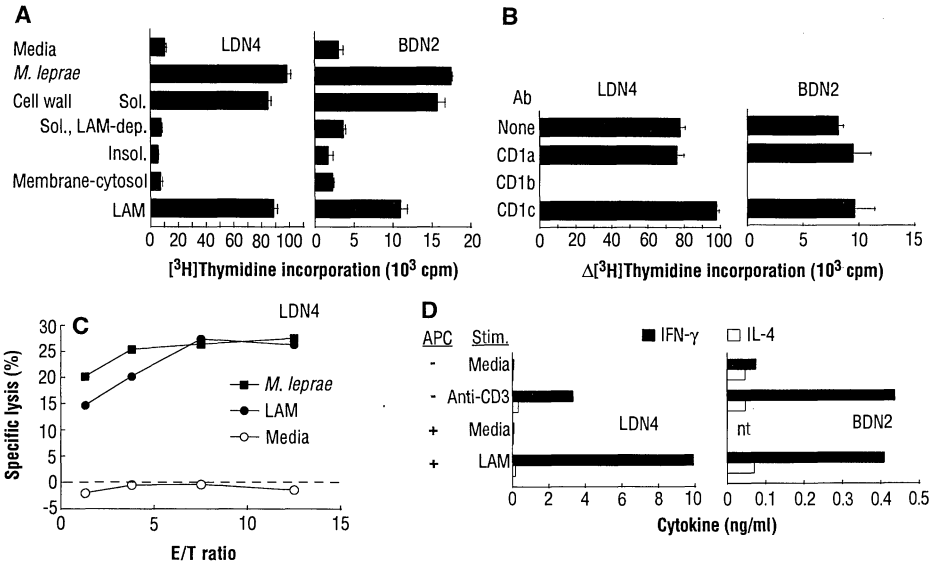

Fig. 1. Recognition of a mycobacterial lipoglycan (LAM) by DN αβ T cells in a CD1-restricted manner (*30*). (**A**) [^{3}H]Thymidine incorporation by DN αβ T cell lines LDN4 and BDN2 in response to *M. leprae* sonicate (*2*), fractionated *M. leprae* (*7*), or purified *M. leprae* LAM (*11*). T cells (1 × 10^4/200 μl) were cultured in the presence of antigen (10 μg/ml of protein or dry weight) and allogeneic CD1-positive APCs (*2*). [^{3}H]Thymidine (0.5 μCi per well) was added during the last 4 hours of culture (72 hours total). Sol., soluble; dep., depleted; and Insol., insoluble. (**B**) CD1 restriction of DN αβ T cells in response to LAM. T cell cultures were prepared as above, except that antibodies to CD1 (Ab) were added to CD1-positive APCs 1 hour before the addition of T cells and antigen. T cell proliferation is expressed as the change in counts per minute of the stimulated cultures minus the counts per minute of the unstimulated cultures. The results shown are representative of three independent experiments. (**C**) DN T cell lysis of LAM-pulsed CD1-positive target cells (*31*). The results are expressed as percent specific lysis {[counts per minute release from the experiment minus counts per minute of the spontaneous release (control)]/(maximal release minus the counts per minute of the control × 100)}. E/T, effector-to-target ratio. (**D**) Cytokine production by CD1-restricted T cells. Cytokine release from DN T cells (1 × 10^5/ml) was measured by enzyme-linked immunosorbent assay (ELISA) after stimulation (Stim.) with antibody to CD3 (anti-CD3; OKT3, 10 μg/ml, Coulter, Hialeah, Florida), or CD1-positive APCs (1 × 10^6/ml) and *M. leprae* LAM (1 μg/ml) or media for 24 hours. IFN-γ (Life Technologies, Gaithersburg, Maryland) and IL-4 (Genzyme, Cambridge, Massachusetts) ELISAs were performed according to the manufacturer's instructions; nt, not tested.

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data demonstrate that a defined mycobacterial lipoglycan is presented to T cells by CD1b.

Like the lipopolysaccharides (LPSs) and lipoteichoic acids (LTAs) of Gram-negative and Gram-positive bacteria, respectively, LAM is a heterogeneous, amphiphilic lipoglycan (Fig. 2A) (*10, 11*). To correlate the structure of LAM with T cell responsiveness, we subjected several of the entities that comprise LAM (which are available as independent products) to degradative treatment, including treatment with protease, α-exomannosidase, and chemical deacylation. These entities include the mixed PIMs, with one Man*p* at position 2 of inositol and up to five more residues at position 6, and LM, which structurally resembles LAM except that it is devoid of the serologically active arabinan (*12*). The purity of LAM, LM, and PIMs was established by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2B), sugar analysis by gas chromatography–mass spectrometry, and fast atom bombardment–mass spectrometry of the peracetylated derivatives; in this analysis, no protein components were detected. LM treated with α-exomannosidase converted the original Man*p* residues of LM to the free form, as shown by its hexitol nature (*13*). In light of the known specificity of the mannosidase for α(1→2)–linked, Man*p*-linked residues, the hexitol must represent α(1→2) Man*p* residues released from the linear α(1-6) Man*p*-linked backbone of LM. We used ^{1}H nuclear magnetic resonance (NMR) to show a marked increase in Man*p* linked to position 6 in the digested product (Fig. 2C).

Proteinase K treatment of LAM had little or no effect on the antigenic activity of LAM (Fig. 3A). The activity of LM was similar to that of LAM for BDN2, which suggests that the arabinan component of LAM is not involved in the stimulation of BDN2 (Fig. 3A). LM stimulated LDN4 cells about half as effectively as did LAM, which suggests some contribution of the polymerized Ara*f* units, although much of the T cell activity was retained in LM. Digestion of LM with α-exomannosidase abrogated much of the activity (Fig. 3A). However, it was evident from the ^{1}H-NMR that some branches were retained on the digested LM, accounting for the residual proliferative activity. These data indicate that the Man*p* residues are critical for T cell responsiveness.

The PIMs (both PIM_2 and PIM_6) represent a partial structure of LAM consisting of the GPI anchor plus portions of the mannan core (*12*). The PIMs induced strong proliferation of BDN2 cells but only weak stimulation of LDN4 cells. However, PIM_2, the minimal structural unit of LAM or LM, did not induce proliferation in any of the DN T cells (Fig. 3A). Thus, these combined proliferation and analytical data indicated that a degree of mannose polymerization and the presence of α(1→2) Man*p* units are required for recognition of LAM by LDN4 and BDN2 cells and that these T cell lines differed slightly in the fine specificity of their recognition of LAM, LM, and the PIMs.

To examine the contribution of the phosphatidylinositol (PI) unit to this form of T cell recognition, we deacylated LAM, releasing palmitic and tuberculostearic (C19, methyl-branched chain) acids from the diacylglycerol unit (dLAM) (*14*). The dLAM had lost all activity (Fig. 3B). The fatty acids recovered after alkali treatment of LAM did not stimulate any of the DN T cells (Fig. 3B), which indicates that the intact diacylglycerophosphate-phospholipid configuration is required for the T cell response.

The response of the DN T cell lines was specific for mycobacterial GPIs, because other lipoglycan products, such as enterobacterial LPSs from *Escherichia coli*, lipophosphoglycan (LPG) from *Leishmania major*, or LTAs from *Streptococcus pyogenes*, did not induce a response (Fig. 3B). Thus, the precise structural features of the mycobacterial GPIs, rather than their general amphipathic nature, are required for recognition by specific T cells. The two T cell lines had different species-specific recognition patterns, in that LDN4 cells proliferated in response to LAM from *M. leprae* but did not respond to that from *M. tuberculosis*, whereas BDN2 cells responded to LAM from both species (Fig. 3B). This difference in specificity may be due to recognition of distinct carbohydrate epitopes, because the lipid units of the two LAMs are identical (*15*). BDN2 cells responded to PIM_6, a GPI with identical structural features among dif-

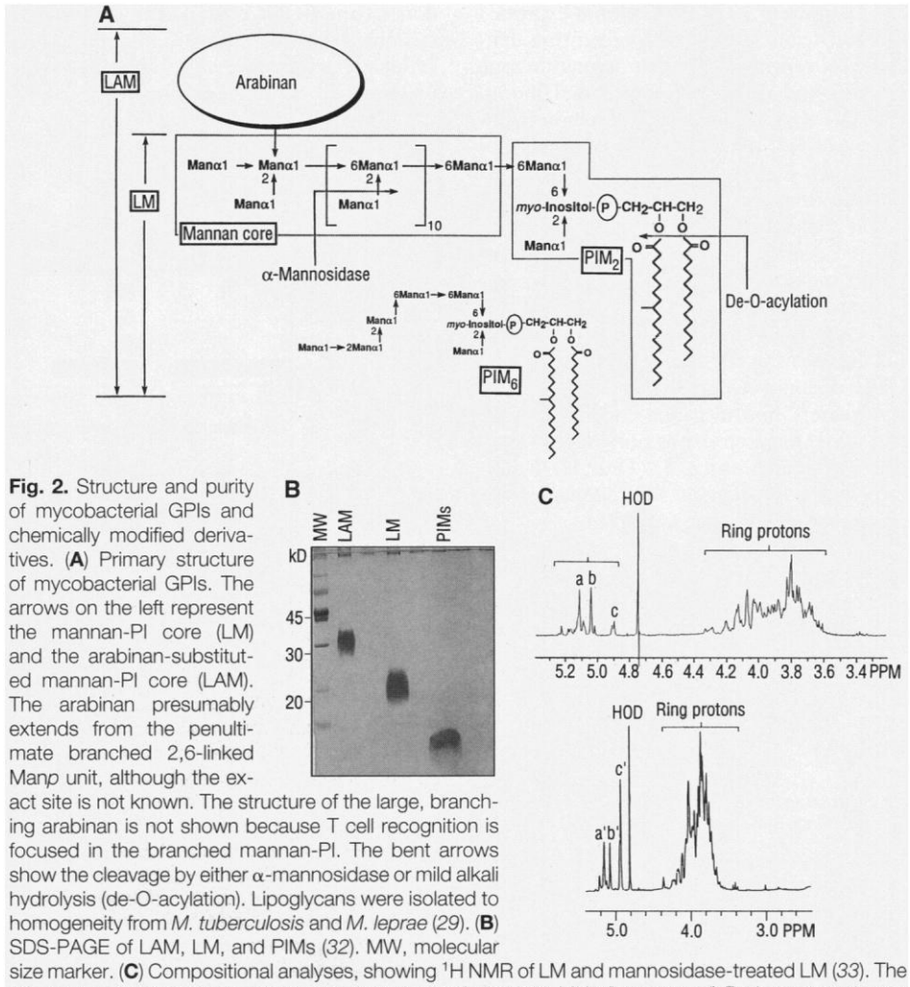

Fig. 2. Structure and purity of mycobacterial GPIs and chemically modified derivatives. (**A**) Primary structure of mycobacterial GPIs. The arrows on the left represent the mannan-PI core (LM) and the arabinan-substituted mannan-PI core (LAM). The arabinan presumably extends from the penultimate branched 2,6-linked Man*p* unit, although the exact site is not known. The structure of the large, branching arabinan is not shown because T cell recognition is focused in the branched mannan-PI. The bent arrows show the cleavage by either α-mannosidase or mild alkali hydrolysis (de-O-acylation). Lipoglycans were isolated to homogeneity from *M. tuberculosis* and *M. leprae* (*29*). (**B**) SDS-PAGE of LAM, LM, and PIMs (*32*). MW, molecular size marker. (**C**) Compositional analyses, showing ^{1}H NMR of LM and mannosidase-treated LM (*33*). The spectra were recorded on a 500-MHz (upper panel) and 300-MHz (lower panel) Bruker spectrometer (*12*). The spectra revealed three sets of anomeric protons (designated by a, b, and c and a′, b′, and c′ in the upper and lower panels, respectively). The a and a′ were assigned to anomeric protons of terminal Man*p*; b and b′ were assigned to H-1 of -2,6- linked Man*p*; and c and c′ to -6-linked Man*p* (*12*). Treatment of LM with α-mannosidase resulted in a concomitant increase in the c′ (6-linked) Man*p* because of the loss of substituted mannoses on the branch. The acylated version was found to be active for T cells (*34*). HOD, deuterium oxide; PPM, parts per million.

ferent mycobacteria. In contrast, the reactivity of LDN4 cells required the entire mannan core, which displays differences in the numbers of mannose linkages and points of branching in different mycobacterial species (*13*). T cell recognition of carbohydrates is consistent with reports of T cell responses to glycopeptides, in which the peptide backbone binds to polymorphic APCs and the T cell recognition depends on the type and distribution of the sugar residues (*16–19*).

To determine if LAM recognition requires intracellular processing for CD1 presentation, as do peptide antigens for MHC, we pulsed the CD1-positive APCs with LAM for varying times (ranging from 30 min to 4 hours), then washed and examined them for the retention of the capacity to stimulate T cells. At least 90 min were required before LAM could be recognized on the APC surface. Moreover, LAM-pulsed APCs retained antigen-presenting capacity after paraformaldehyde or glutaraldehyde fixation (Fig. 4A). In contrast, fixing the APCs before the addition of LAM and the addition of agents that prevent endosomal acidification abrogated the T cell responses (Fig. 4B). These experiments are consistent with the immunolocalization of LAM in LAMP-1$^+$ membranous vacuoles (*20*) and demonstrate that the presentation of LAM requires uptake and localization to an endosomal compartment.

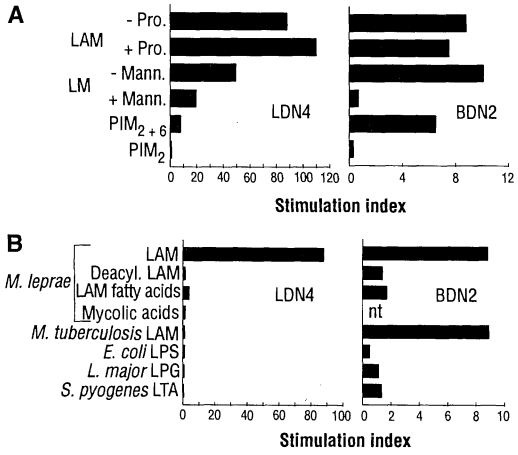

Fig. 3. Structural requirements for DN αβ T cell recognition of LAM. (**A**) Optimal recognition required on the intact mannan core. DN αβ T cells were stimulated as described in Fig. 1 in the presence of LAM, naturally occurring subunits of LAM (LM or PIMs), protease-treated LAM (Pro.), or α-D-mannosidase–digested LM (Mann.). T cell proliferation is expressed as the stimulation index (ratio of the mean counts per minute of the stimulated cultures versus that of the unstimulated cultures). Each compound was tested over a range of concentrations (0.01 to 10 μg/ml); the concentrations stimulating maximal T cell responses are shown. The results shown are representative of three independent experiments (*35*). (**B**) Requirement for an intact PI anchor and specificity of response to LAM versus other lipoglycans. DN αβ T cells were stimulated with *M. leprae* LAM, deacylated (Deacyl.) LAM (*14*), LAM fatty acids, or lipoglycans of other microbial pathogens (*E. coli* LPS serotype 055:B5 Sigma; *L. major* LPG; and *S. pyogenes*) as above; proliferation was measured by [^{3}H]thymidine incorporation. T cell proliferation is expressed as the stimulation index. The lipoglycans were tested at concentrations of 1 and 10 μg/ml, with comparable results (1 μg/ml, as shown). The results shown are representative of three independent experiments; nt, not tested.

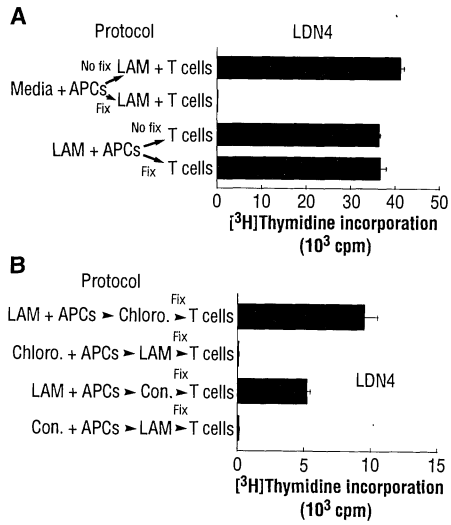

Fig. 4. CD1 presentation of LAM requires internalization and endosomal acidification. (**A**) Glutaraldehyde fixation of APCs inhibited their ability to present LAM to DN αβ T cells. CD1-positive APCs (4×10^6 cells per milliliter) were incubated with (LAM) or without (media) LAM (8 μg/ml) for 4 hours, then washed and treated with (fix) or without (no fix) 0.01% glutaraldehyde (in PBS). APCs (1×10^5) were then added to LDN4 cells (1×10^4), and LAM (1 μg/ml) was added to unpulsed APCs; pulsed APCs were added to LDN4 cells with no further antigen. T cell proliferation was measured by [^{3}H]thymidine incorporation on day 3, and the results are reported as mean counts per minute ± SEM. Results are representative of three independent experiments. (**B**) Endosomal pathway for CD1 presentation of LAM to DN T cells. CD1-positive APCs (4×10^6/ml) were incubated with drugs [chloroquine (Chloro.), 25 mM, Sigma; concanamycin A (Con.) (*36*), 10 nM, Kamiya Biomedical, Thousand Oaks, California] 1 hour before or 90 min after the addition of LAM (8 μg/ml) as indicated, then washed and fixed with 0.01% glutaraldehyde. APCs were then added to DN line LDN4, and proliferation was measured by [^{3}H]thymidine incorporation. Results are expressed as mean counts per minute ± SEM. Results are representative of three independent experiments.

Although CD1b-restricted T cells derived from the PBMCs of healthy donors respond to mycolic acids, a family of long-chain, branched fatty acids of mycobacteria (*4*), LAM did not stimulate mycolic acid–responsive lines (*21*); conversely, mycolic acids did not stimulate the LAM-reactive T cell line LDN4 (Fig. 3B). Although it is not known if LAM, LM, PIMs, and mycolic acids directly bind CD1b, the distinct response patterns of the different CD1-restricted T cell lines indicate antigen presentation and recognition of both fatty acids and glycans, with a likely role for CD1b in binding the lipid portions of these structurally distinct molecules.

These results extend the spectrum of antigens presented by human CD1 molecules and recognized by T cells to include the lipoglycans, which represent an abundant and diverse pool of microbial antigens and virulence determinants. CD1 molecules may transport endosomally targeted lipoglycan antigens of intracellular pathogens to the cell surface, thereby allowing T cell recognition and the killing of infected cells. The in vivo findings that CD1 proteins are highly expressed in the lesions of patients with the self-healing form of leprosy (*22*), and the in vitro findings indicating IFN-γ production and cytotoxicity (*2*), argue for a role for CD1 presentation of microbial lipoglycan antigens in protective immunity.

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6. Cell surface expression of TCRs and accessory molecules on DN T cell lines was determined by flow cytometry. LDN4 and BDN2 cell lines were TCRαβ$^+$, TCRγδ$^-$, CD4$^-$, CD8β$^-$, and weakly positive for CD8α, CD5$^+$, and V_α24$^-$. The ITCR repertoire of LDN4 was examined and found to be clonal with a TCR encoded by a V_β7-D_β1.1-J_β2.1 rearrangement.
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8. In four experiments, the ability of CD1 mAbs to block lysis of LAM-pulsed CD1-positive macrophages was measured. CD1b mAbs blocked lysis by a mean of 62%. In contrast, CD1a mAbs decreased lysis by 14% and CD1c mAbs by 18%.
9. The level of LAM-induced proliferation and IFN-γ release was approximately 10-fold greater for LDN4 as compared to BDN2. However, stimulation by CD3 mAbs also resulted in a 10-fold difference in IFN-γ release. These data would suggest that BDN2 is less responsive not because of lower precursor frequency of lipoglycan-reactive cells but as a result of its lower inherent responsiveness.
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30. DN T cell lines were derived from peripheral blood (BDN2) or a tuberculoid leprosy lesion (LDN4) by immunomagnetic depletion of CD4, CD8, and γδ T cells and culture with *M. leprae* in the presence of PBMCs treated with granulocyte-macrophage colony-stimulating factor (200 U/ml) and IL-4 (100 U/ml) (to induce CD1 expression) (*2*). The following antibodies were used for flow cytometry and blocking experiments: OKT6, CD1a (*23*); WM-25, CD1b (*24*); 10C3, CD1c (*25*); W6/32 HLA-A, B, and C (*26*); DK22, HLA-DR (Dako, Carpenteria, CA); OKT4, CD4 (*23*); OKT8, CD8α (*23*); 2ST8-5H7, CD8β (*27*); WT31, TCRαβ, (Becton-Dickinson, San Jose, CA); and TCRγδ (*28*).
31. Target cells were derived from granulocyte-monocyte colony-stimulating factor– and IL-4–treated PBMCs. Nonadherent cells were discarded, and adherent cells (CD1-positive) were removed with phosphate-buffered saline (PBS) containing 5 mM EDTA (10 min). Target cells (1×10^6 cells per milliliter) were incubated with media, *M. leprae* (2 μg/ml), or *M. leprae* LAM (2 μg/ml) for 16 hours, washed, then labeled with ^{51}Cr. Antibodies to CD1 (5 μg/ml) were added to target cells (4000 cells per 100 μl) and LDN4 T cells for a 5-hour incubation period. Supernatants (75 μl) were harvested and counted in a gamma counter.
32. GPIs were isolated from *M. leprae* by extraction with 50% ethanol and partitioning between phenol and water (*29*). LAM, LM, and PIMs were separated by gel filtration chromatography on Sephacryl S-200 and analyzed (2 μg each) by SDS-PAGE (15% acrylamide) with silver staining (*12*).
33. To improve solubility, we treated both LM and α-mannosidase–treated LM with 0.1 N NaOH for 4 hours at 37°C. The fatty acids were removed by extraction with $CHCl_3$:CH_3OH as described (*29*). The aqueous layers were desalted with Bio-Gel P-2 column chromatography (1 cm by 100 cm; total volume, 90 ml), and 1-ml fractions were collected. The elution position of the salt was measured by conductivity (fractions 70 to 78). The voided fractions (fractions 23 to 28) were pooled, dried, and exchanged with D_2O.
34. For the mannosidase treatment of LM, we solubilized 500 μg of LM in 50 mM ammonium acetate buffer, pH 4.5, and digested it with 2.5 U of α-D-mannosidase (20 μl) from jack bean meal (V-LABS, Covington, LA) for 16 hours at 37°C. The enzyme was denatured by boiling for 2 min and centrifuged to remove insoluble material.
35. For the protease treatment of LAM, proteinase K (0.2 μg) (Boehringer Mannheim, Indianapolis, IN) was added to 250 μg of *M. leprae* LAM in ammonium bicarbonate buffer, and the solution was incubated at 55°C for 1 hour. The mixture was boiled for 2 min to inactivate the enzyme, and the sample was lyophilized.
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Targeted Disruption of Mouse EGF Receptor: Effect of Genetic Background on Mutant Phenotype

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Gene targeting was used to create a null allele at the epidermal growth factor receptor locus (*Egfr*). The phenotype was dependent on genetic background. EGFR deficiency on a CF-1 background resulted in peri-implantation death due to degeneration of the inner cell mass. On a 129/Sv background, homozygous mutants died at mid-gestation due to placental defects; on a CD-1 background, the mutants lived for up to 3 weeks and showed abnormalities in skin, kidney, brain, liver, and gastrointestinal tract. The multiple abnormalities associated with EGFR deficiency indicate that the receptor is involved in a wide range of cellular activities.

The expression pattern and functional analysis of EGFR and its ligands suggest that EGFR is important for embryo development, tissue differentiation, and cellular function (*1*, *2*). The only known genetic alteration is a point mutation ($Egfr^{wa2}$) in the kinase domain that leads to diminished receptor activity (*3*). The phenotype of waved hair and sporadic open eyelids is similar to that of mice deficient for transforming growth factor–α ($Tgf\alpha^{wa1}$) (*4*).

A null allele ($Egfr^{m1Cwr}$) at the *Egfr* locus was created by homologous recombination in 129/Sv-derived D3 embryonic stem cells (Fig. 1A). Seven independently targeted lines were identified (*5*). Germline chimeric males were mated to 129/Sv females to establish a co-isogenic strain carrying the $Egfr^{m1Cwr}$ allele. The allele was then bred into the closed-colony, random-bred CF-1 and CD-1 lines. When homozygous, the mutation resulted in peri-implantation lethality in CF-1 mice, mid-gestation lethality in 129/Sv mice, and perinatal lethality in CD-1 mice. All three phenotypes were fully penetrant and nonoverlapping. Aberrant splicing around the targeted exon was detected joining exon 1 to either exon 3 or exon 5 (Fig. 1B). The former splicing event would create a nonsense protein, whereas the latter retains the reading frame and removes domain 1 of the extracellular region. Although a truncated mRNA is produced, $Egfr^{m1Cwr}$ homozygous pups showed no evidence of an altered EGFR (Fig. 1C) and no indication of EGF-inducible tyrosine phosphorylation.

On a CF-1 background, $Egfr^{m1Cwr}$ homozygous embryos died before embryonic day 7.5 (E7.5) (Table 1). The three mutant embryos recovered at E6.5 consisted of a small mass of unorganized cells. Morphologically normal embryos were observed in 51 implantation sites from $Egfr^{m1Cwr}/+ \times +/+$ control crosses, suggesting that the empty decidua from $Egfr^{m1Cwr}/+ \times Egfr^{m1Cwr}/+$ heterozygous crosses were derived from $Egfr^{m1Cwr}$ homozygous embryos. Preimplantation development proceeded normally, and after transfer of blastocysts to serum-supplemented medium, $Egfr^{m1Cwr}$ homozy-

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