A Haptoglobin-Hemoglobin Receptor Conveys Innate Immunity to Trypanosoma brucei in Humans

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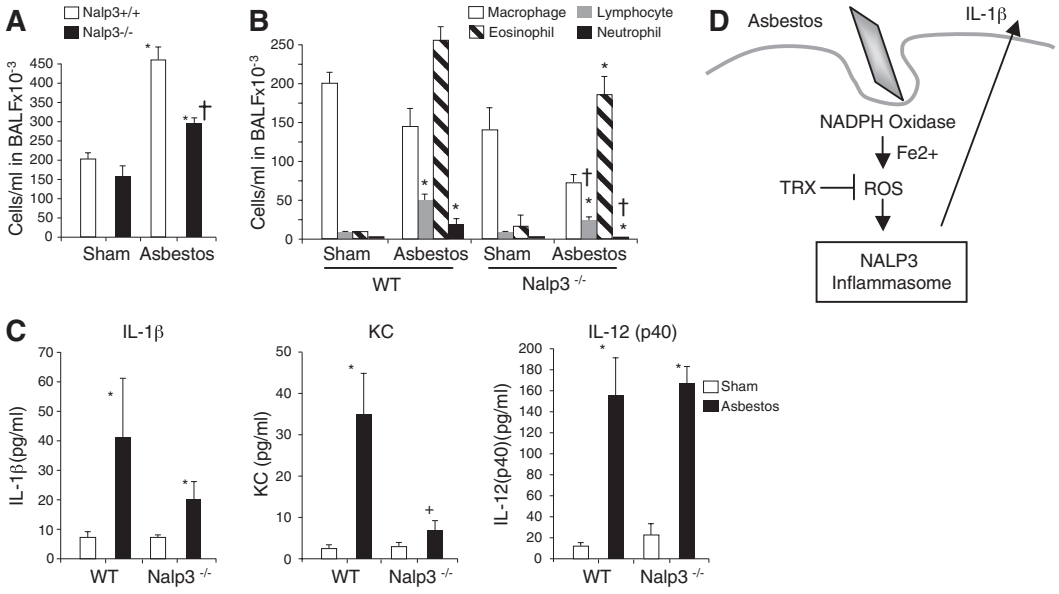

Fig. 4. In vivo inhalation of asbestos results in reduced pulmonary inflammation in Nalp3-deficient mice. (**A**) Total and (**B**) differential cell counts in BALF in 9-day sham- and asbestos-exposed groups. (**C**) Cytokine and/or chemokine levels were measured in BALF of 9-day asbestos-exposed mice. Values are means ± SEM. *Significantly different from sham of same genotype. †Significantly different from wild type. (**D**) A proposed model for asbestos-induced inflammasome activation. See text for details.

(IL-1ra) reduces the proportion of damaged lung (*25*). Silicosis and asbestosis continue to be a common cause of chronic lung disease, despite evidence that they can be prevented by environmental dust control.

Anakinra (IL-1ra) is efficiently used in the clinical treatment of autoinflammatory syndromes (*26*), as well as for gout patients (*27*). The present study suggests that Anakinra may be used to slow down progression of asbestosis, silicosis, and possibly other inflammatory lung diseases.

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A Haptoglobin-Hemoglobin Receptor Conveys Innate Immunity to *Trypanosoma brucei* in Humans

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The protozoan parasite *Trypanosoma brucei* is lysed by apolipoprotein L-I, a component of human high-density lipoprotein (HDL) particles that are also characterized by the presence of haptoglobin-related protein. We report that this process is mediated by a parasite glycoprotein receptor, which binds the haptoglobin-hemoglobin complex with high affinity for the uptake and incorporation of heme into intracellular hemoproteins. In mice, this receptor was required for optimal parasite growth and the resistance of parasites to the oxidative burst by host macrophages. In humans, the trypanosome receptor also recognized the complex between hemoglobin and haptoglobin-related protein, which explains its ability to capture trypanolytic HDLs. Thus, in humans the presence of haptoglobin-related protein has diverted the function of the trypanosome haptoglobin-hemoglobin receptor to elicit innate host immunity against the parasite.

Parasites need to evade both the innate and acquired immunity of their hosts, and this process results from continuous evolution of mutual self-defense mechanisms. Such is the case of human resistance and sensitivity to different subspecies of the African trypanosome *Trypanosoma brucei*. Apolipoprotein L-I (apoL1) is a primate-specific serum apolipoprotein that mainly exists bound to a subset of high-density lipoprotein (HDL) particles that also contain

haptoglobin-related protein (Hpr) (*1*–*3*). ApoL1 protects humans against infection by *T. brucei*, with the exception of subspecies that cause sleeping sickness (*T. brucei* subsp. *rhodesiense* and *T. brucei* subsp. *gambiense*) (*4*, *5*). Trypanosome lysis results from the routing of apoL1 into the lysosome (*4*–*7*), where this protein triggers uncontrolled vacuole swelling due to its anion-selective pore-forming activity in the lysosomal membrane (*7*, *8*). ApoL1 uptake by the parasite is mainly mediated by the Hpr component of the carrier HDL particles termed trypanosome lytic factor 1 (TLF-1) (*9*, *10*). In addition, a minor apoL1 fraction present in distinct Hpr-containing complexes termed TLF-2 enters trypanosomes independently of Hpr (*11*, *12*). Hpr is a primate-specific protein sharing 91% sequence identity with haptoglobin (Hp) (*13*). Both proteins bind hemoglobin (Hb) with high affinity (*14*). However, although Hp-Hb complexes are specifically recognized by the monocyte/macrophage-specific scavenger receptor CD163 for efficient clearance of Hb from blood, Hpr-Hb complexes do not bind to this receptor (*14*, *15*).

Hp has been shown to compete equally as well as Hpr for the uptake of TLF-1 by *T. brucei*, suggesting that the parasite receptor for these particles is unable to discriminate between Hpr and Hp (*9*, *10*). Accordingly, in the present study we observed that both Hp and Hpr were taken up by the parasite, although this required Hb. Conversely, Hb was internalized only when present together with Hp or Hpr (Fig. 1A). To identify the *T. brucei* receptor for Hp-Hb/TLF-1, trypanosome extracts were submitted to affinity chromatography on resins containing immobilized Hp-Hb complexes (*16*). Analysis by mass spectrometry of specifically bound proteins reproducibly revealed the product of gene *Tb927.6.440*, a putative glycosyl-phosphatidyl inositol (GPI)–anchored

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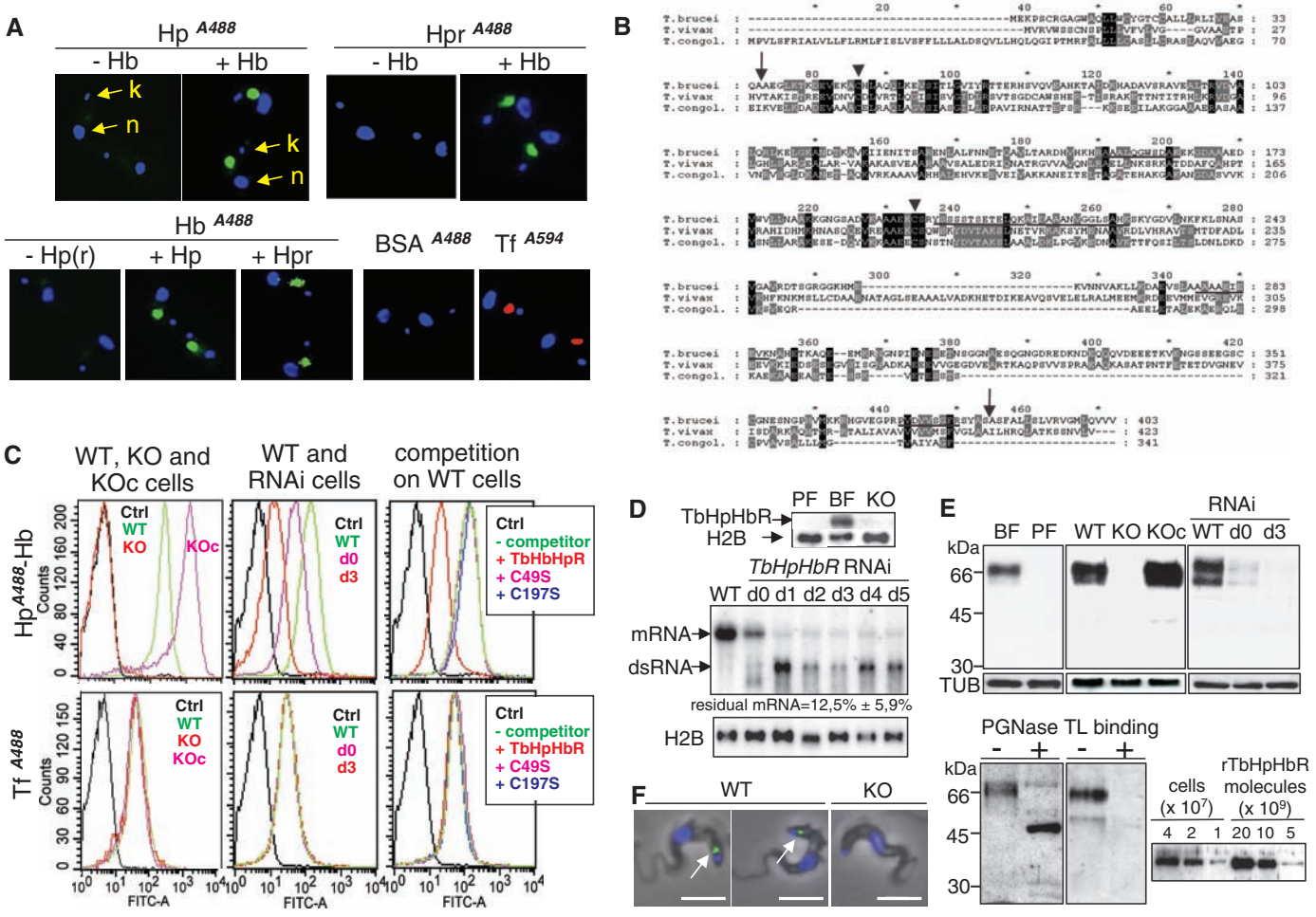

Fig. 1. Identification of TbHpHbR. (**A**) Lysosomal accumulation of Alexa Fluor 488–conjugated Hp, Hpr, Hb, bovine serum albumin (BSA), and Alexa Fluor 594–conjugated Tf, incubated individually or with nonlabeled binding partners in the presence of the protease inhibitor FMK-024. BSA and Tf are respectively negative and positive controls for specific uptake. The blue dots represent 4′,6′-diamidino-2-phenylindole–stained DNA (k, kinetoplast; n, nucleus). (**B**) Alignment of amino acid sequences of Tb927.6.440 and homologs in *T. vivax* and *T. congolense*. Arrows indicate predicted cleavages of N- and C-terminal signal peptides in *T. brucei*. Arrowheads point to conserved cysteines. The underlined peptides were identified by mass spectrometry. (**C**) Flow cytometry analysis of trypanosomes incubated or not (Ctrl) with Alexa Fluor 488–conjugated Hp-Hb or Tf, in the presence or not of a fivefold excess of recombinant TbHpHbR or TbHpHbR mutants (C49S and C197S). RNAi was with or without the addition of doxycyclin (Dox) for 3 days. (**D**) Detection of *TbHpHbR* transcripts from procyclic forms (PF), bloodstream forms (BF), and KO and RNAi cells. dsRNA, double-stranded RNA; d, days after Dox induction; histone H2B mRNA was the loading control. (**E**) Immunodetection of TbHpHbR in Hp-Hb binding extracts from various trypanosome lines. Tubulin (TUB) was the Hp-Hb column-loading control. Extracts from WT BFs were treated (+) or not (–) with peptide-*N*-glycosidase F (PNGase). TbHpHbR was also immunodetected in WT BF extracts bound to tomato lectin (TL) after chromatography with (+) or without (–) competing chito-oligosaccharides. (Bottom right) Comparative TbHpHbR immunodetection in deglycosylated extracts and reference recombinant protein amounts. (**F**) Localization of TbHpHbR by immunofluorescence of WT and KO BFs. Arrows highlight the fluorescent spots. Scale bar, ~10 μm.

surface protein that displays no homology with sequences of known function (Fig. 1B). In *T. brucei* subsp. *brucei*, the single gene *Tb927.6.440* was located at the end of a polycistronic transcription unit on chromosome 6 (fig. S1). It was also present in *T. brucei* subsp. *gambiense* and *T. brucei* subsp. *rhodesiense* but absent from the related kinetoplastids *T. cruzi* and *Leishmania*. In *T. vivax* and *T. congolense*, putative GPI-anchored proteins showed significant similarity [expectation values = 9.7×10^{-10} and 4.9×10^{-16}, respectively] (Fig. 1B). Thus, Tb927.6.440-like sequences were present only in kinetoplastids that exhibited entire development in the bloodstream of their hosts. The involvement of Tb927.6.440 in the binding and uptake of the Hp-Hb complex was next examined using gene knockdown via RNA interference (RNAi), gene knockout (KO), and competition with excess recombinant protein (Fig. 1, C to F, and fig. S2). Under each of these conditions, the uptake of Hp-Hb was inhibited, whereas that of the control protein transferrin (Tf) was not affected (Fig. 1C and fig. S3). Moreover, reintroduction of *Tb927.6.440* by transfection into KO cells [KO complemented (KOc)] restored the uptake of Hp-Hb (Fig. 1C and fig. S3). Thus, experimental evidence suggested that Tb927.6.440 represented the *T. brucei* Hp-Hb receptor (TbHpHbR).

Expression analysis demonstrated that TbHpHbR is a bloodstream stage-specific protein present at 200 to 400 copies per cell (Fig. 1, D and E). The apparent molecular weight (72 kD) was higher than predicted (43.3 kD) because of N-glycosylation (Fig. 1E). The binding of TbHpHbR to tomato lectin (Fig. 1E) revealed the presence of linear chains of poly-*N*-acetyllactosamine, a hallmark of proteins from the endocytic pathway of *T. brucei* (*17*). As was observed for other *T. brucei* receptors (*18*), TbHpHbR localized close to the kinetoplast, in the flagellar pocket region (Fig. 1F).

We next examined the binding parameters of TbHpHbR using surface plasmon resonance (Fig. 2, A to C, and fig. S4). TbHpHbR demonstrated binding properties similar to those of the human functional analog CD163 because it recognized the Hp-Hb complex with high affinity [dissociation constant (K_d) ~ 13×10^{-9} M] but none of the proteins individually. Unlike CD163, however, TbHpHbR bound Hpr-Hb as well as Hp-Hb ($K_d \sim 17 \times 10^{-9}$ M) (Fig. 2A), and the two receptors responded differently to changes in pH and Ca^{++} (Fig. 2B). It seemed likely that the ligand-binding site of TbHpHbR was dependent on a disulfide bridge, because the replacement of either of two conserved cysteines (residues 49 and 197, Fig. 1B) by serine abolished the binding (Fig. 1C). The binding properties of CD163 and TbHpHbR were similar but not identical, because binding of Hp-Hb to CD163 was inhibited by excess TbHpHbR but not to the same extent as seen for the extracellular (soluble) domain of CD163 (Fig. 2C). None of the two TbHpHbR mutants significantly competed in these experiments (Fig. 2C). In vivo, Hp-Hb appeared to be the only essential ligand of TbHpHbR because in $Hp^{-/-}$ mice the growth rates of TbHpHbR KO and wild-type (WT) trypanosomes were similar, whereas in $Hp^{+/+}$ animals, KO parasites grew significantly slower than WTs (Fig. 2D).

Recombinant TbHpHbR, but not the C49S (*19*) mutant, was able to bind TLF-1 in affinity binding assays (*16*) (Fig. 3A). Furthermore, loss of TbHpHbR after RNAi or gene KO (Fig. 1, D to F, and fig. S2) conferred resistance to human HDL-mediated lysis. The lytic activity of normal human serum (NHS) on these parasites was decreased about 200-fold and was comparable to that observed on WT parasites when NHS saturated with competing Hp was used (Fig. 3B). Accordingly, KO parasites were refractory to lysis by TLF-1, but not to lysis by TLF-2, for which Hpr is not involved in uptake (Fig. 3C). In contrast, overexpression of TbHpHbR in KOc cells (Fig. 1, C and E) led to increased sensitivity to NHS (Fig. 3B). Finally, the role of TbHpHbR in TLF-1–mediated lysis was also confirmed by competition experiments in which TLF-1 activity was blocked equally well by excess recombinant TbHpHbR or Hb complexes with recombinant Hp or Hpr but not by Hp or Hpr alone (Fig. 3D).

Trypanosomes seemed to require Hp-Hb because growth rates were reduced in $Hp^{-/-}$ mice (Fig. 2D) or in anhaptoglobinemic [$Hp(r)^{-/-}$] human serum (*10*, *20*), but were restored to normal by addition of exogenous Hp-Hb (Fig. 4A). *TbHpHbR* RNAi or KO cells also grew slower than WT or KOc cells (Figs. 2D and 4A). The growth-promoting effect of Hp-Hb was probably not linked to Hb-derived iron uptake because in *T. brucei* iron is internalized through a specific iron-dependent Tf receptor (*18*), and Tf uptake was not up-regulated in the absence of the Hp-Hb receptor (Fig. 1C). Although African trypanosomes are deficient in heme biosynthesis (*21*), *T. brucei* bloodstream forms contain hemoproteins, such as cytochromes P450 and b5 (*22*–*26*). Therefore, Hb uptake could satisfy the heme requirement of the parasite. Uptake studies of Hp-Hb, either ^{125}I-labeled or containing ^{14}C-labeled heme, performed in WT, KO, and TbHpHbR overexpressor KOc trypanosomes in the presence or absence of the lysosomal protease inhibitor FMK-024, indicated that TbHpHbR allows the intracellular accumulation of heme, whereas the protein carrier undergoes fast degradation (Fig. 4B). Heme accumulation saturated at around 6.5 ng/mg of protein and remained durably cell-associated,

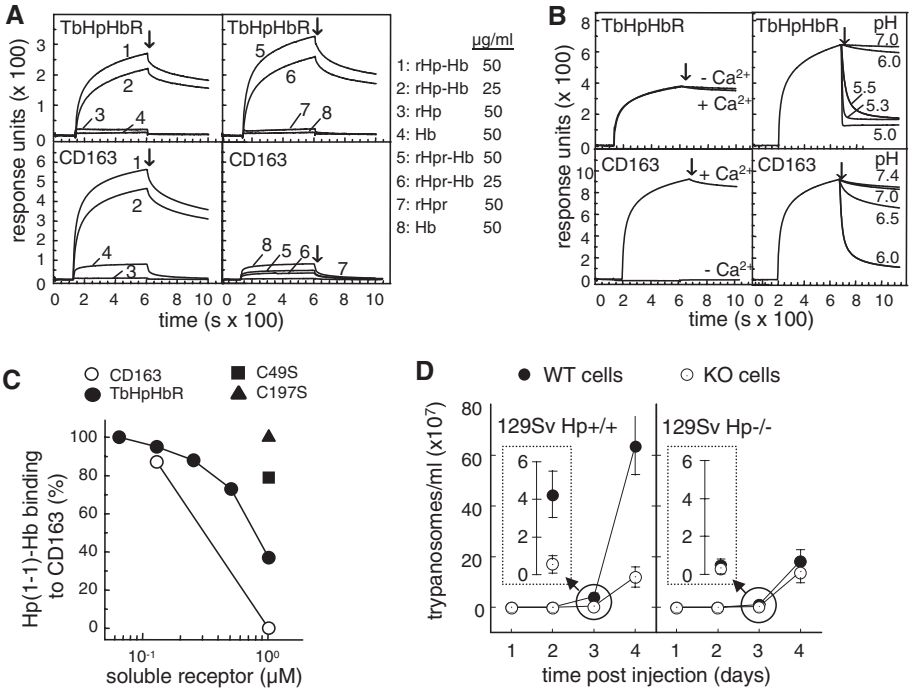

Fig. 2. Binding properties of TbHpHbR. (**A** to **C**) Surface plasmon resonance analysis. (A) Complexes of purified human Hb with purified recombinant Hp (rHp, phenotype 1-1) or Hpr were analyzed for binding to purified recombinant TbHpHbR immobilized on a Biacore sensor chip. For comparison, complexes of Hb with rHp or rHpr were analyzed for binding to immobilized CD163 purified from human spleen. Similar results were obtained with purified Hp (either 1-1 or 2-2 phenotype). (B) Binding of Hp-Hb (25 µg/ml) to immobilized TbHpHbR or CD163, in the presence or absence of 2 mM Ca^{2+} in the flow buffer, or at various pH values. The association phase was recorded at pH 7.4. The arrows indicate the time points for the recording of the dissociation phase, and the pH change. (C) Binding of Hp-Hb (25 µg/ml) to immobilized human CD163 in the presence of increasing concentrations of soluble CD163, TbHpHbR, or TbHpHbR mutants. The effect of soluble receptor on the binding of Hp-Hb to immobilized CD163 is shown relative to the binding recorded in the absence of soluble receptor. rHp and purified Hp were from the 1-1 haplotype. (**D**) Parasitemia by WT and TbHpHbR KO trypanosomes in $Hp^{+/+}$ and $Hp^{-/-}$ 129Sv mice.

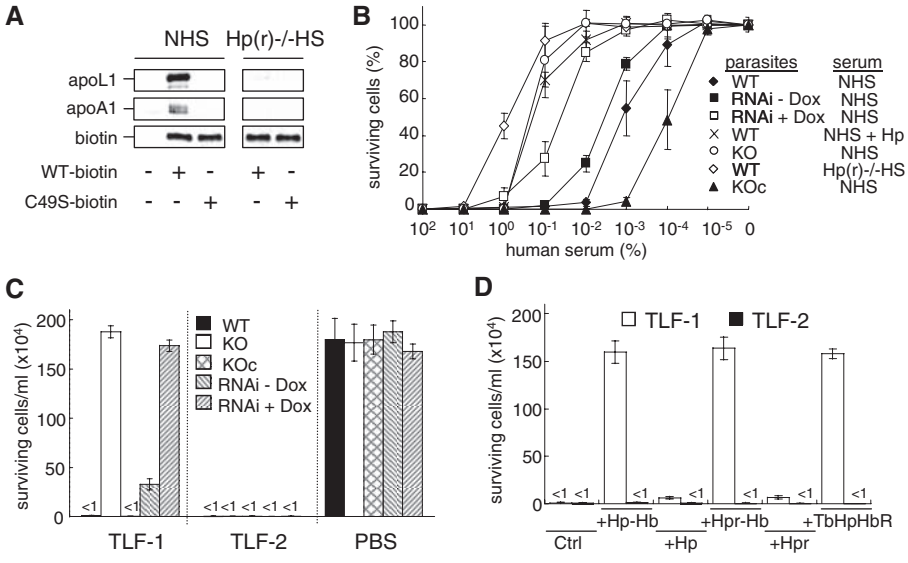

Fig. 3. Involvement of TbHpHbR in trypanolysis. (**A**) Evidence that TbHpHbR binds TLF-1. NHS and anhaptoglobinemic human serum [Hp(r)$^{-/-}$HS] were eluted through streptavidin-agarose beads containing, or not, biotin-conjugated recombinant WT TbHpHbR or TbHpHbR mutant. The bound fraction was analyzed for the presence of apoL1 and apoA1 (TLF-1 markers). (**B**) Trypanolysis assays with human serum. Trypanosomes (10^5/ml) were incubated in HMI-9 medium containing the indicated concentrations of serum. Surviving cells were counted after 24 hours. Dox, 1 μg/ml; Hp, 200 μg/ml. (**C**) Trypanolysis assays with isolated TLF-1 and TLF-2 complexes. The fractions were incubated with 10^6 trypanosomes/ml. Surviving cells were counted after 6 hours. (**D**) Same as in (C), but on WT cells in the presence or absence of the indicated proteins (200 μg/ml each).

with a loss of 16% per generation time (fig. S5A). The steady-state heme content of WT cells isolated from mice was 2.3 ng/mg of protein, whereas heme was undetectable in KO cells (*16*). In trypanosome lysates, heme appeared to be mostly incorporated into hemoproteins because it was recovered in acetone-insoluble material, as occurs with hemoproteins such as Hb but not with free heme (fig. S5B). Accordingly, after uptake at least two ^{14}C-labeled bands could be detected after electrophoresis of protein extracts (fig. S5C). Free ^{14}C-labeled heme was not internalized in bloodstream forms, even when provided with TbHpHbR, whereas it accumulated in the insect-specific procyclic forms that lack TbHpHbR and do not internalize Hp-Hb (Figs. 1D and 4C). Altogether, these data indicate that TbHpHbR directs the internalization of heme carried by the Hp-Hb complex into hemoproteins in order to optimize growth of bloodstream forms. In mice (Swiss or C57BL/6), the growth reduction of KO parasites was alleviated after pretreatment of the animals with the general immunosuppressor drug cyclophosphamide (Fig. 4D). However, this inhibition was conserved in lymphocyte-deprived, severe combined immunodeficient (SCID) mice (BALB/c background) (Fig. 4D). Therefore, we investigated the sensitivity of trypanosomes to macrophages. When incubated with peritoneal macrophages, KO

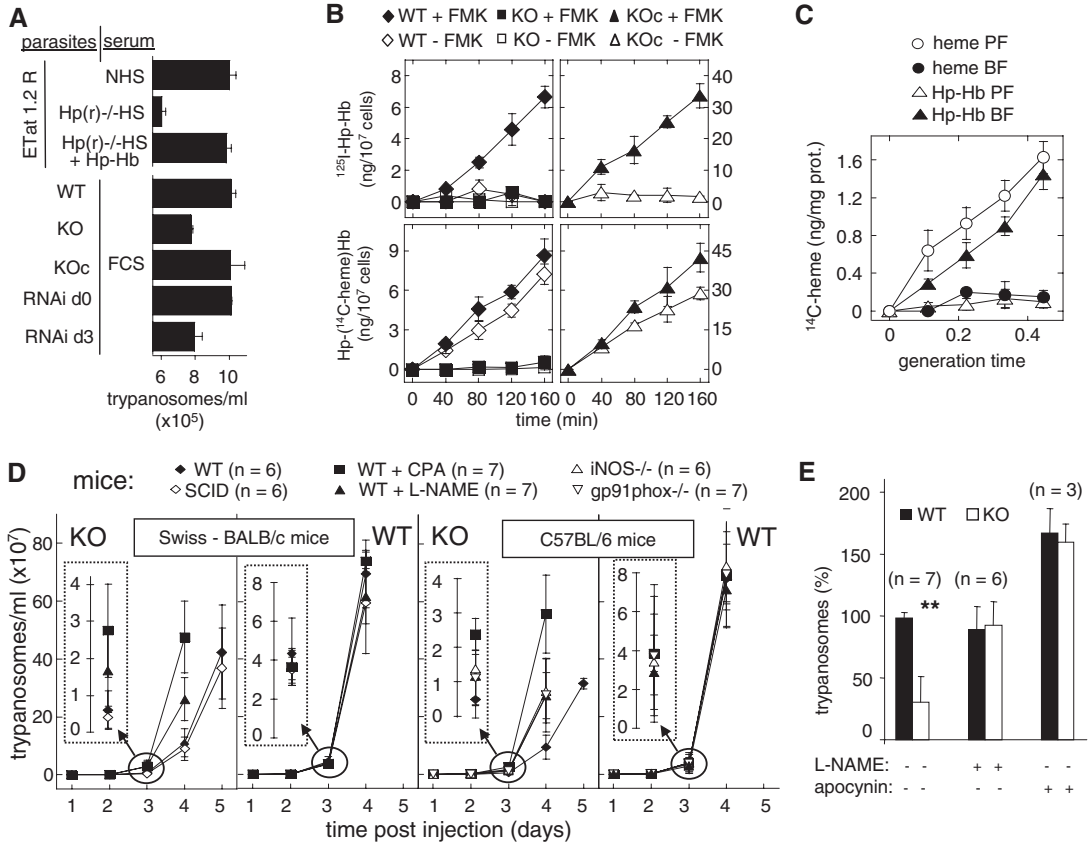

Fig. 4. In vivo function of TbHpHbR (unless indicated, error bars indicate standard deviation from four independent experiments). (**A**) Cell density of various trypanosome lines after 24 hours of incubation in vitro of inoculum (10^5 cells/ml) in sera depleted or not of Hp. Etat 1.2R, *T. brucei* subsp. *rhodesiense* Edinburgh Trypanosoma antigen type 1.2R; d, days; FCS, fetal calf serum. (**B**) Accumulation of radiolabeled Hp-Hb [either ^{125}I–(Hp-Hb) or Hp–(^{14}C-heme)Hb] in 10^7 trypanosomes, monitored in the presence or absence of the lysosomal protease inhibitor FMK-024. (**C**) Accumulation of ^{14}C-heme (ng/mg of protein) from either Hp–(^{14}C-heme)Hb or free ^{14}C-heme in BFs and PFs. The cell doubling time was 6 hours and 18 hours for BFs and PFs, respectively. (**D**) Parasitemia of mice injected intraperitoneally with 10^4 WT or KO trypanosomes, with or without prior administration of cyclophosphamide (CPA) or L-NAME. SCID mice (BALB/c background) were injected as controls. The absence of data at day 5 means that all mice died between days 4 and 5. (**E**) Effect of peritoneal exudate cells (PECs) from Swiss mice on growth of WT and KO parasites, after 48 hours of incubation in the presence or absence of L-NAME (500 μM) or apocynin (250 μM). Under the conditions used, L-NAME reduced NO synthesis by 58.3 ± 16% (n = 5 independent experiments). The trypanosome density was normalized to that of WT cells incubated with nonstimulated PECs. Asterisks indicate significant differences based on unpaired Student's t test (**, $P < 0.01$).

parasites were more affected than WTs (Fig. 4E). This inhibitory effect was substantially relieved when inhibitors of the key enzymes of macrophage oxidative burst were added, either the NO synthase (a nitric oxide producer) inhibitor L-N^G-nitroarginine methyl ester (L-NAME) or the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (a superoxide anion producer) inhibitor apocynin [1-(4-hydroxy-3-methoxyphenyl)ethanone] (Fig. 4E). None of these drugs influenced trypanosome growth in vitro (fig. S6). In support of these data, parasitaemia by TbHpHbR KO parasites was improved to a similar extent either after injection of L-NAME in WT mice or when inducible nitric oxide synthase $(iNOS)^{-/-}$ mice or NAPDH oxidase $(gp91phox)^{-/-}$ mice were used (Fig. 4D). Therefore, TbHpHbR appeared to confer increased resistance to oxidative stress induced by macrophages.

These data suggest that African trypanosomes have evolved a receptor specifically designed to acquire heme from Hp-Hb for incorporation into hemoproteins that both increase the trypanosome's growth rate and resistance to the oxidative response of the host. The mechanism of the resistance to oxidative attack is yet unknown but could involve hemoprotein-mediated modification of membrane lipids (*23*, *25*). In contrast to human CD163, TbHpHbR recognizes Hp-Hb and Hpr-Hb complexes equally well. Therefore, the presence of Hpr on human lytic HDL particles triggered internalization of the Hb-exposed fraction of these particles, which also contain the trypanolytic factor apoL1. This finding now explains the stimulating effect of Hb on trypanolysis as an indirect mechanism (*27*). Thus, in human serum the presence of the Hp-Hb receptor became detrimental instead of protective. In turn, synthesis of the apoL1 physical inhibitor serum resistance–associated protein allowed *T. brucei* subsp. *rhodesiense* to escape this problem and cause human sleeping sickness (*4*, *28*).

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19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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