A Genomic-Based Approach Combining In Vivo Selection in Mice to Identify a Novel Virulence Gene in Leishmania

Wen-Wei Zhang¹, Christopher S. Peacock², Greg Matlashewski¹*

¹ Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada, ²Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom

Abstract

Background: Infection with Leishmania results in a broad spectrum of pathologies where L. infantum and L. donovani cause fatal visceral leishmaniasis and L. major causes destructive cutaneous lesions. The identification and characterization of Leishmania virulence genes may define the genetic basis for these different pathologies.

Methods and Findings: Comparison of the recently completed L. major and L. infantum genomes revealed a relatively small number of genes that are absent or present as pseudogenes in L. major and potentially encode proteins in L. infantum. To investigate the potential role of genetic differences between species in visceral infection, seven genes initially classified as absent in L. major but present in L. infantum were cloned from the closely related L. donovani genome and introduced into L. major. The transgenic L. major expressing the L. donovani genes were then introduced into BALB/c mice to select for parasites with increased virulence in the spleen to determine whether any of the L. donovani genes increased visceral infection levels. During the course of these experiments, one of the selected genes (LinJ32_V3.1040 (Li1040)) was reclassified as also present in the L. major genome. Interestingly, only the Li1040 gene significantly increased visceral infection in the L. major transfectants. The Li1040 gene encodes a protein containing a putative component of an endosomal protein sorting complex involved with protein transport.

Conclusions: These observations demonstrate that the levels of expression and sequence variations in genes ubiquitously shared between Leishmania species have the potential to significantly influence virulence and tissue tropism.

Introduction

Leishmania protozoa are transmitted by the bite of an infected sandfly and cause a spectrum of diseases ranging from self-healing cutaneous lesions to fatal visceral infection [1,2]. There are an estimated 12 million cases in over 80 countries, with an annual incidence of 0.5 million new cases of the visceral leishmaniasis and 2.0 million cases of the cutaneous leishmaniasis [3]. More than 20 different Leishmania species can infect humans. Host health status and genetic background can influence the outcome of infection [4,5] and HIV co-infection has dramatically increased the incidence of visceral leishmaniasis [5]. The major factor that determines the tropism and pathology of Leishmania infection is however the species of Leishmania [1,2]. For example, L. donovani, L. infantum and L. chagasi are closely related members of the L. donovani complex that cause visceral leishmaniasis, which is fatal if not treated. L. major and L. tropica infections usually result in cutaneous lesions that remain localized at the site of the sandfly bite. L. (Viannia) braziliensis causes cutaneous leishmaniasis but can also migrate from the site of initial infection to the nasopharyngeal area resulting in highly destructive mucocutaneous leishmaniasis. The Leishmania genome projects are expected to help identify the genetic differences between these parasites which govern the pathology and tropism of infection caused by the different Leishmania species [6–8].

Our laboratory has previously identified the A2 gene family, which is present in Leishmania species that cause visceral infections including L. infantum and L. donovani but are not present in many of the Leishmania species that cause cutaneous infections including L. major and L. tropica [9,10]. A2 proteins have been shown to be essential for visceral infection with L. donovani in BALB/c mice [11,12]. Cross species transfection of the A2 gene from L. donovani into L. major rendered L. major more virulent in visceral organs but less virulent at cutaneous sites, phenotypes typical of L. donovani [12,13]. This demonstrated that species-specific genes can play a role in virulence and the pathology of Leishmania infection and provided the justification for experimentally studying species-specific genes identified through sequencing of the Leishmania genomes.

We used the genetic information from the completion of the L. major, L. infantum, and L. braziliensis genomes [6,7] to identify genes that could potentially influence the pathology caused by these Leishmania species. Remarkably, out of more than 8000 genes within the Leishmania genome, only about 25 L. infantum-specific
Author Summary

Parasites of the genus Leishmania cause a variety of human diseases that range from destructive skin lesions caused by L. major to visceral infections of the liver and spleen caused by L. donovani that result in death. The Leishmania genes responsible for these different pathologies are not known. In the present study, we used a comparative genome-based approach to introduce and over-express L. donovani genes in L. major to determine whether this results in increased virulence of L. major in visceral organs of infected mice. Through this approach, a novel gene termed Li1040 was identified that is potentially involved in protein transport and was shown to increase pathogenesis in the visceral organs in mice. The Li1040 gene may therefore represent a Leishmania virulence gene that has the potential to regulate the pathology of infection in the mammalian host. These observations help to define how Leishmania causes fatal infections in humans and therefore provide a parasite-specific target for therapy.

Materials and Methods

Comparison of Leishmania infantum and Leishmania major genomes

The detailed comparison of three sequenced Leishmania genomes have been described [7]. However, gene-by-gene comparisons for this study were made in the first release of the L. infantum genome sequence (September 17, 2004), following automatic annotation of translational open reading frames via BLAST analysis and comparison with L. major. This first stage analysis has demonstrated high conservation in both gene content and order (synteny) between the L. infantum and L. major genomes. At the beginning of this study, only up to 20 genes have been identified that are either present in L. infantum and absent in L. major or which are present as complete open reading frames in L. infantum but occur as pseudogenes in L. major. In this study, we chose seven of these potential L. infantum specific genes, which we cloned from L. donovani 15/CI2D using primer sequences derived from the corresponding L. infantum genes. This L. donovani strain causes visceral infection in mice when introduced intravenously. The cloned L. donovani genes were subsequently transfected into L. major for cross species transfection studies (Table 1) although one of these, Li1040 was later identified to be also present in L. major and L. braziliensis.

Construction of Leishmania expression vectors containing A2 tag (pLA2tag) or GFP tag (pLGFP)

To detect L. donovani gene products expressed in L. major, we modified the Leishmania expression vector (pLPneo) [14] by adding a 10 amino acid A2 epitope tag or a GFP tag into its multiple cloning site. Briefly, the pLA2tag vector was constructed by inserting an adapter sequence encoding 10 A2 amino acids (QSVGPLSVGP) and a stop codon flanked by BamH I and Not I sites into the multiple cloning sites (BamH I and Not I) of the pLPneo vector. The adapter sequences are: 5’ GATCCGCAGTCCGTCGCGCCGCCTCTCCGTTGCCGCGGTA GC (plus strain) and 5’ GCCGCCTACGGGCCAAC CGGACGCGGCGACGGACTGGCG (minus strain). pLA2tag vector is therefore suitable for expressing fusion protein with A2 tag at its C-terminus. pLGFP vector was constructed by following two steps: 1) a 820 bp Nhe I and Bcl I fragment containing green fluorescent protein (GFP) gene was removed from pEGFP-C3 vector (BD Biosciences Clontech), 2) the 820 bp Nhe I and Bcl I fragment was ligated into the Xba I and BamH I sites of pLPneo vector, generating pLGFP vector. pLGFP is suitable for expressing GFP fusion proteins with GFP at the N terminus.

Table 1. Leishmania Genes Used in This Study.

| L. infantum | L. major | L. braziliensis | Predicted identity | MW kDa |
|------------|----------|----------------|-------------------|-------|
| LinJ08_V3.0560 | none | LbM08_V2.0590 | Cyclopropane fatty acyl phospholipid synthase | 55.5 |
| LinJ36_V3.0640 | none | LbM35_V2.0690 (pseudogene) | Sec14 cytosolic factor (phosphatidylinositol/phosphatidyl choline transfer protein) | 48.5 |
| LinJ15_V3.1370 | none | LbM15_V2.1300 | Hypothetical protein | 40.7 |
| LinJ32_V3.1040 (Li1040) | LmJ32.0985 (Lm0985) | LbM32_V2.1080 | Hypothetical protein with Vps23 core domain of Tsg101 protein | 45.1 |
| LinJ32_V3.1580 | none | LbM32_V2.1680 (pseudogene) | Hypothetical protein | 28.5 |
| LinJ36_V3.4190 | none | LbM35_V2.4235 (pseudogene) | Hypothetical protein | 78.4 |
| LinJ08_V3.0140 | LmJ08.0135 (pseudogene) | LbM08_V2.0140 (pseudogene) | Hypothetical protein with calcium-binding EF-hand domain | 63.4 |

doi:10.1371/journal.pntd.0000248.t001
PCR amplification of *L. donovani* genes using primers based on the *L. infantum* gene sequences

To facilitate cloning of *L. donovani* genes into pLA2tag or pLGFP vector, a restriction enzyme site (HindIII, BamHI or BglII) was added to the 5′ end of PCR primers. The PCR primers for LinJ32_V3.1040 are 5′cccaagcttCAGTTGAGCTGACACTGCACT and 5′cggatccGTGGGGAAACATCACCTTTGAGCCTG and 5′cggatccTACGGG-GAAGACATCATCTTCTGA and 5′cggatccAAAAATCGTGACGCAATCT. For LinJ36_V3.0640 are 5′cccaagcttCTTGCACATGGAACACTGCACT and 5′cggatccCTTGGGCAAGTTCG and 5′cggatccCTTGGGCAAGTTCG and 5′cggatccAAAAATCGTGACGCAATCT. Primers for LinJ08_V3.0560 are 5′cccaagcttGCGATGGGGCGAATCGACTC and 5′cggatccCTTGGGCAAGTTCG and 5′cggatccAAAAATCGTGACGCAATCT. Primers for LinJ36_V3.1580 are 5′cccaagcttACCAAGCATGGAAAACCGGCCA and 5′cggatccCTTGGGGAAACATCACCTTTGAGCCTG and 5′cggatccCTTGGGCAAGTTCG and 5′cggatccAAAAATCGTGACGCAATCT.

Parasite strains and culture conditions

*L. major* Friedlin V9, *L. donovani* IS/CIID strains were used in this study. Promastigotes were routinely cultured at 27°C in M199 medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 40 mM Hepes (pH 7.4), 0.1 mM Adenine, 5 mg l⁻¹ Haemin, 1 mg l⁻¹ Biotin, 1 mg l⁻¹ Biopetide, 50 U ml⁻¹ Penicillin and 50 µg ml⁻¹ Streptomycin. To determine the Li1040 gene transcript levels in different culture conditions, *L. donovani* and *L. major* promastigotes were also shifted to 37°C, pH 5.5 culture media for 6 hours to mimic the macrophage phagolysosome environment associated with the amastigote stage. Under these conditions, *L. donovani* remains viable and is induced to differentiate into amastigotes, whereas *L. major* remain viable for this 6 hours period, they remain unable to differentiate into amastigotes [13].

Transfection procedure

The procedure for transfection was as previously described [14]. Briefly, 10–20 µg of plasmid DNA was used in each transfection. After electroporation, the *Leishmania* promastigotes were transferred into a drug-free culture medium and the following day, G418 was added to make the final concentration of G418 100 µg ml⁻¹. To avoid selection of spontaneous mutants, pooled transfecants were used for all subsequent studies including mice infections, growth in culture, Southern, Northern and Western blot analysis.

In vivo infection and selection for survival in BALB/c mice spleen

Female BALB/c mice weighing 18–20 g were purchased from Charles River Breeding Laboratories and maintained in the animal care facility under pathogen-free conditions. BALB/c mice were infected by tail vein injection with 1×10⁶ stationary-phase promastigotes in 100 µl PBS per mouse [11–13]. Six weeks post infection, the *in vivo* infection-selected amastigotes were isolated from the spleen as described [13]. The isolated amastigotes were transformed into promastigotes in M199 *L. major* culture medium containing 50 µg ml⁻¹ G418. When the G418-resistant culture was established, the culture was subject to Western blot analysis. To subsequently compare the virulence of plasmid transfecants, BALB/c mice were infected by tail vein injection with 1×10⁶ stationary-phase promastigotes in 100 µl of PBS per mouse. The amastigotes were isolated from infected mice after 4, 6, 8 and 10 weeks of visceral infection respectively. The recovered amastigotes were cultured in promastigote culture medium, and the *Leishmania* parasite burdens were determined by limiting dilution. For cutaneous infections, mice were infected subcutaneously with 5×10⁶ stationary-phase promastigotes in their hind footpads. Disease progression was monitored by weekly caliper measurement of footpad swelling.

Measurement of in vitro growth rates

The growth curves of *Leishmania* transfecants were measured in 96-well plates. Promastigotes in stationary phase were seeded at a concentration of 4×10⁸ ml⁻¹ into wells containing 200 µl of medium with 50 µg ml⁻¹ G418. Each sample was plated in triplicate. OD₅₇₀ values were measured as previously described [16]. The DNA probes were labelled with [α-32P]-dCTP by random priming.

Immunofluorescence microscopy

Indirect immunofluorescence was performed as described [14,17,18]. Anti-A2 monoclonal antibody C9 hybridoma supernatant without further dilution was used as the primary antibody in the immunofluorescence study.

Results

Selection for *Leishmania* genes that increase survival in visceral organs in BALB/c mice

At the beginning of this study, about 20 genes were identified as present in *L. infantum* and absent or were pseudogenes in the *L. major* genome. Among these genes, only a few encode products whose function could be predicted by sequence similarity searches. To investigate whether these species-specific genes are involved in tropism and pathology of *Leishmania* infection, 7 genes were initially selected for expression in *L. major*. This selection included 4 genes intact only in *L. infantum* and 3 genes intact in *L. donovani* and *L. braziliensis* but absent in *L. major*, and 1 gene which was intact in *L. infantum*, *L. braziliensis*, and *L. major* (Table 1). The single gene (Li1040) that is intact in all species was initially classified as absent in *L. major* but during the course of this study was reclassified as present in all *Leishmania* species. It is likely that the Li1040 gene was initially classified as absent in *L. major* during the assembly of the genome because it is flanked by two identical 384 repeat sequences (Figure 1).

*L. infantum* and *L. donovani* both belong to the *L. donovani* species complex. We thus assumed that genes present in *L. infantum* would be largely identical in *L. donovani* and therefore the corresponding *L. donovani* genes were introduced into *L. major*. We carried out PCR amplification of the *L. infantum* ortholog genes from *L. donovani* IS/CIID genomic DNA and ligated them into a *Leishmania* expression vector, pLPneo [14], that was engineered to encode a 10 amino acid *L. donovani* A2 peptide epitope tag at the C terminus as detailed in Methods (Figure 2A). Since *L. donovani* A2 proteins are absent in *L. major* [8,10,11], inclusion of the 10 amino acid A2 peptide epitope tag enabled detection of the *L. donovani* transgene products expressed in *L. major* using an anti-A2 peptide monoclonal antibody C9 hybridoma supernatant without further dilution as used as the primary antibody in the immunofluorescence study.
monoclonal antibody (Mab). Expression of these L. donovani genes in transfected L. major were determined by Western blot analysis with anti-A2 Mabs and revealed that 6 out of the 7 selected genes expressed the corresponding proteins at the predicted molecular weights (Figure 2B, lanes 1–8). All of the transgenic L. major parasites except the LinJ36_V3.4190 transfectant stably expressed detectable levels of the L. donovani ortholog proteins.

We initially performed an in vivo selection in BALB/c mice to determine whether any of the transgenic L. major parasites were better adapted for survival in visceral organs. The transgenic L. major parasites including the vector control (Neo) were pooled, and injected into the tail vein of BALB/c mice. Amastigotes were recovered from the spleens of infected mice 6 weeks following injection, cultured out as promastigotes and subjected to Western blot analysis with anti-A2 epitope tag Mab to determine whether any of the L. donovani transgenes were expressed in the spleen derived parasites. As shown in Figure 2B, lane 9, the transgenic L. major parasites expressing the L. donovani Li1040 ortholog gene were detectable. This suggests that, relative to the other transfectants, the transgenic L. major expressing the L. donovani Li1040 ortholog gene displayed enhanced survival in the spleen.

In addition to the in vivo selection in BALB/c mice, we also performed an in vitro selection in axenic amastigote culture media. The pooled transgenic L. major promastigotes were placed in amastigote culture conditions at pH 5.5 and 37°C for 3 days, conditions that mimic the phagolysosomal compartment of macrophage cells in visceral organs. These culture conditions typically result in loss of viability of L. major, which are unable to adapt and proliferate under these conditions. In contrast, L. donovani differentiate into amastigote like parasites and are able to proliferate under these culture conditions. Following this in vitro selection, some of the transgenic L. major survived and were shifted back to promastigotes culture conditions (pH 7.4, 27°C) to allow them to proliferate. The resulting in vitro selected transgenic L. major was subjected to Western blot analysis with the anti-A2 epitope Mabs to detect expression of the L. donovani transgene products. As shown in Figure 2B lane 10, similar to the in vivo infection selection, the Li1040 protein was the major transgene product detectable in the in vitro selected transgenic L. major. Taken together, the in vivo and in vitro selection protocols resulted in selection for L. major parasites expressing the Li1040 ortholog gene.

Figure 1. Comparison of Li1040 loci in L. infantum and L. major.

Figure 2. L. major transfectants containing L. donovani Li1040 ortholog were selected after in vivo infection and in vitro culture selections. A. Diagram of the pLA2tag vector used to express and detect L. donovani gene products in L. major. B. Expression of L. donovani genes in L. major and in vivo and in vitro selection of L. major transfectants. Individual L. donovani genes were cloned into the expression vector pLA2tag, expressed in L. major and detected by Western blot analysis with anti-A2 epitope tag antibodies (Lanes 1–8). For in vivo selection, the L. major transfectants shown in lanes 1–8 were pooled in equal numbers, injected into the tail vein of BALB/c mice, and 6 weeks later were isolated from the spleen and subjected to Western blot analysis with anti-A2 monoclonal antibodies (Lane 9). For in vitro selection, the L. major transfectants shown in lanes 1–8 were pooled and placed in amastigote growth conditions (pH5.5 and 37°C) for 3 days and then subjected to Western blot analysis with anti-A2 monoclonal antibodies (lane 10). Note: 6 out of these 7 L. donovani genes were expressed in L. major at the expected size indicated by arrows. The L. major transfectants expressing the L. donovani Li1040 ortholog became dominant after both in vivo and in vitro selections (Lanes 9 &10). doi:10.1371/journal.pntd.0000248.g002
The Li1040 ortholog gene increases parasite numbers in visceral organs

The preceding experiments argue that expression of the *L. donovani* Li1040 gene ortholog in *L. major* provided a survival advantage in the spleen of BALB/c mice. It was therefore necessary to directly confirm this by comparing parasite numbers following infection with *L. major* expressing the Li1040 ortholog to the control *L. major* transfectants (Neo) containing the empty vector. Parasite burdens were determined in the liver and spleen after 4, 6, 8 and 10 weeks following infection via the tail vein. Replicate experiments are shown for this analysis to confirm reproducibility since the kinetics and levels of infection can vary considerably between visceral infection experiments. As shown in Figure 3A, expression of the *L. donovani* Li1040 ortholog in *L. major* gave rise to increased parasite numbers in the liver and spleen, which was most prominent at 8 weeks in the liver, and 6 to 10 weeks in the spleen and this was consistent in the duplicate experiments (Figure 3B, C). We also repeated this analysis by over-expressing the Li1040 gene with the A2 epitope tag removed, to rule out the possibility that the 10 amino acid A2 sequence may have been responsible for the increased virulence associated with the expression of the A2 epitope-tagged Li1040. The Li1040 ortholog gene containing no A2-tag was cloned into *Leishmania* expression vector pLPneo and introduced into *L. major*. As shown in Figure 3C, *L. major* transfected with Li1040 gene with no A2-tag displayed similar visceral infection kinetics in BALB/c mice as *L. major* expressing Li1040 containing the A2-tag. This demonstrated that the A2-tag was not responsible for the increased virulence associated with over-expression of the Li1040 protein in *L. major*.

The *L. donovani* Li1040 ortholog gene effect on cutaneous infection and proliferation in culture

Since *L. major* typically causes cutaneous infections, it was necessary to determine whether expression of the *L. donovani* Li1040 ortholog in *L. major* also increased virulence at cutaneous sites or increased proliferation in cultured promastigotes. BALB/c mice were infected subcutaneously in the rear left footpad with *L. major* expressing the Li1040 ortholog and the control vector containing *L. major* and lesion development were measured weekly. As shown in Figure 4A, expression of the *L. donovani* Li1040 ortholog gene in *L. major* did increase the level of cutaneous infection in the footpad although not to the same extent as was...
observed in the liver and spleen. Although the *L. donovani* Li1040 ortholog gene enhanced *L. major* virulence in vivo, it did not provide a growth advantage to promastigotes in culture (Figure 4B).

**Comparison of the *L. infantum* Li1040 ortholog gene in *L. donovani*, *L. major* and *L. braziliensis***

Our original assumption was that *L. infantum*/*L. donovani* specific genes would be the most likely to increase visceral infection when expressed in *L. major*, similar to what we observed previously with the *L. donovani* specific A2 gene [12]. It was therefore unexpected that the *L. donovani* Li1040 ortholog gene, which was also present in *L. major* and *L. braziliensis*, was selected for in the visceral organs of BALB/c mice and increased virulence in the liver, spleen and to a lesser extent in the skin.

The ectopic expression of the *L. donovani* Li1040 ortholog in *L. major* may have increased virulence due to increased levels of expression or to sequence variations resulting in a protein with enhanced function. Sequencing of the *L. donovani* Li1040 ortholog gene revealed that it differed by only 5 nucleotides from the *L. infantum* Li1040 gene and none of these nucleotide changes altered the amino acid sequence (Figure 5). Alignment of the *L. infantum*/*L. donovani* Li1040 protein with the *L. major* and *L. braziliensis* orthologs revealed that Li1040 has 92% identity with the *L. major* ortholog and 82% identity with the *L. braziliensis* ortholog (Figure 5). This is exactly the same percentage (92%) as the genome average amino acid identity between *L. major* and *L. infantum* [5] arguing that there was no selective evolutionary pressure to alter the Li1040 sequence relative to the rest of the genome. The Li1040 ortholog gene is also present in other Kinetoplastids including *Trypanosoma cruzi* (33% identity) and *Trypanosoma brucei* (32% identity) (Figure 5). Interestingly, a Vps23 core domain of the yeast vacuolar protein-sorting protein 23 (Vps23, or Tumor susceptibility gene 101 (Tsg101) in human) was identified between amino acid 267 and 331 of the Li1040 protein (see Figure 5 highlighted sequences). The Vps23/Tsg101 proteins have been shown to be involved in protein sorting from endosomes to lysosomes [19,20] (see more in discussion).

**Endogenous Li1040 ortholog gene expression in *L. donovani* and *L. major***

Considering the above observations, it was necessary to investigate the possibility that the endogenous Li1040 ortholog gene was expressed at higher levels in *L. donovani* than in *L. major*. Total RNA was extracted from wildtype *L. donovani* and *L. major* cultured under promastigote conditions (27°C, pH 7.4) and amastigotes conditions (37°C, pH 5.5) and subjected to Northern blot analysis with the *L. donovani* Li1040 ortholog gene. As shown in Figure 6, the Li1040 ortholog gene was constitutively expressed in *L. donovani* promastigotes and amastigote-like cultures. The expression level of the Li1040 ortholog gene in *L. major* promastigotes was similar to that in *L. donovani* promastigotes. The lower level of expression in the *L. major* amastigotes culture conditions was likely due to reduced viability of some of the *L. major* cells when cultured briefly at 37°C pH 3.5 since the level of the control α-tubulin mRNA was also reduced under these conditions. Compared with the α-tubulin gene, the expression level of Li1040 ortholog gene in both *L. donovani* and *L. major* is low, since only a weak signal was apparent after 2 days of film exposure on the blot and a strong signal was only apparent after 7 days of film exposure. In comparison, a strong signal was apparent for α-tubulin mRNA after less than a day of film exposure with a probe containing the same level of specific radioactivity as the Li1040 ortholog gene probe. Although the level of mRNA is similar, the possibility remains that the Li1040 protein is present in higher levels in *L. donovani* than in *L. major* since the mRNA levels in *Leishmania* generally correlate poorly with the corresponding protein level [21]. Future studies are needed to generate antibodies to the endogenous Li1040 gene product to directly compare protein levels in *L. major* and *L. donovani*.

**Cellular localization of the Li1040 protein in *L. donovani* and *L. major***

Since the sequence analysis of the Li1040 ortholog gene in *L. donovani* and *L. major* showed they are very similar, we sought to determine its cellular localization in these parasites. The *L. donovani* Li1040 ortholog gene was fused with the GFP gene and introduced into *L. donovani* and *L. major*. As shown in Figure 7A, the GFP-Li1040 fusion proteins were expressed at the expected size (791kDa) in *L. donovani* and *L. major*. Fluorescence microscopy revealed that although the GFP-Li1040 ortholog fusion proteins were distributed throughout the cell including the flagella in both *L. major* and *L. donovani* transfectants, the majority of GFP-Li1040 fusion protein appear to be present as cytoplasmic aggregates (Figure 7B). Similar localization of the A2-tagged *L. donovani* Li1040 ortholog protein was shown in transgenic *L. major* using anti-A2 Mabs (Figure 7C). Interestingly, comparable cellular appearance and distribution were reported for the mammalian Tsg101 protein, the potential homolog of *Leishmania* Li1040 [22].
Over expression of the \textit{L. major} Li040 ortholog gene (Lm0985) in \textit{L. major}

The above described experimental analyses suggest that the increased expression level of plasmid derived Li040 may have played a greater role in increasing parasite virulence than the sequence differences between the corresponding \textit{L. donovani} and \textit{L. major} ortholog genes. We attempted to address this issue by over-expressing the \textit{L. major} Lm0985 gene in \textit{L. major}. Lm0985 is the \textit{L. major} ortholog of \textit{L. infantum/donovani} Li1040. As shown in Figure 8A, epitope tagged \textit{L. major} Lm0985 was detectable in Figure 5.
transfected *L. major*. Over-expression of the *L. major* Lm0985 and *L. donovani* Li1040 orthologs in transgenic *L. major* both resulted in increased parasite levels in the liver and spleen at various times following infection (Figure 8B, C). The generally high levels of infection seen with the Li1040 transgenic *L. major* parasites relative to the Lm0985 transgenic parasites could however been due to the higher expression levels of Li1040 relative to Lm0985 (Figure 8A). Taken together these results argue that over-expression of either Lm0985 or Li1040 results in increased parasite virulence. It was also noteworthy that, although there was approximately a 4 fold increase in spleen parasite numbers with the Li1040 gene expressing *L. major* compared to the control Neo *L. major* parasites at 10 weeks following infection (Figure 8C), this difference was less than the previous 3 independent infection experiments shown in Figure 3. This highlights the importance of carrying out multiple repeat independent infection experiments which taken together confirm that over-expression of Li1040 results in increased virulence.

**Discussion**

In the present study we have developed an experimental approach to generate and follow transgenic *L. major* expressing *L. donovani* genes in vivo in BALB/c mice. Rational for this study comes from our previous observations where expression of the *L. donovani* specific A2 gene increased *L. major* survival in visceral organs [12] and we therefore anticipated that additional *L. donovani/L. infantum* specific genes could also increase *L. major* virulence in the visceral organs. *L. major* expressing the *L. donovani* Li1040 ortholog gene was selected for in the spleen of BALB/c mice and further shown to dramatically increase *L. major* parasite numbers in the liver and spleen and to a much lesser extent in the skin. This outcome was somewhat unexpected since the Li1040 ortholog gene was subsequently established to be present in *L. major* and *L. braziliensis* in addition to *L. donovani* and *L. infantum*. This revealed that genes ubiquitously present in different...
Leishmania species could also have a dramatic effect on parasite tropism and virulence.

It is noteworthy that Li1040 expressing transgenic L. major were rapidly selected for in vitro after 3 days when placed under amastigote culture conditions at 37°C and pH 5.5. Overexpression of the Li1040 ortholog however did not enhance promastigotes proliferation in culture at 27°C and pH 7.4 (Figure 4B). This suggests that the Li1040 product enables amastigotes to survive under conditions associated with host macrophage phagolysosomes. This was consistent with the observation that Li1040 ortholog expressing L. major were likewise selected for in vivo after several weeks in the liver and spleen of BALB/c mice (Figure 2). Although these observations suggest that Li1040 plays a greater central role in the amastigote stage, it also appears to be essential for survival as promastigotes since repeated attempts to generate homozygous L. donovani Li1040 gene deletions in promastigotes have so far been unsuccessful.

It was interesting to find that the Li1040 protein contains the Vps23 core domain of the Vps23 and Tsg101 proteins in yeast and human respectively. Vps23 is one of the four protein subunits (Vps23, Vps28, Vps37 and Mvb12) of the yeast ESCRT-I (Endosomal Sorting Complex Required for Transport) complex, which forms the complex driving protein transport from endosomes to lysosomes [19,20]. The core domain of the Vps23 has been shown to be essential for formation of ESCRT-I complex [19,20]. Moreover, the homologs of the three other subunits of ESCRT-I complex (Vps28, Vps37 and Mvb12) also appear to be present in the Leishmania genome (Lin36.V3.5400 encodes a homolog of Vps28, and several potential homologue proteins for Vps37 and Mvb12 are also present in Leishmania, data not shown). Since all 4 ESCRT-I subunits appear to be present in Leishmania, this suggests that, similar to Vps23, Li1040 may also be involved in protein transport. In higher eukaryotes, the Tsg101 (tumour suppressor) appears to be essential for survival as promastigotes since repeated attempts to generate homozygous L. donovani Li1040 gene deletions in promastigotes have so far been unsuccessful.

It is noteworthy that the focus of this study was on genes whose expression was selected for in the parasites isolated from the spleen of BALB/c mice. Although this approach proved successful to identify the ability of the Li1040 gene to increase virulence, this does not rule out the possibility that some of the other species-specific genes can also increase virulence. Since there are relatively few species-specific genes, it may be possible to further study the role of individual genes without performing the in vitro selection used in this study. For example, the cyclopropane fatty acyl phospholipid synthase (CFAS) gene is present in L. infantum and L. braziliensis but not L. major. The CFAS gene in Mycobacterium tuberculosis (Mtb) has been shown to modify cell surface glycolipids, which promotes an inflammatory response and granuloma formation (Table 1) [26]. It would be interesting to determine whether CFAS affects Leishmania surface glycolipid composition and virulence even though it was not enriched for following in vivo selection in mice in this study. The gene encoding Sec14 cytosolic factor is present in L. infantum but is a pseudogene in L. braziliensis and absent in L. major (Table 1) [7]. The Sec14 cytosolic factor has been implicated in the release of secretory vesicles from the transgolgi network [27] and therefore may influence cell-surface molecule expression in L. infantum and affect host-parasite interactions.

These examples and others could be tested individually by transfecting the L. infantum genes into L. major and studying the phenotype of the resulting transgenic parasites. We are currently focusing on additional L. infantum specific genes, which are absent in both L. major and L. braziliensis and introducing these into L. major to assay for changes in virulence as described in this study. The genetic determining factor(s) controlling tropism and virulence could however be widely embedded throughout these different genomes involving a combination of species-specific genes, posttranscriptional regulation and gene polymorphisms. The Li1040 gene identified in this study may be among those playing a major role in virulence and tropism.

Acknowledgments

We wish to thank Deborah Smith for insightful comments during the course of this study.

Author Contributions

Conceived and designed the experiments: WZ GM. Performed the experiments: WZ CP. Analyzed the data: WZ CP. Contributed reagents/materials/analysis tools: CP GM. Wrote the paper: WZ GM.
21. Cohen-Freue G, Holzer TR, Forney JD, McMaster WR (2007) Global gene expression in *Leishmania*. Int J Parasitol 37: 1077–1086.
22. Xie W, Li L, Cohen SN (1998) Cell cycle-dependent subcellular localization of the TSG101 protein and mitotic and nuclear abnormalities associated with TSG101 deficiency. Proc Natl Acad Sci USA 95: 1595–1600.
23. Ruland J, Sirard C, Elia A, MacPherson D, Wakeham A, et al. (2001) p53 accumulation, defective cell proliferation, and early embryonic lethality in mice lacking tsg101. Proc Natl Acad Sci USA 98: 1059–1064.
24. Wagner KU, Krempel A, Qi Y, Park K, Henry MD, et al. (2003) Tsg101 is essential for cell growth, proliferation, and cell survival of embryonic and adult tissues. Mol Cell Biol 23: 150–162.
25. Li L, Cohen SN (1996) Tsg101: a novel tumor susceptibility gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. Cell 85: 319–329.
26. Rao V, Fujiwara N, Porcelli SA, Glickman MS (2005) *Mycobacterium tuberculosis* controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule. J Exp Med 4: 535–543.
27. Bankaitis VA, Malehorn DE, Emr SD, Greene R (1989) The *Saccharomyces cerevisiae* SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. J Cell Biol 108: 1271–1281.