A Trypanosoma brucei Kinesin Heavy Chain Promotes Parasite Growth by Triggering Host Arginase Activity

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Abstract

Background: In order to promote infection, the blood-borne parasite Trypanosoma brucei releases factors that upregulate arginase expression and activity in myeloid cells.

Methodology/Principal findings: By screening a cDNA library of T. brucei with an antibody neutralizing the arginase-inducing activity of parasite released factors, we identified a Kinesin Heavy Chain isoform, termed TbKHC1, as responsible for this effect. Following interaction with mouse myeloid cells, natural or recombinant TbKHC1 triggered SIGN-R1 receptor-dependent induction of IL-10 production, resulting in arginase-1 activation concomitant with reduction of nitric oxide (NO) synthase activity. This TbKHC1 activity was IL-4Rα-independent and did not mirror M2 activation of myeloid cells. As compared to wild-type T. brucei, infection by TbKHC1 KO parasites was characterized by strongly reduced parasitaemia and prolonged host survival time. By treating infected mice with ornithine or with NO synthase inhibitor, we observed that during the first wave of parasitaemia the parasite growth-promoting effect of TbKHC1-mediated arginase activation resulted more from increased polyamine production than from reduction of NO synthesis. In late stage infection, TbKHC1-mediated reduction of NO synthesis appeared to contribute to liver damage linked to shortening of host survival time.

Conclusion: A kinesin heavy chain released by T. brucei induces IL-10 and arginase-1 through SIGN-R1 signaling in myeloid cells, which promotes early trypanosome growth and favors parasite settlement in the host. Moreover, in the late stage of infection, the inhibition of NO synthesis by TbKHC1 contributes to liver pathogenicity.

Introduction

The protozoan flagellate parasite Trypanosoma brucei is responsible for the diseases human sleeping sickness and nagana in cattle. In experimental murine models, the host immune response to this blood-borne pathogen involves antibody production against the Variant Surface Glycoprotein (VSG), as well as interferon-γ (IFN-γ)-mediated activation of macrophages/myeloid cells into cells of the M1 phenotype. These engulf opsonized parasites and synthesize factors that interfere with trypanosome growth including tumor necrosis factor-α (TNF). However, uncontrolled IFN-γ-induced immune responses including TNF and NO production as the infection persists induce tissue pathogenicity and death of the host [1–5]. Induction of IL-10 can attenuate the IFN-γ/M1 response and thereby enables prolonged survival of T. brucei-infected mice [6–8].

Only few T. brucei-derived immunomodulatory factors have been identified. These include the glycosylphosphatidylinositol anchor of VSG, CpG oligodeoxynucleotides and the trypanosome suppressive immunomodulating factor (TSIF) which have been found to induce TNF- and NO-secreting M1 cells [9–12]. Factors...
Author Summary

From the first invasive step onwards, African trypanosomes can efficiently undermine the protective immune response of their mammalian host to favor their survival within the host and successful transmission by its vector, the tsetse fly. Identifying the parasite factors affecting the protective immune response is thus critical to detail the immune evasion mechanisms of these organisms. We report here that during acute infection, a Trypanosoma brucei protein named Kinesin Heavy Chain 1 (TbKHC1) sustains the development of the first (most prominent) peak of parasitaemia in the blood and its control by the host. Mechanistically, TbKHC1 was found to interact with the SIGN-R1 molecule at the surface of immune cells. Hereby, TbKHC1 modulates arginine/NO metabolism in immune cells towards the production by the host of nutrients (polymamines) required for parasite growth via an IL-10-dependent induction of arginase 1 and down-regulation of iNOS activities. Consequently, IL-10/arginase 1 producing myeloid cells from non-infected mice (Fig. 1C). This induction was increased in trypanosomes over-expressing TbKHC1 (Fig. 2A–D), confirming that TbKHC1 is the genuine target of the anti-PRF 2C12 antibody.

Results

A T. brucei kinesin heavy chain induces arginase activity in myeloid cells

T. brucei parasites were found to induce arginase activity in myeloid cells from non-infected mice (Fig. 1A). This induction was maintained when myeloid cells and trypanosomes were separated by a cell-retaining insert, indicating that soluble components from trypanosomes were involved (Fig. 1A). Parasite-released factors (PRF) were prepared under conditions leading to no detectable trypanosome death. PRF induced arginase activity, and this effect was abolished by heat-treatment (Fig. 1A). Monoclonal antibodies raised against T. brucei PRF (2C12) inhibited arginase activity induced by PRF, whereas other antibodies from the same hybridoma fusion (2A3, 2C6, 4B5) or of irrelevant specificity (5F6, 4J5) had no effect (Fig. 1B). The PRF fraction eluted after binding to a 2C12 antibody-based affinity column retained full arginase-inducing activity, confirming that this activity was directly targeted by 2C12 (Fig. 1C).

The 2C12 antibody was used to screen a cDNA expression library and identify the T. brucei arginase-inducing protein. Among the 6 positive clones identified, 5 encoded fragments of the same putative kinesin heavy chain isoform (geneDB accession number Tb927.6.4390), which we termed TbKHC1 for T. brucei kinesin heavy chain 1 (Fig. S1 in Text S1). The protein N-terminal domain contained the highly conserved kinesin motor domain with a typical ATP binding site. The region targeted by 2C12 antibody located downstream from the motor domain and was characterized by a predicted coiled structure (Fig. S1 in Text S1).

TbKHC1 knock-out (KO) parasites were generated from wild-type (WT) T. brucei. In contrast to PRF from WT parasites, PRF from TbKHC1 KO parasites did not trigger arginase activity in myeloid cells from non-infected mice (Fig. 1G).

TbKHC1 partially localizes in the endocytic compartment and is released from the parasite

Plasmodial trypanosomes, differentiating in the bloodstream from proliferative slender forms into quiescent stumpy forms, can induce long-standing infection in mammals and perform cyclical transmission in tsetse flies while differentiating into procyclic forms. In contrast, monomorphic trypanosomes, resulting from prolonged cultivation in vitro, are unable to develop long-lasting infection and cyclical transmission. The TbKHC1 gene was found to be expressed preferentially in slender bloodstream forms (Fig. 2A,B). Parasite immunolabeling with both monoclonal 2C12 and polyclonal antibodies generated against recombinant TbKHC1 (rTbKHC1) vanished in TbKHC1 KO parasites or RNAi-mediated TbKHC1 knock-down (KD) parasites, while it increased in trypanosomes over-expressing TbKHC1 (Fig. 2A–D), confirming that TbKHC1 is the genuine target of the anti-PRF 2C12 antibody.

Both antibodies revealed a scattered protein distribution primarily between the kinetoplast and the nucleus (Fig. 2C,D). TbKHC1 partially co-localized with tomato lectin, which detects linear chains of poly-N-acetyllactosamine typical of the endocytic compartment [16] (Fig. S2A in Text S1). However, TbKHC1 did not bind to tomato lectin in affinity chromatography assay (Fig. S2B in Text S1), suggesting that TbKHC1 is not an endocytic component. Moreover, a fraction of TbKHC1 was reproducibly found in the culture medium of parasites, as revealed by immunoprecipitation of detergent-free supernatants from metabolically 35S-labelled T. brucei (Fig. 2E). In these experiments, trypanosome lysis was unlikely as the supernatants did not contain any evidence of the cytoplasmic markers actin [17] or protein disulfide isomerase 2 [18], but did contain enolase, a protein released from trypanosome-related parasites such as Leishmania [19] (Fig. 2E). TbKHC1 immunoprecipitated from the trypanosome supernatant did not associate with other 35S-labelled proteins (Fig. 2E), as could have been expected in case of release as a complex.

IL-10 and mannose regulate TbKHC1-induced arginase activity

We screened for pathways altering the levels of PRF-induced arginase activity in myeloid cells. Co-incidence with the induction of arginase activity, PRF induced myeloid cells to express the regulatory cytokine IL-10 (Fig. 3A). The arginase activity induced by PRF was inhibited by neutralizing anti-IL-10 antibody and by D-mannose, but not by D-galactose (Fig. 3B).
rTbKHC1 mimicked the arginase-inducing effect of PRF, including its inhibition by 2C12, anti-IL-10R antibodies and D-mannose (Fig. 3C). The increased arginase activity induced by rTbKHC1 associated with increased expression of the *Arg1*, but not *Arg2* gene, as well as impaired expression of the *iNOS* gene (Fig. 3D). Activation of myeloid cells by rTbKHC1 also resulted in increased expression of the *IL-10* gene (Fig. 3D) and protein (Fig. 3C), the latter being inhibited by the 2C12 antibody and D-mannose. Thus, rTbKHC1 interaction with myeloid cells involved a mannose-sensitive event triggering the synthesis of IL-10, resulting in enhanced *Arg1* gene expression and activity, and inhibition of *iNOS* expression.

Although arginase-1 expression is commonly associated with alternatively activated myeloid cell (M2) functions, rTbKHC1 did not trigger *Il4* and *Il13* gene expression in myeloid cells from WT mice (Fig. 3D). Moreover, the induction of *Arg1* and *Il10* genes by rTbKHC1 was conserved in *IL-4Rα* KO mice (Fig. 3E), excluding that IL-10 synergistically enhanced IL-4Rα-induced *Arg1* expression as observed in M2 cells [20,21]. Accordingly, rTbKHC1 did not trigger the expression of M2-associated genes characteristic of African trypanosome infection [22] (Fig. 3F). We also addressed whether the induction of *Arg1* by rTbKHC1 required IL-6 and G-CSF in combination with IL-10 [23]. rTbKHC1 only induced *Il6* gene expression (Fig. 3D). This expression was not affected following addition of anti-IL-10R antibody that inhibited the induction of arginase activity by rTbKHC1 (Fig. 3G), indicating that IL-10 induced by TbKHC1 is the main trigger of *Arg1* expression in myeloid cells.

**Figure 1. Induction of myeloid cell arginase activity by *T. brucei* PRF.** Arginase activity was determined in myeloid cells from non-infected mice incubated with (A) WT parasites separated or not by an insert, or WT parasite-released factors (PRF), (B) PRF from WT parasites in presence of indicated antibodies and (C) PRF from WT or TbKHC1 KO parasites, or material purified on anti-PRF antibody affinity column, in presence of indicated antibodies. Heat treatment was at 45°C for 30 min. Data are means ± SEM of 3–4 individual mice of one representative from 3 independent experiments (A, B), or of triplicate cultures of one representative from 2 independent experiments (C). * p<0.05 compared to non-stimulated (-) cells. doi:10.1371/journal.ppat.1003731.g001
TbKHC1 promotes parasite growth in mice by increasing ornithine availability

In vitro, the parasite growth rate appeared to be identical between WT, TbKHC1 KD, TbKHC1 KO and TbKHC1-rescued parasites obtained by reinsertion of the TbKHC1 gene in one allele of its original locus in the TbKHC1 KO genome (Fig. 4A,B), indicating that TbKHC1 is not required for parasite growth. Moreover, TbKHC1 KO parasites motility or flagellum movement addressed by microscopic examination and sedimentation analysis [24] appeared normal (not shown). TbKHC1 KO parasites were able to perform the full parasite life-cycle, since procyclic transformants could cycle through tsetse flies to produce infective metacyclic forms.

In C57Bl/6 mice, the cumulative parasite load in the first peak of parasitaemia by TbKHC1 KO trypanosomes was reduced by >70% as compared to WT parasites (Fig. 4C). This reduced TbKHC1 KO parasite load was also observed under natural infection conditions in which infected tsetse flies were allowed to feed on mice (Fig. 4D). Reinsertion of TbKHC1 in TbKHC1 KO parasites reverted early parasitaemia to WT levels (Fig. S3 in Text S1).

As compared to infection with WT parasites, at day 7 post-infection (p.i.) with TbKHC1 KO parasites, spleen myeloid cells showed a reduced induction of arginase activity that coincided with increased NO2 accumulation, likely reflecting a shift towards NOS activity (Fig. 4E,F). Moreover, the induction of arginase

Figure 2. Expression and localization of T. brucei TbKHC1. (A) Detection of TbKHC1 transcripts by Northern blotting of RNA from procyclic forms (PF), bloodstream (BF) slender (SL) and stumpy (ST) forms, BF TbKHC1 knock-out (KO), overexpressor (OV) and TbKHC1 knock-down (RNAi) trypanosomes (d: days after doxycyclin induction; ds RNA: double-stranded RNA; histone H2B mRNA: loading control). (B) Detection of TbKHC1 with anti-rTbKHC1 polyclonal antibody in whole extracts from the indicated trypanosome forms (Tub: Tubulin loading control). (C) Parasite staining with anti-PRF monoclonal 2C12 antibody. Nucleus (N) and kinetoplast (K) are stained in blue with 4',6-Diamidino-2-phenylindole (DAPI). (D) Localization of TbKHC1 on WT, KO and OV trypanosomes with anti-rTbKHC1 polyclonal antibody. (E) Immunoprecipitation of TbKHC1, actin, enolase and PDI-2 from 35S-metabolically labelled WT parasite total extracts (1) or supernatants (2).

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activity was not observed in infected mice treated with a neutralizing anti-IL-10R antibody (Fig. 4E). Furthermore, mice infected with \textit{TbKHC1} KO parasites exhibited lower IL-10 serum levels than mice infected with WT parasites (Fig. 4G). The reduction of IL-10 secretion in \textit{TbKHC1} KO-infected mice could account for the higher inhibition of arginase activity observed in the presence of anti-IL-10R antibodies in these mice (Fig. 4E).

In \textit{TbKHC1} KO-infected mice, it is unlikely that the reduction of the first peak of parasitaemia resulted from increased NO production. Indeed, inhibition of NOS activity by N-(G)-nitro-L-arginine methyl ester (L-NAME) or absence of \textit{iNOS} activity in \textit{iNOS} KO mice did not affect \textit{TbKHC1} KO parasitaemia, while it strongly reduced WT parasitaemia (Fig. 5A,B). The lower parasitaemia in \textit{TbKHC1} KO-infected mice might rather result from reduced arginase activity, which would limit nutrient availability for the parasite. Indeed, this enzyme converts L-arginine to L-ornithine, a precursor of polyamines that are required for trypanosome growth [13]. Accordingly, in mice

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**Figure 3. Mechanism of arginase activity induction by \textit{T. brucei} PRF and \textit{rTbKHC1.}** Myeloid cells from non-infected WT (A-D,F, G) or IL-4R\(\alpha\) KO (E) mice were incubated with PRF or \textit{rTbKHC1}. (A) IL-10 production induced by PRF. (B) Arginase activity induced by PRF incubated with indicated antibodies or sugars. (C) Arginase activity and IL-10 secretion induced by \textit{rTbKHC1} incubated with indicated antibodies or sugars. (D–G) Relative expression level of M2 genes after incubation with \textit{rTbKHC1}. In F, M2 IL-10-dependent genes are indicated, and in G, \textit{rTbKHC1} was incubated with the indicated antibodies. Data are means ± SEM of 3 individual mice of one representative from 3 independent experiments. *p<0.05 compared to non-stimulated (-) cells.

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lacking arginase-1 in myeloid cells/macrophages following a cross between Arg1loxP-targeted mice and LysMCre or Tie2Cre deleter mice [21], WT parasitaemia dropped to that of TbKHC1 KO parasitaemia (Fig. 5C,D). Moreover, treatment of mice with L-ornithine increased the cumulative parasite load to a greater extent in TbKHC1 KO- than in WT-infected mice (~94% vs ~43%, respectively; Fig. 5E). In addition, spermine levels tended to increase although without reaching statistical significance in spleen myeloid cells and blood from mice infected with WT but not TbKHC1 KO parasites (Fig. S4A in Text S1). Furthermore, an
Figure 5. Effects of TbKHC1 on T. brucei parasitaemia. WT and TbKHC1 KO parasitaemias were monitored in various mice and conditions: (A) WT mice treated with L-NAME or D-NAME; (B) iNOS KO and WT mice; (C,D) myeloid cell Arg1 KO mice (KO1 = LysMcreArg1lox/lox; KO2 = Tie2creArg1lox/lox) and controls (Arg1lox/lox); (E,F) WT mice treated with L-ornithine, D-mannose or D-galactose; (G) MMR KO and WT mice; (H) SIGN-R1 KO and control (Ctrl) mice. Data are means ± SEM of 4 individual mice of one representative from 3 independent experiments. * p < 0.05 comparing D-NAME and L-NAME treated mice (A) or WT and iNOS KO mice (B) infected with WT parasites; ¥ p < 0.05 comparing Arg1lox/lox and LysMcreArg1lox/lox or Tie2creArg1lox/lox mice infected with WT parasites; # p < 0.05 comparing L-ornithine treated and non treated mice infected with TbKHC1 KO parasites; £ p < 0.05 comparing D-mannose and D-galactose-treated mice infected with WT parasites; £ p < 0.05 comparing SIGN-R1 KO and control mice infected with WT parasites.

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increase in L-ornithine production, coinciding with the consumption of L-arginine and the induction of N-acetylputrescine production, was observed in the supernatant of myeloid cells from non-infected mice activated in vitro with rTbKHC1 (Fig. S4B in Text S1).

The effect of TbKHC1 on parasite growth involves the myeloid cell receptor SIGN-R1
Since the in vitro induction of arginase activity by PRF and rTbKHC1 was inhibited by D-mannose (Fig. 3B,C), we treated infected mice with D-mannose. This treatment reduced WT parasitaemia to the level of TbKHC1 KO parasitaemia, but did not significantly affect TbKHC1 KO parasitaemia (Fig. 5F). To follow up on this in vivo finding and the in vitro observation that arginase activation by PRF and rTbKHC1 was inhibited by D-mannose as well as by an antibody recognizing coils of TbKHC1 (Fig. 1B, 3C), we monitored the course of WT- and TbKHC1 KO-infection in mice deficient for myeloid cell receptors able to bind both mannose and peptidic coils, namely MMR (CD206) and SIGN-R1 (CD209b), using appropriate congenic control mice [25–27]. In MMR KO mice, WT and TbKHC1 KO parasitaemia were not affected as compared to infection in WT mice (Fig. 5G). In SIGN-R1 KO animals the differential control of the first peak of parasitaemia between WT and TbKHC1 KO parasites vanished, due to the reduction of the WT parasite level to that of TbKHC1 KO parasites (Fig. 5H). Thus, either the absence of SIGN-R1 in infected mice or the absence of TbKHC1 in the parasite decreased T. brucei parasitaemia similarly. These data suggested that TbKHC1 interacts with the SIGN-R1 receptor. To evaluate this hypothesis, we tested the in vitro activity of rTbKHC1 on myeloid cells from SIGN-R1 KO mice. In contrast to results obtained with myeloid cells from control mice, in myeloid cells from SIGN-R1 KO mice rTbKHC1 did not stimulate IL-10 and Arg1 gene expression (Fig. 6A) and did not increase arginase activity (Fig. 6B). Conversely, in myeloid cells from MMR KO mice rTbKHC1 induced expression of Il10 and Arg1 genes as in WT mouse myeloid cells (Fig. S5 in Text S1). Therefore, TbKHC1 appeared to trigger arginase activity through SIGN-R1-mediated signaling.

TbKHC1 increases liver pathogenicity and reduces host survival by NO-dependent mechanisms
The survival time of TbKHC1 KO-infected mice was significantly prolonged as compared to WT-infected animals (Fig. S6 in Text S1). Death of mice infected with African trypanosomes is related to systemic inflammatory response syndrome (SIRS) that causes multiple organ failure [28]. Liver myeloid cells are critical to the clearance of trypanosomes from the bloodstream [29], hence maintaining liver integrity is fundamental to the host’s ability to clear trypanosomes. At day 30 p.i. several indicators of liver damage differed between WT- and TbKHC1 KO-infected mice: (i) the serum levels of the liver damage marker alanine aminotransferase (ALT) were lower in TbKHC1 KO- than in WT-infected mice; (ii) anoxic infarcts were observed in none of the TbKHC1 KO-infected mice, but in 6 out of 8 WT-infected mice; (iii) lobular and portal mononuclear cell infiltrates were milder in all TbKHC1 KO- than in WT-infected mice (Table 1, Fig. 6C). Concomitantly, NO production was higher in TbKHC1 KO- than in WT-infected mice, while IL-10 production was not induced and similar in all infected experimental groups (Fig. 6D). A moderate increase in iNOS mRNA expression level was also observed in myeloid cells from mice infected with TbKHC1 KO parasites (4.2±1.5 vs 1.32±0.33 fold in mice infected with WT parasites, n = 3; p = 0.08). These data suggested that increased NO production protected liver integrity in TbKHC1 KO-infected mice. Accordingly, L-NAME treatment or knock-out of iNOS activity in TbKHC1 KO-infected mice reduced the survival time and increased ALT levels to those found during WT parasite infection (Table 1). L-NAME treatment or absence of iNOS activity in WT-infected mice did not affect the survival time, although it reduced the serum levels of ALT (Table 1). Therefore, a threshold of NOS activity, achieved in TbKHC1 KO- but not in WT-infected mice, might be required during T. brucei infection to protect liver integrity, thereby contributing to extended host survival. This hepatoprotective effect of NO was not due to difference in parasite load in mice infected with WT and TbKHC1 KO parasites (Fig. S7 in Text S1).

The survival times and ALT levels in WT and TbKHC1 KO-infected mice were similar in Arg1-deficient mice and control mice (Table 1). However, treatment of WT-infected mice with L-ornithine slightly increased the serum level of ALT, while in TbKHC1 KO-infected mice L-ornithine treatment reduced the survival time and increased ALT levels to those found during WT parasite infection (Table 1). Therefore, L-ornithine as a polyamine precursor favored the development of liver injury and negatively affected the survival of T. brucei-infected mice.

As in the liver, brain injury characteristic of African trypanosomiasis was also lower in TbKHC1 KO- than in WT-infected mice. Indeed, no parasites could be detected in the choroid plexus of TbKHC1 KO-infected mice, sharply contrasting with the presence of numerous parasites, associated with immune infiltrates, in the choroid plexus of WT-infected mice (Fig. S8 in Text S1).

The survival times as well as ALT, NO and IL-10 levels in WT- and TbKHC1 KO-infected mice were similar in SIGN-R1 KO and control mice (Table 1, Fig. 6E), suggesting that in late infection TbKHC1 signaling no longer operates primarily through SIGN-R1.

Discussion
We report that a particular isoform of the large KHC family of T. brucei is released by the trypanosome and influences both parasite growth and host pathogenicity. T. brucei contains 51 sequences encoding kinesin-like proteins. The T. brucei isoform described here appears to be an orphan member without predicted function [30]. That a KHC isoform could be released in the extracellular environment is not unprecedented, since a KHC-like protein has been reported as secreted from the coagulating gland in rat [31]. TbKHC1 did not appear to be contained in high molecular weight complexes released from the parasite, for instance in exosomes as occurs in T. cruzi [32] and Leishmania [33]. Our immunoprecipitation data, rather, suggested that TbKHC1 is free in the medium, but the mechanism of release remains unclear.

TbKHC1 did not appear to be essential for T. brucei cellular proliferation or differentiation.
In vivo, TbKHC1 triggered arginase activity and, in contrast to the other documented T. brucei immunomodulators VSG, DNA or TSIF [9,10,12], inhibited NO synthesis by myeloid cells. These effects involved a region rich in coils in the TbKHC1 C-terminal domain, since they were inhibited by an antibody specifically recognizing the coiled region of TbKHC1.

Arg1 is often recognized as a prototypic M2 myeloid cell marker induced following STAT6 activation by IL-4/IL-13/IL-4Rα signaling. However, Arg1 can be expressed by M1 cells in a STAT3-dependent pathway triggered by IL-10, IL-6 and G-CSF [21,23]. In agreement, rTbKHC1 induced the expression of Il10.
Figure 6. SIGN-R1 receptor contribution to myeloid cell activation and liver injury. (A,B) Effects of rTbKHC1 on myeloid cells from non-infected control (Ctrl) or SIGN-R1 KO mice (A: relative expression of Arg1, Arg2, Il10 and iNOS genes; B: arginase activity in presence of indicated antibodies or sugars). Data are means ± SEM of 3 individual mice of one representative from 3 independent experiments. * p<0.05 compared to non-stimulated (-) cells. (C) Effects of rTbKHC1 on liver injury: microscopic analysis (hematoxylin-eosin staining, magnification 20×) of sections from WT- and TbKHC1 KO-infected mice at day 30 p.i. Anoxic infarcts (ai), periportal (>>) and lobular (>) mononuclear cell infiltrates were representative of 8 animals tested in 2 independent experiments. (D) Spontaneous NO and IL-10 secretions in spleen myeloid cell supernatants from WT- and TbKHC1 KO-infected mice treated with D-NAME or L-NAME (day 30 p.i.). (E) Idem as D in SIGN-R1 KO and control (Ctrl) mice. Data are means ± SEM of 3–4 individual mice of one representative from 3 independent experiments. ▼ p<0.05 comparing WT or TbKHC1 KO- infected mice to non-infected mice; £ p<0.05 comparing L-NAME and D-NAME treatment in WT- or TbKHC1 KO-infected mice; # p<0.05 comparing WT- and TbKHC1 KO-infected mice.

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and Il6 genes in myeloid cells, and this neither induced IL-1Rα
nor the expression of genes associated with M2 activation.
Since Il6 expression was still induced in the presence of the
Arg1 expression inhibitor anti-IL-10R antibody, we concludethe
rTbKHC1-induced Arg1 expression primarily depended on
IL-10 production.

In mice, TbKHC1 activity appeared to promote parasite
growth in the early stage of infection. Reduced parasitaemia
induced by TbKHC1 KO trypanosomes was associated with
increased NO production, but did not directly result from this
increase. Indeed, inhibition of NO production by L-NAME
treatment or in iNOS KO mice did not improve TbKHC1 KO
growth. Rather, in agreement with previous studies [34] L-NAME
treatment or absence of iNOS activity in mice actually increased
the control of WT parasitaemia. Together, these experiments
suggested that the negative/braking effect of NO on the control of
WT parasitaemia requires the expression/activity of TbKHC1.
We propose that TbKHC1-mediated induction of host arginase
activity fuels the polyamine pathway of the host. In bloodstream
forms of T. brucei the arginase gene and activity are absent, but when exogenous L-
ornithine is lacking in body niches these parasites can use
arginase to produce L-ornithine through an arginase-independent
mechanism [35,36]. However, the differential growth-promoting
effect of exogenous L-ornithine during infection by WT or
TbKHC1 KO parasites suggests that this arginase-independent
mechanism is insufficient to provide all the amines necessary for
the polypeptide pathway. Similarly, the related intracellular
parasite Leshmania also requires host arginase induction for
optimal growth, despite the presence of an arginase gene [37].
Therefore, optimal production of L-ornithine/polyamines for
T. brucei growth would rely on host arginase activity induced by
TbKHC1. In accordance, in two models of mice deficient for
arginase-1 in myeloid cells/macrophages, WT T. brucei growth was
reduced to the level observed with TbKHC1 KO parasites.

In T. brucei-infected mice, the TbKHC1-mediated effect on
parasitaemia appeared to involve the myeloid cell receptor SIGN-
R1. Indeed, improved parasite growth control was observed after
removal of TbKHC1 from the parasite or removal of
SIGN-R1 from the host. Moreover, rTbKHC1-mediated induction of
arginase activity was lost in myeloid cells from SIGN-R1 KO
mice. The involvement of SIGN-R1 in TbKHC1-mediated
IL-10 and arginase production is in keeping with previous data
demonstrating the anti-inflammatory role of SIGN-R1 in various
diseases [38–40]. Although LPS and intracellular mycobacteria
infection can induce Arg1 in a MyD88/IL-10 dependent manner
[21,23], we can exclude that TbKHC1 is simultaneously a ligand
for SIGN-R1 and a trigger for MyD88 signaling in our
extracellular infection model since the differential infection
phenotype between WT and TbKHC1 KO T. brucei was conserved in
MyD88 KO mice (data not shown).

D-mannose was found to decrease parasite growth during the
first peak of parasitaemia by WT but not TbKHC1 KO parasites,
and it inhibited rTbKHC1-mediated induction of IL-10 and
arginase by myeloid cells. Similarly, the 2C12 antibody, which is
specific to the coiled region of TbKHC1, inhibited IL-10
production and arginase activity triggered by rTbKHC1. Man-
nose-specific receptors such as SIGN-R1 and MMR are known to
interact with coiled proteins, the complement protein C1q and
collagen respectively [26,27]. Therefore, it is possible that
mannose binding to SIGN-R1 could affect the binding of
TbKHC1 peptidic coils to a second binding site of this receptor.

After the parasite growth-promoting effect occurring during the
first peak of parasitaemia, a distinct effect of TbKHC1 consisted in
reducing the survival time of infected mice while contributing to
liver injury. Given the similar in vitro growth properties between
parasites expressing or not TbKHC1, the mechanism by which
this protein influenced the host survival time is likely to be
independent from differences in parasite virulence. Parasite load
throughout the infection, survival and pathogenicity are independent
traits in experimental and natural infection with African

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**Table 1. Survival and liver pathology in T. brucei WT- and TbKHC1 KO- infected mice.**

| Mice                  | Survival time (days, Mean ± SEM) | # animals tested | ALT (day 30 p.i.) (U/ml, Mean ± SEM) | # animals tested |
|-----------------------|----------------------------------|------------------|--------------------------------------|------------------|
|                       | WT                               | TbKHC1 KO        |                                      |                  |
| D-Name treated        | 42±6.1                           | 79±10           | 573±95                               | 80±69           |
| L-NAME treated        | 45±5.1                           | 46±5.6          | 344±120                              | 216±51          |
| iNOS control          | 42±8.0                           | 83±15           | 618±124                              | 110±69          |
| iNOS KO               | 38±14                            | 42±6.1          | 494±99                               | 545±53          |
| Untreated             | 42±9.0                           | 66±12           | 783±65                               | 94±79           |
| L-Ornithine treated   | 40±6.9                           | 46±3.9          | 649±123                              | 325±45          |
| Arg1 control*         | 35±4.8                           | 74±10           | 808±316                              | 195±177         |
| LysM*Arg1 KO          | 37±4.4                           | 71±13           | 582±142                              | 270±146         |
| Tie2*Arg1 KO*         | 39±7.6                           | 76±9.5          | 1130±295                             | 160±80          |
| MMR control           | 37±2.3                           | 69±18           | 555±234                              | 193±173         |
| MMR KO                | 43±11                            | 59±11           | 558±111                              | 145±114         |
| SIGN-R1 control       | 38±10                            | 81±18           | 1010±300                             | 270±10          |
| SIGN-R1 KO control    | 29±6.0                           | 95±12           | 745±187                              | 360±50          |

*Arg1lox/lox mice;  
LysM*Arg1lox/lox mice;  
Tie2*Arg1lox/lox mice.  
*p<0.05 comparing TbKHC1 KO- and WT- infected mice;  
#p<0.05 comparing treated and control treated infected mice.

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trypanosomes [2,4,6–8,28]. Therefore, the survival time difference between WT- and \(TbKHC1\) KO-infected mice is primarily due to differences in tissue damage inflicted by inflammatory immune responses, rather than differences of parasite load in the first peak of parasitaemia. NO contributes to peripheral pathogenicity induced by African trypanosomes, while IL-10 exerts counter-pathogenic activity [2,3]. In late stages of infection, IL-10 production was unaffected by the absence of TbKHC1, while NO production (and iNOS expression) was lower when TbKHC1 was present. Moreover, the reduction of liver injury in \(TbKHC1\) KO-infected mice was erased by inhibition of NO synthesis upon L-NAME treatment or by infecting \(iNOS\) KO mice. Thus, TbKHC1-mediated depletion of NO might contribute to the liver damage associated with WT parasite infection. Accordingly, NO plays a pivotal role in vascular tone, and decreased NO bioavailablity contributes to pathogenesis in chronic infectious diseases like malaria, and in liver ischemia reperfusion injury [41,42]. Our observation that after the first peak of parasitaemia parasite load was similar in WT- and \(TbKHC1\) KO-infected mice, even upon inhibition of NO production, supports the notion that blood parasite load is not a major contributor to liver pathogenicity.

Besides NO, polyamines contribute to the pathogenicity of \(T. brucei\) infection [43]. Since our data suggest that induction of arginase activity by TbKHC1 could sustain the production of polyamines, these molecules could contribute to increase liver injury in WT- as well as in \(TbKHC1\) KO-infected mice fed with L-ornithine.

In the late stage of infection, the effects of TbKHC1 did not appear to depend on SIGN-R1 any longer, since the absence of SIGN-R1 did not affect the survival time and liver injury in WT- and \(TbKHC1\) KO-infected mice. A major difference between early and late infection is the evolution of the immune response driven by the continuous release of immunomodulatory/inflammatory factors by the parasite, such as VSG and TSIF, which induce pathogenic TNF-\(\alpha\) and IFN-\(\gamma\) [1,4,9] while switching off the IL-10 production [7]. The evolving context of host-parasite interaction could affect TbKHC1 signaling, and indeed the myeloid cell activation state and corresponding expression of receptors is known to vary during the time course of \(T. brucei\) infection [44]. Therefore, we cannot exclude that in infected SIGN-R1 KO mice, compensation by other myeloid cell receptors influences the immune response and outcome of pathogenicity in the late stage of the disease.

In conclusion, we have identified an unexpected function for a KHC of extracellular \(T. brucei\), which is SIGN-R1-mediated activation of myeloid cells to produce IL-10 leading to increase in arginase activity, resulting in promotion of parasitic growth in the host.

**Methods**

**Ethic statement**

Experiments, maintenance and care of mice complied with guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS n°123) and were approved by the Ethical Committee for Animal Experiments of the Université Libre de Bruxelles, Brussels, Belgium (laboratory accreditation number LA2500402).

**Mice, trypanosomes and infection protocol**

WT C57Bl/6 mice were from Harlan, \(iNOS\) KO C57Bl/6 mice from Jackson Laboratory. \(MMIR\) KO, \(IL4Rx\) KO, control \(Ag^\text{pro/wo}\), \(L_gM^\text{Agr}^+/\text{lox}\) and \(Tcz2^\text{Agt}^+/\text{lox}\) mice (C57BL/6 background), SIGN-R1 KO and congenic control mice (BALB/c background) were bred in-house.

**T. brucei bloodstream monomorphic “single-marker”** [45] and pleomorphic AnTat 1.1E (ETATRO1125 strain) were used. Mice were infected i.p. with 2,000 AnTat 1.1E parasites [6]. Parasitaemia was monitored by tail blood puncture. When required, mice were injected i.p. 2 h before infection with 2 mg L-NAME, then 1 mg/ml L-NAME was added to drinking water. The less active enantiomer D-NAME was used as control. For L-ornithine treatment, mice received 2 mg i.p. 2 h before infection, and then were given 5 mg/ml in drinking water. For D-mannose or D-galactose treatment, mice received 50 mg i.p. 2 h before infection, then every 2 days. Sugar treatment caused massive inflammation and the animals were euthanized 12 days p.i. To neutralise IL-10 activity, mice were treated once with 200 mg control antibody (rat IgG1, BD Biosciences) or 1BL.3a anti-IL-10R antibody at day 6 p.i. Injections of anti-IL-10R antibody at day 2, 4 and 6 p.i. killed WT- and \(TbKHC1\) KO-infected mice within 48 h, as reported [6].

**Parasite released factors, antibodies and PRF immunoaffinity column**

PRF was prepared as described [15]. Monoclonal antibodies were obtained following an immunoenzymatic screening that selected antibodies directed against PRF, followed by a test based on inhibition of arginase activity induction. \(\text{rTbKHC1}\) C-terminal fragment was used for production of rabbit polyclonal antibodies. PRF was incubated overnight with 2CG1 monoclonal antibody coupled to Sepharose 4B CNBr resin at 4°C in carbonate buffer (\(\nu/\nu\)). After loading on a column and washings, bound material was eluted with glycine-HCl (pH 3), then glycine-NaOH (pH 10), dialysed for 24 h against milliQ water, lyophilised and solubilized at 1 mg/ml in 50 mM phosphate buffer, 1 M NaCl (pH 6.2). The residual level of endotoxins was below 2 U/\(\mu\)g (LAL assay, Biowhittaker).

**cDNA expression library screening**

Approximately 10^9 plaques from a bloodstream \(T. b. gambiense\) LiTaT1.3 cDNA expression library (lambda ZAP express, Stratagene) were screened by incubation with the mouse 2CG1 monoclonal antibody. Clones targeted by an anti-mouse antibody/alkaline phosphatase conjugate were identified by incubation at pH 9.5 with the chromogenic substrate NBT/BCIP. Reactive clones were plaque purified by 2 additional rounds of screening. The positive clones were processed to obtain the circular pBK CMV plasmid version of the phage, according to the manufacturer instructions.

**TbKHC1 KD and KO parasites**

For \(TbKHC1\) KD, a 500 bp fragment from the \(TbKHC1\) open reading frame (ORF) was amplified by polymerase chain reaction (PCR) using the KD primers (Table S1 in Text S1), digested with \(Xho\)I and \(Ban\)HI and ligated into the p2T7-177TITA plasmid [46]. The vector was linearized with \(Nod\)I and transfected into multimorphic bloodstream forms. Stable transformants were selected with 1 \(\mu\)g/ml hygromycin. Double stranded RNA production was induced by addition of 1 \(\mu\)g/ml doxycycline. For \(TbKHC1\) KO parasites, both alleles of the complete \(TbKHC1\) ORF were replaced in procyclic forms by genes encoding resistance to neomycin and bleomycin, following transfection of these genes flanked by 75 bp sequences from the \(TbKHC1\) UTRs. Primers used for PCR amplification of the resistance genes were KOneo
and KObleo (Table S1 in Text S1). Stable transformants were selected with 15 μg/ml genetin and 2.5 μg/ml phleomycin. Pleomorphic parasites as described [47].

Rescue of TbKHC1 KO parasites
TbKHC1 gene and UTRs were PCR-amplified (Rescue primers, Table S1 in Text S1) and cloned into pCR-XL-TOPO vector (Invitrogen). The neomycin resistance cassette was extracted from pLew11thyg5" [45] with ApaI and Sst restriction enzymes and cloned into the ApaI and EcoRV digestion of the pCR-XL-TOPO-TbKHC1 clones presenting the 5’ sequence of TbKHC1 gene next to the pUC origin. pCR-XL-TOPO-TbKHC1-Neo was linearized at AvaII and transfected in vivo into TbKHC1 KO pleomorphic parasites as described [47].

TbKHC1 overexpressing trypanosomes
TbKHC1 ORF was amplified by PCR from the initial pBK-CMV plasmid clone 4 (primers OV, Table S1 in Text S1) digested with EcoRV and HindIII, and cloned in the pTSArib vector [48]. The construct was linearized with BglII before transfection.

rTbKHC1

The full length TbKHC1 and a 2,253 bp TbKHC1 fragment were amplified by PCR from the initial pBK-CMV plasmid clone 4 using primers FL and C-term (Table S1 in Text S1). The products were digested with Sall and NotI and cloned in-frame in pET 21d (Novagen). rTbKHC1 was extracted from IPTG-induced E. coli and purified on Ni-NTA agarose (Qiagen) using 60% isopropanol and additionally purified by HPLC Superdex 200 (10/300 GL, Pharmacia) in 50 mM phosphate buffer, 1 M NaCl (pH 6.2).

The residual level of endotoxins was below 2 U/μg (LAL assay, BioWhittaker).

Immunofluorescence assay
Fixed parasites (4% formaldehyde in PBS, 10 min, 20°C) spread on poly(L-lysine)-coated slides were permeabilized (0.1% Triton X-100 in Tris-buffered saline 5 min, 20°C) and incubated with 5% BSA. TbKHC1 was detected with the 2C12 monoclonal (dilution 1/200) and Alexa 480-conjugated anti-mouse or anti-rabbit IgG (dilution 1/1000).

Metabolic labelling
Washed trypanosomes were incubated 15 min at 37°C at a density of 2×10^7 cells/ml in IMDM methionine-free medium supplemented with 10% FCS, 0.07 mM L-arginine, 0.2 mM L-glutamine, 0.04 mM L-leucine, 0.1 μM L-arginine, 0.05 mM D-glucose, 30 mM Heps, 1 mg/ml BSA, 0.5 mM adenosine and 5 μg/ml catalase. Then, a mix of 35S-labelled methionine and cysteine was added (100 μCi/ml). After 1 h labelling, cells were washed and incubated for 2 h at 37°C as a density of 2×10^7/ml.

Trypanosomes were centrifuged and lysed in 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% NP40, complete protease inhibitors (Roche).

Tomato lectin chromatography
Fractionation of trypanosome extracts on tomato lectin affinity column was performed as described [16].

Preparation of myeloid cells
Non elicited peritoneal cells were collected in 0.34 M sucrose. Spleen homogenates were incubated 10 min in cold 0.83% NH4Cl/0.01 M Tris-HCl, pH 7.2 to lyse erythrocytes. Peritoneal and spleen cells were washed twice in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 5×10^-5 M 2-mercaptoethanol, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM non essential amino acids; all from Invitrogen Life Technologies) and counted by trypan blue exclusion. To prepare myeloid cells, peritoneal cells adjusted to 5×10^6 cells/ml in complete medium were dispensed by 1 ml in 6-well plates and spleen cells adjusted to 10^6 cells/ml were dispensed by 10 ml in 10-cm tissue culture dishes. After 3 h at 37°C in 5% CO2, cells were washed five times with warm complete medium to remove non adherent cells. Adherent cells were collected in cold complete medium using a scraper, washed and viability was determined by trypan blue exclusion (usually >95%). Cells (CD11b+ for >90% as determined by flow cytometry, data not shown) were suspended in complete medium at 10^5 cells/ml.

In vitro culture of myeloid cells
Peritoneal myeloid cells from non infected mice (5×10^7/ml) were cultured in 24-well plates in presence of 5×10^7/ml trypanosomes, 80 μg/ml PRF, 3 μg/ml chetate of anti-PRF antibody affinity column or 3 μg/ml rTbKHC1. When required, antibodies (monoclonal anti-PRF, JESS-2A5 anti-IL-10 (BD Biosciences), 1B.5a anti-IL-10R or appropriate isotype-matched antibodies (3 μg/ml), D-mannose or D-galactose (50 mM) were added to PRF- or rTbKHC1-stimulated cells. Unless specified, gene expression, arginase activity and NO2 and IL-10 secretions were determined after 24 h of culture. Alternatively, spleen myeloid cells from infected mice (10^6/ml) were cultured at day 7 or 30 p.i. and stored for arginase activity determination or cultured for 24 h before determining spontaneous NO2 and IL-10 production in culture supernatants.

Myeloid cell metabolite extraction, HPLC-MS analysis and data processing
Spleen myeloid cells prepared as described above were cultured in modified complete medium (2% FCS, no 2-mercaptoethanol, no antibiotic) in 24-well plates in presence of 3 μg/ml rTbKHC1.

For data processing, LC-MS raw data were processed using IDEOM software (version 17) [50] which is a composite of XCMS [51], mzMatch [52], R and Microsoft Excel packages. Firstly, XCMS detected peaks with a peak width between 10 and 100 seconds; signal to noise ratio of 3 and mass deviation between scans of 2 parts per million (ppm). MzMatch then matched the peaks between the two samples, comparing their retention times.
(RT) and ion densities before calculating any deviations. Peaks were identified according to their mass-to-charge ratio and RT.

Gene expression analysis

Gene expression analysis determined in blood serum and myeloid cell homogenates. Spermine levels (Spermine ELISA Kit, Antibodies-online) in accordance to the manufacturers’ protocols. Reaction. IL-10 was quantified with a specific sandwich ELISA quantification (reflecting NO production) was assayed by a Griess reaction. In activation of L-arginine, the Kinetoplastida. Annu Rev Microbiol 46: 695–729.

Cumulative parasitemia load, statistical analysis

GraphPad Prism 4.0 software was used to determine the cumulative parasitemia load (Area Under Curve function) and the statistical significance (Two-way ANOVA).

Author Contributions

Conceived and designed the experiments: GDM AB EP PV. Performed the experiments: GDM AB SD LL PU YM JVDA GC MH PH SS PC BS. Contributed reagents/materials/analysis tools: JVDA JLB ANJM LB. Wrote the paper: AB EP GDM PV.

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Supporting Information

Text S1 Contains Table S1 (Primers used to generate genetically modified parasites) and Table S2 (Primers used for gene expression analysis in myeloid cells) as supplement to Experimental procedures. The Text S1 contains also Fig. S1 (Schematic primary structure of T. brucei TbKHC1), Fig. S2 (Localization of T. brucei TbKHC1), Fig. S3 (Characterization and growth pattern of TbKHC1-rescued T. brucei), Fig. S4 (Effect of T. brucei infection on metabolites involved in L-arginine pathway), Fig. S5 (Effect of rTbKHC1 on Arg1 and Arg10 gene expression in MMR KO mice), Fig. S6 (Effect of TbKHC1 on the survival of T. brucei infected mice), Fig. S7 (Effects of absence of iNOS gene activity on T. brucei parasitemia) and Fig. S8 (Effects of TbKHC1 on cerebral injury in T. brucei infected mice), as well as their legends. (DOCX)

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