**An invariant *Trypanosoma vivax* vaccine antigen inducing protective immunity**

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**Abstract**

Trypanosomes are protozoan parasites that cause infectious diseases including human African trypanosomiasis (sleeping sickness), and nagana in economically-important livestock animals1,2. An effective vaccine against trypanosomes would be an important control tool, but the parasite has evolved sophisticated immunoprotective mechanisms including antigenic variation3 that present an apparently insurmountable barrier to vaccination. Here we show using a systematic genome-led vaccinology approach and a murine model of *Trypanosoma vivax* infection4 that protective invariant subunit vaccine antigens can be identified. Vaccination with a single recombinant protein comprising the extracellular region of a conserved cell surface protein localised to the flagellum membrane named “invariant flagellum antigen from *T. vivax*” (IFX) induced long-lasting protection. Immunity was passively transferred with immune serum, and recombinant monoclonal antibodies to IFX could induce sterile protection and revealed multiple mechanisms of antibody-mediated immunity, including a major role for complement. Our discovery identifies a vaccine candidate for an important parasitic disease that has constrained the socioeconomic development of sub-Saharan African countries5 and provides evidence that highly protective vaccines against trypanosome infections can be achieved.

**Main**

African trypanosomiasis is an infectious disease caused by unicellular parasites of the genus *Trypanosoma* that are transmitted by the bite of an infected tsetse fly. In humans, trypanosome infections cause sleeping sickness: a deadly disease that threatens the lives of millions of people living in over 30 sub-Saharan countries6. Some species of trypanosome also infect important livestock animals such as cattle, goats and pigs causing the wasting disease nagana which affects the livelihoods of people relying on these animals for milk, food and draught power1. Approximately three million cattle die from this disease every year resulting in an estimated direct annual economic impact of many hundreds of millions of dollars7 and represents a major barrier for the socioeconomic advancement of many African countries. Nagana (animal African trypanosomiasis) is primarily caused by *T. vivax* and *T. congolense* and is currently managed with drugs, but resistance is increasing8. Previous attempts to develop subunit vaccines against African trypanosome infections have highlighted the difficulties in overcoming the immune evasion strategies evolved by these parasites to enable them to survive in host blood9. These strategies include antigenic variation: the serial expression of an abundant allelically-excluded variable surface glycoprotein (VSG), and the rapid removal of surface-bound antibodies by hydrodynamic sorting3,10. It is widely thought that the VSG forms a constantly-changing impenetrable surface coating that sterically shields other surface proteins from host antibodies leading to chronic infections characterised by oscillating parasitaemia; however, a careful analysis of recent structural information suggests that this model may not fully explain the protective role of the VSGs11. We therefore hypothesized that the VSGs may also subvert natural immunity by preventing the acquisition of high titre antibody responses to protective antigens suggesting that eliciting prophylactic unnatural host immunity by vaccination could be achieved. We now report the identification of a conserved cell surface protein that we term “invariant flagellum antigen from *T. vivax*” (IFX) that, when used as a subunit vaccine in a murine model of *T. vivax* infection, is capable of eliciting highly protective immunity.

**Results**

**IFX induces immunity to *T. vivax***

To identify subunit vaccine candidates for *T. vivax,* we established a genome-led vaccinology approach using a bioluminescent murine infection model4 (Fig. 1a). We determined that adoptive transfer of ~100 parasites intravenously into BALB/c hosts resulted in an acute reproducible infection that permitted sensitive and accurate parasitaemia quantification using light-based imaging12. We selected subunit vaccine candidates by searching the *T. vivax* genome13 for genes encoding predicted cell surface and secreted proteins that are likely to be accessible to vaccine-elicited antibodies. We selected 60 candidates using the following criteria: 1) they did not belong to paralogous gene families to minimise the risk of functional redundancy in different mammalian hosts14; 2) contained >300 amino acids in their predicted extracellular region and so are likely to be accessible at the cell surface; and, 3) had evidence of expression in the blood stages15 (Supplementary Table 1). Gene sequences encoding the entire predicted extracellular region were synthesised and cloned into a mammalian protein expression plasmid containing an exogenous secretion peptide and purification tags. Candidates were expressed as soluble recombinant proteins in HEK293 cells to increase the chances that structurally-critical posttranslational modifications were appropriately added. Of the 60 expression plasmids tested, 39 yielded sufficient protein after purification for vaccination trials (Extended Data Fig. 1a). For vaccination, we used a prime and two boost regime using alum as an adjuvant to bias host responses towards humoral immunity. To reduce any systemic adjuvant-elicited effects on disease progression, vaccinated animals were rested for a minimum of four weeks following the final boost before parasite challenge (Extended Data Fig. 1b, c). *T. vivax* loses virulence once removed from donor animals, and so to avoid confounding effects due to the loss of parasite viability during the infection procedure, we ensured that infections were comparable in control animals challenged before and after the vaccinated animals (Extended Data Fig. 1b).

Elicited antibody titres to each antigen were determined with the vast majority (90%) having mean half-maximal responses at serum dilutions greater than 1:10,000 (Extended Data Fig. 1d). We found that of the 39 antigens tested, 34 had no effect on the infection parameters relative to controls (Fig. 1b, Extended Data Fig. 2). Statistically significant effects on parasite growth was observed with four antigens (Fig. 1b): two candidates (V2, V8) exhibited a slight, and one (V31) a longer delay to the ascending phase of parasitaemia (Fig. 1b, c), and one (V23) showed no detectable parasites in all five vaccinated animals (Fig. 1b, d). For each of the four candidates, experiments were repeated using independent protein preparations and larger cohorts of animals. The two candidates that induced a slight delay (V2, V8) did not replicate and so were not pursued further (Extended Data Fig. 3a). Candidate V31 reduced the rate of parasite multiplication once more, inducing improved protection with 9/15 animals surviving until day 16 post infection (Extended Data Fig. 3a). Again, V23 vaccination elicited robust protection, and longitudinal sampling of these animals showed that 10 out of the 15 animals were protected beyond at least day 170 (Extended Data Fig. 3a, b). Dissection of protected animals several months after infection revealed no detectable extravascular reservoirs of parasites (Extended Data Fig. 4). Based on these and subsequent findings, we propose to name the V23 candidate (TvY486\_0807240) IFX for “invariant flagellum antigen from *T. vivax*”.

**IFX localises to the flagellum**

IFX is a previously uncharacterised type I cell surface glycoprotein containing a short (18 amino acid) cytoplasmic region that does not include any known protein domains and has no paralogs (protein sequence identity >25%) within *T. vivax*, nor homologs in other sequenced *Trypanosoma* spp. genomes. To begin the functional characterisation of IFX, we asked whether it had a specific localisation in blood-stage parasites. Immunocytochemistry showed that staining was localised along the length of the flagellum and loosely concentrated in discrete puncta (Fig. 2a). Using immunogold electron microscopy, IFX was enriched at the boundaries of where the flagellum is attached to the cell body; in different sections, these clusters were either uni- or bilaterally located (Fig. 2b). In mid-sagittal sections, IFX was located along the length of the flagellum membrane and concentrated in discrete clusters at the points where the flagellum was in close apposition to the cell membrane; specifically, the gold particles were located between the flagellum and cell body membranes (Extended Data Fig. 5a-f). These data demonstrated IFX was localised to the flagellum membrane and particularly enriched as continuous or punctuated bilateral stripes along the flagellum, bordering the region where the flagellum is attached to the parasite cell body, suggesting a structural role in maintaining flagellar function.

**Antibodies to IFX passively protect**

To determine the immunological mechanisms of IFX-mediated protection, we first demonstrated that antibodies contributed to immunity by transferring immune serum from IFX-vaccinated animals to naïve recipients which inhibited parasite growth in a dose-dependent manner (Fig. 3a). Depletion of CD4 and CD8-positive T-lymphocytes and NK1.1-positive natural killer cells in IFX-vaccinated mice did not affect protective efficacy demonstrating these cell types were not direct executors of immunity once established (Extended Data Fig. 6). To further investigate the role of antibodies in immunity using an independent approach, we selected six hybridomas secreting monoclonal antibodies (mAbs) to IFX. Out of the six mAbs selected, three affected parasite growth when used in passive protection experiments (Fig. 3b). We determined the approximate location of the mAb binding sites on IFX and quantified their binding affinities but did not observe a simple positive correlation between their protective efficacy and either the location of their epitope or binding affinity (Extended Data Figure 7). The inhibitory effects of one antibody (8E12) titrated with dose (Fig. 3c).

**Multiple mechanisms of anti-IFX protection**

Isotyping the mAbs to IFX revealed that they were all of the IgG1 subclass, which in mice, do not effectively recruit immune effector functions such as complement or bind activating Fc receptors with high affinity16 suggesting that direct antibody binding to IFX affected parasite viability. To establish the role of Fc-mediated immune effectors in anti-IFX antibody protection, we selected a mAb, 8E12, that gave intermediate protective effects, and by cloning the rearranged antibody variable regions, switched the mAb isotype from IgG1 to IgG2a (Extended Data Fig. 8). We observed that the 8E12-IgG2a mAbs had a significantly higher potency compared to the 8E12-IgG1 when used in passive transfer experiments (Fig. 4a), and titrating this antibody showed that three doses of 50 micrograms or more conferred sterile protection (Fig.4b, Extended Data Fig. 9a). This demonstrated that recruitment of antibody-mediated immune effectors were important for parasite neutralisation and to quantify their relative contributions, we engineered three further mAbs which each lacked the binding sites for C1q (ΔC1q), FcRs (ΔFcR), or both (ΔC1qΔFcR)17 (Extended Data Fig. 8c). When used in passive protection experiments, we observed that mutation of the C1q binding site almost completely reversed the inhibition of parasite growth demonstrating that C1q-mediated complement recruitment was a major protective mechanism (Fig. 4c, Extended Data Fig. 9b). Mutating the FcR binding site also relieved the inhibition of parasite growth, but to a lesser extent, while mutation of both C1q and FcR sites inhibited growth with a similar potency as the IgG1 isotype (Fig. 4c, Extended Data Fig. 9b). These experiments revealed that anti-IFX antibodies inhibited parasite multiplication by several immune mechanisms dominated by the recruitment of complement.

**IFX is highly conserved across isolates**

To further assess and develop IFX as a potential vaccine target, we tested appropriate routes of administration and other adjuvants that would bias antibody responses towards more protective isotypes. Of two selected adjuvants that have previously been used in veterinary vaccines and can be delivered subcutaneously, we found that the saponin-based adjuvant Quil-A elicited consistent antibody titres equivalent to the protective responses induced by alum, of which a large proportion were of the IgG2 isotypes (Extended Data Fig. 10a, b); these mice were potently protected against parasite challenge (Fig. 5a, Extended Data Fig. 10c). One potential challenge with subunit vaccines is that the genes encoding antigens eliciting protective immune responses in natural infections can be subject to diversifying selection, potentially leading to strain-specific immunity limiting the usefulness of the vaccine18. We therefore analysed the IFX gene sequence in 29 cosmopolitan *T. vivax* genomes and showed that it was highly conserved by comparison to other surface antigens. We observed only a single non-synonymous polymorphism in 2/29 strains (Fig. 5b) and both of these were heterozygotes, and so the frequency of the mutation across all strains was very low (0.058), demonstrating that IFX is almost completely invariant within the parasite population. This high level of sequence conservation across isolates suggests that it is not a target of host immune responses and consistent with this, sera from naturally-infected cattle were not immunoreactive to IFX (Fig. 5c). Finally, a successful vaccine must be able to elicit long-lasting protection and so we repeatedly challenged IFX-vaccinated mice over 100 days after receiving their final immunisation. We observed that mice remained fully protected including when parasites were delivered subcutaneously (Fig. 5d).

**Discussion**

We have shown that it is possible to elicit apparently sterile protection to an experimental trypanosome infection with a subunit vaccine corresponding to the ectodomain of an invariant cell surface parasite protein named IFX. The localisation of IFX to the boundaries of where the flagellum is in contact with the parasite cell body suggests it performs a role in flagellar structure and function. Our demonstration that antibodies are required for immunity raises questions about the immunoprotective mechanisms employed by trypanosomes, and importantly, their vulnerabilities that can be exploited to develop vaccines. The inhibition of parasite growth by antibodies to IFX suggest that the *T. vivax* VSG surface coat cannot fully shield it from antibody binding, and that anti-IFX antibodies are not removed by endocytosis within the flagellar pocket from the parasite surface with sufficient rapidity to prevent antibody-mediated immune effector recruitment. The finding that the IFX gene sequence was highly conserved across parasite isolates and that sera from infected cattle living in endemic regions were not immunoreactive to IFX suggest that natural parasite infections in some species can subvert host immunity to avoid eliciting protective antibody responses. These mechanisms could include perturbations of the B-cell compartment which have been described in experimental models of other trypanosome species19–21, or that the IFX protein may not be suitably presented to the host immune system in the context of a natural infection. Preliminary experiments to translate these findings to goats did not show protection22 and highlighted the need to develop infection models that are suitable for vaccine testing, and a greater understanding of which antibody isotypes and adjuvants elicit the necessary immune effector recruitment mechanisms. The discovery of an antigen that can elicit protection to a trypanosome infection provides optimism and a technical roadmap that could be followed to identify vaccine antigens not just for other trypanosome species, but also parasites that have thus far proved intractable to vaccine development. Finally, IFX represents a very attractive vaccine candidate for an important livestock disease that has been a major barrier to the socioeconomic development of sub-Saharan Africa.

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**Figure legends**

**Figure 1 | V23/IFX induces protective immunity in a *Trypanosoma vivax* infection model. a,** Schematic illustrating the genome-led vaccinology approach. **b,** Summary of mean parasitaemia (*n* ≥ 3 animals) quantified by bioluminescence in cohorts of vaccinated mice challenged with *T. vivax*. One candidate, V58, although having lower mean parasitaemia relative to controls on day 8 rebounded on day 9 and so was not considered further. **c**, **d**, Bioluminescent images of adjuvant-only control and mice vaccinated with V23 and V31 five and eight days post infection (Dpi)*.*

**Figure 2 | IFX is expressed on the *Trypanosoma vivax* flagellum membrane and concentrated at the periphery of flagellum-cell body contact. a,** Immunofluorescence staining of *T. vivax* with rabbit anti-IFX antiserum (red, left panels) or control pre-immune serum (right) counterstained with DAPI (blue) demonstrates localisation of IFX to the flagellum. Scale bars = 5 µm. **b,** Immunogold electron microscopy using an anti-IFX mouse monoclonal localised IFX to the borders of where the flagellum is in contact with the parasite cell body in transverse sections (black arrows in leftmost images) compared to isotype-matched control (right). Scale bars = 100 nm. Representative images of at least two independent experiments are shown.

**Figure 3 | Passive transfer of immunity to *Trypanosoma vivax* infections with anti-IFX antibodies. a,** Dose-dependent inhibition of *T. vivax* by adoptive transfer of sera from IFX-vaccinated mice relative to unimmunised control sera. Groups of five animals were compared by one-way ANOVA with Sidak post-hoc test; \* P ≤ 0.05, \*\*\*\* P ≤ 0.0001. **b**, Three of six anti-IFX IgG1-isotype mAbs (6B3, 8E12 and 3D12) each given at a dose of 3 x 100 μg passively protect against *T. vivax* infection relative to an isotype-matched control. Groups of five animals were compared by one-way ANOVA with Sidak post-hoc test; \*\*\*\* P ≤ 0.0001. **c**, Passive protection of the 8E12-IgG1 mAb is dose dependent. Parasitaemia was quantified at day 5 using bioluminescence and data points represent individual animals; bars indicate mean ± SD, groups of five mice were compared by one-way ANOVA with Sidak post-hoc test; \*\* P ≤ 0.01, \*\*\*\* P ≤ 0.0001; background bioluminescence threshold is indicated by grey shading.

**Figure 4 | Multiple mechanisms of antibody-mediated anti-IFX immunological protection dominated by complement recruitment.** **a,** Anti-IFX 8E12-IgG2a mAbs passively protect more potently against *T. vivax* infection than 8E12-IgG1 mAbs. **b**, Dose titration of 8E12-IgG2a mAb compared to isotype-matched control. **c,** Passive transfer of 8E12-IgG2a mAbs containing mutations that prevent binding to C1q (ΔC1q), FcRs (ΔFcR) or both (ΔC1qΔFcR) relieved the inhibition of parasite multiplication to differing degrees demonstrating multiple mechanisms of antibody-based immunological protection including a major role for complement. Parasitaemia was quantified at day 5 using bioluminescence, data points represent individual animals and grey shading indicates background bioluminescence; bars indicate mean ± SD. One of two independent experiments with very similar outcomes is shown.

**Figure 5 | IFX is highly conserved and can elicit long-lasting immunity to *Trypanosoma vivax* infections. a,** Comparing veterinary adjuvants using subcutaneous delivery demonstrates that Quil-A is as effective as the IFX/alum intraperitoneal positive control. Parasitaemia was quantified at day 5 using bioluminescence, data points represent individual animals and grey shading indicates background bioluminescence; bars indicate mean ± SD. **b,** Parasite population genetic analysis show *IFX* is highly conserved compared to other genes; mean synonymous (Ds) and nonsynonymous (Dn) substitution densities are shown + SD where appropriate. **c,** IFX is not immunogenic in the context of a natural infection. Immunoreactivity to the indicated proteins in sera from Cameroonian (C, *n*=10), Kenyan (K, *n*=10) or uninfected UK control cattle (U, *n*=6); bar indicates median. **d,** IFX/alum-vaccinated mice were protected from two intravenous and one final subcutaneous *T. vivax* challenge given over 100 days following the final booster immunisation.

**Methods**

**Mouse strains and ethical approvals**

All animal experiments were performed under UK Home Office governmental regulations (project licence numbers PD3DA8D1F and P98FFE489) and European directive 2010/63/EU. Research was ethically approved by the Sanger Institute Animal Welfare and Ethical Review Board. Mice were maintained under a 12-hour light/dark cycle at a temperature of 19-24°C and humidity between 40 to 65%. The animals used in this study were 6 to 14 week-old female *Mus musculus* strain BALB/c which were obtained from a breeding colony at the Research Support Facility, Wellcome Sanger Institute.

**Cell lines and antibodies**

Recombinant proteins and antibodies used in this study were expressed in HEK293E23 or HEK293-6E24 kindly provided by Yves Durocher (NRC, Montreal). Neither cell line was authenticated but were regularly tested for mycoplasma (Surrey Diagnostics, UK) and found to be negative. The antibodies used in this study are detailed below. Primary antibodies: six anti-IFX mouse monoclonal antibodies were selected and validated in this study: 6B3, 8C9, 3D12, 2H3, 10E2 and 8E12 from hybridomas all secreting IgG1 isotypes. A rabbit polyclonal antibody to the entire ectodomain of IFX was generated by Cambridge Research Biochemicals and validated by ELISA against the recombinant IFX ectodomain. The 8E12 antibody was cloned and expressed recombinantly as a mouse IgG2a isotype as described below. Mouse isotype control antibodies were: IgG1 (MOPC-21, BE0083, BioXcell) and IgG2a (C1.18.4, BE0085, BioXcell). Antibodies used for *in vivo* leukocyte cell depletion were: anti-mouse Cd4 (clone GK1.5, BP0003-1, BioXcell), anti-mouse Cd8 (clone 2.43, BP0061, BioXcell), anti-mouse Nk1.1 (clone PK136, BE0036, BioXcell), and control anti-keyhole limpet hemocyanin (clone LTF-2, BP0090, BioXcell). Antibodies used for protein quantification for ELISAs were mouse monoclonal anti-His (His-Tag mAb, 70796, EMD-Millipore), and biotinylated mouse anti-rat Cd4 (clone OX68). OX68 was purified from the spent tissue culture media from the hybridoma which was a kind gift from Professor Neil Barclay (University of Oxford). Secondary antibodies used were goat anti-mouse alkaline phosphatase conjugated secondary (A3562, Sigma-Aldrich) and rabbit anti-bovine alkaline phosphatase conjugated secondary (A0705 Sigma-Aldrich). Mouse antibody isotypes were determined using the mouse monoclonal antibody isotyping kit (ISO2-KT Sigma-Aldrich).

**Vaccine target identification and expression**

The *T. vivax* genome was searched for proteins encoding predicted type I, GPI-anchored and secreted proteins using protein feature searching in TriTrypDB25. The regions corresponding to the entire predicted extracellular domains of *T. vivax* cell-surface and secreted proteins from the Y486 strain were determined by using transmembrane26 and GPI-anchor27 or signal peptide28 prediction software. Protein sequences encoding the predicted extracellular domain and lacking their signal peptide, were codon-optimized for expression in human cells and made by gene synthesis (GeneartAG, Germany and Twist Bioscience, USA). The sequences were flanked by unique NotI and AscI restriction enzyme sites and cloned into a pTT3-based mammalian expression vector23 between an N-terminal signal peptide to direct protein secretion and a C-terminal tag that included a protein sequence that could be enzymatically biotinylated by the BirA protein-biotin ligase29 and a 6-his tag for purification30. The ectodomains were expressed as soluble recombinant proteins in HEK293 cells as described31,32. To prepare purified proteins for immunisation, between 50 mL and 1.2 L (depending on the level at which the protein was expressed) of spent culture media containing the secreted ectodomain was harvested from transfected cells, filtered and purified by Ni2+ immobilised metal ion affinity chromatography using HisTRAP columns using an AKTAPure instrument (GEHealthcare, UK). Proteins were eluted in 400 mM imidazole as described33, and extensively dialysed into HBS before being quantified by spectrophotometry at 280 nm. Protein purity was determined by resolving one to two micrograms of purified protein by SDS-PAGE using NuPAGE 4–12 % Bis Tris precast gels (ThermoFisher) for 50 minutes at 200 V. Where reducing conditions were required, NuPAGE reducing agent and anti-oxidant (Invitrogen) were added to the sample and the running buffer, respectively. The gels were stained with InstantBlue (Expedeon) and imaged using a c600 Ultimate Western System (Azure biosystems). Purified proteins were aliquoted and stored frozen at -20 °C until used. Where enzymatically monobiotinylated proteins were required to determine antibody titres by ELISA, proteins were co-transfected with a secreted version of the protein biotin ligase (BirA) as described32, and extensively dialysed against HEPES-buffered saline and their level of expression determined by ELISA using a mouse monoclonal anti-His antibody (His-Tag mAb, 70796, EMD Millipore) as primary antibody and a goat anti-mouse alkaline phosphatase-conjugated secondary (A3562, Sigma-Aldrich).

**Vaccine formulation and administration**

For the initial screening of antigens, aliquots of purified protein for immunisation were thawed, diluted, and mixed 50 % v/v with alhydrogel adjuvant 2 % (InvivoGen) for two hours at room temperature. For each antigen, groups of five 6 to 8-week old female BALB/c mice were immunised intraperitoneally using a prime and two boosts strategy using the amounts of protein documented in Extended Data Table 1. For retesting those antigens which had shown some effect in the preliminary screen, one group of 15 animals received three intraperitoneal immunisations of the query protein adjuvanted in alum using similar amounts as used in the initial screen (Extended Data Table 1); a control group, also 15 animals, received the adjuvant alone. For evaluating different IFX vaccine adjuvant formulations, groups of five mice received three immunisations of 50 μg IFX adjuvanted with either alhydrogel, Montanide ISA 201 VG, or Quil-A in a total volume of 200 μL. IFX was formulated with Montanide ISA 201 VG according to the manufacturer's instructions using a stirrer to create the water-in-oil emulsion. IFX was mixed in a 1:1 (v/v) ratio with Quil-A adjuvant using a 0.5 mg mL-1 solution. IFX/Montanide and IFX/Quil-A vaccines were administered subcutaneously at two different injection sites (100 μL per site), and IFX/alhydrogel formulation was administered intraperitoneally.

**Quantification of serum antibody titres by ELISA**

To determine the serum antibody responses to immunised proteins, blood biopsies were collected between ten to twelve days after the final immunisation from the tail of each animal and clotted for two hours at room temperature. Cells were removed by centrifugation, the serum collected, supplemented with sodium azide to a final concentration of 2 mM as a preservative and stored at -20 °C until use. Cattle sera were donated from archived material at the University of Liverpool, originally collected from natural *T. vivax* infections in Cameroon (Northwest state), and Kenya (Western state) where the infection was positively identified by thick blood smear and the parasite identified as *T. vivax* using the VerY Diag field test.

To determine the antibody titre against an antigen of interest, individual sera were initially diluted 1:1000 and then six four-fold serial dilutions in PBST/2 % BSA were prepared. These dilutions were pre-incubated overnight at room temperature with 100 µg mL-1 of purified rat Cd4d3+4-BLH protein to adsorb any anti-biotin/his tag antibodies. Sera were transferred to streptavidin-coated ELISA plates on which the biotinylated target antigen was immobilised. To ensure that all anti-tag antibodies were adsorbed, binding of the lowest dilution of antisera was also tested against biotinylated rat Cd4d3+4-BLH protein similarly immobilised on the ELISA plate to confirm the absence of any anti-tag immunoreactivity34. Sera were incubated for one hour at room temperature followed by three washes with PBST before incubating with an anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich) used as a 1:5000 dilution for one hour. Following three further washes with PBST, 100 µL of 1 mg mL-1 Sigma 104 phosphatase substrate was added and substrate hydrolysis quantified at 405 nm using a plate reader (Spark, Tecan). To quantify immunoreactivity to *T. vivax* antigens in the context of natural infections, cattle sera were diluted 1:800 in PBST/2 % BSA and incubated for two hours at room temperature with biotinylated ectodomains of V2, V53, IFX or control rat Cd200, adsorbed on the microtitre plate. Following three washes with PBST, a secondary rabbit anti-bovine IgG antibody (A0705, Sigma-Aldrich) diluted 1:20,000 was incubated for one hour and washed three times with PBST before adding colorimetric phosphatase substrate and acquiring absorbance readings as described above.

**Antibody isotyping**

Isotyping of the monoclonal antibodies and polyclonal sera responses was performed using the Mouse Monoclonal Antibody Isotyping Kit (ISO2-KT, Sigma-Aldrich), according to the manufacturer’s instructions. Briefly, the biotinylated ectodomain of the IFX protein was immobilised on a streptavidin-coated plate, incubated with sera diluted 1:1000 in PBST/2 % BSA or hybridoma supernatants, washed in PBST before adding isotype-specific goat anti-mouse secondary antibodies diluted 1:1000. Binding was quantified with an alkaline-phosphatase-conjugated rabbit anti-goat tertiary antibody (1:5000, Sigma-Aldrich) followed by a colourimetric phosphatase substrate, and hydrolysis products quantified by absorbance readings at 405 nm.

***Trypanosoma* parasite strain and maintenance**

A transgenic form of *Trypanosoma vivax* genetically engineered to ubiquitously express the firefly luciferase enzyme35 was kindly provided by Paola Minoprio, Institut Pasteur, Paris. The parental strain of this parasite is the IL1392 line derived from the Y486 strain used for genome sequencing13 and is fully documented by Chamond *et al*.12. Parasites were initially recovered from a frozen stabilate by intraperitoneal administration into two BALB/c female mice. Parasites were maintained by weekly serial blood passage in wild type female BALB/c mice by taking a blood biopsy, quantifying living parasites in PBS/20 mM D-glucose by microscopy and infecting four naive mice intravenously. During the course of the project, two further aliquots of frozen parasites were thawed and then used for infection challenges, no significant differences in the kinetics of infection were observed. Luciferase-expressing *T. congolense* parasites were a kind gift from Bill Wickstead and Caterina Gadelha, University of Nottingham, and maintained by weekly serial intravenous blood passage in wild type female BALB/c mice.

***Trypanosoma vivax* infections**

For infection challenges, bloodstream forms of *T. vivax* parasites were obtained from the blood of an infected donor mouse at the peak of parasitaemia, diluted in PBS/20 mM D-glucose, quantified by microscopy and used to infect mice by intravenous injection. While establishing the infection model in our facility, we observed that the *T. vivax* parasite was labile and gradually lost virulence once removed from living mice. To reduce the possibility of any artefactual protective effects being due to the loss of parasite virulence during the challenge procedure, we screened the protective effects of antigens in a cohort design. Each cohort contained six cages of five animals: four cages contained mice immunised with a different query subunit vaccine candidate, and the other two cages contained control mice immunised with adjuvant alone. Vaccinated animals were rested for four to eight weeks after the final immunisation to mitigate any possible non-specific protective effects elicited by the adjuvant. During the infection procedure, the mice in the control cages were challenged first and last, and the data from the cohort only used if the infections in the control mice from the two cages were comparable. During the infection procedures, parasites were outside of a living mouse for no more than 40 minutes. Mice were normally challenged by intravenous delivery of 102 (cohorts 1-7, 10-11) to 103 (cohorts 8 and 9) parasites for the initial screening and passive transfer protection experiments, but were also challenged intraperitoneally during the establishment of the model and subcutaneously when investigating the duration of protection. The animals were not randomised between cages and the operator was not blinded to the group condition. Occasionally, individual infected mice within a group unexpectedly exhibited only background levels of bioluminescence which was attributed to the injected luciferin substrate not distributing from the site of delivery, possibly due to mislocalization of the injection bolus; in these instances, these animals were excluded from the analysis. This occurred 12 times out of 1650 injections (0.7%) in screening cohorts 3, 5, 6, and 9. Groups were compared using bioluminescence quantification as a proxy for parasitaemia and one-way ANOVA with Dunnett’s post-hoc test unless specified.

**Quantification of *Trypanosoma vivax* infections by bioluminescent *in vivo* imaging**

The luciferase substrate D-luciferin (potassium salt, Source BioScience, UK) was reconstituted to 30 mg mL-1 in Dulbecco’s PBS (Hyclone), filter-sterilised (0.22 µm) and stored in aliquots at -20 °C. Aliquots were thawed and administered to animals at a dose of 200 mg kg-1, by intraperitoneal injection ten minutes before bioluminescence acquisitions. The mice were given three minutes of free movement before being anaesthetized with 3% isoflurane and placed in the imaging chamber where anaesthesia was maintained for acquisition. An average background bioluminescence measurement was determined by luciferin administration in five female BALB/c mice and calculating the mean whole-body bioluminescence; where appropriate, this value is indicated as a light grey shading on bioluminescence plots. To determine long-term persistence of the parasites in different organs of infected mice, animals were administered with luciferin, imaged, and then euthanised with an overdose of anaesthetic. Mice were then perfused with PBS until the perfusion fluid ran clear, the organs dissected, arranged on a petri dish, and bathed in PBS containing 20 mM glucose and 3.3 mg mL-1 luciferin for imaging. Emitted photons were acquired by a charge coupled device (CCD) camera (IVIS Spectrum Imaging System, Perkin Elmer). Regions of interest (ROIs) were drawn and total photons emitted from the image of each mouse were quantified using Living Image software version 4.7.4 (Xenogen Corporation, Almeda, California), the results were expressed as the number of photons sec-1. Bioluminescence values were exported and plotted in Prism GraphPad version 8.0.2 which was also used for test of statistical significance where needed. Where necessary, peripheral parasitaemia was quantified by direct microscopic observation as previously described12. Briefly, five microliters of blood obtained from the tail vein were appropriately diluted in PBS containing 20 mM glucose and parasite counts were expressed as number of parasites per blood millilitre.

**Passive transfer of immunity**

To obtain sufficient sera for adoptive transfer experiments, fifty 6 to 8-week-old female BALB/c mice were immunised intraperitoneally three times with 20 μg of purified IFX adjuvanted in alum, with each immunisation separated by two weeks. Nine days after the final immunisation, sera were collected as above, aliquoted, and stored at -20 °C until use. For passive transfer experiments, groups of ten to fourteen-week-old female BALB/c mice were dosed three times with either sera or purified monoclonal antibodies on three consecutive days; three hours after the second dosing, mice were challenged intravenously with 102 *T. vivax* parasites. When using immune serum for passive transfer protection experiments, doses of 100 and 200 μL of sera from either IFX-vaccinated mice or non-immunised control mice were administered. For monoclonal antibodies, the purified antibody was diluted to the required dose in PBS and 200 μL administered intravenously. Control isotypes antibodies used were MOPC-21 for the IgG1 isotype and C1.18.4 for the IgG2a isotype (both from BioXcell). The serum half-life for mouse IgG1 and IgG2a are known to be between 6 to 8 days36.

***In vivo* cell depletion**

Groups of five mice were immunised three times with 50 µg doses of purified IFX to induce protective immunity to *T. vivax*. To deplete immune animals of defined leucocyte lineages, animals within each group were depleted by intra-peritoneal administration of lineage-specific monoclonal antibodies using standard procedures. Briefly, NK cells were depleted by four injections of 500 µg of the PK136 mAb that targets the Nk1.1 glycoprotein at days -5, -1, 0 and 2 post-infection. Mouse CD4 and CD8 T-lymphocytes were depleted by one intraperitoneal 750 µg injection of the mAbs targeting Cd4 (clone GK1.5) or Cd8 (clone 2.43) receptors, respectively, the day prior of the infection. The LFT-2 mAb (750 µg) was used as an isotype-matched control antibody. Mice were challenged with 102 *T. vivax* parasites and parasitaemia quantified using bioluminescent imaging as described above.

**Trypanosome genomic sequence analysis**

To identify if IFX had any homologues in other *Trypanosome* species, the entire IFX sequence was analysed with Interproscan which showed that it does not contain any known protein domains, other than the predicted N-terminal signal peptide and transmembrane helix. Comparison of the predicted IFX protein sequence with all the other sequenced *Trypanosoma* spp. genomes in TriTrypDB25 (<https://tritrypdb.org>) using tBLASTx returned no significant matches; moreover, comparison of a Hidden Markov Model of the IFX protein sequence with all *T. brucei, T. congolense* and *T. cruzi* proteins using HMMER also produced no matches demonstrating IFX is unique to *T. vivax*. To confirm that IFX is present in a single-copy, the IFX protein sequence was compared with a six-frame translation of the genome sequence using tBLASTn to identify any sequence copies (annotated or not) with >98% amino acid identity typical of allelic variation.

Illumina sequencing reads from 29 clinical strains isolated from Nigeria, Togo, Burkina Faso, The Gambia, Ivory Coast, Uganda and Brazil were mapped to the *T. vivax* Y486 reference sequence using BWA37 before SNPs were called using the GATK4 analysis toolkit38. Indels and variant positions with QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5 or ReadPosRankSum < -8.0 were excluded to produce a final list of 403,190 SNPs. Individuals were classified as missing if allele calls were supported by fewer than three reads. Coding SNPs and synonymous/non-synonymous codon alterations were identified by comparison to the reference annotation using a custom Biopython script; pi-values were calculated on a per site basis using vcftools39. Genes selected for comparison were *V31* (TvY486\_0003730), two *VSG*s (TvY486\_0031620, TvY486\_0040490), *ISG* (TvY486\_0503980), *HpHbR* (TvY486\_0040690) and the “housekeeping” gene *GAPDH* (TvY486\_1006840).

**Electron microscopy**

*T. vivax* parasites were resuspended in 1 % formalin in PBS for 30 minutes (all steps at room temperature), washed three times in PBS, blocked with PBS/glycine followed by 5 % foetal calf serum for 30 minutes and then incubated with a mouse monoclonal antibody to IFX (clone 8E12) for 1 hour. After rinsing, the parasites were incubated with goat anti-mouse IgG preadsorbed to 10 nm gold particles (ab27241 Abcam) for 30 minutes, washed, and fixed in a mixture of 2 % formalin and 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 minutes. After washing again, the parasites were post-fixed in 1 % osmium tetroxide for 30 minutes, dehydrated in an ethanol series, embedded in epoxy resin and 60 nm ultrathin sections were cut on a Leica UC6 ultramicrotome, contrasted with uranyl acetate and lead citrate and examined on a 120 kV FEI Spirit Biotwin using a Tietz F4.16 CCD camera. The density of anti-IFX gold particle staining was determined by counting the number of gold particles per micron of membrane on both sagittal and transverse sections. Membrane lengths were determined using the segmented line function in ImageJ version 1.45s and known image scaling factor. To assign dorsal and ventral sectors, a line was drawn across (transverse) or along (sagittal) the flagellum midpoint.

**Anti-IFX antibody selection and characterisation**

To raise polyclonal antisera against IFX, the entire ectodomain of IFX was expressed and purified and injected into rabbits (Cambridge Research Biochemicals, Billingham, UK). The sera were purified on Hi-Trap Protein G HP columns (GE Healthcare) according to the manufacturer’s instructions. Hybridomas secreting monoclonal antibodies to IFX were selected using standard protocols as described40. In brief, the SP2/0 myeloma cell line was grown in advanced DMEM/F12 medium (Invitrogen, CA, USA) supplemented with 20 % fetal bovine serum, penicillin (100 U mL-1), streptomycin (100 μg mL-1) and L-glutamine (2 mM). Following spleen dissection and dissociation, 108 splenocytes were fused to 107 SP2/0 myeloma in 50 % PEG (PEG 1500, Roche, Hertfordshire, UK), using standard procedures. The resulting hybridomas were plated over ten 96-well plates and initially grown in advanced DMEM/F12 medium (Invitrogen) supplemented with 20 % fetal bovine serum, penicillin (100 U mL-1), streptomycin (100 μg mL-1) and L-glutamine (2 mM) before addition of hypoxanthine-aminopterin-thymidine (HAT) selection medium 24 hours after the fusion. After 11 days, hybridoma supernatants were harvested to determine the presence of antibodies reacting to the IFX protein using an ELISA-based method as previously described40. Six wells (2H3, 3D12, 6B3, 8C9, 8E12, and 8F10) containing hybridoma colonies secreting antibodies that reacted with IFX but not a control protein containing the same purification tags were identified and cultured for a further four days in HAT-selection medium. Hybridoma cells from each of the positive wells were cloned by limiting dilution over two 96-well plates at a density of 0.5 cells per well and grown in HAT-free SP2/0 conditioned medium. Eleven days later, twelve wells corresponding to each of the seven clones were selected and tested again by ELISA for reactivity to the IFX protein; three positive wells per clone were chosen for a second round of dilution cloning in the conditions described above. After a final test for reactivity to IFX, a single well from each of the seven positive clones was expanded and adapted to grow in Hybridoma-SFM serum-free medium (Thermo Fisher).

To determine the location of the anti-IFX monoclonal antibody epitopes, subfragments of the IFX ectodomain corresponding to the boundaries of predicted secondary structure (M1-T251, M1-S472, S135-T251 and N442-S535) were designed, produced by gene synthesis and cloned into a mammalian expression plasmid with an enzymatically biotinylated C-terminal tag (Twist Biosciences, USA). Biotinylated proteins were expressed as secreted recombinant proteins in HEK293 cells as described above and dialysed to remove free D-biotin. Biotinylated IFX fragments were immobilised on a streptavidin-coated plate and binding of the six mouse monoclonal antibodies was tested by ELISA and detected with an alkaline-phosphatase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) as previously described40. Binding of a rabbit polyclonal antibody raised to the entire ectodomains of IFX (Cambridge Research Biochemicals) was used as a positive control for each of the subdomains, and detected with an alkaline-phosphatase-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch).

For affinity-purification of monoclonal antibodies from hybridoma culture supernatants, spent supernatants were supplemented with 0.1 M sodium acetate, pH 5.0 immediately before purification on a HiTrap Protein G HP 1 mL column (GE Healthcare) using an AKTA pure instrument. Elution was performed in 0.1 M glycine, pH 2.7 followed by immediate neutralisation with 1 M Tris-HCl, pH 9.0. Purified antibodies were extensively dialysed against PBS and stored at 4 °C until use. To capture antibodies on streptavidin-coated sensor chips for biophysical interaction analysis, 300 µg of purified monoclonal antibodies were chemically biotinylated using a 20-fold molar excess of sulfo-NHS-biotin (ThermoFisher) for two hours at room temperature; to remove excess biotin the solutions were dialysed against 5 L PBS for 16 hours.

**Antibody affinity by surface plasmon resonance**

Antibody affinities were determined by SPR essentially as described41 using a Biacore 8K instrument (GE Healthcare, Chicago, IL). To measure antibody interaction affinity rather than avidity, between 400 to 600 RU of biotinylated anti-IFX monoclonal antibodies were immobilised on a streptavidin-coated sensor chip prepared using the Biotin CAPture kit (GE Healthcare); a biotinylated mouse monoclonal antibody (OX68) was used as a non-binding control in the reference flow cell. The entire ectodomain of IFX was used as the analyte which was first purified and resolved by size exclusion chromatography on a Superdex 200 Increase 10/300 column (GE Healthcare, Chicago, IL) in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05 % v/v P20 surfactant) just prior to use in SPR experiments to remove any protein aggregates that might influence kinetic measurements. Increasing concentrations of two-fold dilutions of the entire ectodomain of IFX as a soluble analyte were injected at 30 μL min-1 for a contact time of 120 s and dissociation of 600 s. Both kinetic and equilibrium binding data were analyzed in the manufacturer's Biacore 8K evaluation software version 1.1 (GE Healthcare, Chicago, IL) and plotted in Prism GraphPad version 8.0.2. All experiments were performed at 37 °C in HBS-EP.

**Antibody cloning, isotype switching, mutagenesis, and purification**

To switch the isotype of the 8E12 anti-IFX monoclonal antibody from IgG1, it was first necessary to amplify the genes encoding the rearranged light and heavy variable regions from the hybridoma; this was performed essentially as described40. Briefly, total RNAs were extracted from the cloned 8E12 hybridoma using the RNAqueous-micro total RNA isolation kit (Ambion) followed by reverse transcription with Superscript III (Thermo Fisher). PCR products encoding the rearranged heavy and light chain regions were individually amplified using sets of degenerate oligonucleotides and then assembled in a subsequent fusion PCR using a linker fragment to create a single PCR product containing both the rearranged light and heavy chains, as previously described42. The fusion PCR product was ligated using the NotI and AscI restriction sites into an expression plasmid obtained from Addgene (plasmid # 114561) in frame with the mouse constant IgG2a heavy chain43. Competent *E.coli* were transformed and purified plasmids used in small-scale transfections of HEK293 cells to identify those plasmids encoding functional antibodies as described44.

To perturb the recruitment of immune effectors in the murine IgG2a recombinant antibody and retain serum half-life, we mutated the C1q and FcR binding sites in the IgG2a constant heavy chain by site-directed mutagenesis as described17. Mutation to the binding site of Fcγ receptors (ΔFcR) was achieved by introducing the L234A and L235A substitutions using primers FcRf - 5’ GCACCTAACGCTGCAGGTGGACCATCCG 3’ and FcRr - 5’ TGGTCCACCTGCAGCGTTAGGTGCTGGGC 3’. To abrogate C1q binding (ΔC1q), a single amino-acid change P329A was introduced using primers C1qf – 5’ CAAAGACCTCGCTGCGCCCATCGAGAGAACC 3’ and C1qr – 5’ GATGGCGCAGCGAGGTCTTTGTTGTTGACC 3’. In both cases, antibody mutagenesis was achieved by first amplifying 20 ng of an expression vector containing the mouse constant IgG2a heavy chain with each oligonucleotide separately for nine cycles (denaturation for 45 seconds at 94 °C; annealing for 40 seconds at 58 °C; elongation for 7 minutes and 30 seconds at 72 °C), using the KOD Hot Start DNA polymerase (Merck). Amplification reactions performed with complementary oligonucleotides were then mixed, 0.5 µL KOD Hot Start DNA polymerase was added to the reaction, and the amplification was resumed for a further 18 cycles. At the end of the reaction, half of the PCR reaction was digested with 20 U DpnI enzyme (New England Biolabs), which specifically cleaves methylated strands from the parental plasmid, for 3 hours at 37 °C before transforming 5 µL into TOP 10 chemically-competent bacteria (Invitrogen). Mutations were confirmed in selected clones by DNA sequencing. To generate a double mutant lacking both the C1q and FcR binding sites (ΔC1qΔFcR), site-directed mutagenesis was performed as described above on an expression plasmid containing the FcR mutation, using the set of oligonucleotides designed for C1q mutagenesis. Both single mutants and the double mutant backbones were doubly digested with NotI and AscI restriction enzymes and the fusion PCR product encoding the variable regions of the 8E12 recombinant antibody described above cloned into them, plasmids purified and verified by sequencing.

Antibodies were produced by transfecting HEK293 cells with plasmids encoding the recombinant 8E12-IgG2a monoclonal antibody with the wild-type IgG2a heavy chain, single mutants that lacked C1q and FcR binding, and the double mutant. Six days after transfection, the cell culture supernatant was harvested and the recombinant antibodies were purified on a HiTrap Protein G HP 1mL column, according to the manufacturer’s instructions as described40.

**Data availability**

Annotated *T. vivax* genome data were obtained from TriTrypDB (<https://tritrypdb.org>). All data generated or analysed during this study are included in this published article and/or available from the corresponding author on reasonable request.

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**Author contributions**

D. A. and G.J.W. designed the study. D.A. prepared proteins, and performed immunisations and parasite challenges with help from S.C., C.B., H.O. and K.H. C.C. selected hybridomas, cloned the 8E12 mAb, did the isotype switching, mutagenesis and purification. D.A.G. performed immunogold labelling. C. T. generated hybridomas. F.G. and M.K. performed SPR experiments and analysis. A. R.-R., C. W. D. and A. P. J. provided and analysed *T. vivax* genome sequences and provided infected cattle sera. D.A. and G.J.W. prepared the manuscript with comments from all co-authors.

**Competing interests**

D.A and G.J.W. are named inventors on two patent applications relating to this research.

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**Extended Data Figure legends**

**Extended Data Figure 1 | *T. vivax* vaccine candidate antigens, organisation of the protection screen and antibody titres. a,** Vaccine candidates were expressed as soluble recombinant proteins in HEK293 cells, purified, and resolved by SDS-PAGE to determine protein integrity and purity. For uncropped gel images, see Supplementary Figure 1. **b,** Mice were vaccinated with a protein-in-alum formulation using a prime and two boost regime and rested before challenge with the luciferase-expressing *T. vivax* parasite line and parasitaemia quantified using bioluminescent imaging. Vaccine candidates were tested in cohorts containing two control cages which were infected first and last to ensure any effect on the reduction of parasite multiplication was not confounded by the loss of parasite virulence. **c**, Parasite multiplication was identical in animals treated with adjuvant alone compared to naive mice. A group of five mice were immunised three times with alum alone (filled circles) and rested for 6 weeks and the infection with bioluminescent *T. vivax* was compared to naive mice (open circles). Data points represent individual mice; grey shading indicates background bioluminescence. **d,** Serum dilutions for the half-maximal responses for each antigen. Data points are individual mice and bars represent mean ± SD; *n* = 5. ND = not determined, and grey stars indicate no detectable response.

**Extended Data Figure 2 | Summary of systematic genome-led reverse vaccinology screen that identified subunit vaccine candidates for *Trypanosoma vivax*.** Bioluminescence is used as a proxy for parasitaemia and is shown for each mouse in the indicated days post-challenge. Candidates are identified using their “V number” and organised into their screening cohorts. The two cages of adjuvant-only controls for each cohort are highlighted in grey. The majority of candidates had no effect on the ascending phase of parasitaemia, and are left unshaded. Three candidates (V2, V8, V31) that had a statistically significant effect on infection are highlighted in pale yellow, and the candidate that elicited strong protection (V23) is highlighted in pale green. Data points are bioluminescence readings from individual animals. The occasional reductions in parasitaemia followed by rebound after day 8 are likely to be due to protective anti-VSG responses and selection of an antigenically distinct variant.

**Extended Data Figure 3 | Replication of strong protective effects in a larger group of IFX/V23-vaccinated mice.** **a**, Quantification of replicate vaccinations and *T. vivax* infections with four antigens showing protective effects in the initial screen. V23 and V31 vaccinations replicated, but not V2 and V8. Parasitaemia was quantified on day six using bioluminescence; data points represent individual animals and bars indicate mean ± SD. ns = not significant, \*\*\*\* P ≤ 0.0001 two-tailed t-test; grey shading indicates background bioluminescence thresholds. **b**, Fifteen mice were immunized with purified soluble IFX/V23 recombinant protein adjuvanted in alum and challenged with transgenic luciferase-expressing *T. vivax*. Parasitaemia was quantified on the indicated days after parasite challenge using bioluminescence; controls are a cohort of 15 animals treated with adjuvant only. Ten out of the 15 mice were protected until at least day 170. Data points represent individual animals and grey shading indicates bioluminescence thresholds of uninfected mice. Where animals had to be euthanized for health reasons thought to be unrelated to the infection, this is indicated by a cross.

**Extended Data Figure 4 | Parasites do not detectably persist in the organs, adipose tissue, or dermis of IFX/V23-vaccinated *Trypanosoma vivax*-challenged mice.** An IFX/V23-immunised mouse was challenged with luciferase-expressing transgenic *T. vivax* parasites and protection from infection relative to controls was established. The animal was rested and nine months later, injected with luciferin to detect residual parasites using bioluminescence. No bioluminescent signals above background were detected in vaccinated animals either when the whole animal was imaged, or within the dissected organs, adipose deposits and dermis of IFX/V23-immunised (top panels). An unimmunised animal was used 8 days after parasite challenge as a positive control (lower panels).

**Extended Data Figure 5 |** **IFX staining is specific to *T. vivax* and concentrated at the boundary of the flagellum-cell body contact.** **a**, Immunogold electron microscopy using an anti-IFX mouse monoclonal antibody localised IFX to clusters along the length of the flagellum in mid-sagittal sections (white arrows and bars); (**b**) is a zoom of box in (**a)**, showing IFX located between the flagellum and cell membranes. **c**, Anti-IFX particle staining density was quantified along the membrane interface of the ventral flagellum/cell body (IVC), dorsal flagellum (DF) and cell body area on sagittal and transverse sections. Individual data points are shown; bars represent means. Control electron micrographs of *T. vivax* parasites stained with an isotype-matched control mouse IgG1 antibody (**d**) or goat anti-mouse coated gold particles alone (**e**) showing no accumulation of gold particles. **f,** *T. congolense* parasites were stained with anti-IFX rabbit polyclonal sera (left) or control preimmune sera (right) followed by fluorescently conjugated anti-rabbit secondary (red) and counterstained with DAPI (blue). No staining of the parasites was observed demonstrating antibody specificity. Flag. = flagellum; fm = flagellar membrane; cm = parasite cell membrane. Scale bars represent 100 nm (**a, b**), 150 nm (**d, e**), and 8 µm (**f**). Representative images of at least two independent experiments are shown.

**Extended Data Figure 6 |** **Depletion of CD4 and CD8-positive T-lymphocytes and NK cells in IFX-vaccinated mice prior to parasite challenge do not affect IFX-mediated protective efficacy.** Groups of mice were vaccinated with IFX, rested, and then either NK cells (*n*=4) or CD4- (*n*=5) or CD8-positive (*n*=4) T-lymphocytes were depleted using lineage-specific monoclonal antibodies (antibody clone names indicated) before challenging with luciferase-expressing *T. vivax* parasites. Cell-depleted animals showed no significant difference to control animals treated with an isotype-matched control antibody (LTF-2). The virulence of parasites was confirmed by showing robust infections in naïve mice in the same experiment. Parasitaemia was quantified on day 5 using bioluminescence, grey shading indicates bioluminescence thresholds of uninfected mice; data points represent individual animals and bars indicate mean ± SD.

**Extended Data Figure 7 | Identification of the binding epitopes and affinities of a panel of mouse monoclonal antibodies recognising *T. vivax* IFX.** **a**, Schematic showing the N- and C-terminal boundaries of four fragments of the IFX ectodomain. **b**, Identification of the epitope locations for the anti-IFX monoclonal antibodies. The entire ectodomain (EE) (*n*=5) and derived fragments (1 to 4) (*n*=3) were expressed as enzymatically biotinylated soluble recombinant proteins in HEK293 cells, immobilised on streptavidin-coated microtitre plates, and the binding of each of the anti-IFX monoclonal antibodies quantified by ELISA. The hybridoma secreting mAb 8F10 was not successfully cloned and therefore not further investigated. Bars represent means ± SD. **c**, Schematic interpretation of the antibody binding data to the IFX ectodomain fragments showing the approximate locations of the antibody epitopes. **d**, Quantification of the equilibrium binding affinity of the anti-IFX monoclonal antibodies by surface plasmon resonance. Five of the anti-IFX monoclonal antibodies were chemically biotinylated and immobilized on a streptavidin-coated senor chip and the binding to serial dilutions of purified soluble IFX ectodomains measured. The binding affinity for each of the antibodies at equilibrium (*K*D) was calculated by fitting the binding data (inset) to a simple 1:1 binding isotherm. *K*D values represent mean ± SD using seven analyte dilutions from one experiment. There was no simple positive correlation between the antibody binding affinity and protective efficacy. Antibody affinities were plotted against percentage parasite inhibition in passive transfer experiments at day 5 post-infection (mean ± SD; *n*=5).

**Extended Data Figure 8 | Recombinant antibody cloning, isotype switching, and mutation of antibody effector recruitment sites of the anti-IFX 8E12 hybridoma.** **a**, The rearranged variable light and heavy regions of the anti-IFX 8E12 monoclonal antibody were amplified and assembled by fusion PCR using a “joining” fragment before being subcloned into a mammalian protein expression plasmid containing the mouse IgG2a heavy chain. 12/15 colonies expressed functional anti-IFX antibodies and 3 selected clones contained identical VH and VL sequences. The 8E12-IgG2a antibody was produced by transfection of HEK293 cells. For uncropped gel images see Supplementary Figure 1. **b**, The binding affinity of the 8E12 monoclonal antibody for IFX is unaffected after isotype switching. The biophysical binding parameters of the 8E12 monoclonal antibody for IFX were determined by SPR as both the hybridoma-expressed IgG1 (left panel) and recombinant IgG2a (right panel). Serial dilutions of the purified complete ectodomain of IFX were injected for two minutes over the biotinylated antibodies immobilised on a streptavidin-coated sensor chip and left to dissociate. Equilibrium binding constants were calculated by fitting the binding data to a Langmuir binding isotherm and found to be essentially equivalent. **c**, Mutation of the C1q and FcR recruitment sites on the 8E12-IgG2a heavy chain. The specified mutations which are known to abrogate binding to either C1q or FcR were made on the recombinant 8E12-IgG2a plasmid using site directed mutagenesis. Mutations were made individually (ΔC1q and ΔFcR) and together (ΔC1qΔFcR). Each of the three mutant antibodies were expressed, purified, and IFX-binding activity normalised to the parent 8E12-IgG2a and 8E12-IgG1 by ELISA.

**Extended Data Figure 9 | The anti-IFX 8E12-IgG2a monoclonal antibody with abrogated immune effector recruitment sites reveals highly potent protection due to multiple mechanisms of immunological protection including a major role for complement. a,** Groups of five mice were injected three times intravenously with the indicated doses of purified anti-IFX 8E12-IgG2a monoclonal antibody and challenged with the luciferase-expressing transgenic *T. vivax* parasites. Control is an isotype-matched mouse IgG2a monoclonal antibody. A cross indicates where a single animal had to be removed from the study on day 16 for health reasons thought to be unrelated to the infection. **b,** Groups of five mice were administered three times intravenously with either 50 μg (left panel) or 100 μg (right panel) of purified anti-IFX 8E12-IgG2a monoclonal antibody containing mutations in immune effector recruitment binding sites and challenged with luciferase-expressing transgenic *T. vivax* parasites. Mutations prevented binding to C1q (ΔC1q), FcRs (ΔFcR) or both (ΔC1qΔFcR) and were compared to non-mutated 8E12-IgG2a, 8E12-IgG1 and both isotype-matched IgG2a and IgG1 controls. In all panels, data points represent individual animals and grey shading indicates bioluminescence thresholds of uninfected mice; dashed lines indicate survival within each group. Reductions in parasitaemia followed by rebounds after day 8 post infection are likely to be due to the development of protective host antibody responses directed to the dominant variable surface glycoprotein (VSG) within the parasite population and selection of an antigenically distinct variant. One of two independent experiments with very similar outcomes is shown.

**Extended Data Figure 10 | IFX adjuvanted in Quil-A and delivered subcutaneously induces consistent and isotype-balanced anti-IFX titres that are highly protective. a,** Groups of five mice were immunised with the purified ectodomain of IFX adjuvanted in alum, Quil-A and Montanide ISA 201 VG (Mont.) using a prime and two-boost regime either intraperitoneally (alum i.p.) or subcutaneously (Quil-A and Montanide s.c.). Half-maximal anti-IFX titres were determined by ELISA. Bars represent means ± SD. IFX/Quil-A administered subcutaneously was able to elicit anti-IFX antibody titres that were as high as IFX/alum delivered intraperitoneally. **b,** Quantification of different anti-IFX antibody isotypes elicited by the different adjuvants. IFX/Quil-A was able to induce a larger proportion of IgG2 isotype subclasses. Data points represent individual mice (*n*=5) and bars are mean ± SD. **c,** Increased protection to *T. vivax* challenge using Quil-A in a protein-in-adjuvant vaccine formulation. Fourteen mice were immunized subcutaneously with purified soluble IFX recombinant protein adjuvanted in Quil-A and challenged with transgenic luciferase-expressing *T. vivax*. Parasitaemia was quantified on the indicated days after parasite challenge using bioluminescence; controls are a cohort of 14 animals treated with adjuvant only. Data points represent individual animals and grey shading indicates bioluminescence thresholds of uninfected mice. Crosses indicate where individuals had to be removed from the study for health reasons thought to be unrelated to the infection. Note that the smaller bioluminescence peaks in four mice corresponding to high bioluminescent readings between days 16 and 24 were caused by bleed-through of bioluminescence signal from the mouse that eventually succumbed to infection.