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Human Dendritic Cells Exhibit a Pronounced Type I IFN Signature following Leishmania major Infection That Is Required for IL-12 Induction

Michelle A. Favila,* Nicholas S. Geraci,* Erliang Zeng,† Brent Harker, † David Condon,* Rachel N. Cotton,* Asha Jayakumar,* Vinita Tripathi,*† and Mary Ann McDowell*  

Leishmania major–infected human dendritic cells (DCs) exhibit a marked induction of IL-12, ultimately promoting a robust Th1-mediated response associated with parasite killing and protective immunity. The host cell transcription machinery associated with the specific IL-12 induction observed during L. major infection remains to be thoroughly elucidated. In this study, we used Affymetrix GeneChip (Affymetrix) to globally assess the host cell genes and pathways associated with early L. major infection in human myeloid-derived DCs. Our data revealed 728 genes were significantly differentially expressed and molecular signaling pathway revealed that the type I IFN pathway was significantly enriched. Addition of a neutralizing type I IFN decoy receptor blocked the expression of IRF7 and IL-12p40 during DC infection, indicating the L. major–induced expression of IL-12p40 is dependent upon the type I IFN signaling pathway. In stark contrast, IL-12p40 expression is not elicited by L. donovani, the etiological agent of deadly visceral leishmaniasis. Therefore, we examined the gene expression profile for several IFN response genes in L. major versus L. donovani DC infections. Our data revealed that L. major, but not L. donovani, induces expression of IRF2, IRF7, and IFIT5, implicating the regulation of type I IFN–associated signaling pathways as mediating factors toward the production of IL-12. 

The online version of this article contains supplemental material.

Abbreviations used in this article: CAPE, caffeic acid phenyl ester; CT, cycle threshold; DC, dendritic cell; IRF, IFN regulatory factor; MeV, Multiple Experiment Viewer; PCA, principal component analysis; qRT-PCR, quantitative RT-PCR.

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elucidate the modulated host DC transcriptional mechanisms that may influence the robust IL-12 induction observed following L. major infection. We demonstrate that during early infection (2, 4, 8, and 24 h), L. major–infected DCs exhibit a distinct type I IFN–associated transcriptomic signature, including the upregulation of IRF2, IRF9, STAT1/2, and IFNAR. In the presence of a neutralizing type I IFN pathway inhibitor, B18R, a reduction of IL-12p40 and secreted IL-12p70 is observed. Moreover, IRF2, IRF7, and IFIT5 were upregulated in L. major infections compared with L. donovani infections; this trend is analogous to the IL-12p40 gene expression elicited by these two species. Combined, these data indicate that the L. major–induced IL-12 response is dependent upon a type I IFN response.

Materials and Methods

Parasite strains

The following parasites strains were used for this study: L. major strain FV1 (MHOM/IL/80/Friedlin) and L. donovani strain 1S (MHOM/SD/00/1S). All parasites were cultured in log phase and expanded to stationary phase 7 d prior to infection day. Infective-stage metacyclic promastigotes were isolated from 3- to 4-d stationary-phase cultures using a standard Ficoll density-gradient purification protocol (11, 12). All parasites tested negative for mycoplasma (PCR detection; Takara) and tested below the limits of detection for endotoxin (∼0.25 U/ml) (Limulus amoebocyte assay; Endosafe, Charles River Laboratories, Charleston, SC).

DC generation

Monocytes were isolated from healthy human donor buffy coats (Central Indiana Regional Blood Center, Indianapolis, IN) by enriching for CD14+ cells using a magnetic bead separator system (AutoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes from each donor were plated in six-well plates (Costar-Corning) at a concentration of 106 cells/2 ml RPMI 1640 complete media (10% FBS, 2 mM L-glutamine 100 U/ml, and 1% Pen/Strep/antimycotics) and supplemented with recombinant IL-4 (40 U/ml; PeproTech, Rocky Hill, NJ), GM-CSF, and granulocyte-macrophage stem cell factor (1000 U/ml; PeproTech) on days 0, 3, and 6 to allow differentiation into immature DCs. DCs were harvested and washed 1 d before infection to remove any residual cytokines. Flow cytometry analysis for DC-associated marker CD1a was assessed to confirm a homogenous population of immature DCs. Metacyclic infective-stage promastigotes were isolated according to previously described methods (11) and opsonized in 5% normal human serum to promote host cell phagocytosis. DCs were infected at a concentration of 10 parasites per 1 DC, and the cocultures of L. major–infected DCs were harvested at 2, 4, 8, and 24 h postinfection. Slides were prepared at the conclusion of each experiment, Diff-Quick stained (Fisher Scientific, Pittsburgh, PA), and visualized by light microscopy. For each infection sample, the infection percentages (percent infected DCs) were determined by counting the number of uninfected versus infected DCs (100 total). The parasite index was determined by counting the total number of intracellular parasites per 100 cells.

RNA isolation and microarray hybridization

For the microarray gene chip hybridizations, L. major–infected human monocyte-derived DC cultures from three individual donor peripheral blood buffy coats were assessed for gene expression at time points 2, 4, 8, and 24 h postinfection, including one uninfected control one per donor for a total of 15 arrays. RNA was extracted from each infection sample using an RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocols for spin column RNA isolation and DNase treatment. The quality of RNA peaks was assessed by examining the integrity of rRNA using an Agilent 2100 RNA Bioanalyzer (Agilent Technologies, Santa Clara, CA) reporting a good RNA yield and no degradation. Labeling and hybridization to human HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA) containing ∼54,000 probe sets (47,000 transcripts representing 38,500 genes) was performed according to the manufacturer’s protocol. Briefly, cDNA was synthesized from 2 μg total RNA using the Gene Chip Expression 3’ Amplification IVT Labeling kit (Affymetrix) and processed to generate biotinylated cRNA. Purified cRNA was fragmented in 5× fragmentation buffer (200 mM Tris-acetate [pH 8.1], 500 mM potassium acetate, and 150 mM magnesium acetate) at 94°C for 35 min prior to fragmentation. Arrays were washed and stained with R-PE streptavidin and scanned with the Gene Chip Scanner 3000 (Affymetrix). All arrays met the acceptable limit of detection for the ∼100 housekeeping genes present on each chip. Microarray chip processing and subsequent data analyses were performed at the University of Notre Dame Genomics and Bioinformatics Core Facility (University of Notre Dame).

Microarray data analysis: statistical analysis, hierarchical clustering, and pathway analysis

The Affymetrix data files (.cel) were processed using Bioconductor software package (http://www.bioconductor.org/; Seattle, WA) (13). Probe set signal intensities were subjected to background correction by GCRMA (Genespring GX 7.0) normalization across all 15 chips (14). The mean fluorescence intensity was derived from a log2 transformation of the raw data. Probe sets with present call in at least 7 of the 15 chips were retained, which filtered our list of 54,000 probe sets down to ∼14,000. Statistical significance was determined using a one-way ANOVA and Benjamini-Yekutieli multiple correction test to control false discovery rates (15). A final set of 84 candidate probe sets (representing 728 genes) were identified as significantly expressed in at least one time point compared with noninfected samples (p < 0.05). Microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE42088. The post-ANOVA set of genes with significant differential expression over time was analyzed by the hierarchical clustering algorithm in the Gene-E data visualization software package (http://www.broadinstitute.org/cancer/software/Gene-E/) using the Euclidean distance metric and average linkage methods. Prior to clustering, the postdata were normalized to expression values of matching uninfected samples and adjusted proportionally to fit a defined scale (−3 to 3). Clustering enabled global visual examination of postinfection gene expression patterns and identification of groups of genes with similar temporal profiles. Pathway analysis using the GeneGo MetaCore Analytical Suite (http://genego.com; GeneGo, St. Joseph, MI) was also performed to identify significantly enriched pathways. A principal component analysis (PCA) was performed by sample clustering with median centering and 10 KNN imputations using the Multiple Experiment Viewer (MeV) v.4.8.1, part of the open-source TM4 Microarray Software Suite (PubMed Identifier: 16939790 and 12613259).

Quantitative RT-PCR

A total of 10 genes were selected for quantitative RT-PCR (qRT-PCR) validation from our original 848 candidates based on gene ontology, immunomodulatory features, and a literature survey. All human primer sequences were designed by Integrative DNA Technologies (http://www.idtdna.com/scitools/scitools.aspx) and used at a concentration of 5 μm per reaction (Supplemental Table I). To validate the selected genes’ expression patterns, we performed qRT-PCR with SYBR Green PCR Master Mix (Applied Biosystems) on the donor cDNAs used for microarray hybridization in addition to cDNA derived from additional donors under the same infection conditions described above. All cDNA was generated according to the manufacturer’s protocols for the SuperScript III First Strand Synthesis System kit (Invitrogen, Carlsbad, CA). Total RNA from uninfected or Leishmania-infected DCs was isolated using an RNAeasy kit (Qiagen), and 1 μg RNA per infection sample was used to generate cDNA using SuperScript III Synthesis (Invitrogen). For each gene, relative numbers of mRNA copies were determined by the ΔΔ cycle threshold (Ct) method (16). Briefly, experimental Ct values were normalized to hypoxanthine phosphoribosyltransferase (HPRT) Ct values and depicted as fold change over uninfected samples (calibrator). The formula used is: ΔCt = ΔCt(expperimental) − ΔCt(calibrator)), where ΔCt equals Ct(expperimental) − Ct(HPRT).

Additional statistical analyses

Statistical tests were performed using either Origin Pro 8.5.1 (OriginLab, Northampton, MA) or GraphPad Prism version 5.0 (GraphPad, San Diego, CA). For statistical analysis, data were first assessed with Kolmogorov-Smirnov test of normality using Dallal-Wilkinson-Lillie for p values. Data sets that did not fit a Gaussian distribution were log10 transformed prior to subsequent analysis. Further analyses included two-way ANOVA followed by Bonferroni multiple comparisons posttest or Student t test. Values were considered significant at p < 0.05. A Pearson correlation test was used to address validated gene expression values.

Type I IFN and NF-κB pathway inhibition assays

Immature DCs were treated with a type I IFN pathway inhibitor, B18R (eBioscience, San Diego, CA), a vaccinia virus–encoded protein that binds soluble type I IFNs including IFN-α, -β, -κ, and -ω, ultimately blocking their capacity to bind to endogenous IFN-specific receptors. A final concentration of 100 ng/ml B18R (resuspended in PBS [pH 7.2], 1% BSA, and

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150 mM NaCl) was added to 10^6 DCs in 2 ml culture media 1 h prior to L. major infection. The NF-κB pathway was blocked by pretreating DCs with 52 μM caffeic acid phenyl ester (CAPE) prior to infection. Uninfected and infected samples were harvested at 8 h postinfec-
tion and processed for cDNA extraction. Secreted IL-12p70 was detected in supernatants by ELISA using paired Abs for IL-12p70 (Pierce, Rockford, IL).

**Results**

**Microarray analysis**

A previous microarray study examined the genetic profile of Leishmania-infected monocyte-derived DCs at 16 h postinfection representing the expression profile of seven donor RNAs pooled together (9). In the current study, we further investigate the L. major–infected DC transcriptional profile and expand upon the current expression data by assessing the transcriptome at early time points postinfection. The cRNAs for each L. major–infected time point (2, 4, 8, and 24 h), including an uninfected sample (2 h), were extracted and hybridized to 15 individual human HG-U133 Plus2.0 Affymetrix microarray gene chips (Affymetrix), in which each chip represented the expression profile for one donor at a given infection time point. Each microarray gene chip encompasses ~54,000 probe sets representing ~38,500 human genes. Aliquots for each L. major–infected DC sample were prepared and Diff-Quick stained (Fisher Scientific) for visualiza-
tion by light microscopy. The percentage of infected cells (average ~61%) (Fig. 1A) and parasite indices (average ~300 parasites/100 cells) (Fig. 1B) were comparable across all infection samples. Additionally, an RNA Bioanalyzer (Agilent Technologies) was employed to assess the quality of RNA and measure the signal intensities of Leishmania–specific peaks (representing 16S and 23S rRNAs). Each infected sample exhibited similar peak heights, suggesting the parasite loads were equivalent (data not shown). The hybridized arrays underwent a GCRMA correction, elimi-
nating background noise values, and probe set signals were then normalized across all 15 gene chips using Gene Spring 7.3.1 (Agilent Technologies). The 54,000 probe sets were then filtered based on a present call in at least 7 of the 15 chips, and the resulting 14,000 transcripts were analyzed by one-way ANOVA (Benjamini-Yekutieli, p < 0.05) with the grouping parameters set as time and infection status (uninfected or infected). A final set of 848 probe sets (representing 728 genes), which exhibited a 2-fold change difference in at least one infected time point compared with uninfected samples, were considered significant and used for further analysis. The data set was analyzed using a hierarchical clustering algorithm, grouping probe sets/gene sets based on average expression and time and dividing the expression values for the infected time points over the expression values for the uninfected samples. The resulting heat map including the selected genes for qRT-PCR validation is presented (Fig. 2, left panel). A complete table for the 848 probe sets corresponding to our generated heat map including raw expression values and fold changes is available in the Gene Expression Omnibus database under accession num-
ber GSE42088. At 2 h, only 15 transcripts exhibited a significant change of at least 2-fold difference compared with uninfected. The number of significant differentially expressed gene transcripts changed with time: 149 at 4 h, 398 at 8 h, and 291 at 24 h. These results suggested that L. major does not significantly impact gene expression until later time points.

**Microarray validation**

A total of 10 genes were selected from our microarray data set and validated by qRT-PCR in at least three new donors independent from the original donors used for the microarray hybridization. Fold change derived from the array versus validated qRT-PCR are presented (Fig. 2A–J). These genes were selected based on their immune ontology, temporal pattern exhibited, and several genes were chosen for their potential association with IL-12 regulation, as L. major induces IL-12 expression in human DCs. For example, STAT1 (Fig. 2E), IRF7 (Fig. 2F), and IRF2 (Fig. 2G) belong to the type I IFN pathway that has been well characterized in response to viral and bacterial infections (16, 17). IFIT3, which is also re-
ferred to as ISG58, (Fig. 2B), belongs to a family of genes for which expression is strongly induced by IFNs and pathogenic molecular patterns (18). Genes associated with either NF-κB signaling, including MALTI (Fig. 2H) (19, 20) and TRIM8 (Fig. 2I) (21), or MAPK signaling pathways, such as MAP3K1 (MEKK1) (Fig. 2A) (22), were also selected. Several genes were chosen randomly, including CITED2 (Fig. 2J), RAB23A (Fig. 2D), and ARF6 (Fig. 2C); the latter two are associated with intracel-
ular trafficking and endocytosis (23, 24). Our qRT-PCR analysis demonstrates that the gene-expression patterns from the donors used for the microarrays correspond to the expression values from several independent donors used for qRT-PCR validation. To de-
termine the strength of the linear relationship between our arrays and the quantified qRT-PCR values, a Pearson corre-
ation test was applied for each individual time point. Both the 4- and 8-h time points (Fig. 3B, 3C) display significant correlation (r < 0.05), and although the 2- and 24-h time points (Fig. 3A, 3D) did not reach statistical significance, the microarray values and qRT-PCR values exhibit similar kinetic trends for all 10 genes assayed (Fig. 2A–J). To assess the variation of gene expression between the individual donors, we employed a PCA using the MeV v4.8.1.TM4 Micro-
array Software Suite (Fig. 4). The three-dimensional plot dem-
strates that the expression profiles for each donor are grouped based on infection state and time (uninfected, 2, 4, 8, and 24 h).

**CITED2 and MAP3K1 are differentially regulated by Leishmania species**

It has been well established that IL-12 production elicited by human DCs is parasite species specific, in which generally
**FIGURE 2.** Hierarchical clustering analysis and qRT-PCR validation for *L. major*–infected DCs. Employing the GENE-E data visualization software package, a hierarchical clustering algorithm was applied to the 848 significant probe sets (representing 728 genes), grouping probe sets based on expression values and time (2, 4, 8, and 24 h). The corresponding heat map was generated (left panel). Genes that are upregulated (red) or downregulated (green) are depicted. Ten genes were selected for validation by qRT-PCR analysis: MAP3K1 (A), IIFT5 (B), ARF6 (C), RAB22A (D), STAT1 (E), IRF7 (F), IRF2 (G), MALT1 (H), TRIM8 (I), and CITED2 (J). For each gene, fold change was calculated using the raw expression values for the three donors used for the microarray (ARRAY; open shapes). To validate the gene expression patterns, DCs derived from three additional donors were analyzed for the selected genes’ expression at 2, 4, 8, and 24 h by qRT-PCR (closed shapes). Different shapes represent individual donors. The ΔΔCT method was employed to determine the fold change of infection over uninfected samples (qRT-PCR).

*L. major* induces IL-12 and *L. donovani* does not (5, 10). The IL-12p40 promoter contains an Ets (TTTCCT) and an NF-kB (GAAATTCCCC) consensus sequence that, upon transcription factor activation, mediates the gene transcription of IL-12p40 (25). Interestingly, our microarray data set included MALT1, which encodes for MALT1, a protein forming a scaffold with BCL10 and CARMA1 (CARMA1/BCL10/ MALT1 complex) that activates NF-kB–mediated cytokine expression in immune cells (26). In addition, CITED2, which was downregulated by *L. major* infection (Fig. 2J), encodes for a protein that represses Ets-mediated gene activation (27, 28). We evaluated the expression of both MALT1 and CITED2 in *L. major*– and *L. donovani*–infected DCs to determine whether these genes may be associated with *Leishmania* species–specific induction of IL-12p40 expression. Both *L. major* and *L. donovani* slightly upregulated MALT1 expression at 4 h postinfection (Fig. 5A), indicating that regulation of this factor cannot account for *Leishmania* infections. Our data indicated that TRIM8 expression is downregulated by infection with both *L. major* and *L. donovani* (Fig. 5C) and MAP3K1 expression was reduced by *L. donovani* but upregulated by *L. major* at 24 h postinfection (Fig. 5D).

**Inhibition of the type I IFN response blocks *L. major*–induced IL-12 expression in DCs**

In an effort to identify biological pathways that were related to *L. major* infection, expression data were processed by the Metacore software suite system, which scores and ranks pathways enriched in our data by the proportion of pathway associated genes with significant expression values. Ultimately, this allows the visualization of canonical pathways significantly enriched for our given data set. The top pathways are provided in Table I. Functional pathways belonging to the immune response category, including “antiviral actions of IFNs,” “IFN-gamma signaling,” “IL-1 signaling,” and “oncostatin M signaling via MAPK in human cells,” were significantly enriched (p < 0.05). However, the most striking observation was the enrichment of the IFN-α/β signaling pathway or type I IFN response pathway. The pathway map associated with the IFN-α/β signaling pathway included several genes from our candidate set (IRF2, IRF9, STAT1, and IFN-α/β receptor) as indicated by the thermometer symbols (Supplemental Fig. 1).
Type I IFNs were first recognized for their role in viral infections and recently in parasitic and bacterial infections (17, 30). We previously demonstrated that *L. major* induces human DC expression of IFN-β and IDO, an immunomodulatory enzyme that promotes establishment of infection through the suppression of lymphocyte proliferation (31). Interestingly, the induction of IFN-β expression during earlier time points indicated that IFN-β may act in an autocrine manner to directly mediate IDO upregulation during *L. major* infection.

Our original objective was to determine the genes and pathways that may be associated with IL-12 regulation; therefore, we employed a type I IFN inhibitor, B18R, to directly address the role of the type I IFNs on *L. major* induction of IL-12p40 transcription in human DCs. B18R is a vaccinia virus–encoded protein or decoy type I IFN receptor that has a high affinity for soluble type I IFNs (IFN-α, -β, -κ, and -δ), essentially sequestering soluble type I IFNs and blocking their ability to engage type I IFN–initiating receptors (32). Immature DCs were cultured in the presence or absence of the B18R recombinant protein 1 h prior to *L. major* infection, harvested at 8 h postinfection, and mRNA levels were determined by qRT-PCR. In the presence of the B18R, the expression of IRF7, a gene induced by the type I IFN response (33), was significantly reduced (Fig. 6A), whereas the expression of TNF-α was not inhibited (Fig. 6B), indicating the effectiveness of the recombinant protein to sufficiently neutralize a response in a type I IFN–specific manner. Treatment with B18R blocked *L. major*–induced transcription of IL-12p40 (Fig. 6C). IL-12 is a member of a heterodimeric family of cytokines including IL-23 and IL-27, for which the bioactive form is dependent on the covalent linkage of the IL-12p40 and IL-12p35 subunits. Interestingly, IL-12p35 was not reproducibly modulated by blocking type I IFN signaling (Fig. 6D); however, secretion of *L. major*–induced IL-12p70 was inhibited in the presence of B18R (Fig. 6F). IL-12p40 can dimerize with p19 to produce IL-23, and inhibition of type I IFNs results in lower levels of IL-23p19 (Fig. 6E), indicating *L. major*–mediated IL-12 and IL-23 production is regulated by the type 1 IFN pathway.

Type I IFN–induced genes are differentially expressed in *L. major*– or *L. donovani*–infected human DCs

It has been well established that IL-12 production elicited by human DCs is parasite species specific, in which generally *L. major* induces IL-12, and *L. donovani* does not (5, 10) (Fig. 7A). We previously demonstrated that *L. major* induces the expression of IFN-β at 4 and 8 h postinfection (31); therefore, we assessed the
expression of both type I IFNs in response to L. major or L. donovani infection and found no significant differential expression of IFN-α (Fig. 7B). However, L. major induced IFN-β at 4, 8, and 24 h postinfection (Fig. 7C). To further investigate the role of type I IFNs in response to Leishmania infections, we quantified the expression of three genes known to be regulated through type I IFN signaling—IRF2, IRF7, and IFIT5 (Fig. 6D–F)—in both L. major- and L. donovani-infected DCs. L. major infection generally induced IRF gene expression at 8 h postinfection, whereas L. donovani did not induce transcription of these IRF genes. These trends were analogous to the IL-12p40 host cell expression differences observed between these two infecting species. The percentage of infected DCs (Fig. 7G) and the parasite indices (Fig. 7H) were comparable between Leishmania species and across all infection time points, indicating sufficient parasite uptake for the respective infections.

Previously, we demonstrated that inhibition of NF-κB activation with CAPE prior to L. major infection blocked IL-12 expression (10). To determine the necessity of NF-κB for L. major–induced IFN-β production and signaling, we pretreated human DCs with CAPE and assessed IFN-β (Fig. 8A) and IRF7 (Fig. 8B) expression 8 h postinfection. As with IL-12 expression, both IFN-β and IRF7 mRNA levels dropped drastically in L. major–infected DCs pretreated with CAPE compared with infected cells treated with the DMSO control.

Discussion

Resistance to intracellular Leishmania infections requires a robust cellular Th1 response that is largely driven by the ability of DCs to produce IL-12p70, the biologically active dimer, which is controlled at the level of IL-12p40 transcription. It has been well established that human DCs exhibit a spectrum of IL-12 responses depending on the Leishmania-infecting species, strain, and parasite life-cycle stage (5, 10). It is particularly interesting that L. major–infected human DCs exhibit an enhancement of IL-12p40 induction compared with other species. We therefore employed microarray-based transcript expression analysis on L. major–infected monocyte-derived DCs in an effort to identify genes and pathways that may be associated with this heightened response. Our objective was to gain insight into the transcriptional mechanisms that control the development of a protective immune response to L. major infections.

Several studies have used microarray technologies to study global gene expression profiles of host cells during Leishmania infection in both murine models (34–36) and human systems (9, 37–39). In this study, we build upon a previously published microarray dataset that explored the transcriptional profile of L. major–infected DCs, representing seven donors pooled together at a single time point of infection (16 h) (9), by analyzing the expression profiles of three additional independent donors across early infection time points (2, 4, 8, and 24 h). We demonstrate that individual donors’ gene-expression profiles exhibit similar spatial orientations for the individual infection time points, indicating that the donor–donor variability that often skew human data are not an issue for our particular data set (Figs. 2, left panel, 4). As ex-

Table I. Significantly enriched pathways for L. major–infected human DCs

<table>
<thead>
<tr>
<th>Pathway Name</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>Immune response_Antiviral actions of IFNs</td>
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<td>Immune response_IFN-α/β signaling pathway</td>
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<td>Oxidative phosphorylation</td>
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<td>Apoptosis and survival_TNFRII signaling pathway</td>
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<td>Immune response_EVT3 affect on CSF1-promoted macrophage differentiation</td>
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</tr>
<tr>
<td>Immune response_IL-1 signaling pathway</td>
<td>2.7E-04</td>
</tr>
<tr>
<td>Development_Angiotensin signaling via STATs</td>
<td>3.0E-04</td>
</tr>
<tr>
<td>Ubiquinone metabolism</td>
<td>3.1E-04</td>
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<tr>
<td>Immune response_Oncostatin M signaling via MAPK in mouse cells</td>
<td>5.0E-04</td>
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<tr>
<td>wtcFCTR and delta508-CFTR traffic/Generic schema (norm and CF)</td>
<td>6.1E-04</td>
</tr>
<tr>
<td>Immune response_Oncostatin M signaling via MAPK in human cells</td>
<td>6.8E-04</td>
</tr>
<tr>
<td>Transcription_Transcription regulation of amino acid metabolism</td>
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<tr>
<td>Development_Role of HDAC and calcium/ calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis</td>
<td>9.8E-04</td>
</tr>
</tbody>
</table>

Pathway analysis consists of matching gene identifications of possible targets for the “common,” “similar,” and “unique” sets with gene IDs in functional ontologies in MetaCore. The probability of a random intersection between a set of identifications the size of the target list with ontology entities is estimated in p values of hyper-geometric intersection. The lower p values mean higher relevance of the entity to the dataset, which shows in a higher rating for the entity.
neutralization of the type I IFN pathway blocks *L. major*–induced IL-12p40 transcription in DCs. DCs were cultured in the presence of a type I IFN–neutralizing decoy receptor, B18R, 1 h prior to *L. major* infection. The gene expression for IRF7 (n = 8) (A), TNF-α (n = 5) (B), IL-12p40 (n = 8) (C), IL-12p35 (n = 4) (D), and IL-23p19 (n = 4) (E) was assessed by qRT-PCR at 8 h postinfection. The ΔΔCt method was employed to determine the fold change of infection over uninfected samples. (F) Secreted IL-12p70 was assessed by ELISA (n = 3). *Statistical significance (p ≤ 0.05, Student t test).

One of the major observations from our study revealed that the type I IFN pathway is significantly enriched during *L. major* infection (Supplemental Fig. 1, Table I). It has been widely accepted that type I IFNs can exhibit a protective or deleterious immune effect on viral-infected host cells (17). Their potential immunomodulatory role during nonviral infections, including *Leishmania*, has also been reported (9, 30). A previous murine study reported that the host type I IFNs control *L. mexicana* infection by increasing early IFN-γ production in a STAT4-dependent manner (40). Another group demonstrated that *L. donovani* selectively recruits IRF7 to the parasite-containing phagosome mediating an NO-independent, antimicrobial effector function in resident murine splenic macrophages (41) and also established the importance of IRF7 in mounting an effective acquired cellular immune response to *L. donovani*–infected Kupffer cells (42). Upregulation of IFN-β transcripts has been observed during *L. guayensis* infection, but is attributed to the virus-containing parasite’s ability to induce response upon TLR3 receptor engagement (38). For *L. major* infection, transient neutralization of IFN-α/β prior to infection inhibits parasite metastasis to visceral organs in resistant mouse strains (43). Furthermore, prophylactic treatment with IFN-β reverses progressive disease and augments IL-12 production in susceptible mouse strains (44). Although these previous reports suggest a role for type I IFNs during murine *L. major* infection, a necessity for resistance has not been defined, and few studies have assessed these cytokines during human infections. We recently demonstrated that *L. major* induces IFN-β expression (4 h postinfection) in human DCs and that early production of this type I IFN may act in autocrine-dependent manner to subsequently drive IDO induction (31). In human macrophages, it has been reported that type I IFNs impair antileishmanicidal activity (45) through superoxide dismutase 1/RNA-dependent protein kinase signaling mechanisms that become activated downstream of TLR2 engagement (46). Our pathway analysis revealed the type I IFN pathway was significantly enriched during *L. major* infection, and we therefore evaluated the applicability of these findings by examining the role of this pathway in our DC model system. Inhibition of the type I pathway, via a neutralizing decoy receptor B18R, resulted in reduction of IL-12p40 mRNA levels (Fig. 5A) and secreted IL-12p70 production (Fig. 6F). Furthermore, human DCs infected with *L. donovani* exhibited relatively low levels of IFR2, IFR7, and IFIT5 (Fig. 7), altogether suggesting the type I IFNs are important regulators of the specific IL-12 induction observed in *L. major* infections. TNF-α expression was upregulated in the presence of B18R, suggesting that in contrast to IL-12 expression, type I IFNs may inhibit TNF-α signaling. However, the mechanism has yet to be defined. We demonstrated that type I signaling is necessary for *L. major* induction of both IL-12p40 and IL-23p19 (Fig. 6). Although the role of IL-12 in resistance to *Leishmania* infection is well established, the relevance of IL-23 is less clear. Although increased levels of IL-23 production from human peripheral blood macrophages are associated with healing forms of *L. major* infection (47), higher levels of IL-23 are present in *L. major*–infected susceptible BALB/c mice compared with resistant C57BL/6 mice (48). Furthermore, IL-23 may be a contributing factor for disease progression in human post-kala-azar dermal leishmaniasis caused by *L. donovani* (49). Although it has been long established that the type I IFNs have a regulatory role in IL-12 production, the mechanism by which *L. major* targets type I IFNs to enhance human IL-12 induction remains to be elucidated. It is possible that soluble IFN-α and IFN-β may be interacting with host cell-surface receptors in an autocrine fashion to augment IL-12 activities, as it has been established that type I IFNs affect IL-12p70 production via an autocrine feedback loop (50).

Interestingly, our significant gene set included several genes, *CITED2* and *MAP3K1*, for which the encoded proteins may participate in the signaling events, leading to IL-12p40 transcriptional activation. Our qRT-PCR analyses revealed that mRNA levels of *CITED2* and *MAP3K1* (Fig. 7A–C) were differentially expressed between *L. major* and *L. donovani* infections. *CITED2* has been characterized as a negative regulator of gene function. One study reported that *CITED2* competes with ETS1 for binding to p300,
ultimately dissociating ETS1 from the ETS genomic target docking sequence, part of the IL-12p40 gene promoter, consequentially repressing gene activation (27, 28, 51–54). In this study, we demonstrate that infection with L. major resulted in a marked decrease of CITED2, suggesting that downregulation of CITED2 may allow IL-12p40 transcription in response to L. major infection. Furthermore, we demonstrated that MAP3K1 expression increases over time in L. major–infected DCs compared with L. donovani infection. Although the role of MAP3 kinases remains controversial, it is possible that during L. major infection, MAP3K1 could participate in the induction of type I IFNs and subsequent enhancement of IL-12 activity.

We previously demonstrated that in the presence of an NF-κB inhibitor (CAPE), a reduction of mRNA levels was observed for IL-12, which allows us to conclude that NF-κB family members control gene activation during L. major infection in human DCs (10). In this study, we demonstrate that NF-κB signaling also is necessary for IFN-β production in response to L. major infection, suggesting that the lack of IL-12 gene activation in our previous study may have been due, in part, to the lack of IFN-β–mediated signaling.

Altogether, we present a global genome-wide perspective on the effects of L. major species in human DCs during an early time course of infection. This body of work also provides a platform for further investigations toward functionally characterizing candidate

![FIGURE 7. L. major and L. donovani differentially modulate the expression of IL-12p40 and the type I IFN genes. DCs were infected with either L. major or L. donovani parasites and analyzed for gene expression at 4, 8, and 24 h by qRT-PCR. L. major induced the expression of IL-12p40 (n = 19) (A), IFN-α (n = 7) (B), IFN-β (n = 8) (C), IRF2 (n = 5) (D), IRF7 (n = 5) (E), and IFIT5 (n = 5) (F), which was assessed in contrast to L. donovani infection. The ΔΔCt method was employed to determine the fold change of infection over uninfected samples. Mean values of individual donors ± SEM are presented. Aliquots from L. major–or L. donovani–infected DCs were prepared for Diff-Quick analysis (Fisher Scientific). For each infection time point (4, 8, and 24 h), the percentage of infected cells (% DC infected) (G) and the parasite index (# parasites/100 cells) (H) are presented. Mean values of individual donors ± SEM are presented. *Statistical significance between L. major and L. donovani infections (p ≤ 0.05, two-way ANOVA, Bonferroni multiple comparisons posttest).](http://www.jimmunol.org/)

![FIGURE 8. Inhibition of NF-κB blocks type I IFN signaling in response to L. major–infection. DCs were pretreated with 52 μM CAPE for 1 h prior to infection with L. major. Gene expression of IFN-β (A) and IRF7 (B) was assessed by qRT-PCR at 8 h postinfection. The ΔΔCt method was employed to determine the fold change of infection over uninfected samples. *Statistical significance (p ≤ 0.05, Student t test; n = 3).](http://www.jimmunol.org/)
genes that may be important for regulating the induced IL-12 transcriptional response characteristic of L. major infections. We contribute to the growing body of literature regarding the influence of type I IFNs on Leishmania infectivity by highlighting the potential involvement of the type I IFNs in augmenting the L. major-induced IL-12 transcription in DCs. Also, we have identified two candidates, CITED2 and MAP3K1, that may be targeted during infection and could potentially influence the variation in IL-12 activities exhibited depending on the infecting species. Further investigations exploring the function of these genes and their association with IL-12 regulation are currently underway. Ultimately, the data provide important insights for the factors that regulate early establishment of L. major infection.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. The Type I IFN pathway is significantly expressed in L. major infected DCs. Expression data was processed by the Metacore software suite system yielding enriched pathways. One representative pathway, the IFN alpha/beta pathway map is displayed (top). This pathway map was created by Thomson Reuters scientists using high quality manual curation process based on published peer-reviewed literature. Thermometer symbols indicate relative expression levels of genes that encode the protein within the pathway. A legend for representative symbols is provided (bottom).
### Supplemental Table 1. Human Primer Sequences for qRT-PCR analysis

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