Use of Genetic Profiling in Leprosy to Discriminate Clinical Forms of the Disease

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Leprosy presents as a clinical and immunological spectrum of disease. With the use of gene expression profiling, we observed that a distinction in gene expression correlates with and accurately classifies the clinical form of the disease. Genes belonging to the leukocyte immunoglobulin-like receptor (LIR) family were significantly up-regulated in lesions of lepromatous patients suffering from the disseminated form of the infection. In functional studies, LIR-7 suppressed innate host defense mechanisms by shifting monocyte production from interleukin-12 toward interleukin-10 and by blocking antimicrobial activity triggered by Toll-like receptors. Gene expression profiles may be useful in defining clinical forms of disease and providing insights into the regulation of immune responses to pathogens.

For many pathogens, host responses can vary considerably, with some individuals proving resistant to infection whereas others display a high level of susceptibility. The clinical manifestations found in leprosy form a spectrum that corresponds with the type of immune response to the pathogen, *Mycobacterium leprae* (*1*). At one end of the spectrum, the tuberculoid form (T-lep) is characterized by limited, self-curing disease with few bacteria and local expression of type 1 cytokines characteristic of strong cell-mediated immunity (*2*, *3*). In contrast, lepromatous leprosy (L-lep) patients present clinically with disseminated lesions and high bacterial loads. In this case, expression of type 2 cytokines in lesions typically associated with humoral immunity and suppression of cell-mediated immunity predominates. In order to accurately stage the disease, skin lesions of leprosy patients are biopsied, and previous studies have indicated that differences in patients' immune responses could be discerned from examination of lesion biopsy specimens but not from peripheral blood (*4*). Taking advantage of the recent data made available for the human genome, we compared patterns of expression of ~12,000 genes directly in lesions taken from leprosy patients.

Skin biopsy specimens were obtained from six T-lep and five L-lep patients who had been diagnosed and classified according to the clinical and histopathologic criteria of Ridley (*1*). We analyzed gene expression data from these biopsy samples with the use of two unsupervised learning algorithms, principal component and hierarchical clustering analysis. Principal component analysis (*5*) of the gene-sample matrix was used first to identify patients displaying similar trends in gene expression (Fig. 1A). All five L-lep patients formed a group characterized by negative values for the principal component. In contrast, five of six T-lep patients had positive values, with only patient T6 having a negative value, suggesting a gene expression pattern more like that of the L-lep patients.

Hierarchical clustering was next performed to explicitly group together patient samples showing similar gene expression patterns (*6*). The clustering algorithm identified two main groups consisting of T-lep and L-lep patients, respectively (Fig. 1C). Consistent with the principal component analysis, one patient (T6) was clustered with the L-lep group. Because clustering analysis was not consistent with previous clinical and histologic classification, we reexamined the patient record and the histology of a biopsy taken on the same day as the specimen used for the present study. Contrary to the original classification, we found that the presentation was in fact consistent with L-lep. Upon correction of the classification of patient T6 to L-lep (L6), the principal component analysis (Fig. 1B) and the hierarchical clustering (Fig. 1C) showed a clear and totally consistent distinction between T-lep and L-lep patients. The ability of gene expression analysis to identify the clinical T-lep and L-lep classifications, and to expose the misclassified sample, demonstrates the potential of this approach and reveals a striking difference in gene expression profiles between lesions of T-lep and L-lep patients.

To determine whether the observed differences in gene expression between the T-lep and L-lep patients were statistically significant, we performed a permutation analysis in which the number of genes below each P value threshold in the clinically relevant T-lep or L-lep grouping was compared to all other 461 possible patient groupings (permutated groups). With the use of this approach, we determined that fewer than 1% of the permutated groupings displayed more differential gene expression than the correct T-lep–L-lep grouping (Fig. 2A). For example, at $P = 0.01$ the T-lep–L-lep grouping had more differentially expressed genes (1970) than both the mean (458) and the top 1% (1544) of the permutated groupings. This established that, despite the relatively small number of samples examined in this study, the differences in gene expression observed between T-lep and L-lep patients are not likely to have resulted from chance.

To evaluate whether gene expression profiles are sufficiently robust to correctly assign the subclasses of unknown samples, we performed supervised class prediction analysis as follows: We identified patterns of gene expression among all but one of the samples to predict the class of the withheld sample, known as leave-one-out cross-validation, with the use of a weighted gene-voting algorithm (*7*). With the use of the clinically defined T-lep–L-lep patient grouping, the algorithm predicted the classes of all 11 samples correctly [100% accuracy; 8 out of 11 had high confidence (prediction strength > 0.4)] (*8*, *9*). This result

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was statistically significant, because fewer than 1% (4/462) of the possible patient groupings performed as well as the clinically defined T-lep–L-lep grouping at classifying the withheld sample (Fig. 2B). Taken together, our results from both unsupervised and supervised prediction algorithms demonstrate that T-lep and L-lep lesions are distinct at the level of gene expression and suggest that it may be possible to develop reliable biomarkers to improve patient diagnosis and classification. The ability to accurately classify patients by itself predicts the clinical course of disease, because L-lep patients are more likely to develop one or more reactional states that cause patient morbidity.

Within the lesion, the host response to the pathogen remains localized to the tissue granuloma. The difference in the gene expression profiles between the two leprosy subclasses suggested that their differences in disease manifestation may reflect two opposing gene expression programs that influence the type of host response (*10*). The most pronounced differences in T-lep and L-lep gene expression profiles were among genes within the immune response family (Fig. 3). The gene expression profiles were consistent with previous data showing that type 1 cytokines associated with cell-mediated immunity predominate in T-lep lesions, whereas type 2 cytokines predominate in L-lep lesions (*2*, *11*, *12*). For example, genes encoding the type 1 cytokines lymphotoxin-α, interleukin (IL)-7, and IL-15 were comparatively up-regulated in T-lep lesions, as well as genes encoding CD1b and signaling lymphocytic activation molecule (SLAM), two molecules previously linked to cell-mediated immunity and type-1 cytokine production in these patients (*13*–*15*). In contrast, L-lep lesions differentially expressed the type 2 cytokines transforming growth factor–β and IL-5, as well as IL-4 and IL-10, although the differences in gene expression for the latter two genes between the patient groups were not statistically significant (P not ≤ 0.05) and were instead confirmed by quantitative polymerase chain reaction (qPCR) (*8*). As part of the type 2 pattern, L-lep lesions also exhibited marked up-regulation of genes related to humoral immunity, including immunoglobulin (Ig) heavy and light chains and molecules involved in B cell activation.

Although the presence of type 2 cytokines has been shown to correlate with immunologic unresponsiveness in L-lep patients (*2*, *3*), the elevated expression of inhibitory receptors in L-lep lesions, including several members of the leukocyte immunoglobulin-like receptor (LIR) family (*16*) (Fig. 3), may also contribute to immunologic unresponsiveness in these patients. LIR-7 (ILT-1/LILRA2) (*17*) was the most differentially expressed of the LIR genes identified, up-regulated 5.4-fold in L-lep relative to T-lep lesions. We confirmed the expression of LIR-7 in lesions from 12 additional leprosy patients by qPCR (Fig. 4A), demonstrating marked up-regulation in six out of six L-lep lesions relative to the T-lep samples (*18*). Because the expression pattern of Toll-like receptors (TLRs) and LIR-7 in leprosy lesions is reciprocal (*19*), we investigated the relationship between these two different receptors. Activation of monocytes by using TLR2/1 and TLR4 ligands down-regulated LIR-7 expression (Fig. 4B), suggesting that the relatively reduced expression of LIR-7 in T-lep lesions may be because of the elevated local expression and activation of TLRs in these patients.

In light of these observations, we determined whether activation of LIR-7 alters TLR-induced innate immune responses. Activation of TLRs triggers the release of IL-12 and IL-10, two cytokines whose balance influences the nature of the adaptive T cell response. TLR-induced production of IL-12 was dramatically reduced in the presence of antibodies against LIR-7 (Fig. 4C), whereas IL-10 release was either unaffected (TLR2/1) or augmented (TLR4) in these cells, thus increasing the ratio of IL-10 to IL-12. These data suggest that LIR-7 activation can divert the balance of cytokines produced during the innate response away from the pro-inflammatory program. In the context of infection with an intracellular

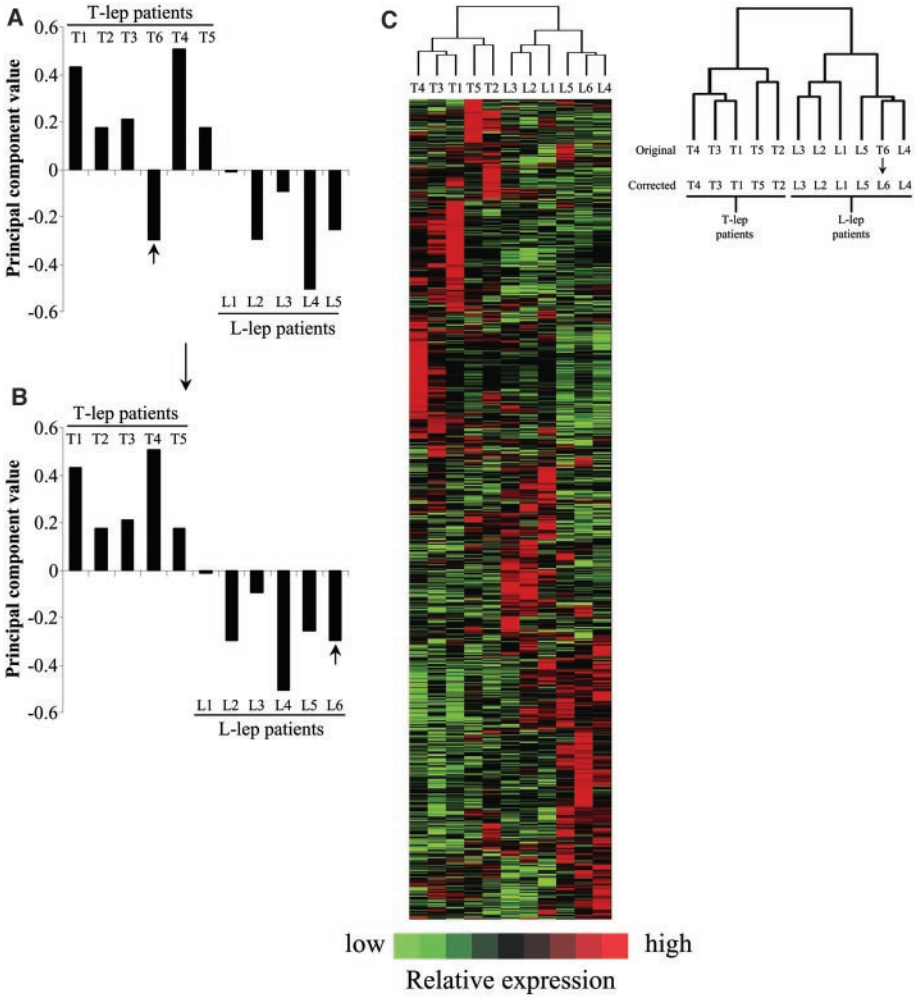

Fig. 1. Two unsupervised data analyses separate leprosy patients into clinically relevant subclasses on the basis of their gene expression patterns. (**A**) Principal component analysis shows that the gene expression pattern of T-lep patients is distinct from the L-lep patients (*9*). All T-lep patients (T1 to T6) except patient T6 (marked with an arrow) formed a group with positive values for the principal component, whereas L-lep patients (L1 to L5) had negative values and formed a second group. (**B**) After verification that patient T6 had been misclassified, we regrouped that patient within the correct L-lep subclass and labeled as L6 (marked by an arrow). (**C**) Hierarchical clustering analysis (*9*) is consistent with the principal component analysis, dividing the samples into two distinct groups that cluster on separate branches of a dendrogram. The original and corrected clinical subclass of each patient is shown.

pathogen, this would result in suppression of cell-mediated immunity and immune unresponsiveness (*20*).

To determine whether LIR-7 affects TLR-induced antimicrobial responses (*21*), we assessed antimicrobial activity by measuring colony-forming units of *M. tuberculosis*–infected alveolar macrophages. Relative to untreated controls, TLR activation alone resulted in a nearly 60% antimicrobial activity; however, pretreatment with antibodies to LIR-7 reduced the antimicrobial activity to 20%. The blockade of antimicrobial activity was not observed with an isotype control antibody or

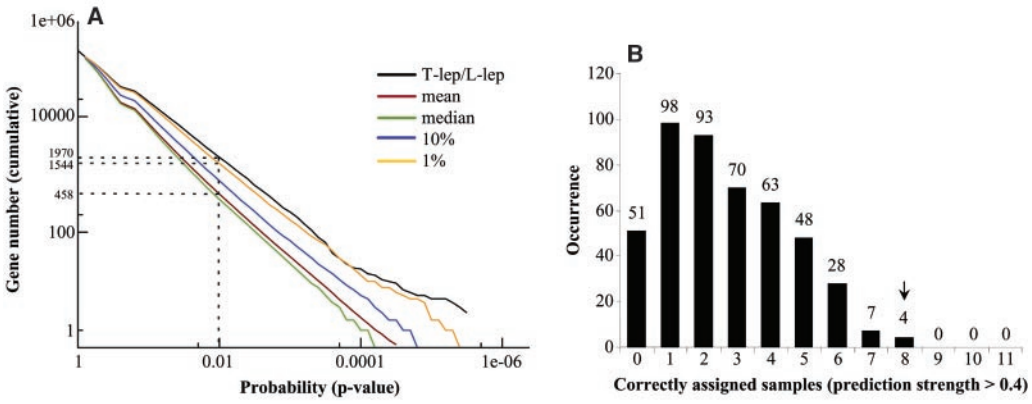

Fig. 2. Two supervised analyses coupled to permutation analysis indicate that the gene expression differences between T-lep and L-lep patient groups are statistically significant. (**A**) Determination of the number of differentially expressed genes between subgroups. The cumulative number of genes (*y* axis) with Student's *t* test *P* values less than various threshold levels (*x* axis) was calculated for the clinically relevant T-lep–L-lep grouping and plotted (black). Every possible permutated grouping was also generated and tested (*9*). We plotted the mean (red), median (green), 10th percentile (blue), and first percentile (yellow) number of genes below a given *P* value among the permutated groupings and compared these to the correct T-lep–L-lep grouping. (**B**) Prediction accuracy using leave-one-out cross-validation and weighted gene-voting (*9*). With the use of the clinically defined T-lep–L-lep grouping, the weighted gene-voting algorithm correctly assigned the subclasses of 8 out of 11 samples with high confidence (prediction strength > 0.4). This result is statistically significant, because fewer than 1% of all possible groupings (4/462, indicated by the arrow) were able to correctly assign eight samples with a prediction strength > 0.4, demonstrating that the differences in gene expression between T-lep and L-lep patients can be used to predict the subclasses of the disease.

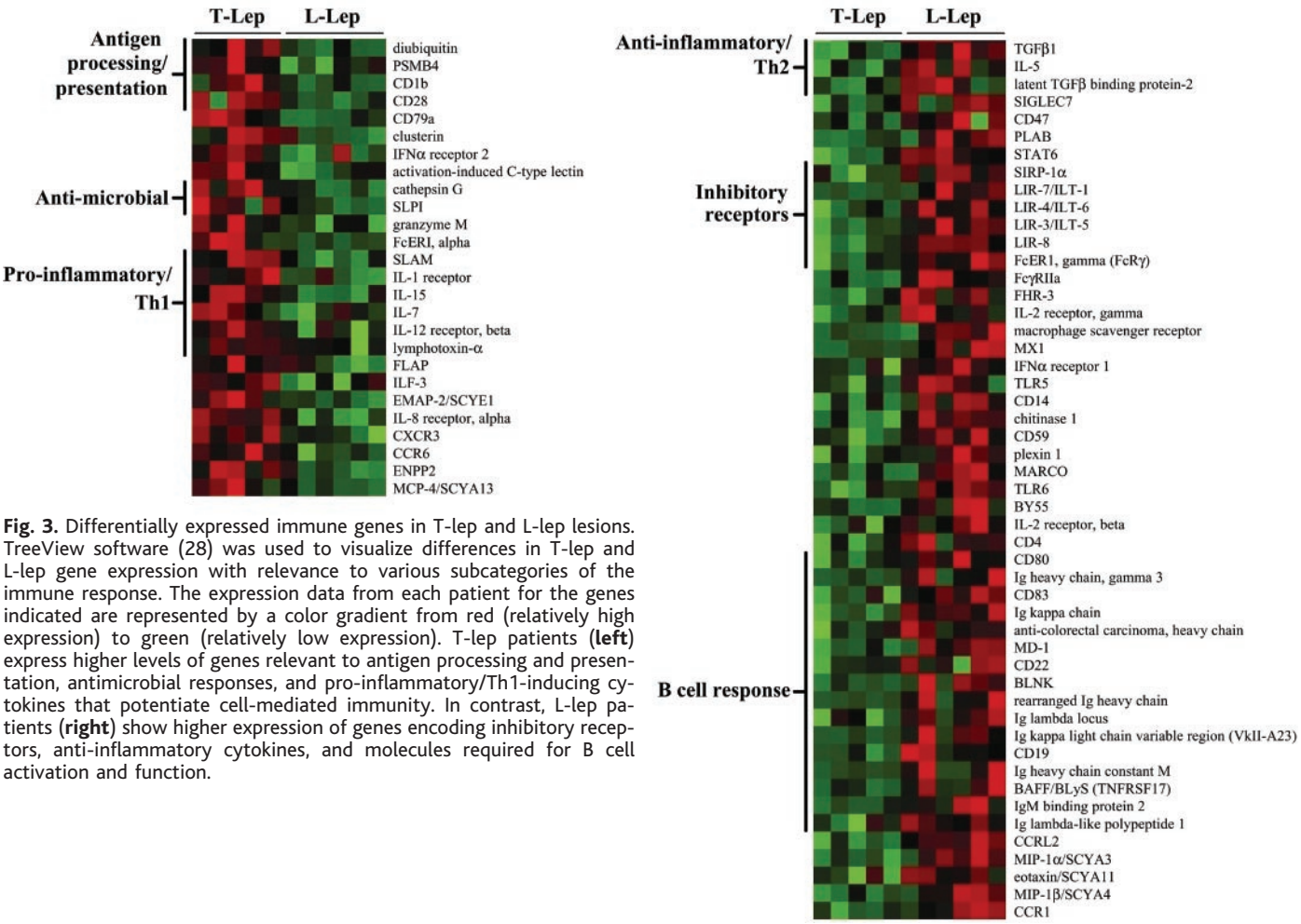

Fig. 3. Differentially expressed immune genes in T-lep and L-lep lesions. TreeView software (28) was used to visualize differences in T-lep and L-lep gene expression with relevance to various subcategories of the immune response. The expression data from each patient for the genes indicated are represented by a color gradient from red (relatively high expression) to green (relatively low expression). T-lep patients (**left**) express higher levels of genes relevant to antigen processing and presentation, antimicrobial responses, and pro-inflammatory/Th1-inducing cytokines that potentiate cell-mediated immunity. In contrast, L-lep patients (**right**) show higher expression of genes encoding inhibitory receptors, anti-inflammatory cytokines, and molecules required for B cell activation and function.

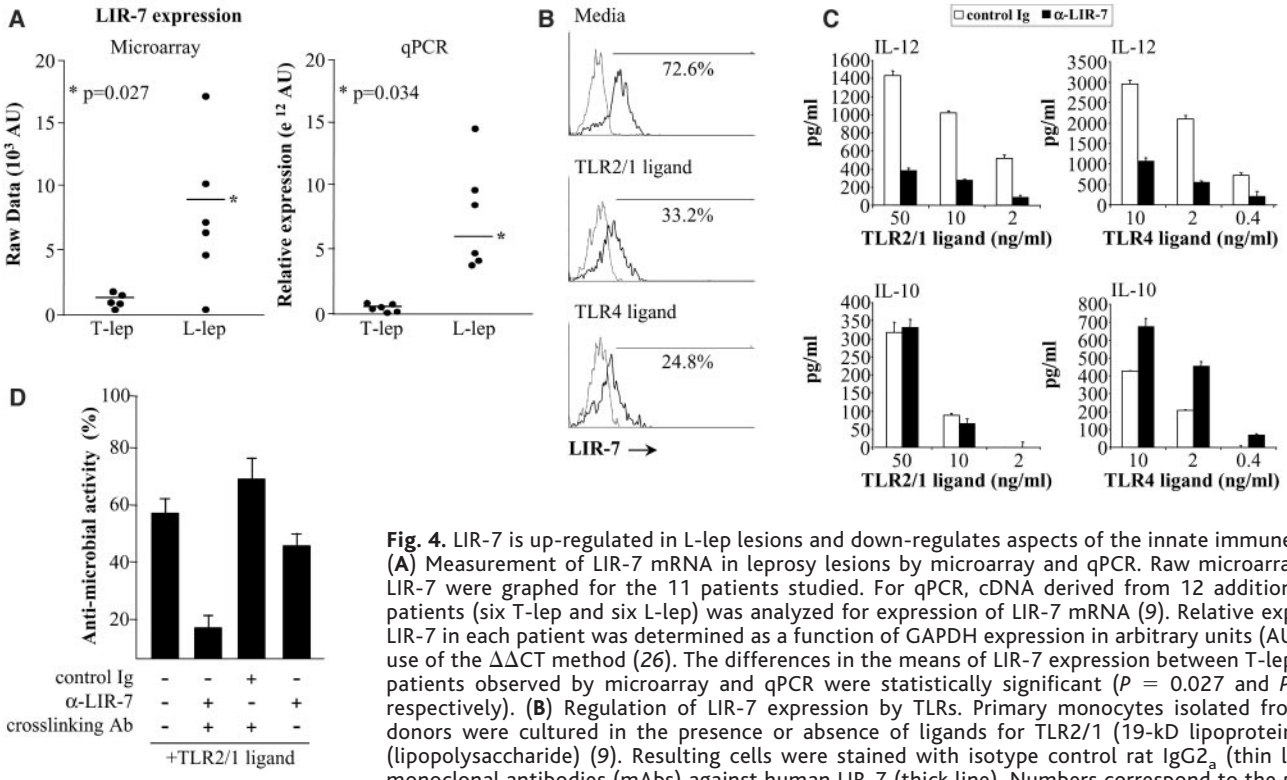

Fig. 4. LIR-7 is up-regulated in L-lep lesions and down-regulates aspects of the innate immune response. (**A**) Measurement of LIR-7 mRNA in leprosy lesions by microarray and qPCR. Raw microarray data for LIR-7 were graphed for the 11 patients studied. For qPCR, cDNA derived from 12 additional leprosy patients (six T-lep and six L-lep) was analyzed for expression of LIR-7 mRNA (*9*). Relative expression of LIR-7 in each patient was determined as a function of GAPDH expression in arbitrary units (AU) with the use of the ΔΔCT method (*26*). The differences in the means of LIR-7 expression between T-lep and L-lep patients observed by microarray and qPCR were statistically significant ($P = 0.027$ and $P = 0.034$, respectively). (**B**) Regulation of LIR-7 expression by TLRs. Primary monocytes isolated from healthy donors were cultured in the presence or absence of ligands for TLR2/1 (19-kD lipoprotein) or TLR4 (lipopolysaccharide) (*9*). Resulting cells were stained with isotype control rat $IgG2_a$ (thin line) or rat monoclonal antibodies (mAbs) against human LIR-7 (thick line). Numbers correspond to the percentage of LIR-7$^+$ cells relative to the isotype control. (**C**) LIR-7 modulates the balance of IL-12 and IL-10. Primary monocytes were preactivated with isotype control (white) or LIR-7 (black) mAbs, followed by goat crosslinking secondary antibodies against rat IgG, as previously described (*27*), and cultured in media containing interferon-γ and serially diluted amounts of ligands for TLR2/1 or TLR4 (*9*). Supernatants were analyzed for production of IL-12 and IL-10 by enzyme-linked immunosorbent assay. Error bars represent ± standard error of the mean (SEM), and data are representative of at least five experiments. (**D**) LIR-7 inhibits TLR-induced antimicrobial activity. *M. tuberculosis*–infected alveolar macrophages were activated with isotype control or LIR-7 mAbs followed by a TLR2/1 ligand to induce antimicrobial activity (*9*, *21*). After 72 hours, lysates were plated and colony-forming units (CFU) were counted after 21 days. Antimicrobial activity represents the percent reduction in CFU compared with untreated controls (CFU in untreated cultures = 15×10^5 CFU). Error bars show ±SEM, and data are representative of three independent experiments.

in the absence of crosslinking secondary antibodies. These data indicate that activation of LIR-7 interferes with TLR-induced antimicrobial responses.

In previous studies of microbial infection, microarray analyses have been used to characterize responses of normal peripheral blood mononuclear cells to distinct classes of pathogens in vitro (*22*, *23*) or mice challenged with different antigens (*24*). In this study, we have defined gene expression patterns associated with an ongoing immune response in lesions of human leprosy. Our data support the view that modern genomics can reveal the sets of genes that correlate with protective responses or inappropriate responses leading to disease progression and tolerance, providing unanticipated insights into pathogenesis and targets for therapy.

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10. The tissue granuloma is largely composed of monocytes and lymphocytes. By immunohistologic analysis, there were similar percentages of monocytes in T-lep compared to L-lep lesions, although the frequency of lymphocytes is somewhat greater in T-lep lesions (*25*). The greatest differences between lesions of the patient groups are in the CD4:CD8 T cell ratio and the degree of differentiation of cells of the monocyte-macrophage series. The difference in gene expression profiles likely reflects two opposing gene programs but could also be because of somewhat different cellular compositions or the consequence of the growth of the bacilli.
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18. The percentages of LIR-7+ peripheral blood monocytes were similar in normal donors (68% ± 7.8, $n = 3$), T-lep donors (54% ± 4.7, $n = 3$), and L-lep donors (61% ± 9.7, $n = 3$). We were not able to obtain the antibody against LIR-7 from Amgen, Incorporated, for tissue labeling because the parties have been unable to agree to a mutually acceptable material transfer agreement.
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Supporting Online Material

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Materials and Methods

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