Vector transmission regulates immune control of *Plasmodium* virulence

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Abstract

Defining mechanisms by which *Plasmodium* virulence is regulated is central to understanding the pathogenesis of human malaria. Serial blood passage of *Plasmodium* through rodents[1-3], primates[4] or humans[5] increases parasite virulence, suggesting that vector transmission regulates *Plasmodium* virulence within the mammalian host. In agreement, disease severity can be modified by vector transmission[6-8], which is assumed to 'reset' *Plasmodium* to its original character[3]. However, direct evidence that vector transmission regulates *Plasmodium* virulence is lacking. Here we utilise mosquito transmission of serially blood passaged (SBP) *Plasmodium chabaudi chabaudi*[9] to interrogate regulation of parasite virulence. Analysis of SBP *P.c. chabaudi* before and after mosquito transmission demonstrates that vector transmission intrinsically modifies the asexual blood-stage parasite, which in turn, modifies the elicited mammalian immune response, which in turn, attenuates parasite growth and associated pathology. Attenuated parasite virulence associates with modified expression of the *pir* multi-gene family. Vector transmission of *Plasmodium* therefore regulates gene expression of probable variant antigens in the erythrocytic cycle, modifies the elicited mammalian immune response, and thus regulates parasite virulence. These results place the mosquito at the centre of our efforts to dissect mechanisms of protective immunity to malaria for the development of an effective vaccine.

The definitive host for mammalian *Plasmodium* is the Anopheline mosquito. Within this vector, a complex series of developmental events, including fertilisation and meiosis, culminates in invasion of the salivary glands by infective sporozoites, which are transmitted to the mammalian host via mosquito bite. Sporozoites deposited in the dermis migrate to the liver, invade hepatocytes and undergo further developmental processes prior to the release of merozoites that invade erythrocytes. The subsequent erythrocytic cycle is entirely responsible for the morbidity and mortality associated with malaria. The complexity of the *Plasmodium* life cycle has led to much of the basic biology of the blood-stage infection being studied in isolation, with *in vivo* experiments largely initiated via direct injection of infected erythrocytes. However, serial blood passage of *Plasmodium* increases parasite virulence[1-5], suggesting that regulation of *Plasmodium* virulence is an inherent consequence

of vector transmission[3]. This could result indirectly from vector control of inoculum size or the passage of large parasite populations through extreme bottlenecks, although these consequences of mosquito transmission are not thought to be major determinants of disease severity[8,10]. Alternatively, vector transmission may regulate *Plasmodium* virulence by intrinsically modifying the parasite and its interaction with the mammalian host. In this context, the immune response elicited by *Plasmodium* influences disease severity[11], and can therefore dictate parasite virulence. The interrelationship between the vector, parasite and mammalian immune system could thus underpin the pathogenesis of malaria.

To study regulation of *Plasmodium* virulence we developed routine mosquito transmission of SBP *P.c. chabaudi*[9], a rodent malaria parasite that exhibits many characteristics associated with the pathogenesis of human infection[12]. This allowed us to directly compare SBP parasites before and after vector transmission. Accordingly, mice were infected with SBP *P.c. chabaudi* AS either by injection of parasitised erythrocytes (pE) or mosquito bite (see Methods Summary). Following mosquito transmission, asexual blood-stage parasite growth was attenuated (Fig. 1a), and a low-grade, recrudescing infection with extended chronicity was established (Supplementary Fig. 1). Attenuated parasite growth in the erythrocytic cycle was not influenced by dose (ref.[9] and Supplementary Fig. 2) or, importantly, by the pre-erythrocytic-stages of infection, as attenuated parasite growth was similarly observed when mice were injected with pE derived from recently mosquito transmitted (MT) parasite lines (Fig. 1b). Similar results were observed with cloned parasites derived from SBP *P.c. chabaudi* AS (Supplementary Fig. 3), and with the hypervirulent *P.c. chabaudi* CB (Supplementary Fig. 4). Mosquito transmission therefore attenuated the asexual blood-stage parasite. As expected, serial blood passage of MT *P.c. chabaudi* AS rapidly increased parasite growth (Supplementary Fig. 5). Mice infected with *P.c. chabaudi* AS via mosquito bite did not exhibit the severe hypothermia, cachexia or hepatic cellular damage that was observed during the acute phase of infection in mice injected with SBP parasites, although they still exhibited severe anaemia despite attenuated parasite growth (Fig. 1c-f). Mosquito transmission therefore reduced disease severity in the mammalian host. Despite attenuated parasite growth and reduced pathogenicity, MT *P.c. chabaudi* AS elicited robust, long-term protection to reinfection with homologous or heterologous blood-stage parasites (Fig. 1g and Supplementary Fig. 6). Thus, vector transmission regulates the virulence of *Plasmodium* by intrinsically modifying the asexual blood-stage parasite, without influencing the capacity of the mammalian host to acquire robust immunity to reinfection.

The pathogenesis of malaria is complex and influenced by the mammalian immune system; dysregulated immune reactions can directly promote severe disease[11], whereas an apposite response can enhance parasite clearance without promoting pathology[13]. The immune response induced by *Plasmodium* can therefore define its virulence. Throughout the erythrocytic cycle the spleen is the major anatomical site associated with the developing immune response[14], and mice infected with *P.c. chabaudi* AS via mosquito bite developed marked splenomegaly with rapid recruitment of inflammatory monocytes (Supplementary Fig. 7-8). Importantly, following mosquito transmission there was enhanced expansion of activated $CD8^+$ and $CD8^-$ dendritic cells, which present malaria-specific antigens and stimulate $CD4^+$ T cell proliferation[15], in the acute phase of infection (Fig. 2a and Supplementary Fig. 9). Correspondingly, the magnitude of the effector $CD4^+$ T cell response, which orchestrates innate and adaptive immune control of blood-stage parasite growth[13], was also enhanced following mosquito transmission, and the memory $CD4^+$ T cell population exhibited a predominantly effector memory phenotype (Fig. 2b and Supplementary Fig. 10). Infection with MT *P.c. chabaudi* AS also increased the magnitude of the class-switched malaria-specific antibody response, a central component of erythrocytic immunity[11] (Fig. 2c-d). Mosquito transmission therefore enhanced antibody production in the chronic phase of infection, subsequent to enhanced innate and adaptive

cellular responses early in infection. Conversely, mosquito transmission attenuated systemic inflammation during the acute phase response, with decreased circulating levels of pro-inflammatory cytokines and chemokines associated with severe disease[11,16] (Fig. 2e). Thus, vector transmission intrinsically modifies the asexual blood-stage parasite and transforms the mammalian immune response elicited during the erythrocytic cycle.

Parasite growth and pathogenicity are, in part, determined by host susceptibility. Infection of susceptible mouse strains with *P.c. chabaudi* AS via mosquito bite causes severe disease and death[9], demonstrating that vector transmission does not limit the potential virulence of the asexual blood-stage parasite. We therefore addressed whether attenuated parasite virulence in C57BL/6 mice infected with MT *P.c. chabaudi* AS was a consequence of the transformed host immune response. Immunodeficient mice were injected with SBP *P.c. chabaudi* AS, or with an equivalent number of pE derived from a recently MT line. Disruption of the innate and adaptive immune responses, through depletion of $CD4^+$ T cells, or depletion of the entire adaptive arm of the immune system, led to a virulent and fatal acute phase infection (Fig. 3). Attenuation of parasite virulence by mosquito transmission was therefore dependent upon an intact mammalian immune response and, furthermore, independent of parasite growth rate (Supplementary Fig. 11). Immune control of parasite virulence therefore resulted directly and exclusively from modified innate and adaptive immune responses elicited by, and directed against, the blood-stage parasite. Vector transmission of *Plasmodium* thus intrinsically modifies the asexual blood-stage parasite, which in turn, modifies the elicited mammalian immune response, which in turn, regulates parasite virulence.

Defining parasite gene expression in the erythrocytic cycle after vector transmission is thus central to understanding the pathogenesis of malaria. We therefore performed genome-wide RNA sequencing on *P.c. chabaudi* AS, directly comparing blood-stage parasites before and after mosquito transmission. This allowed us to identify a set of *Plasmodium* virulence genes that direct the elicited mammalian immune response (Fig. 4). Vector transmission modified expression of approximately 10% of the entire genome in the late trophozoite stage parasite (Supplementary Tables). The majority of genes upregulated following mosquito transmission encoded exported proteins, with the potential to access and modulate the mammalian immune system. Importantly, parasite gene expression was most intensely regulated within the sub-telomeric large multi-gene families, with preferential regulation of the *pir* multi-gene family (termed *cir* in *P.c. chabaudi*) (Supplementary Fig. 12). 123 of 200 *cir* genes (61.5%) were differentially expressed following mosquito transmission, with 114 *cir* genes (57%) upregulated. Furthermore, the most upregulated gene following serial blood passage was identified as the most highly expressed *cir* gene (PCHAS_110030) in mice infected with SBP *P.c. chabaudi* AS (ref.[17], Fig. 4 and Supplementary Fig. 12). Serial blood passage therefore selected for dominant *cir* gene expression, whilst mosquito transmission revoked the selected expression hierarchy and promoted a generalised increase in *cir* expression across the parasite population. We therefore uncover a direct association between *pir* gene expression and *Plasmodium* virulence, and demonstrate that vector transmission regulates expression of probable antigenic variants[18], as previously hypothesised[19,20]. Vector transmission of *Plasmodium* thus regulates parasite gene expression in the erythrocytic cycle and, consequently, regulates immune control of *Plasmodium* virulence.

Vector transmission will inherently regulate *Plasmodium* virulence within the mammalian host. Recombination of distinct parasite genotypes within the mosquito is likely to be fundamental for the evolution of virulence[21]. The results of this study reveal that vector transmission also regulates *Plasmodium* virulence by modifying parasite gene expression, and therefore the mammalian immune response, in the erythrocytic cycle. This is likely the outcome of a combination of distinct regulatory processes acting at multiple stages of the

parasite life cycle, in both the mosquito vector and mammalian host. It is therefore important to delineate the timing and mechanism(s) of regulation of parasite gene expression, in the context of the complete *Plasmodium* life cycle, to understand the molecular regulation of parasite virulence. Attenuation of parasite virulence following mosquito transmission associates with modified expression of the *pir* multi-gene family, which is conserved from rodent to human *Plasmodium*[17]. Importantly, vector transmission of cultured *P. falciparum* similarly modifies the composition and frequency of *var* gene expression[22]. Regulation of antigenic variants by vector transmission is therefore universal[19,20,22], and vector transmission will therefore universally regulate immune control of *Plasmodium* virulence. The interrelationship between the vector, parasite and mammalian immune system thus underpins the pathogenesis of malaria.

Methods

Mice

Inbred wild-type, major histocompatibility complex class II knock-out (MHC II KO)[23] and recombination activation gene-1 knock-out (RAG KO)[24] C57BL/6 mice were bred under specific pathogen-free conditions at NIMR. All experiments were performed in accordance with UK Home Office regulations (PPL 80/2358) and approved by the ethical review panel at NIMR. Experimental mice were age- and sex-matched, housed under reverse light conditions (light 19.00-07.00: dark 07.00-19.00) at 20-22°C, and had continuous access to mouse breeder diet and water. Measurements of clinical pathology were taken at 16.00 hours: core body temperature was measured with a rectal thermometer; body weight was calculated relative to a baseline measurement taken on day −2; and erythrocyte density was determined on a VetScan HMII haematology system (Abaxis). To measure liver enzymes, plasma was analysed on a VetScan Chemistry Analyzer, using a Mammalian Liver Profile reagent rotor (Abaxis).

Enumeration of blood-stage parasites by Real-Time PCR

Whole blood was isolated 20 hours after liver merozoite egress, when parasites were at the late trophozoite stage of development and within the first cycle of schizogony. Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction[25], and reverse transcribed by PCR at 42°C using 75U MuLV Reverse Transcriptase and 2.5μM Random Hexamer primers (both Applied Biosystems) per sample. Parasites were quantified by Real-Time PCR, comparing *P.c. chabaudi* AS 18S rRNA copy number between samples and a standard curve of pE prepared at the late trophozoite stage of development. The reaction mix contained TaqMan Universal PCR Master Mix (Applied Biosystems), 300 M forward primer (5 -AAGCATTAAATAAAGCGAATACATCCTTAT-3), 300 M reverse primer (5 -GGGAGTTTGGTTTTGACGTTTATGCG-3) and 50 M probe ([6FAM]CAATTGGTTTACCTTTTGCTCTTT[TAM]). Real-Time PCR amplification was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), with a temperature profile as follows: 50°C for 2 minutes, followed by 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Parasite numbers were determined per 100μl whole blood; total circulating parasites were then calculated for each mouse based on their weight and, therefore, their estimated circulating blood volume.

Flow cytometry

Single cell suspensions of splenocytes were prepared, erythrocytes lysed, and cells enumerated on a haemocytometer. Cells were stained with monoclonal antibodies (CD3 Biotin or PerCP-Cy5.5 (145-2C11); CD4 Pacific Blue (RM4-5); CD8 Pacific Blue (53-6.7); CD11b Pacific Blue (M1/70); CD11c APC (N418); CD44 FITC (IM7); I-A^b FITC (AF6-120.1); Ly-6G PE (1A8); NK-1.1 Biotin (PK136); TER-119 Biotin (all from

BioLegend)) (CD19 Biotin (1D3); CD62L APC (MEL-14); CD127 PE (A7R34) (all from eBioscience)) (Ly-6C ALEXA FLUOR 647 (ER-MP20) (from AbD Serotec)) or irrelevant isotype-matched monoclonal antibodies as negative controls. PerCP/Cy5.5-conjugated Streptavidin (BD Biosciences) was used secondary to biotinylated antibodies. For phenotypic analysis, samples were acquired on a CyAn (Beckman Coulter), and data were analysed with FlowJo software (TreeStar).

Antibodies and cytokines

Malaria-specific antibodies were measured in plasma by ELISA: 96 well PolySorp plates (Nunc) were coated with 50μg/ml parasite lysate prepared from pE isolated from C57BL/6 mice infected with SBP *P.c. chabaudi* AS; 2-fold serial dilutions of plasma from uninfected and hyper-immune mice were used as negative and positive controls, respectively, for experimental samples; alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, IgG2c, IgG2b and IgG3 (all from SouthernBiotech) were used for detection. Samples were developed with 1mg/ml 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma) and OD was measured at 405 m. Antibody concentrations are presented as arbitrary units (AU) relative to hyper-immune plasma. Cytokines were measured in plasma by LEGENDplex Luminex custom assay (BioLegend).

Plasmodium RNA preparation

C57BL/6 mice were infected with SBP *P.c. chabaudi* AS via injection of infected erythrocytes, or via mosquito bite. Parasites were isolated at exactly 20 hours into the seventh cycle of the blood-stage infection, at the late trophozoite stage of development, as follows: whole blood was depleted of leukocytes by Plasmodipur filtration (EuroProxima); erythrocytes were centrifuged at 400xg for 10 minutes and lysed with 0.15% (w/v) saponin (Sigma); samples were centrifuged at 1000×g for 5 minutes and washed with PBS; parasites were resuspended in TRIzol (Life Technologies) and snap-frozen on dry ice. We prepared 3 biological replicates of SBP *P.c. chabaudi* AS from 8 mice each, and 2 biological replicates of MT *P.c. chabaudi* AS from 30 mice each. RNA was extracted as described[26], resuspended in water and DNA removed with a TURBO DNA-*free* Kit (Applied Biosystems), according to the manufacturer's instructions. RNA quantity/quality was determined on an Agilent 2100 Bioanalyzer RNA 6000 Nano chip.

Amplification-free RNA-seq libraries

PolyA+ transcripts were selected from 10μg total RNA using Sera-Mag Oligo(dT)-coated Magnetic Particles (Thermo Scientific). RNA was diluted with water to a volume of 130μl and fragmented to approximately 200 nucleotides using Covaris Adaptive Focused Acoustics technology (settings: 5% Duty Cycle; Intensity 5; 200 Cycles per burst for 60 seconds). The RNA was ethanol precipitated and resuspended in 10μl water. First strand cDNA was synthesised with Random Hexamer primers and SuperScript II Reverse Transcriptase (Life Technologies), following the manufacturer's instructions. Second strand cDNA synthesis, end repair and dA-tailing were performed using the NEBNext mRNA library kit for Illumina (New England Biolabs), eluting in a final volume of 15μl. Sequencing templates were prepared by mixing 15μl cDNA, 5μl 33μM adaptors (based on the published adaptor[27] with the addition of barcode sequences; oligos supplied by Integrated DNA Technologies), 25μl Quick Ligation buffer and 5μl Quick DNA ligase (both from New England Biolabs) and incubating for 15 minutes at 25°C. Excess adaptors were removed with 2 rounds of clean up with 50μl of Agencourt AMPure XP Beads (Beckman Coulter). Final libraries were eluted in 30μl water, visualised on an Agilent Bioanalyzer 2100 High Sensitivity DNA chip and quantified by qPCR. A pool of the 5 indexed libraries was sequenced on an Illumina HiSeq2000, with 100bp paired-end reads.

Analysis of RNA expression

Paired-end RNA sequencing reads were mapped to the *P.c. chabaudi* AS reference genome (September 2012 release: ftp://ftp.sanger.ac.uk/pub/pathogens/P_chabaudi/ September_2012/) using Tophat[28] v1.4.1, with appropriate fragment size parameters and maximum intron size 10000. Read counts per gene were calculated using in-house Perl scripts and non-uniquely mapping reads were excluded. Six genes with less than 20% unique coding sequence (kmer = 100) were excluded from the analysis (PCHAS_073210; PCHAS_083750; PCHAS_100020; PCHAS_113280; PCHAS_113290; PCHAS_130130). Differential gene expression between SBP and MT *P.c. chabaudi* AS was determined using DESeq[29]; the three SBP *P.c. chabaudi* AS replicates were compared against the two MT *P.c. chabaudi* AS replicates to determine genes upregulated in blood-stage parasites following mosquito transmission, and vice versa to determine genes upregulated following serial blood passage. In both cases a corrected p-value cutoff of 0.01 was applied. The resulting gene lists were categorised into '*cir*' (based on published annotation[17]), '*Pc-fam*' (based on GeneDb annotation by Ulrike Boehme at the Wellcome Trust Sanger Institute, with minor reannotation), 'Exported' (based on known biology or ExportPred[30] prediction), 'Other known function' and 'Unknown function'. For those genes within the category 'Other known function', we sub-categorised genes based on enriched biological process GO terms using TopGO[31]; a p-value cutoff of 0.01 was applied. We independently added 'glideosome' as a sub-category.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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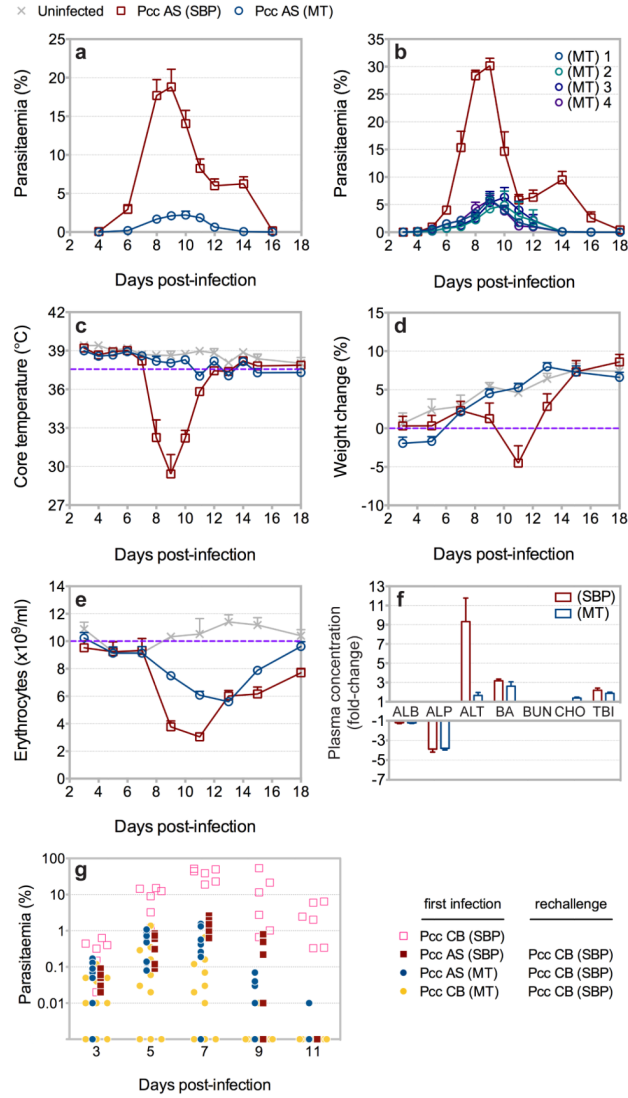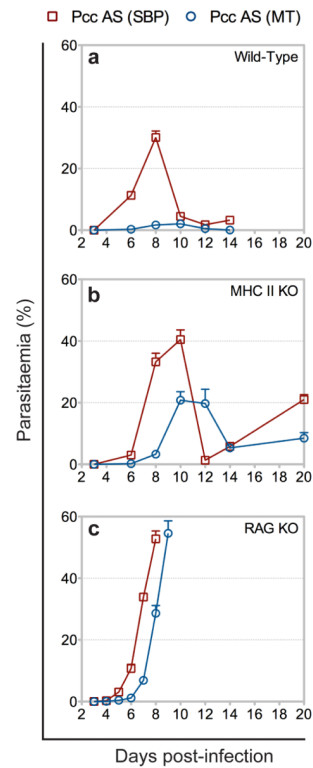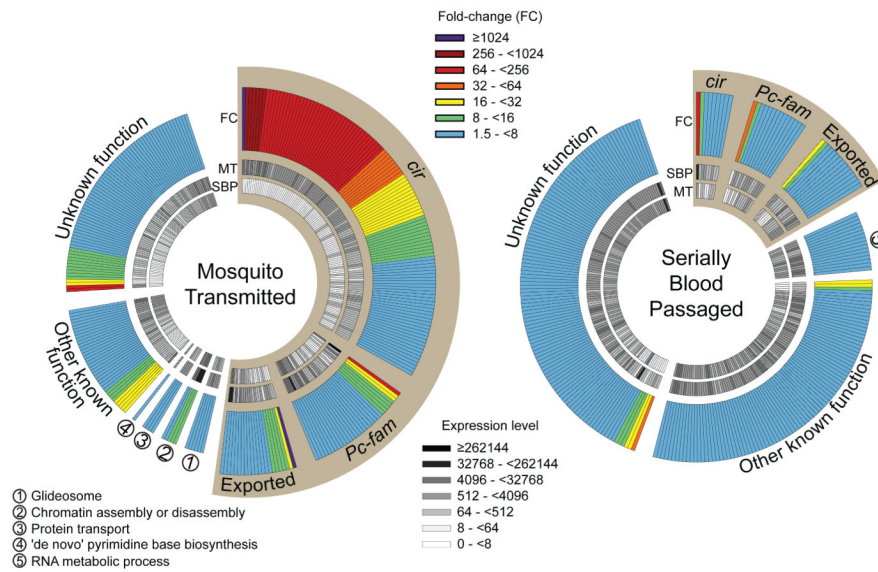
Figure 1.

Mosquito transmission of *P.c. chabaudi* AS attenuates virulence. **a**, Parasitaemia of C57BL/6 mice injected with 10^5 SBP *P.c. chabaudi* AS (Pcc AS) or infected with Pcc AS via mosquito bite. **b**, Parasitaemia of C57BL/6 mice injected with 10^5 SBP Pcc AS or injected with 10^4 or 10^5 pE derived from one of four recently MT lines of Pcc AS. **c**-**e**, Temperature (**c**), weight (**d**) and erythrocyte count (**e**) of C57BL/6 mice injected with 10^5 SBP Pcc AS or infected with Pcc AS via mosquito bite. **f**, Liver enzyme concentration on day 10 post-infection in plasma of C57BL/6 mice injected with 10^5 SBP Pcc AS or infected with Pcc AS via mosquito bite. Data presented as fold-change relative to uninfected control mice. (<u>ALB</u>umin; <u>AL</u>kaline <u>P</u>hosphatase; <u>ALa</u>nine amino <u>T</u>ransferase; <u>B</u>ile <u>A</u>cids, <u>B</u>lood <u>U</u>rea <u>N</u>itrogen; <u>CHO</u>lesterol; <u>T</u>otal <u>BI</u>lirubin). **g**, Parasitaemia of C57BL/6 mice injected with 10^6 SBP *P.c. chabaudi* CB (Pcc CB) as a first infection (open symbols), or as a rechallenge (closed symbols) 90 days after injection with 10^5 SBP Pcc AS or infection with Pcc AS or CB via mosquito bite. (n = 3-20 mice per group; data presented as mean with SEM).
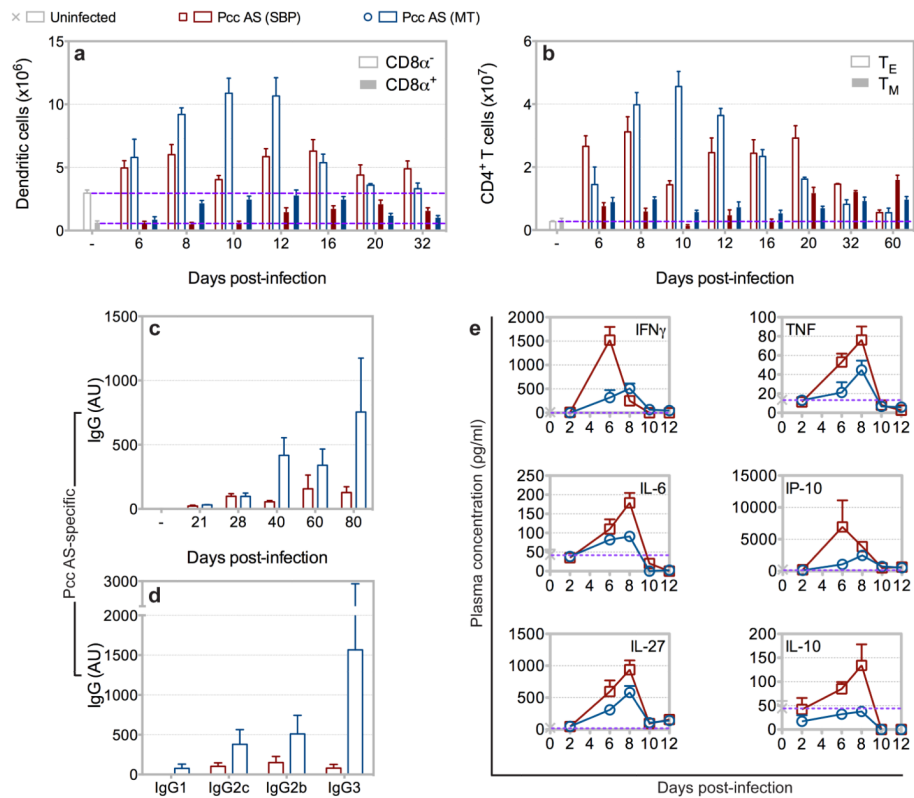
Figure 2.
Mosquito transmission of *P.c. chabaudi* AS transforms the elicited mammalian immune response. **a-b**, Number of CD8^{−} (open bars) and CD8^{+} (closed bars) dendritic cells (**a**), and effector (T_E) (open bars) and memory (T_M) (closed bars) CD4^{+} T cells (**b**) in spleens of C57BL/6 mice injected with 10^5 SBP Pcc AS or infected with Pcc AS via mosquito bite. **c-d**, Plasma concentration of total parasite-specific IgG throughout infection (**c**) and parasite-specific IgG subclasses on day 80 post-infection (**d**) in C57BL/6 mice injected with 10^5 SBP Pcc AS or infected with Pcc AS via mosquito bite. Data presented as arbitrary units (AU) relative to hyper-immune plasma. **e**, Plasma cytokine concentration in C57BL/6 mice injected with 10^5 SBP Pcc AS or infected with Pcc AS via mosquito bite. (n = 3-5 mice per group per time-point; data presented as mean with SEM).

Figure 3.
Transformed innate and adaptive immune responses attenuate *P.c. chabaudi* AS virulence. **a-c**, Parasitaemia of wild-type (**a**), CD4^{+} T cell deficient (MHC II KO) (**b**), and B and T cell deficient (RAG KO) (**c**) C57BL/6 mice injected with 10^5 SBP Pcc AS or injected with 10^5 pE derived from a recently MT line of Pcc AS. (n = 4-6 mice per group; data presented as mean with SEM).

Figure 4.
Mosquito transmission of *P.c. chabaudi* AS modifies parasite gene expression in the erythrocytic cycle. C57BL/6 mice were injected with 10^5 SBP Pcc AS or infected with Pcc AS via mosquito bite. Parasites were isolated after 6 cycles of the blood-stage infection, and at the late trophozoite stage of development (98.3% (0.76%) and 97.0% (1.41%) trophozoites for SBP and MT samples, respectively (mean with SD)). Total parasite RNA was extracted and sequenced. Those genes differentially expressed between SBP and MT Pcc AS were determined; genes identified as significantly upregulated in blood-stage parasites following mosquito transmission (left) versus serial blood passage (right) are shown. Each segment represents one gene, and genes are categorised according to the function of their product and ranked based on fold-change (outer circle). The DESeq-normalised expression levels for each gene are also shown (inner circles). Sepia wedges highlight genes whose products are predicted to be exported, or otherwise accessible to the mammalian immune system.