INFECTION AND IMMUNITY, May 2010, p. 2138–2145 Vol. 78, No. 5
0019-9567/10/$12.00 doi:10.1128/IAI.01102-09


# Upregulated Expression of B-Cell Antigen Family Tandem Repeat Proteins by *Leishmania* Amastigotes▽†

Yasuyuki Goto,[1,2,3]* Darrick Carter,[2,3] Jeffrey Guderian,[2] Noboru Inoue,[1] Shin-Ichiro Kawazu,[1] and Steven G. Reed[2]

*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan[1]; Infectious Disease Research Institute, 1124 Columbia Street, Suite 400, Seattle, Washington 98104[2]; and Protein Advances Inc., 1102 Columbia Street, Suite 107, Seattle, Washington 98104[3]*

Received 30 September 2009/Returned for modification 10 November 2009/Accepted 10 February 2010

**Proteins with tandem repeat (TR) domains have been found in various protozoan parasites, and they are often targets of B-cell responses. Through systematic analyses of whole proteomes, we recently demonstrated that two trypanosomatid parasites, *Leishmania infantum* and *Trypanosoma cruzi*, are rich in antigenic proteins with large TR domains. However, the reason that these proteins are antigenic was unclear. Here, by performing molecular, immunological, and bioinformatic characterizations of *Leishmania* TR proteins, we found two possible factors affecting the antigenicity of these proteins; one factor is their fundamental composition as TR proteins, and the other is regulation of their expression by parasites. Enzyme-linked immunosorbent assays (ELISAs) using recombinant proteins revealed that the copy number of the repeat affects the affinity of binding between antigens and antibodies, as expected based on thermodynamic binding kinetics. Other than containing TR domains, the TR proteins do not share characteristics, such as sequence similarity or biased cellular location predicted by the presence of a signal sequence(s) and/or a transmembrane domain(s). However, the TR proteome contained a higher percentage of proteins upregulated in amastigotes than the whole proteome, and upregulated expression of a TR protein seemed to affect its antigenicity. These results indicate that *Leishmania* parasites actively utilize the TR protein family for parasitism in mammalian hosts.**

Genes encoding proteins with tandem repeat (TR) domains, defined here as two or more copies of an amino acid sequence, have been found in a variety of organisms ranging from prokaryotes to higher animals. Although the functions of TR proteins are disparate and not confined to a single type of protein and although common stages of expression or cellular localization are not consistently observed, one feature of these proteins appears to be shared: they are often potent B-cell antigens. The immunological significance of TR proteins during bacterial infections has been reported previously (1, 30), and even some cancer antigens to which patients show antibody responses contain TR domains (35, 39). TR proteins also often serve as targets of B-cell responses in protozoan parasites (8, 10, 11, 14, 15, 26, 32, 34). Although for long time only a few TR antigens of *Leishmania* species were identified (8, 10, 17, 19, 50), our recent studies demonstrated that there are more TR proteins with serological significance in these parasites (23, 24). Many novel TR proteins were identified by performing a computational search of the *Leishmania* genome (24), and the same approach also demonstrated that there is immunological dominance of such proteins in *Trypanosoma cruzi* (22). Although the results of these bioinformatic studies showed that TR proteins constitute a family of proteins that share immunological characteristics, the mechanism remained unknown.

The total number of TR proteins in parasites is not necessarily higher than the total number of TR proteins in other organisms, but trypanosomatid parasites, including *Leishmania* and *Trypanosoma*, are rich in proteins with TR domains that include a larger number of repeats (22, 24). Here we produced recombinant proteins made up of increasing numbers of copies of the repeat sequence from two *Leishmania infantum* TR proteins, evaluated the reactivities of these proteins with plasma samples from visceral leishmaniasis (VL) patients, and demonstrated that antibody binding to the antigen is stronger as the copy number increases. Interestingly, an analysis of proteome data obtained with isobaric tags for relative and absolute quantification (iTRAQ) recently reported by Rosenzweig et al. (46) revealed a bias in expression of the TR proteins toward amastigotes, the developmental stage in mammalian hosts. These results indicate that *Leishmania* parasites actively utilize members of an antigenic protein family for parasitism in vertebrate hosts.

## MATERIALS AND METHODS

**Computational search for TR protein genes.** DNA sequence data for *L. infantum* CDS version 3.0 (44) were obtained from GeneDB (28; www.genedb.org). Tandem Repeats Finder, a program for locating and displaying TR in DNA sequences (7; http://tandem.bu.edu/trf/trf.html), was used to identify TR protein genes as previously described (22, 24). In this study, genes were considered TR protein genes if the scores from the Tandem Repeats Finder analysis were 500 or higher.

**Sequence analyses of *L. infantum* TR proteins.** The biochemical properties of each *L. infantum* TR protein were analyzed to determine (i) the molecular mass of the protein, its isoelectric point, and the presence of a signal sequence and a transmembrane domain; (ii) the known antigenicity and/or functions of the protein by BLAST searches using both DNA and deduced amino acid sequences with the NCBI database; and (iii) the sequence similarity of the protein to other *L. infantum* proteins by a BLAST search of the GeneDB database. Biochemical characteristics, such as the isoelectric point, the molecular weight, and the pres-

* Corresponding author. Mailing address: Inada-cho, Obihiro, Hokkaido 080-8555, Japan. Phone: 81-155-49-5800. Fax: 81-155-49-5643. E-mail: ygoto@obihiro.ac.jp.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 16 February 2010.

ence of predicted signal peptides and transmembrane domains, were obtained from TriTrypDB (http://TriTrypDB.org) (2).

**Antigen preparation.** PCR cloning of Li73r1 and Li73r2 was performed using primers matching both ends of the 219-bp repeat unit as previously described (23). The resulting PCR products produced ladder bands corresponding to one or multiple copies of the repeat (23). PCR-amplified products corresponding to either one or two copies of the 219-bp unit were used for cloning in the pET28 vector (EMD Biosciences, San Diego, CA). The same PCR amplification strategy was used to clone two and four copies of the rK39 repeat (39r2 and 39r4, respectively). Li73r8.7 was PCR amplified as a single band using primers corresponding to regions just outside the TR domain. PCR-cloned genes were also cloned into the pET28 vector. *Escherichia coli* Rosetta was transfected with pET28 plasmids containing individual genes, and recombinant proteins were purified as soluble proteins using Ni-nitrilotriacetic acid (NTA) agarose (Qiagen Inc., Valencia, CA) as previously described (23). TR2, the entire TR domain of LinJ29_V3.0110, was produced in a previous study as rLinJ29.0110TR (24). K39, TSA, and LmSTI1 were also produced in previous studies (10, 51, 52). A partial repeat domain of LinJ03_V3.0260, LinJ11_V3.0070, LinJ21_V3.0920, LinJ22_V3.1160, LinJ25_V3.1100, LinJ27_V3.0500, LinJ32_V3.3350, or LinJ33_V3.3230 was produced as a recombinant protein in previous studies (23, 24). Nucleotides encoding a partial repeat domain of LinJ05_V3.0380, LinJ14_V3.1540, LinJ16_V31550, LinJ19_V3.1680, LinJ20_V3.1210, LinJ26_V3.2180, LinJ27_V3.0250, LinJ32_V3.2370, or LinJ34_V3.2360 were synthesized by Blue Heron Biotechnology, Inc. (Bothell, WA), and recombinant proteins were produced using the pET28 vector system and Ni-NTA agarose as described above for other TR proteins. All of the antigens used in this study had endotoxin levels of less than 100 endotoxin units (EU)/mg, as determined by a *Limulus* amebocyte lysate test (Cambrex Corporation, East Rutherford, NJ).

**Infection of mice.** All mice were maintained in the Infectious Disease Research Institute (IDRI) animal care facility under specific-pathogen-free conditions and were treated in accordance with the regulations and guidelines of the IDRI Animal Care and Use Committee. BALB/c mice and BALB/c nude mice (6 to 8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and maintained under specific-pathogen-free conditions. The *Leishmania major* Friedlin strain was kindly provided by David Sacks (National Institutes of Health, Bethesda, MD) and was maintained as promastigotes as previously described (6). Both BALB/c mice and BALB/c nude mice were infected subcutaneously in the left hind footpad with $2 \times 10^5$ *L. major* metacyclic promastigotes. These mice were sacrificed 8 weeks after infection, and serum samples were collected for immunological examination.

**Antibody ELISAs.** Human antibody enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (22). Plasma samples were collected from Brazilian visceral leishmaniasis (VL) patients and healthy Brazilian people after written consent was obtained for use for research purposes (25), and they were used at a 1:200 dilution. Collection and use of these human samples were approved by the Institutional Review Board ethics committee at Universidade Federal da Bahia (25). Mouse sera were diluted 1/100 and then diluted further using 5-fold serial dilution up to 1:7,812,500 before they were added to plates (100 μl/well). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgG3 (Southern Biotech, Birmingham, AL) were used as secondary antibodies. Reciprocal endpoint titers for individual antigens were calculated with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) using a cutoff value of 0.1. Endpoint titers for samples were recorded as $<100$ if the optical densities of the samples were less than the cutoff value at a 1:100 dilution or as $>7{,}812{,}500$ if the optical densities of the samples were more than the optical density for a 1:7,812,500 dilution. In these cases, titers of 100 or 7,812,500 were used when graphs were constructed. For both human and mouse ELISAs, 200 ng/well of individual recombinant antigens or 1 μg/well of soluble leishmanial antigen (SLA) was used to coat the plates unless specifically indicated otherwise.

**iTRAQ data analysis.** Changes in the levels of expression of TR proteins were evaluated by analyzing iTRAQ data reported by Rosenzweig et al. (46). A total of 1,712 proteins detected by the iTRAQ analysis were searched for *L. infantum* TR proteins listed in Table S1 in the supplemental material. K39 and K26, two of the *L. infantum* TR proteins detected by iTRAQ, were examined to determine their expression in both promastigotes and amastigotes to validate the iTRAQ data.

**Western blotting.** Western blotting and preparation of *L. infantum* promastigotes and amastigotes were performed as previously described (20). Sera from rabbits immunized six times with either recombinant K39, K26, A2, Li73r1, TSA, LmSTI1, CPB, or KMP11 formulated in Freund's incomplete adjuvant were used as the primary antibody.

## RESULTS

**Number of repeats affects binding of antibodies to TR antigens.** As described in previous studies (22, 24), TR protein genes were identified in *L. infantum* using the Tandem Repeats Finder (7). In this study, genes were considered TR protein genes if the scores from the Tandem Repeats Finder analysis were 500 or higher. The cutoff value used (500) was likely to eliminate genes with repeat domains less than 250 bp long. Sixty-four genes were identified as TR protein genes when we initially analyzed *L. infantum* genome version 2 (24). We analyzed the latest version of the *L. infantum* genome (version 3) in a previous study, and 56 genes were identified as genes in this category (22), because some of the TR protein genes identified in the previous version no longer exist after further annotation. It was found that one gene, LinJ28_V3.2370, which had a score of 1,398, was missed by the program the last time. Therefore, a total of 57 genes were identified as genes encoding TR proteins (see Table S1 in the supplemental material).

Large TR domains with high repeat copy numbers tend to be found in TR proteins from trypanosomatid parasites, including *Leishmania* (22). To assess the influence of copy number on a TR's antigenicity, we compared the reactivity of plasma samples from human VL patients to the reactivity of recombinant proteins with various repeat copy numbers. We used LinJ16_V31760 from *L. infantum* as a model antigen. The LinJ16_V31760 gene is a gene consisting of 9,225 bp, and it contains a single TR domain composed of 8.7 copies of a 219-bp repeat (Fig. 1A). An orthologue is present in *L. major* (LmjF16.1660), and it has a high overall level of similarity to LinJ16_V31760; the major difference is the copy number in the repeat (Fig. 1A). The 8.7-copy TR domain of the LinJ16_V31760 protein is comprised of a 73-amino-acid (aa) repeat that is highly conserved. Although only repeats 5 and 6 are identical, the residues at only 6 amino acid positions are different in different copies in the conserved sequence (Fig. 1B). To examine how the difference in the amino acid sequences among copies affects antibody recognition of the 73-aa repeat, 3 recombinant proteins with a single copy of each repeat having different sequences were produced (Li73r1, Li73r1′, and Li73r1″, corresponding to repeats 5, 3, and 2, respectively [Fig. 1C; see Fig. S1 in the supplemental material]). The results of an ELISA performed with human VL patient plasma samples demonstrated that the reactivities of the three proteins were comparable (Fig. 2A).

Next, two recombinant proteins having two copies of the 73-aa repeat (repeats 5 and 6) (Li73r2) or all 8.7 copies of the 73-aa repeat (Li73r8.7) were produced (see Fig. S1 in the supplemental material) and evaluated to determine their antibody binding. Human VL patient plasma showed stronger reactivity with proteins with more copies of the 73-aa repeat (Fig. 2B). Increased reactivity of multiple-copy TR was also found when proteins were tested with plasma from healthy controls (Fig. 2B). The dominance of Li73r8.7 over Li73r1 was observed even when only 50 ng/well of Li73r8.7 (25% of the amount usually used) was used (Fig. 2C). The effect of copy number on antibody reactivity was not restricted to the 73-aa repeat. Similar copy number-dependent increases in reactivity with the 39-aa repeat from rK39, the defined diagnostic anti-

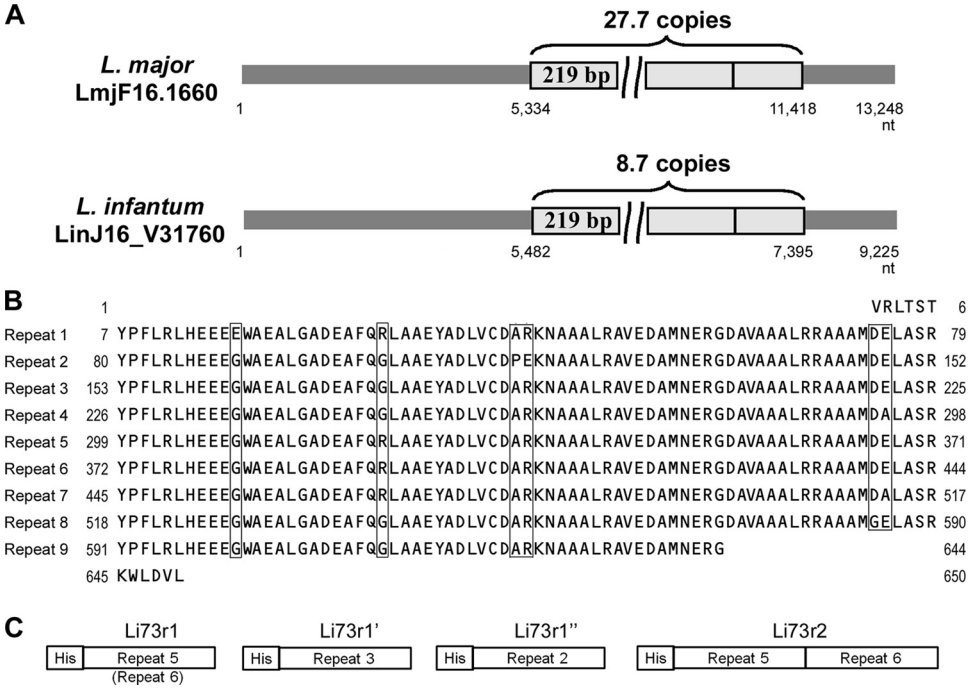


FIG. 1. Construction of proteins with different numbers of repeats. (A) Diagrams of two 73-aa repeat genes, LmjF16.1600 from *L. major* and LinJ16_V31760 from *L. infantum*. (B) Deduced amino acid sequence of the entire TR domain and flanking 6-aa sequences of LinJ16_V31760. The fragment shown was PCR amplified and cloned as Li73r8.7. (C) Schematic diagrams of Li73 recombinant proteins. Li73r8.7 was composed of the entire amino acid sequence shown in panel B flanked by the same 20-aa His tag present at the N terminus in other recombinant proteins.

gen used in commercially available tests, were also observed (Fig. 2D).

**Antibody responses to TR proteins are different from antibody responses to non-TR proteins.** TR proteins share a characteristic with T-cell-independent antigen type 2 (TI-2) proteins as multivalent antigens. TI-2 proteins, which are molecules with repetitive epitope motifs, can activate B cells in the absence of major histocompatibility complex (MHC) class II-restricted T-cell help by cross-linking of antigen receptors (49). To examine the involvement of T cells in antibody responses to TR proteins, we used a murine model of *Leishmania* infection. BALB/c and nude mice with a BALB/c background were infected with *L. major*, and sera were collected after 8 weeks. As shown in Fig. 3A, IgG specific to both non-TR antigens (LmSTI1 and TSA) and TR antigens (Li73r2 and TR2) as well as to crude parasite antigen (LmSLA), was detected in BALB/c mice. Regardless of the antigen characteristics (TR or non-TR), the antibody responses were largely dependent on T cells as the antibody titers with all of the antigens tested were lower in nude mice (Fig. 3B). However, there was a difference in the antibody subclass patterns between TR and non-TR proteins. The IgG responses to TSA and LmSTI1 in *L. major*-infected BALB/c mice were dominated by IgG1, whereas comparable levels of IgG1 and IgG2a were found for Li73r2 and TR2 (Fig. 3C); statistical analysis revealed significant differences in IgG1/IgG2a ratios between TR proteins and non-TR proteins (Fig. 3D). A high level of IgG3 was detected in antibodies directed to TR2 (Fig. 3C).

**No apparent biochemical similarity in *Leishmania* TR proteins.** To explore the functional similarity of the 57 TR proteins of *L. infantum*, biochemical characteristics and the sequence similarity of these proteins were examined. As previously reported for TR proteins based on an older version of the *L. infantum* genome (24), TR protein data from the latest version of the *L. infantum* genome showed that the TR protein molecules were larger than the proteins in the rest of the proteome, and the frequency of proteins with lower isoelectric points was higher for the TR protein group (see Fig S2 in the supplemental material). A high prevalence of charged amino acids (D, E, K, and R) with an emphasis on acidic amino acids was observed in TR proteins, and these amino acids were more concentrated in the repeat domains (24). Other than these factors, the TR proteins did not appear to be dominated by groups sharing biochemical functions or biased cellular locations, except for proteophosphoglycan (7 proteins). The TR domains of the 57 proteins were not dominated by certain repeat motifs; their TR motifs were variable in length (22), and no conserved sequence motifs were found to dominate the 57 proteins. Although the percentage of proteins with predicted signal peptides was slightly higher for TR proteins than for the whole proteome (25% and 18%, respectively) (Table 1), the difference did not indicate a significant bias in the cellular location of the TR proteins in the proteome. The percentages of transmembrane domains in the protein sets were comparable for the TR proteins analyzed and the whole proteome (Table 1).

**Upregulation of TR expression in amastigotes.** To search for other characteristics of *L. infantum* TR proteins relevant to their antigenicity, the levels of expression of these proteins in amastigotes (the developmental stage in mammalian hosts)

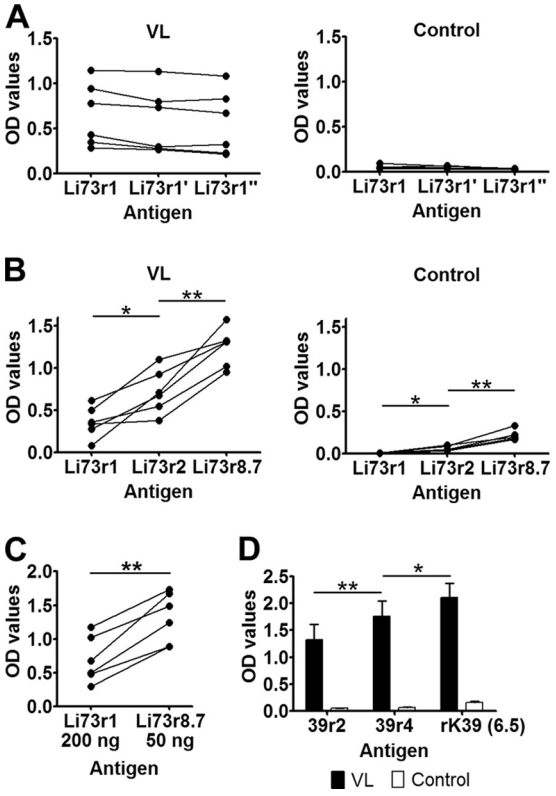


FIG. 2. Copy number of the TR affects the affinity between antigen and antibody. (A) Single-copy 73-aa repeat proteins with different sequences (Li73r1, Li73r1′, and Li73r1″) were examined by ELISA using sera from human VL patients (left) and healthy controls (right). Symbols connected by a line indicate ELISA results obtained with an individual plasma sample. (B) Proteins with different copy numbers of the 73-aa repeat (Li73r1, Li73r2, and Li73r8.7) were examined by ELISA, as described above for panel A. (C) Comparison of ELISA reactivities for 200 ng/well of Li73r1 and 50 ng/well of Li73r8.7. (D) Proteins with different copy numbers of the 39-aa repeat (39r2, 39r4, and the original rK39 protein containing 6.5 copies) were examined by ELISA as described above for panel A. OD, optical density. *, $P < 0.05$ by *t* test; **, $P < 0.01$ by *t* test.

were examined by analyzing proteomic data recently obtained by Rosenzweig et al. using isobaric tags for relative and absolute quantification (iTRAQ) (46). In the study of Rosenzweig et al., *Leishmania donovani* was used for iTRAQ analysis, and the peptide sequences identified were compared with those from the *L. infantum* database using BLAST analysis. Therefore, although the results shown here are based on gene identities in *L. infantum*, the data are data for *L. donovani*. Because both *L. donovani* and *L. infantum* belong to the *L. donovani* complex, are genetically very similar, and cause VL, we thought that the *L. donovani* data would be applicable to *L. infantum*. The iTRAQ data were generated using an axenic differentiation model (3); *L. donovani* promastigotes (time zero during transformation), amastigotes (after 144 h), and transforming parasites (2.5 h, 5 h, 10 h, 15 h, and 24 h) were analyzed for protein expression. A total of 1,712 proteins were detected by iTRAQ in the study. As differentiation progressed, increased numbers of proteins showed levels of expression that were different (either upregulated or downregulated) from those in promastigotes. After 144 h of differentiation, 31% of the proteins in the whole proteome were upregulated ($\log_2$ change $\geq 0.5$), the expression of 33% of the proteins was unchanged ($-0.5 < \log_2$ change $< 0.5$), and 36% of the proteins were downregulated ($\log_2$ change $\leq -0.5$) (Table 2). Based on a mean for the whole proteome, protein expression appeared to be consistent during differentiation (Fig. 4A). Of the 57 TR proteins of *L. infantum*, 28 were detected by iTRAQ (see Table S2 in the supplemental material). In contrast to the data for the whole proteome, 50% of the proteins in the TR proteome were upregulated and the expression of 45% of the proteins was unchanged, whereas only 5% of the proteins were downregulated after 144 h of differentiation (Table 2). When the average change in expression of the 28 TR proteins was analyzed, the TR proteins showed increased expression in amastigotes, and the increase was significant from 15 h onward during differentiation (Fig. 4A). The iTRAQ data were verified by Western blotting. An overall correlation between the iTRAQ data and the Western blotting data was observed, as observed by Rosenzweig et al. (Fig. 4B; see Fig. S3 in the supplemental material); K39 and K26 showed increased expression in amastigotes as determined by Western blotting (Fig. 4B). Although neither A2 (one of the previously well-characterized TR proteins of the *L. donovani* complex) nor LinJ16_V31760 (Li73) was not detected in the iTRAQ study, these two proteins also showed increased expression in amastigotes as determined by Western blotting (Fig. 4B). Kinetoplastid membrane protein-11, which showed slightly decreased expression ($\log_2$ change, $-0.23$) when iTRAQ was used, showed decreased expression in amastigotes when Western blotting was used (Fig. 4B). To examine the influence of upregulated expression in amastigotes on the antigenicity of a TR protein, the ELISA reactivities with human VL patient sera and the iTRAQ values of individual TR proteins were compared. When the data were plotted, a positive correlation was observed between antigenicity and amastigote expression, although it was not significant (Fig. 5A). The correlation was statistically significant ($P < 0.01$; $r = 0.70$; Pearson correlation test) after two outliers in the TR proteome were excluded (Fig. 5B).

## DISCUSSION

TR proteins have been implicated in the ability of protozoan parasites to influence host immune responses and to contribute to parasite survival. In fact, many TR proteins in the proteomes of *L. infantum* and *T. cruzi* are antigenic (22, 24). Here we found that TR proteins of *Leishmania* parasites can induce antigen-specific humoral responses in a manner different than non-TR proteins and that parasites express these proteins at higher levels in amastigotes, the developmental stage in mammalian hosts. In both natural mammalian hosts and experimental animals, B cells and immunoglobulins have a negative impact on controlling *Leishmania* parasites (12, 29, 33, 38, 42, 45, 47). Hypergammaglobulinemia is one of the major manifestations in humans with VL (9). Passive administration of anti-*Leishmania* IgG exacerbates *Leishmania* infection through induction of interleukin-10 (IL-10) production by macrophages (38). Even opsonization, which eliminates pathogens in most infections, is beneficial for *Leishmania* amastigotes as they are designed to proliferate in macrophages (27). Therefore, accelerating the induction of antigen-specific IgG using molecules

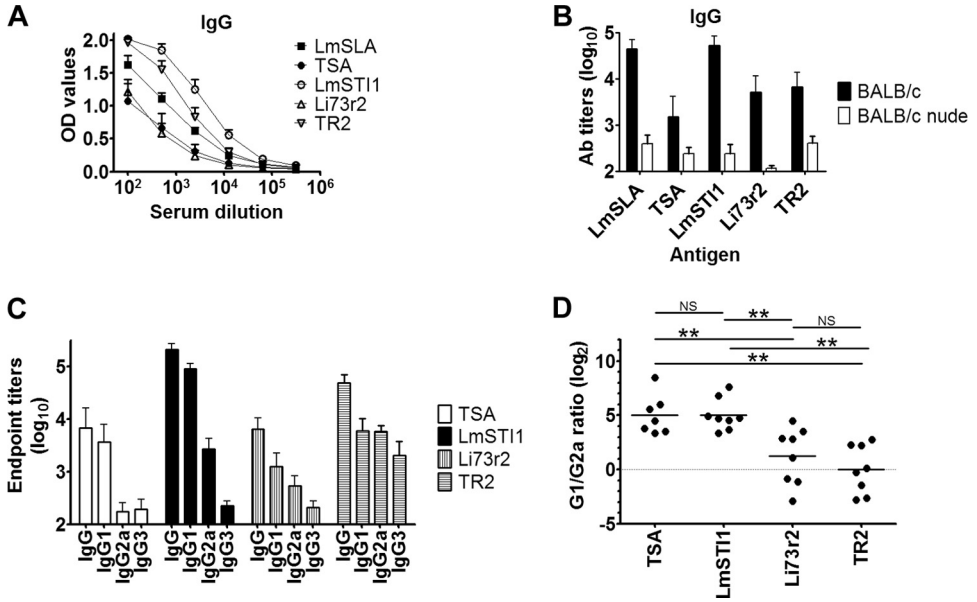


FIG. 3. Antibody responses to TR antigens are distinct from the antibody responses to non-TR antigens. (A) Total IgG responses of *L. major*-infected BALB/c mice to a crude antigen (LmSLA) and defined antigens (TSA, LmSTI1, Li73r2, and TR2). (B) Comparison of total IgG responses to the antigens for BALB/c and nude mice, both infected with *L. major*. (C) IgG subclasses of *L. major*-infected BALB/c mice for the four defined antigens. (D) IgG1/IgG2a ratios for individual antigens (D). OD, optical density; Ab, antibody; NS, not significant. **, $P < 0.01$ by *t* test.

such as TR proteins might be beneficial for survival of *Leishmania* in mammalian hosts. The presence of an immunoglobulin binding protein in *Leishmania* parasites (13) indicates that IgG induced by a TR protein can bind to the parasite surface and serve as an IL-10 inducer even when the antigen is not expressed on the cell surface. It has been reported previously that a synthesized octamer of the repeat unit from one of the *Leishmania* TR proteins can induce a disease-exacerbating TH2 response, whereas the monomers did not do this (37). A2, one of the *Leishmania* TR proteins, was shown to be a virulence factor of *L. donovani*, the causative agent of VL. This protein has more than 40 copies of a 10-amino-acid repeat, whereas A2 in *L. major*, which causes cutaneous leishmaniasis, has only 1 copy of the repeat, suggesting that multiple repeats in the A2 protein may play a role in visceralization of the parasites (55, 56). A2 does not have any enzymatic activities that have been characterized, and all that is known about this virulence factor is that it exhibits amastigote-specific expression (54) and is able to induce an antibody response (19).

Even if *Leishmania* TR proteins have biochemical functions other than immunological dominance, these functions are likely to be divergent due to the lack of unifying sequence characteristics. The only function characterized for all TR proteins other than proteophosphoglycans and polyubiquitins is serological reactivity. TR protein genes are probably generated by replicational slippage and gene conversion (53). Since replicational slippage may occur by chance, the generation of TRs is also random; selective pressure after insertion results in maintenance, amplification, or deletion of the gene. If positive selection occurs due to added biochemical functionality, a group of TR proteins selected during a period of evolution would be expected to have some shared domain structures. In fact, a large portion of TR proteins from *Mycobacterium tuberculosis* and *Homo sapiens* belong to functional protein families with structural conservation (22). In contrast, neither sequence similarity nor a bias in predicted cellular location was found for *Leishmania* TR proteins, suggesting that this parasite retained the TR protein genes because of the product's antigenic effect rather than because of a specific biochemical function. Predominance of strongly charged and hydrophilic amino acids in a TR domain could be a sign of immunological selection as these amino acids contribute to surface exposure of the TR domain on a protein molecule. Therefore, any such sequence could be useful for inducing a humoral response by the mammalian host as long as it contains multiple repeats.

TABLE 1. Characteristics of *L. infantum* TR proteins

| Proteins | Total no. (% of all proteins) | No. with Tandem Repeats Finder scores of ≥500 (% of all proteins) |
|---|---|---|
| All | 8,216 | 57 |
| Proteins with signal peptides | 1,452 (18) | 14 (25) |
| Proteins with a transmembrane domain(s) | 1,481 (18) | 11 (19) |

Two major factors that impact the interaction between antigens and B-cell receptors (BCR) are the affinity and valence of epitopes on the antigen. The affinity of an antigen for the BCR is reflected in the outcome of B-cell stimulation and differentiation in both T-cell-dependent and -independent settings (4, 36, 43, 48). Because the same amount of proteins was used to coat plates for ELISAs, the number of available epitopes was not the factor that resulted in differences in ELISA absorbance among proteins with different copy numbers of repeats. For example, 10 molecules of the one-repeat protein have the same amount of protein as 5 molecules of the

TABLE 2. Changes in *L. infantum* TR proteins from the promastigote stage to the amastigote stage

| Proteins | % with change from zero time (promastigotes) to 144 h (amastigotes) of[a]: | | | | | | |
|---|---|---|---|---|---|---|---|
| | $\text{Log}_2$ change $\leq -2$ | $-2 < \log_2$ change $\leq -1$ | $-1 < \log_2$ change $\leq -0.5$ | $-0.5 < \log_2$ change $< 0.5$ | $0.5 \leq \log_2$ change $< 1$ | $1 \leq \log_2$ change $< 2$ | $\text{Log}_2$ change $\geq 2$ |
| All | 3 | 18 | 15 | 33 | 17 | 11 | 3 |
| TR | 0 | 5 | 0 | 45 | 15 | 20 | 15 |

[a] The percentages were calculated as follows: (number of proteins with values in the range)/(number of proteins in the corresponding proteome) × 100.

two-repeat protein, and both have a total of 10 repeat units. Therefore, the results obtained may have been due to differences in avidities between an antigen and an antibody as recruitment of multiple binding sites on a single antigen would enhance the measured binding avidity. In trypanosomatid parasites, including *Leishmania* and *Trypanosoma* species, there are more TR proteins with large repeat domains (22). To explore the influence of repeat copy number on the avidity of antigens for immunoglobulins, we produced recombinant proteins composed of different numbers of each TR unit and focused on two antigens: LinJ16_V31760 (Li73) and rK39. In both cases, binding of the TR protein to antibodies increased as the copy number increased. Together, the TR proteins of *Leishmania* parasites may be able to induce humoral responses through multiple mechanisms that non-TR proteins with only monovalent molecular epitopes cannot use.

In this study, the levels of IgG2a to both Li73 and TR2 and the levels of of IgG3 to TR2 were remarkable in *L. major*-infected mice. A high level of IgG2a is one of the characteristics of polyclonal B-cell activation (41), and the IgG3 response in mice to the TR2 antigen is indicative of unique B-cell activation. It has been reported that ordinary soluble protein antigens induce primarily IgG1, while TI-2 antigens stimulate IgG2 in humans and IgG3 in mice (40, 49). Taken together, although the antibody responses to TR proteins were largely T cell dependent, findings obtained in this study indicate that there is a TI-2-like B-cell activation pathway for induction of anti-TR antibody responses. In fact, in human VL patients the TR antigen rK39 detects IgG2, but the non-TR antigen SMT does not detect IgG2 (21). In addition, anti-K39 antibodies can be detected even in VL patients with AIDS, whereas the levels of antibodies to crude antigen are drastically decreased in such patients (31). *trans*-Sialidase of *T. cruzi* is a TR enzyme known to induce T-cell-independent B-cell activation through its TR domain (18), and interestingly, heterologous expression of the *T. cruzi trans*-sialidase in *L. major* enhances virulence (5). Because *trans*-sialidase is naturally not thought to have any biochemical functions in *Leishmania* parasites as the enzyme is not present in the parasite (44), these studies further support the hypothesis that TRs are important for parasite survival through immunological mechanisms independent of other biochemical functions.

Protein families that have immunological functions that enable parasitism in mammalian hosts have been found in protozoan parasites. These families include the variant surface glycoproteins of African trypanosome parasites and erythrocyte membrane protein 1 of *Plasmodium falciparum*. These proteins are involved in antigenic variation important for parasite evasion of the immune system (16). Many pathogens can be cleared by antibody-mediated immune responses and must have proteins that contribute to escape from recognition by antibodies. In order for such proteins to maximally serve as a basis for antigenic variation, they should have a well-regulated expression system, such as phase variation, and there must be mutually exclusive expression (16). For *Leishmania* parasites certain B-cell populations and immunoglobulin levels can have a negative impact on control of infection (33, 38, 45). Therefore, *Leishmania* may utilize TR proteins to skew the entire

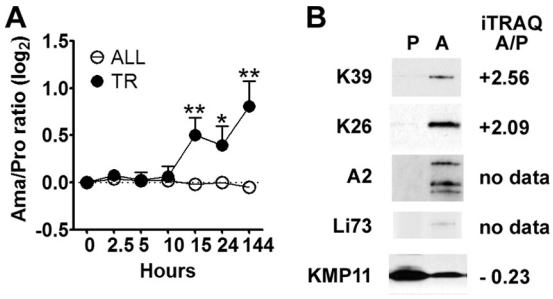


FIG. 4. Upregulated expression of *L. infantum* TR proteins in amastigotes. (A) Mean values for fold changes in levels of expression of all 1,712 proteins (ALL) and 28 *L. infantum* TR proteins (TR), both detected by iTRAQ. (B) Western blot analysis of expression of K39, K26, A2, Li73, and KMP11 by both promastigotes and amastigotes. Ama and A, amastigotes; Pro and P, promastigotes. *, $P < 0.05$ by *t* test; **, $P < 0.01$ by *t* test.

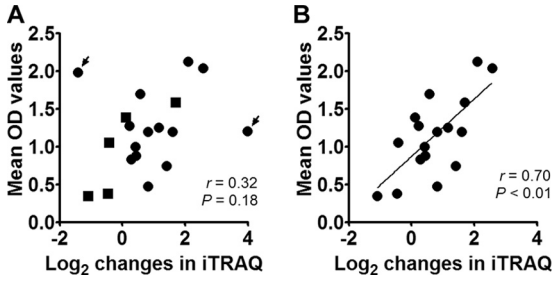


FIG. 5. Relationship between the expression pattern of a protein and its antigenicity. (A) TR proteins which were detected by iTRAQ were analyzed to determine their antigenicity by ELISA using human VL patient sera. The data for individual proteins were plotted (●) based on the $\log_2$ difference in expression between promastigotes (zero time) and amastigotes (144 h) using the iTRAQ data (*x* axis) and the mean optical density (OD) determined by the ELISA (*y* axis). For some proteins (LinJ20_V3.1210, LinJ26_V3.2180, LinJ27_V3.0250, LinJ32_V3.2370, and LinJ34_V3.2360) there were no iTRAQ values at 144 h (■); the values for the last available time point were used in these cases (see Table S2 in the supplemental material). Pearson *r* and *P* values from a correlation test are shown. (B) The correlation test was performed again after exclusion of two outliers indicated by arrows in panel A. OD, optical density.

immune response, and since this does not encompass periodic escape, the most favorable strategy would be to express the skewing protein families at the same time at high levels. We found that expression of many TR proteins is upregulated in amastigotes, strongly supporting such a strategic use of this family. In contrast to *Leishmania* species rich in large TR proteins, *P. falciparum* has a lot of small TR proteins (22). Assuming that larger TR proteins have greater B-cell-activating ability, TR proteins may have different functions in *Leishmania* and *Plasmodium* parasitism. The limited number of strongly expressed long TR proteins overwhelm the response in one case, and an uncountable number of decoys perturb the response in the other case. Therefore, it would be intriguing to look at the role of TR proteins in the context of each parasite's mechanism of survival.

## ACKNOWLEDGMENTS

Sequence data for *L. infantum* were produced by the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute and were obtained from GeneDB (http://www.genedb.org). We thank Alex Picone for technical assistance.

This study was supported by World Health Organization grant A60524, by National Institutes of Health grant AI25038, by Bill and Melinda Gates Foundation grant 39129, and by a grant to the Obihiro University of Agriculture & Veterinary Medicine Global COE program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

## REFERENCES

1. **Allred, D. R., T. C. McGuire, G. H. Palmer, S. R. Leib, T. M. Harkins, T. F. McElwain, and A. F. Barbet.** 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc. Natl. Acad. Sci. U. S. A. **87:**3220–3224.
2. **Aslett, M., C. Aurrecoechea, M. Berriman, J. Brestelli, B. P. Brunk, M. Carrington, D. P. Depledge, S. Fischer, B. Gajria, X. Gao, M. J. Gardner, A. Gingle, G. Grant, O. S. Harb, M. Heiges, C. Hertz-Fowler, R. Houston, F. Innamorato, J. Iodice, J. C. Kissinger, E. Kraemer, W. Li, F. J. Logan, J. A. Miller, S. Mitra, P. J. Myler, V. Nayak, C. Pennington, I. Phan, D. F. Pinney, G. Ramasamy, M. B. Rogers, D. S. Roos, C. Ross, D. Sivam, D. F. Smith, G. Srinivasamoorthy, C. J. Stoeckert, Jr., S. Subramanian, R. Thibodeau, A. Tivey, C. Treatman, G. Velarde, and H. Wang.** 2010. TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. *38*(Database issue)**:**D457-D462.
3. **Barak, E., S. Amin-Spector, E. Gerliak, S. Goyard, N. Holland, and D. Zilberstein.** 2005. Differentiation of *Leishmania donovani* in host-free system: analysis of signal perception and response. Mol. Biochem. Parasitol. **141:**99–108.
4. **Batista, F. D., and M. S. Neuberger.** 1998. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. Immunity **8:**751–759.
5. **Belen Carrillo, M., W. Gao, M. Herrera, J. Alroy, J. B. Moore, S. M. Beverley, and M. A. Pereira.** 2000. Heterologous expression of *Trypanosoma cruzi* trans-sialidase in *Leishmania major* enhances virulence. Infect. Immun. **68:**2728–2734.
6. **Belkaid, Y., E. Von Stebut, S. Mendez, R. Lira, E. Caler, S. Bertholet, M. C. Udey, and D. Sacks.** 2002. $CD8^+$ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with *Leishmania major*. J. Immunol. **168:**3992–4000.
7. **Benson, G.** 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. **27:**573–580.
8. **Bhatia, A., N. S. Daifalla, S. Jen, R. Badaro, S. G. Reed, and Y. A. Skeiky.** 1999. Cloning, characterization and serological evaluation of K9 and K26: two related hydrophilic antigens of *Leishmania chagasi*. Mol. Biochem. Parasitol. **102:**249–261.
9. **Boelaert, M., B. Criel, J. Leeuwenburg, W. Van Damme, D. Le Ray, and P. Van der Stuyft.** 2000. Visceral leishmaniasis control: a public health perspective. Trans. R. Soc. Trop. Med. Hyg. **94:**465–471.
10. **Burns, J. M., Jr., W. G. Shreffler, D. R. Benson, H. W. Ghalib, R. Badaro, and S. G. Reed.** 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. Proc. Natl. Acad. Sci. U. S. A. **90:**775–779.
11. **Burns, J. M., Jr., W. G. Shreffler, D. E. Rosman, P. R. Sleath, C. J. March, and S. G. Reed.** 1992. Identification and synthesis of a major conserved antigenic epitope of *Trypanosoma cruzi*. Proc. Natl. Acad. Sci. U. S. A. **89:**1239–1243.
12. **Buxbaum, L. U., and P. Scott.** 2005. Interleukin 10- and Fcgamma receptor-deficient mice resolve *Leishmania mexicana* lesions. Infect. Immun. **73:**2101–2108.
13. **Campos-Neto, A., I. Suffia, K. A. Cavassani, S. Jen, K. Greeson, P. Ovendale, J. S. Silva, S. G. Reed, and Y. A. Skeiky.** 2003. Cloning and characterization of a gene encoding an immunoglobulin-binding receptor on the cell surface of some members of the family Trypanosomatidae. Infect. Immun. **71:**5065–5076.
14. **Coppel, R. L., A. F. Cowman, R. F. Anders, A. E. Bianco, R. B. Saint, K. R. Lingelbach, D. J. Kemp, and G. V. Brown.** 1984. Immune sera recognize on erythrocytes *Plasmodium falciparum* antigen composed of repeated amino acid sequences. Nature **310:**789–792.
15. **Dame, J. B., J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, et al.** 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science **225:**593–599.
16. **Deitsch, K. W., S. A. Lukehart, and J. R. Stringer.** 2009. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. Nat. Rev. Microbiol. **7:**493–503.
17. **Dillon, D. C., C. H. Day, J. A. Whittle, A. J. Magill, and S. G. Reed.** 1995. Characterization of a *Leishmania tropica* antigen that detects immune responses in Desert Storm viscerotropic leishmaniasis patients. Proc. Natl. Acad. Sci. U. S. A. **92:**7981–7985.
18. **Gao, W., H. H. Wortis, and M. A. Pereira.** 2002. The *Trypanosoma cruzi* trans-sialidase is a T cell-independent B cell mitogen and an inducer of non-specific Ig secretion. Int. Immunol. **14:**299–308.
19. **Ghedin, E., W. W. Zhang, H. Charest, S. Sundar, R. T. Kenney, and G. Matlashewski.** 1997. Antibody response against a *Leishmania donovani* amastigote-stage-specific protein in patients with visceral leishmaniasis. Clin. Diagn. Lab. Immunol. **4:**530–535.
20. **Goto, Y., A. Bhatia, V. S. Raman, S. E. Vidal, S. Bertholet, R. N. Coler, R. F. Howard, and S. G. Reed.** 2009. *Leishmania infantum* sterol 24-c-methyltransferase formulated with MPL-SE induces cross-protection against *L. major* infection. Vaccine **27:**2884–2890.
21. **Goto, Y., L. Y. Bogatzki, S. Bertholet, R. N. Coler, and S. G. Reed.** 2007. Protective immunization against visceral leishmaniasis using *Leishmania* sterol 24-c-methyltransferase formulated in adjuvant. Vaccine **25:**7450–7458.
22. **Goto, Y., D. Carter, and S. G. Reed.** 2008. Immunological dominance of *Trypanosoma cruzi* tandem repeat proteins. Infect. Immun. **76:**3967–3974.
23. **Goto, Y., R. N. Coler, J. Guderian, R. Mohamath, and S. G. Reed.** 2006. Cloning, characterization, and serodiagnostic evaluation of *Leishmania infantum* tandem repeat proteins. Infect. Immun. **74:**3939–3945.
24. **Goto, Y., R. N. Coler, and S. G. Reed.** 2007. Bioinformatic identification of tandem repeat antigens of the *Leishmania donovani* complex. Infect. Immun. **75:**846–851.
25. **Goto, Y., R. F. Howard, A. Bhatia, J. Trigo, M. Nakatani, E. M. Netto, and S. G. Reed.** 2009. Distinct antigen recognition pattern during zoonotic visceral leishmaniasis in humans and dogs. Vet. Parasitol. **160:**215–220.
26. **Gruber, A., and B. Zingales.** 1993. *Trypanosoma cruzi*: characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease. Exp. Parasitol. **76:**1–12.
27. **Guy, R. A., and M. Belosevic.** 1993. Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages. Infect. Immun. **61:**1553–1558.
28. **Hertz-Fowler, C., C. S. Peacock, V. Wood, M. Aslett, A. Kerhornou, P. Mooney, A. Tivey, M. Berriman, N. Hall, K. Rutherford, J. Parkhill, A. C. Ivens, M. A. Rajandream, and B. Barrell.** 2004. GeneDB: a resource for prokaryotic and eukaryotic organisms. Nucleic Acids Res. **32:**D339–D343.
29. **Hoerauf, A., M. Rollinghoff, and W. Solbach.** 1996. Co-transfer of B cells converts resistance into susceptibility in T cell-reconstituted, Leishmania major-resistant C.B-17 scid mice by a non-cognate mechanism. Int. Immunol. **8:**1569–1575.
30. **Hollingshead, S. K., V. A. Fischetti, and J. R. Scott.** 1986. Complete nucleotide sequence of type 6 M protein of the group A Streptococcus. Repetitive structure and membrane anchor. J. Biol. Chem. **261:**1677–1686.
31. **Houghton, R. L., M. Petrescu, D. R. Benson, Y. A. Skeiky, A. Scalone, R. Badaro, S. G. Reed, and L. Gradoni.** 1998. A cloned antigen (recombinant K39) of *Leishmania chagasi* diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. J. Infect. Dis. **177:**1339–1344.
32. **Ibanez, C. F., J. L. Affranchino, R. A. Macina, M. B. Reyes, S. Leguizamon, M. E. Camargo, L. Aslund, U. Pettersson, and A. C. Frasch.** 1988. Multiple *Trypanosoma cruzi* antigens containing tandemly repeated amino acid sequence motifs. Mol. Biochem. Parasitol. **30:**27–33.
33. **Kane, M. M., and D. M. Mosser.** 2001. The role of IL-10 in promoting disease progression in leishmaniasis. J. Immunol. **166:**1141–1147.
34. **Koenen, M., A. Scherf, O. Mercereau, G. Langsley, L. Sibilli, P. Dubois, L. Pereira da Silva, and B. Muller-Hill.** 1984. Human antisera detect a *Plas-*

*modium falciparum* genomic clone encoding a nonapeptide repeat. Nature **311:**382–385.
35. **Kotera, Y., J. D. Fontenot, G. Pecher, R. S. Metzgar, and O. J. Finn.** 1994. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. Cancer Res. **54:**2856–2860.
36. **Kouskoff, V., S. Famiglietti, G. Lacaud, P. Lang, J. E. Rider, B. K. Kay, J. C. Cambier, and D. Nemazee.** 1998. Antigens varying in affinity for the B cell receptor induce differential B lymphocyte responses. J. Exp. Med. **188:**1453–1464.
37. **Liew, F. Y., S. M. Millott, and J. A. Schmidt.** 1990. A repetitive peptide of *Leishmania* can activate T helper type 2 cells and enhance disease progression. J. Exp. Med. **172:**1359–1365.
38. **Miles, S. A., S. M. Conrad, R. G. Alves, S. M. Jeronimo, and D. M. Mosser.** 2005. A role for IgG immune complexes during infection with the intracellular pathogen *Leishmania*. J. Exp. Med. **201:**747–754.
39. **Mollick, J. A., F. S. Hodi, R. J. Soiffer, L. M. Nadler, and G. Dranoff.** 2003. MUC1-like tandem repeat proteins are broadly immunogenic in cancer patients. Cancer Immun. **3:**3.
40. **Mond, J. J., A. Lees, and C. M. Snapper.** 1995. T cell-independent antigens type 2. Annu. Rev. Immunol. **13:**655–692.
41. **Montes, C. L., E. V. Acosta-Rodriguez, M. C. Merino, D. A. Bermejo, and A. Gruppi.** 2007. Polyclonal B cell activation in infections: infectious agents' devilry or defense mechanism of the host? J. Leukoc. Biol. **82:**1027–1032.
42. **Padigel, U. M., and J. P. Farrell.** 2005. Control of infection with *Leishmania major* in susceptible BALB/c mice lacking the common gamma-chain for FcR is associated with reduced production of IL-10 and TGF-beta by parasitized cells. J. Immunol. **174:**6340–6345.
43. **Paus, D., T. G. Phan, T. D. Chan, S. Gardam, A. Basten, and R. Brink.** 2006. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. J. Exp. Med. **203:**1081–1091.
44. **Peacock, C. S., K. Seeger, D. Harris, L. Murphy, J. C. Ruiz, M. A. Quail, N. Peters, E. Adlem, A. Tivey, M. Aslett, A. Kerhornou, A. Ivens, A. Fraser, M. A. Rajandream, T. Carver, H. Norbertczak, T. Chillingworth, Z. Hance, K. Jagels, S. Moule, D. Ormond, S. Rutter, R. Squares, S. Whitehead, E. Rabbinowitsch, C. Arrowsmith, B. White, S. Thurston, F. Bringaud, S. L. Baldauf, A. Faulconbridge, D. Jeffares, D. P. Depledge, S. O. Oyola, J. D. Hilley, L. O. Brito, L. R. Tosi, B. Barrell, A. K. Cruz, J. C. Mottram, D. F. Smith, and M. Berriman.** 2007. Comparative genomic analysis of three Leishmania species that cause diverse human disease. Nat. Genet. **39:**839–847.
45. **Quinnell, R. J., O. Courtenay, M. A. Shaw, M. J. Day, L. M. Garcez, C. Dye, and P. M. Kaye.** 2001. Tissue cytokine responses in canine visceral leishmaniasis. J. Infect. Dis. **183:**1421–1424.
46. **Rosenzweig, D., D. Smith, F. Opperdoes, S. Stern, R. W. Olafson, and D. Zilberstein.** 2008. Retooling *Leishmania* metabolism: from sand fly gut to human macrophage. FASEB J. **22:**590–602.
47. **Sacks, D. L., P. A. Scott, R. Asofsky, and F. A. Sher.** 1984. Cutaneous leishmaniasis in anti-IgM-treated mice: enhanced resistance due to functional depletion of a B cell-dependent T cell involved in the suppressor pathway. J. Immunol. **132:**2072–2077.
48. **Shih, T. A., M. Roederer, and M. C. Nussenzweig.** 2002. Role of antigen receptor affinity in T cell-independent antibody responses in vivo. Nat. Immunol. **3:**399–406.
49. **Vos, Q., A. Lees, Z. Q. Wu, C. M. Snapper, and J. J. Mond.** 2000. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. Immunol. Rev. **176:**154–170.
50. **Wallis, A. E., and W. R. McMaster.** 1987. Identification of *Leishmania* genes encoding proteins containing tandemly repeating peptides. J. Exp. Med. **166:**1814–1824.
51. **Webb, J. R., A. Campos-Neto, P. J. Ovendale, T. I. Martin, E. J. Stromberg, R. Badaro, and S. G. Reed.** 1998. Human and murine immune responses to a novel *Leishmania major* recombinant protein encoded by members of a multicopy gene family. Infect. Immun. **66:**3279–3289.
52. **Webb, J. R., D. Kaufmann, A. Campos-Neto, and S. G. Reed.** 1996. Molecular cloning of a novel protein antigen of *Leishmania major* that elicits a potent immune response in experimental murine leishmaniasis. J. Immunol. **157:**5034–5041.
53. **Wickstead, B., K. Ersfeld, and K. Gull.** 2003. Repetitive elements in genomes of parasitic protozoa. Microbiol. Mol. Biol. Rev. **67:**360–375.
54. **Zhang, W. W., H. Charest, E. Ghedin, and G. Matlashewski.** 1996. Identification and overexpression of the A2 amastigote-specific protein in *Leishmania donovani*. Mol. Biochem. Parasitol. **78:**79–90.
55. **Zhang, W. W., and G. Matlashewski.** 1997. Loss of virulence in *Leishmania donovani* deficient in an amastigote-specific protein, A2. Proc. Natl. Acad. Sci. U. S. A. **94:**8807–8811.
56. **Zhang, W. W., S. Mendez, A. Ghosh, P. Myler, A. Ivens, J. Clos, D. L. Sacks, and G. Matlashewski.** 2003. Comparison of the A2 gene locus in *Leishmania donovani* and *Leishmania major* and its control over cutaneous infection. J. Biol. Chem. **278:**35508–35515.

*Editor:* J. F. Urban, Jr.