Down-regulation of 7SL RNA Expression and Impairment of Vesicular Protein Transport Pathways by Leishmania Infection of Macrophages*

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The parasitic protozoan Leishmania specifically manipulates the expression of host macrophage genes during initial interactions, as revealed by mRNA differential display reverse transcription-PCR and cDNA microarray analyses. The genes that are down-regulated in mouse (J774G8) or human (U937) macrophages upon exposure to Leishmania include small RNA transcripts from the short interspersed element sequences. Among the short interspersed element RNAs that are down-regulated is 7SL RNA, which is the RNA component of the signal recognition particle. Because the microbiciphal functions of macrophages profoundly count on vesicular protein transport processes, down-regulation of 7SL RNA may be significant in the establishment of infection by Leishmania in macrophage phagolysosomes. To evaluate whether down-regulation of 7SL RNA results in inhibition of signal recognition particle-mediated vesicular protein transport processes, we have tested and found that the targeting of proteins to the endoplasmic reticulum and plasma membrane and the secretion of proteins by macrophages are compromised in Leishmania-infected J774G8 and U937 cells. Knocking down 7SL RNA using small interfering RNA mimicked the effect of exposure of macrophages to Leishmania. The overexpression of 7SL RNA in J774G8 or U937 cells made these cells resistant to Leishmania infection, suggesting the possible biological significance of down-regulation of 7SL RNA synthesis in the establishment of infection by Leishmania. We conclude that Leishmania down-regulates 7SL RNA in macrophages to manipulate the targeting of many proteins that use the vesicular transport pathway and thus favors its successful establishment of infection in macrophages.

Pathogenic microbes often develop strategies to evade host immune responses, learn to adapt to the host environment, and/or manipulate the host system to make it more hospitable for the establishment of infection and propagation (1–3). Any microbe interacting with the mammalian immune system is subjected to ultimate phagocytosis by blood mononuclear phagocytes or tissue macrophages, and it is then cytolyzed to death inside macrophage phagolysosomes (2–4). The parasitic protozoan Leishmania has the uncanny ability to evade the immune reactions of macrophages and is able to establish infection inside macrophage phagolysosomes and to propagate inside tissue macrophages (1–3). To survive inside the macrophage and to escape immunity, Leishmania has developed mechanisms that deactivate macrophage immune functions, including inhibition of the respiratory burst and interleukin-12 and nitric oxide synthesis and down-regulation of major histocompatibility complex class II molecules as well as promotion of the synthesis of inhibitory cytokines such as transforming growth factor-β and interleukin-10 and induction of the suppressor of cytokine signaling (5, 6). Cytokines and especially γ-interferon are essential contributors to macrophage activation to promote the effective killing of parasites (5, 6).

Many of the functions carried out by macrophages, including destruction of intracellular parasites such as Leishmania, are enhanced by their activation by extracellular factors. Proper targeting of the receptor molecules specific for those activating factors on the cell surface is thus very important for the optimal function of macrophages. We hypothesize that, in the early stages of infection, Leishmania manipulates the expression of cell-surface receptors of the macrophages, making the cells unable to respond to their external stimuli.

It has become increasingly apparent that, in addition to their phagocytic and immunomodulatory properties, macrophages have an extensive secretory capability that includes secretion of protein molecules such as lysozymes, lysosomal acid hydrolases, neutral proteases, lipases, arginase, protease inhibitors, phospholipase inhibitors, complement factors, blood coagulation factors, and several cytokines (7, 8). Macrophage secretion involves synthesis on the rough endoplasmic reticulum (ER),1 cotranslational glycosylation and translocation into the lumen of the ER, transport to the Golgi, and vesicular transport to the plasma membrane. The fusion of the secretory vesicle membrane and plasma membrane then occurs with the re-establishment of the membrane bilayer structure, thereby maintaining the integrity of the cell while expelling vesicle contents to the external milieu (9). The secretion of many cytokines by macrophages is of particular importance because those cytokines interact with macrophages and T- or B-cells and assist in the control and fine-tuning of the immune response. Our proposal is that Leishmania successfully establishes infection in macrophages by down-regulating the biosynthesis of 7SL RNA, which in turn results in inhibition of protein targeting into the ER

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1 The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition particle; DD-RT, differential display reverse transcription; siRNA, small interfering RNA; ECFP, enhanced cyan fluorescent protein; MBSA, maleylated bovine serum albumin; BSA, bovine serum albumin.

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lumen and plasma membrane and secretion of protein molecules by macroprophages. The signal recognition particle (SRP) is a ribonucleoprotein machine that delivers certain nascent polypeptides to specific recognition components on the cytoplasmic face of the ER membrane for translocation of secretory or membrane proteins (10, 11). The RNA component of the SRP (7SL RNA) contains two elements related to the human and rodent Alu families of interspersed repetitive DNA sequences connected by a unique sequence, the S domain (11). 7SL RNA associates with six proteins in mammalian cells termed SRP72, SRP68, SRP54, SRP19, SRP14, and SRP9 (11).

All proteins destined for the secretory pathway must first be targeted to the ER. In mammalian cells, this targeting reaction primarily occurs cotranslationally via the SRP pathway. Proteins targeted to the cell membrane for either secretion or integration typically have an N-terminal signal peptide that directs them to their destinations. As the nascent polypeptide chain exits the ribosome, it is recognized and bound by the SRP. The SRP-ribosome complex then binds to the SRP receptor, which resides in the target membrane. Once bound, the paused translation machinery docks with a protein-translocating pore, or channel, and the SRP releases the signal peptide (10, 11). Translation resumes with the growing peptide integrating into or passing through the membrane. The SRP pathway is essential in all organisms examined to date except the yeast Saccharomyces cerevisiae (12).

To substantiate our hypothesis that Leishmania actively manipulates macroprophages in the initial phases of its establishment of infection, we studied the differential expression of genes in cultured macroprophages exposed to virulent Leishmania promastigotes (the insect form of the parasite) compared with unexposed macroprophages. Exposure of cultured mouse macrophages to virulent Leishmania promastigotes for 2–6 h caused significant decreases in the levels of small RNA transcripts from short interspersed element sequences in the cells (13). We report here the data from our differential display reverse transcription (DD-RT-PCR) and cDNA microarray analyses showing the differential expression of specific abundant mRNAs in Leishmania-exposed macroprophages. We hypothesize that the altered expression of these genes may lead to the non-apoptotic growth arrest of host cells favoring the parasitism of Leishmania. We further characterize the down-regulation of one of the short interspersed element RNAs, 7SL RNA, in Leishmania-exposed macroporphages and present evidence that this down-regulation leads to inhibition of vesicular protein transport in macroporphages, perhaps favoring leishmanial parasitism in the phagolysosomes of these cells. We also show that the overexpression of 7SL RNA in macroporphages confers resistance to the cells against Leishmania infection, at least in vitro.

MATERIALS AND METHODS

Leishmania, Trypanosoma, and Macrophages—The promastigotes and amastigotes of Leishmania amazonensis (LV78), Leishmania major (Friedlin), and Leishmania donovani (DD8) were obtained from Dr. Minu Chaudhuri (Department of Microbiology, Meharry Medical College). We used the mouse macrophage cell line J774G8 and the human monocytic leukemia cell line U937 in our study. A mouse promastigote cell line J774G8 and the human monocytic leukemia cell line U937 in our study. These cells were grown in tissue culture flasks in RPMI 1640 medium with 20% heat-inactivated (56 °C, 30 min) fetal bovine serum at 37 °C as described (13, 14). U937 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (10 ng/ml) for 24 h as described (17). The macrophages were incubated with the parasite cells at a macrophage:parasite ratio of 1:10 at 37 °C for 2–6 h for exposure experiments (13–15, 18).

DD-RT-PCR and Microarray Analyses—Comparative gene expression analyses of unexposed and virulent L. amazonensis-exposed J774G8 cells were done by DD-RT-PCR as well as mouse cDNA microarray analyses similar to those described previously (13, 19, 20). Total RNA was isolated from subconfluent cultured cells using TRIzol reagent (Invitrogen). For isolation of RNA from unexposed macroporphages, we added an equivalent number of parasite cells after addition of TRIzol to the macroporphages so that the contribution of Leishmania RNA, if any, in the subsequent microarray hybridization was countered. The RNA quality was checked by formaldehyde-agarose gel electrophoresis. DD-RT-PCR analysis was performed as described previously (13). For cDNA microarray analysis, total RNAs (40 μg) from exposed and unexposed cells were labeled in reverse transcription reactions (SuperScript II kit, Invitrogen) with Cy5-labeled dCTP and Cy3-labeled dCTP (Amersham Biosciences), respectively (19, 20). Four microarrays were used for this study. In every second replicate experiment, the fluorescent deoxyribonucleotides were swapped. Purified cDNA probes labeled with Cy3 and Cy5 were mixed per pair and hybridized to mouse cDNA microarray chips (Mouse Research Genetics, Inc., Providence, RI) with Affymetrix Genechip Microarray Shared Resource. The slides were scanned with a GenePix 4000A microarray scanner (Axon Instruments, Inc., Foster City, CA), and the images were analyzed using GenePix Pro 3 software. A uniform scale factor was applied to normalized signal intensities between Cy5 and Cy3. Flagged spots and spots with an average intensity below 2.5-fold above the background were not retained for further analysis. The log2(Cy5/Cy3) ratio of the other spots was calculated for each slide. To compare the results from the different samples, data from each slide were normalized in log space to have a mean of 0 and an S.D. of 1 by using the Cluster program (21). Genes with significant changes in mRNA levels were identified using the SAM (significant analysis of microarrays) procedure (22), a validated statistical technique for identifying differentially expressed genes across high density microarrays. This procedure provides a list of “significant” genes and an estimate of the false discovery rate, which represents the percentage of genes that could be identified by chance (22).

Primer Extension and Nuclear Run-on Analyses—The levels of 7SL RNA in the control and parasite-exposed macroporphages were determined by primer extension analysis using avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) according to the protocol supplied by the manufacturer. β-Actin mRNA levels were evaluated as a normalization control. The primers used were as follows: 7SL RNA, 5′-ATGCCGAACCTTAGTGCCG-3′; and β-actin, 5′-TACACGTAAGC TGTCTACAGCA-3′. The primers were end-labeled with 10-32P-ATP and T4 polynucleotide kinase (23). Nuclear run-on analysis was performed as described (23).

PCR Evaluation of 7SL RNA Levels—Total RNA was isolated from cells and analyzed for 7SL RNA or β-actin by RT-PCR analysis (Amersham Biosciences). β-Actin was used as a loading control. Total RNAs (5 μg) were treated with DNase I (Promega Corp.) and reverse-transcribed with SuperScript II and random hexamers as primers. The resulting first-strand cDNAs were used as templates to amplify 7SL RNA (157 bp; 5′-GGAGTCTCGGCGCTGTAATGTC-3′ and 5′-ATCCG CGGGAATTTTGGAC-3′) and β-actin (353 bp, 5′-GCCTCTGCTGCA CAACGCGTCT-3′ and 5′-CAACATCTGTGGTGATCCTCTC-3′).

Knockdown of 7SL RNA Gene Expression—The small interfering RNAs (siRNAs) for human 7SL RNA were designed based on the nucleotide sequence (GenBank™ accession number X04248) using the software available at the Invitrogen web site (maidenewriter.invitrogen.com/sirna) and custom-synthesized by Invitrogen. The nucleotide sequences of the siRNA pair (SR) and its respective control (SRC) are as follows (the number indicates the location of the sequence in 7SL RNA; see Ref: 24): SR110, 5′-UCCGCAGAUUGUCCGCCAU-3′/5′-UGGC CGAUCUUAGGGCGCA-3′; and SRC110, 5′-UCCCAUAGUUG CGGCCCAU-3′/5′-AUGGCCGAACUUAAUGUGGA-3′. We used Trans- Messenger reagent and protocols (Qiagen Inc.) for the transfection of siRNAs. The siRNAs were purified from the siRNA-treated cells using TRizol reagent and treated with DNase I before RT-PCR analysis (19, 20).

Overexpression of 7SL RNA in Cultured Macrophages—The expression of 7SL RNA in cultured macrophages was assessed by RT-PCR analyses (13–15, 18). The small interfering RNAs (siRNAs) for human 7SL RNA were designed based on the nucleotide sequence (GenBank™ accession number X04248) using the software available at the Invitrogen web site (maidenewriter.invitrogen.com/sirna) and custom-synthesized by Invitrogen. The nucleotide sequences of the siRNA pair (SR) and its respective control (SRC) are as follows (the number indicates the location of the sequence in 7SL RNA; see Ref: 24): SR110, 5′-UCCGCAGAUUGUCCGCCAU-3′/5′-UGGC CGAUCUUAGGGCGCA-3′; and SRC110, 5′-UCCCAUAGUUG CGGCCCAU-3′/5′-AUGGCCGAACUUAAUGUGGA-3′. We used Trans- Messenger reagent and protocols (Qiagen Inc.) for the transfection of siRNAs. The siRNAs were purified from the siRNA-treated cells using TRizol reagent and treated with DNase I before RT-PCR analysis (19, 20).
Manipulation of Macrophage Biology by Leishmania

GGAAGGTTAGACCGGCGGCTCGGATT
gene product in plasmid pSUPER (19) at the BglII/HindIII site to obtain plasmid pSUPER-75L. We mutated the 7SL RNA gene in pSUPER-75L simultaneously (G193A, G194A, and G198A) to create a nonfunctional 7SL RNA. These Gly residues are highly conserved across the genera and are essential for the function of 7SL RNA (25, 26). We used the QuikChange site-directed mutagenesis reagents and protocols (Stratagene, La Jolla, CA) (27) to accomplish the mutagenesis. Mutation was verified by nucleotide sequencing. We named the mutated plasmid pSUPER-75Ls, pSUPER-75Lr, or pSUPER-75L+1 and pcDNA3.0 were cotransfected at a ratio of 10:1 into cultured macrophages using Lipofectamine 2000 (Invitrogen) as described (27). Control cells were transfected only with pcDNA3.0, which contains the G418 resistance gene. Recombinant cells were selected with G418 (200 μg/ml). To overexpress 7SL RNA in cells that express either enhanced cyan fluorescent protein (ECFP) or secreted alkaline phosphatase and that are resistant to G418, we cotransfected them with pSUPER-75L or pSUPER-75Ls and pTK-Hyg (a selection vector that confers hygromycin resistance; Clontech) and selected for cells that were resistant to both G418 (200 μg/ml) and hygromycin (200 μg/ml). Control cells were transfected only with pTK-Hyg. The overexpression of 7SL RNA in the transfected cells was evaluated by RT-PCR as described above.

Evaluation of Protein Targeting to the Plasma Membrane—To evaluate the effect of Leishmania- or siRNA-induced knockdown of 7SL RNA in macrophages on the targeting of proteins to the ER, we employed a commercially available plasmid construct (Clontech) designed to test this cellular process. The Living Color ER targeting vector pECFP-ER (Clontech) encodes a fusion protein consisting of ECFP, the ER targeting sequence (KDEL) cloned at the 3′-end. ECFP-ER is a soluble protein that localizes in the lumen of the ER in transfected cells. We stably transfected macrophages with the pECFP-ER plasmid. Cultured J774G8 or undifferentiated U937 cells (6-well tissue culture plates) were transfected with the pECFP-ER plasmid (1 μg) using Lipofectamine 2000 (27). Stable transfectants were obtained after selection with G418 (200 μg/ml). U937 cells were differentiated before exposure to virus, while J774G8 cells described above were cultured without parasite cells in antibiotic-free medium at a macrophage/parasite ratio of 1:10 continuously for 5 days. 100 macrophages without parasite cells in antibiotic-free medium at the end of the incubation, the cell monolayer was washed twice with phosphate-buffered saline and then twice with 1 ml of RPMI 1640 medium containing 20% FBS, 50 μg/ml gentamicin, and 200 μg/ml G418 (200 μg/ml). Transfected cells were selected with G418 (200 μg/ml). Secreted alkaline phosphatase activity in the growth medium and cell extracts was quantitated by the Quantigreen fluorescence assay (Promega). We employed DD-RT-PCR and cDNA microarray techniques to evaluate whether, at the early stage of exposure to host cells, Leishmania induces differential gene expression in mouse macrophage cell line J774G8. Our data suggest that indeed the exposure of macrophages to Leishmania induces the up- and down-regulation of many genes (Table I). These inductions are reproducible and specific, as these changes were not revealed when J774G8 cells were exposed to avirulent promastigotes of L. amazonensis or to T. brucei procyclines (data not shown). We positively identified and validated 22 differentially regulated genes in virulent L. amazonensis-exposed J774G8 cells by independent RNA evaluation techniques such as RNase protection assays and real-time and end-point RT-PCR (Table I). The majority of these genes were detected by both DD-RT-PCR and cDNA microarray analysis (Table I). Some of them were detected either by cDNA microarray analysis or by DD-RT-PCR analysis (Table I). Details of the evaluations of all these differentially expressed genes and the significance of their differential expression individually or as a group in leishmanial parasitism of macrophages are under study. We report here the significance of the down-regulation of 7SL RNA in macrophages by Leishmania infection.

The genes that were significantly down-regulated in Leishmania-exposed J774G8 cells include DNA primase p49 (catalytic subunit), replication protein A p14, proliferating cell nuclear antigen, p160 c-Myb-binding protein, RAD50, cycdin-dependent kinase regulatory protein-1, p70 S6 kinase, cycdin-dependent kinase-2, and a protein of the replication origin recognition complex (Table I). Up-regulated genes include histone H1.1 and protein phosphatase 1A. Apoptotic marker genes that were down-regulated include Pdcd8 (programmed cell death 8; apoptosis-inducing factor) and mNapor (mouse neuroblastoma apoptosis-related RNA-binding protein; an apoptosis-inducing ELAV-type RNA-binding protein) (Table I). The
profiling may indicate non-apoptotic growth arrest of the Leishmania-exposed J774G8 cells at the non-dividing stage.

**Significant Inhibition of the Expression of 7SL RNA in Leishmania-exposed Macrophages**—An important observation we have made during our previous differential gene expression analysis is the down-regulation of the short interspersed element RNAs induced in J774G8 cells by short exposure (2–6 h) to *Leishmania* (13). One of the short interspersed element RNAs in mammals is 7SL RNA, which is the RNA component of the SRP (10, 11). The nucleotide sequence of 7SL RNA is highly conserved between mouse and human. Thus, we were able to use the same primers for primer extension and RT-PCR analyses of 7SL RNA in both J774G8 and U937 cells. We found that 7SL RNA was down-regulated in mouse as well as human macrophages during exposure to virulent *Leishmania* promastigotes (Fig. 1A). U937 cells were differentiated to macrophages before exposure to virulent *L. amazonensis* promastigotes. The down-regulation of 7SL RNA in J774G8 cells by virulent *L. amazonensis* promastigotes further reduced the levels of 7SL RNA when compared to the levels in control untransfected cells.

**TABLE 1**

| Regulated gene                  | Change (increase or decrease) |
|---------------------------------|------------------------------|
| Up-regulated                    |                              |
| Histone H1.1*                   | 10–11                        |
| Protein phosphatase 1A*         | 3–5                          |
| Transcription elongation factor SII* | 3–4                     |
| Branched-chain ketoacid dehydrogenase | 5–6                  |
| E1, α-polypeptide*              | 5–6                          |
| Syndecan-3 precursor protein*   | 3–4                          |
| Fibrillin-2*                    | 6–7                          |
| Down-regulated                  |                              |
| DNA primase p49*                | 3–5                          |
| Replication protein A p14*      | 4–5                          |
| Proliferating cell nuclear antigen* | 8–11                |
| p160 c-Myb-binding protein*     | 4–6                          |
| RAD50*                          | 3–5                          |
| Cdk regulatory protein-1*       | 4–5                          |
| p70 S6 kinase*                  | 5–7                          |
| Cdk2*                           | 7–8                          |
| Replication origin recognition protein* | 5–6                |
| Pdc8 (AIF)*                     | 4–5                          |
| mNapor*                         | 4–5                          |
| Glycine transporter-1*          | 6–7                          |
| Ceruloplasmin*                  | 6–8                          |
| T-complex protein-1, ζ-subunit* | 6–8                          |
| Glvr1 mRNA*                     | 6–7                          |
| 7SL RNA*                        | 15–18                        |

* Detected by both microarray and DD-RT-PCR analyses.  
* Detected only by cDNA microarray analysis.  
* Detected only by DD-RT-PCR analysis.

**A. Primer Extension Analysis**

**B. Nuclear Run-On Analysis**

**C. RT-PCR with RNA from J774G8 cells**

FIG. 1. Evaluation of the levels of 7SL RNA in unexposed and *Leishmania*-exposed macrophages. **A**. primer extension analysis of 7SL RNA in J774G8 and differentiated U937 cells that were either unexposed or exposed to virulent *L. amazonensis* promastigotes for 2 h before RNA isolation. β-Actin was used as a loading control. **B**, nuclear run-on analysis of 7SL RNA in J774G8 cells that were either unexposed or exposed to virulent *L. amazonensis* promastigotes, showing apparent transcriptional regulation of the 7SL RNA gene in *Leishmania*-exposed macrophages. β-Actin was used as a control. **C**, RT-PCR analysis of the levels of 7SL RNA in J774G8 cells that were unexposed or exposed for 2 h to virulent *L. amazonensis*, *L. major*, or *L. donovani* promastigotes or to avirulent *L. amazonensis* (La) promastigotes. β-Actin was used as a normalization control.

**Mouse genes up- or down-regulated in J774G8 cells after 2 h of exposure to virulent *L. amazonensis* promastigotes**

| CDK, cyclin-dependent kinase; AIF, apoptosis-inducing factor. |

**7SL RNA Is Knocked Down or Overexpressed in Macrophages**

**In Attempts to Mimic or Alleviate, Respectively, the Action of Leishmania**—To mimic the action of *Leishmania* in knocking down the 7SL RNA levels, we treated macrophages (J774G8 and differentiated U937 cells) with siRNA specific to 7SL RNA. Compared with the cells treated with control siRNA containing several mismatches, the siRNA specific to 7SL RNA was able to ablate the expression of this RNA in J774G8 cells (Fig. 2A). Similar results were obtained with differentiated U937 cells (data not shown). To evaluate whether the consequences of leishmanial knockdown of 7SL RNA may be alleviated by the overexpression of 7SL RNA in macrophages, we developed stable J774G8 and undifferentiated U937 cell lines in which 7SL RNA was overexpressed from the H1 RNA promoter. The RT-PCR data indicated that J774G8 cells that were stably cotransfected with pSUPER-7SL and pcDNA3.0 had 2–3-fold higher 7SL RNA levels than the cells that were not transfected or those that were transfected with pcDNA3.0 alone (Fig. 2B). We obtained similar results with undifferentiated U937 cells (data not shown). To overexpress a 7SL RNA that is not functional, we made three point mutations in the 7SL RNA gene in pSUPER-7SL and stably transfected the macrophage cell lines with this mutated plasmid along with pcDNA3.0. RT-PCR analysis suggested that mutated 7SL RNA was expressed in the stably transfected cells in an amount comparable with that in the cells transfected with the wild-type construct (Fig. 2B). Exposure of the 7SL siRNA-treated J774G8 cells to virulent *L. amazonensis* promastigotes for 2 h further reduced the levels of 7SL RNA (Fig. 2C). On the other hand, exposure of macrophages overexpressing 7SL RNA from the H1 RNA promoter to virulent *L. amazonensis* promastigotes for 2 h decreased only endogenous 7SL RNA synthesis, and the 7SL RNA levels remained at least similar to those in the control untransfected cells (Fig. 2D).

**Leishmania-induced Down-regulation of 7SL RNA Impairs the Targeting of Proteins to the ER in Macrophages**—To under-
stand the consequences of the down-regulation of 7SL RNA in J774G8 cells by *Leishmania*, we tested the targeting of proteins to the ER. The pECFP-ER plasmid encodes a fusion protein consisting of ECFP containing the ER targeting sequence of calreticulin. J774G8 cells permanently expressing this protein were developed. These cells were exposed to virulent *L. amazonensis* promastigotes for 4 h. The expression of ECFP in the ER was monitored by fluorescence microscopy. *Leishmania* exposure appeared to inhibit (by 50–80%) the ER targeting of ECFP to the ER (Fig. 3, a/H11032 and b/H11032). Knockdown of 7SL RNA with a specific siRNA in the ECFP-ER-expressing cells similarly inhibited the expression of ECFP fluorescence (Fig. 3c/H11032).

On the other hand, the overexpression of 7SL RNA in these cells alleviated the effect of *Leishmania* exposure (Fig. 3d/H11032). The alleviating effect of overexpressed 7SL RNA depends upon the functionality of this RNA, as the overexpression of mutated 7SL RNA instead failed to alleviate the effect of *Leishmania* exposure on the targeting of ECFP to the ER (data not shown). The disappearance of ECFP in the *Leishmania*-exposed cells is probably due to the proteasomal degradation of the untargeted protein, as pretreatment of the ECFP-ER-expressing J774G8 cells with the proteasome inhibitor MG132 (1 μM) for 24 h before *Leishmania* exposure prevented the disappearance of the fluorescence (data not shown).

*Leishmania*-induced Down-regulation of 7SL RNA Decreases the Levels of Receptor Protein Molecules on the Macrophage Cell Surface—One of the major roles of the SRP is to help in the targeting of receptor proteins to the plasma membrane. To understand the effect on the level of a receptor on the cell surface, *Leishmania*-exposed (4 h) and unexposed J774G8 cells were assayed for 125I-MBSA binding to the scavenger receptors at 4 °C for 5 h with different concentrations of the ligand. The levels of scavenger receptor AII were reduced by 2–3-fold on the surface of *Leishmania*-exposed J774G8 cells (Fig. 4A). A similar down-regulation of the scavenger receptor concentration was observed when J774G8 cells were treated with 7SL siRNA (as in C) or between cells with no overexpression or with either wild-type (Wt) or mutated (as in D) 7SL RNA overexpression were statistically significant (p < 0.001).
scavenger receptor levels in J774G8 cells or differentiated U937 cells (data not shown).

_Leishmania_-induced Knockdown of 7SL RNA Inhibits the Secretion of Proteins by Macrophages—Another consequence of the down-regulation of 7SL RNA expression should be the impairment of the secretion of protein molecules by macrophages. To evaluate the effect of _Leishmania_-induced reduction of 7SL RNA levels on the secretion of proteins by J774G8 cells, we stably transfected J774G8 cells with the pSEAP2-Control plasmid. These cells were exposed for 4 h to virulent _L. amazonensis_ promastigotes, and the levels of thermostable alkaline phosphatase (coded by the plasmid) secreted in the growth medium were assayed and compared with those in the uninfected cells using a fluorescent substrate (Clontech). Alkaline phosphatase secretion was inhibited by 60–85% upon _Leishmania_ exposure (Fig. 5A). On the other hand, exposure of the transfected cells to _T. brucei_ procyclics did result in any significant change in alkaline phosphatase secretion (Fig. 5A). Inhibition of alkaline phosphatase secretion occurred only when the macrophages were exposed to virulent promastigotes of _Leishmania_, but not when the corresponding avirulent stocks were used (Fig. 5A). The degree of inhibition of alkaline phosphate secretion depended upon the time of exposure to _Leishmania_ promastigotes (Fig. 5B) and on the macrophage/parasite ratio (Fig. 5C). Knockdown of 7SL RNA by siRNA also decreased alkaline phosphatase secretion, whereas mismatched control siRNA had no effect (Fig. 5D). _Leishmania_-induced inhibition of alkaline phosphatase secretion by J774G8 cells was alleviated in cells overexpressing wild-type 7SL RNA, but not in those expressing mutated 7SL RNA (Fig. 5E). These experiments were repeated with U937 cells, and we obtained similar results (data not shown).

siRNA-mediated Knockdown of 7SL RNA in Macrophages Transiently Helps Avirulent _Leishmania_ Promastigotes to Establish Infection—Avirulent _Leishmania_ promastigotes could not down-regulate the expression of 7SL RNA promastigotes or to _T. brucei_ procyclics; B, effect of siRNA-induced knockdown of 7SL RNA without any exposure to the _Leishmania_ promastigotes; C, effect of the overexpression of wild-type (Wt) or mutated (Mt) 7SL RNA, followed by a 4-h exposure to virulent _L. amazonensis_ promastigotes. The _B_∞ for the binding of 125I-MBSA to the scavenger receptor on the cell surface was determined by Scatchard analysis.
A. Effect of exposure to different parasite cells

[Graph showing inhibition of alkaline phosphatase activity from J774G8 cells exposed to different parasite cells.]

B. Effect of the length of exposure

[Graph showing the effect of the length of exposure on alkaline phosphatase activity.]

C. Effect of the parasite/macrophage ratio

[Graph showing the effect of the parasite/macrophage ratio on alkaline phosphatase activity.]

D. Effect of 7SL siRNA

[Graph showing the effect of 7SL siRNA on alkaline phosphatase activity.]

E. Effect of parasite exposure on 7SL RNA overexpressed cells

[Graph showing the effect of parasite exposure on 7SL RNA overexpressed cells.]

Fig. 5. Inhibition of the secretion of alkaline phosphatase from J774G8 cells exposed for 4 h to virulent \textit{L. amazonensis} promastigotes. \textit{A}, effect of exposure to different parasite cells. Stably transfected J774G8 cells were exposed to equivalent numbers of avirulent, virulent (\textit{vLam}), and heat-killed (100 °C for 10 min) virulent \textit{L. amazonensis} (\textit{Lam}) promastigotes or \textit{T. brucei} procyclics for 4 h before alkaline phosphatase assay with the cell-free supernatant. \textit{B}, effect of the length of exposure of recombinant J774G8 cells to avirulent or virulent \textit{L. amazonensis} promastigotes on the secretion of alkaline phosphatase. \textit{C}, effect of the number of avirulent or virulent \textit{L. amazonensis} promastigotes/recombinant J774G8 cell on the secretion of alkaline phosphatase. \textit{D}, effect of knockdown of 7SL RNA by siRNA on the secretion of recombinant alkaline phosphatase. \textit{E}, effect of exposure of recombinant J774G8 cells overexpressing either wild-type or mutated 7SL RNA to virulent \textit{L. amazonensis} promastigotes on the secretion of alkaline phosphatase. Data are expressed as a percent of the data obtained with unexposed or untreated J774G8 cells. The results are the means ± S.E. (\(n=12\)). The differences in the levels of alkaline phosphatase activities between virulent and avirulent \textit{L. amazonensis}-exposed cells (\textit{A}), between 7SL siRNA-treated and control siRNA-treated cells (\textit{D}), and between wild-type and mutated 7SL RNA-overexpressing cells (\textit{E}) were statistically significant (\(p < 0.001\)).
promastigotes (Fig. 6, A and B). The establishment of infection by the avirulent promastigotes was indicted by the increased number of infected macrophages in the population (Fig. 6, A and B). On the other hand, their inability to grow in the macrophages was indicated by the lower number of amastigotes/100 macrophages (Fig. 6, A and B). These data thus indicate that transient ablation of 7SL RNA expression may be beneficial for the establishment of infection by Leishmania promastigotes, but the ability of the transformed amastigotes to multiply inside the phagolysosomes of the infected macrophages may be determined by other phenotypes of the parasite.

DISCUSSION

The molecular events involved in the parasite-host interactions between Leishmania and macrophages have captivated scientists for the last half-century. The mechanism of the successful establishment of infection by Leishmania in phagolysosomes of human or rodent macrophages is far from clear. It is apparent from the studies of several scientists that Leishmania takes an active part in manipulating the cell biology of the host.
macrophages to make them more hospitable to infection (32). The results from this study show that virulent *Leishmania* promastigotes indeed specifically altered the transcriptome profile of the macrophages at the early stages of their encounter with the host cells. The ability of the promastigotes to manipulate macrophage gene expression in such a way may determine whether they will be able to establish infection in the host cells. The reproducible changes in the transcriptome profile induced by short (2 h) exposure to virulent *Leishmania* promastigotes indicated non-apoptotic growth arrest of the cells at the non-dividing stage. Detailed analysis of the individual regulated genes is needed before we will be able to precisely determine their roles in the *Leishmania*-macrophage interactions. We do not know at present the molecule(s) in virulent *Leishmania* promastigotes responsible for induction of the changes in the transcriptome of the macrophages. Abundant parasite surface molecules, such as the major surface proteinase gp63 and lipophosphoglycan (33), did not individually bring about these changes. It is possible that a combinatorial display of several molecules on the parasite surface and their simultaneous and/or sequential/progressive interactions with the macrophage surface molecules may bring about these changes.

We are intrigued by the significant decrease in the level of 7SL RNA in macrophages exposed to the virulent (but not avirulent) *Leishmania* promastigotes. The mechanism of this regulation remains elusive at present. We know from our nuclear run-on experiment that the regulation is at the transcriptional level. The 7SL RNA gene is transcribed by RNA polymerase III (34–36). Our preliminary experiments suggested that the gene internal promoters of RNA polymerase III, such as that of the tRNAs, and the gene external promoters of RNA polymerase III, such as that of the H1 RNA, are not affected by *Leishmania* exposure. The mammalian 7SL RNA gene has outside as well as inside transcriptional elements (34–36). Because *Leishmania* specifically inhibits transcription of the 7SL RNA gene, it may not inhibit RNA polymerase III transcription in general. The specific molecular target in this RNA polymerase III transcription pathway, which is manipulated by exposure of macrophages to *Leishmania* to down-regulate the 7SL RNA gene promoter, has yet to be identified.

The SRP is a soluble ribonucleoprotein complex that was originally identified as an important intermediary in the transport of proteins into the secretory pathway in mammalian cells (37).
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During translation, the 54-kDa polypeptide subunit of SRP (SRP54) binds to hydrophobic targeting signals that are found in both the presecretory and integral membrane proteins (38). The targeting signals are generally either N-terminal signal sequences (39) or, in the case of many membrane proteins that lack discrete signal peptides, the first transmembrane segment (40). Subsequently, the SRP targets the ribosome-nascent chain complexes to the ER, where an interaction between SRP54 and a heterodimeric SRP receptor catalyzes the release of the nascent polypeptides and their insertion into a translocation channel or “translocon” (37–41). In the final step of the targeting cycle, the SRP dissociates from the ER membrane. In mammalian cells, the entry of the vast majority of proteins into the secretory pathway is completely dependent on the SRP targeting pathway. Partial entry of the vast majority of proteins into the secretory pathway results in a “translocon” (37–41). In the final step of the targeting cycle, the serum requires the presence of a translocon for translocation. The translocon is a protein complex that consists of multiple proteins, including SRP54, SRP68, SRP71, and SRP72, which are bound to the ER membrane.

The down-regulation of 7SL RNA synthesis in macrophages may not be the primary event of Leishmania-macrophage interactions, and it is not the only molecular event that happens as a result of the parasite-host encounter. However, it could be a pivotal event that translates the hostile behavior of the macrophages toward Leishmania into moderate hospitable interactions. It is also possible that several secondary and tertiary biochemical changes happen inside and outside of the infected macrophages as a direct or indirect result of the down-regulation of 7SL RNA biosynthesis, which may in turn make the macrophages more vulnerable to leishmanial parasitism.

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