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Low levels of mammalian TGF- β 1 are protective against malaria parasite infection, a paradox clarified in the mosquito host

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Abstract

Nitric oxide (NO), derived from catalysis of inducible NO synthase (iNOS), limits malaria parasite growth in mammals. Transforming growth factor (TGF)- β 1 suppresses iNOS in cells *in vitro* as well as *in vivo* in mice, but paradoxically severe malaria in humans is associated with low levels of TGF- β 1. We hypothesized that this paradox is a universal feature of infection and occurs in the mosquito *Anopheles stephensi*, an invertebrate host for *Plasmodium* that also regulates parasite development with inducible NO synthase (AsNOS). We show that exogenous human TGF- β 1 dose-dependently regulates mosquito AsNOS expression and that parasite killing by low dose TGF- β 1 depends on AsNOS catalysis. Furthermore, induction of AsNOS expression by TGF- β 1 is regulated by NO synthesis. These results suggest that TGF- β 1 plays similar roles during parasite infection in mammals and mosquitoes and that this role is linked to the effects of TGF- β 1 on inducible NO synthesis. .

Index descriptors

Plasmodium falciparum; *Anopheles stephensi*; malaria; protozoan; mosquito; nitric oxide synthase

Introduction

Isoforms of mammalian TGF- β are involved in immune responses to various pathogens including *Plasmodium* (Omer et al., 2000). The TGF- β superfamily includes the prototypical TGF- β s, which function both as cytokines and growth factors, along with activins, nodal and bone morphogenetic proteins that regulate a broad array of cellular responses including proliferation, differentiation, migration and apoptosis. During malaria parasite infection, TGF- β 1 plays a pivotal role in controlling parasite load and the host immune response and, as such, has been suggested to maintain an “immunological balance” during parasite infection (Omer et al., 2000).

In mice, low levels of TGF- β 1 promote parasite clearance early in infection, while later in infection higher levels of TGF- β 1 are anti-inflammatory and minimize host immunopathology (Omer and Riley, 1998 and Omer et al., 2000). Similarly, experimental manipulation of

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circulating levels of this cytokine in mice by injection of recombinant TGF- β 1 or via antibody neutralization indicated that high early or low late TGF- β 1 levels were associated with uncontrolled parasitemia and increased pathology (Tsutsui and Kamiyama, 1999 and reviewed in Omer et al., 2000). Two studies of natural infection in patients in Gabon (Perkins et al., 2000) and Thailand (Wenisch et al., 1995) and one study of experimental human infection (Walther et al., 2005 and Walther et al., 2006) corroborate these animal studies. Specifically, severe late-stage infection was associated with significantly reduced serum levels of TGF- β 1 (7.6 pg/ml, Perkins et al., 2000; 14 pg/ml, Wenisch et al., 1995) relative to levels in healthy controls (16 pg/ml, Perkins et al., 2000; 63 pg/ml, Wenisch et al., 1995), suggesting that uncontrolled immunopathology exacerbated clinical disease. In other work, a longitudinal study of sporozoite-induced infection of *P. falciparum* in humans noted that early upregulation of TGF- β 1 and high levels of bioactive TGF- β 1 (up to 400 pg/ml or 9.2-fold above baseline) at parasite emergence from the liver were associated with the most rapid parasite growth (Walther et al., 2005 and Walther et al., 2006).

A key target in mammalian cells of TGF- β 1 regulation during inflammation is inducible nitric oxide synthase (iNOS; (Vodovotz, 1997 and Vodovotz et al., 2004). In this context, the primary role of TGF- β 1 (at doses as low as 100 pg/ml) is to decrease iNOS induction (Vodovotz et al., 1993). The regulation of iNOS by TGF- β 1 can occur at the transcriptional, translational and post-translational levels (Vodovotz, 1997).

During *Plasmodium* infection in human and mice, NO inactivates sporozoites during liver invasion and can also inactivate circulating gametocytes (Mellouk et al., 1994 and Naotunne et al., 1993). Nitric oxide has also been linked to the development of cerebral malaria, the most severe form of human infection, with most studies reporting a positive association between high NO levels and increased pathology in late stage disease (Clark and Cowden 2003). In contrast, Anstey et al., (1996) documented that high NO levels were associated with reduced malaria severity and low parasite density and Hobbs et al., (2002) showed that higher NO production was associated with less severe malaria but with no differences in parasitemia levels. As such, studies of NO levels in human malaria have revealed both protective and pathological effects. A possible explanation for these findings is that parasite clearance early in infection requires NO synthesis, which if uncontrolled contributes to immunopathology in late stage disease (reviewed in Riley et al., 2006).

We propose that certain aspects of the TGF- β 1 – NO paradox can be clarified by examining the interactions of these factors in the mosquito host. The control of malaria parasite infection by inducible NO synthesis in a mosquito was first described in *Anopheles stephensi*, a major malaria vector in India, southeast Asia and parts of the Middle East (Luckhart et al., 1998). The response is catalyzed by *A. stephensi* NO synthase (AsNOS) (Luckhart and Li 2001 and Luckhart and Rosenberg, 1999) and results in the synthesis of inflammatory levels of toxic reactive nitrogen intermediates (Luckhart et al., 1998 and Peterson et al., 2007). Previous work revealed that low levels of human TGF- β 1 (2 pg/ml, 200 pg/ml) provided in blood inhibited malaria parasite growth in *A. stephensi*, while a higher dose (2000 pg/ml) did not affect parasite growth, suggesting an anti-inflammatory or dampening effect on mosquito defenses against parasite infection (Luckhart et al., 2003).

Based on our previous observations, we hypothesized that TGF- β 1 would exert dichotomous, dose-dependent effects on AsNOS expression *in vivo* and on AsNOS catalytic activity during malaria parasite infection in the mosquito. Our data indicated that while both low and high doses of human TGF- β 1 could induce AsNOS expression, only the lower doses resulted in higher, sustained induction of anti-parasitic AsNOS. Our data also confirmed that catalytic activity of AsNOS regulates AsNOS induction and TGF- β 1-associated killing of developing parasites. Furthermore, our studies suggest that the mosquito model not only affirms a role for

TGF- β 1 in maintaining immunological balance, but also directly connects this balance with TGF- β 1 regulation of NOS.

Materials and Methods

Laboratory reared 3- to 5-day-old female *A. stephensi* mosquitoes used in the present study were maintained as described previously (Luckhart et al., 2003). Human TGF- β 1 (Cell Signaling Technology, Danvers, MA), L-NAME (Sigma-Aldrich, St. Louis, MO) and D-NAME (Sigma-Aldrich) stock solutions were prepared in phosphate-buffered saline (PBS; Cellgro, Mediatech, Herndon, VA) and diluted directly into an artificial bloodmeal for feeding. For all experiments, the artificial bloodmeal was provided via a Hemotek feeding system (Discovery Workshops, Accrington, Lancaster, UK) and was comprised of washed human type O positive erythrocytes (Interstate Blood Bank, Memphis, TN) diluted in heat-inactivated compatible human serum. In previous work, we determined that heat-inactivation, followed by multiple freeze-thaw cycles eliminated detectable active TGF- β 1 in the serum (Luckhart et al., 2003). Mosquitoes were allowed to feed on the artificial bloodmeal for 30 min, and then blood-filled midguts were dissected into PBS (Cellgro) and transferred immediately into TRI-reagent (Invitrogen, Carlsbad, CA) for RNA extraction. Total RNA from 20 mosquito midguts pooled from each treatment group were extracted as per manufacturer's instructions. Analyses of TGF- β 1 dose-dependence (Fig. 1A) and the effects of L-NAME on TGF- β 1-induced *AsNOS* expression (Fig. 1B) were each replicated with five separate cohorts of *A. stephensi*; each cohort was subdivided into four treatment groups. Relative *AsNOS* transcript abundance was calculated as normalized ΔC_T values using a comparative cycle threshold method of analysis (User Bulletin 2, ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, CA) with S7 ribosomal protein gene expression as the housekeeping gene control. Primers, probes and thermal cycling conditions were described previously (Crampton and Luckhart 2001). Briefly, *AsNOS* primers and probe included the forward primer NOSF2 (5'-GACCAAACCGGTCATCCTGAT-3'), the reverse primer NOSR2 (5'-GGAATCTTGCAGTCAACCATTTC-3') and the Taqman® probe AsNOSP (6FAM-CACCGTTCCGTTCTGGCA-TAMRA). The ribosomal S7 primers and probe included the forward primer S7F2 (5'-GAAGGCCTTCCAGAAGGTACAGA-3'), the reverse primer S7R2 (5'-CATCGGTTTGGGCAGAATG-3') and the Taqman® probe S7P (VIC-AGAAGTTCTCCGGCAAGCACGTCGT-TAMRA). Real-time PCR data represented in Fig. 1A were not normally distributed and were analyzed by Kruskal-Wallis test for overall significance and by Wilcoxon rank test for pairwise comparisons. Real-time PCR data represented in Fig. 1B were normally distributed and analyzed by ANOVA for overall significance and by Student-Neuman-Keuls for pairwise comparisons.

To determine the effects of human TGF- β 1 on malaria parasite development, female *A. stephensi* were fed on cultured mature stage V *P. falciparum* NF54 gametocytes. The gametocyte culture was diluted with human erythrocytes and heat-inactivated serum and mixed with 2 pg/ml or 200 pg/ml recombinant human TGF- β 1 with or without 1 mg/ml L-NAME or 1 mg/ml D-NAME immediately prior to feeding. Control mosquitoes were provided an infectious bloodmeal supplemented with an equivalent volume of PBS. After feeding, mosquitoes were maintained at 24–28°C and 70–80% relative humidity under a 12 h light/dark cycle for seven days. On day 7, mosquito midguts were dissected in PBS and stained with mercurochrome to count *P. falciparum* oocysts. Parasite infection data were normally distributed and were analyzed by ANOVA for overall significance and by Student-Neuman-Keuls for pairwise comparisons.

Results and Discussion

In *A. stephensi*, malaria parasite infection induces biphasic expression of *AsNOS* in the midgut epithelium at 6 h and 36–48 h after infection (Luckhart et al., 2003). In the context of parasite development, induction at 6 h coincides with sexual stage parasite fertilization and the formation of mobile zygotes (ookinetes) in the blood mass, while induction at 36–48 h coincides with ookinete escape from the blood mass via direct penetration of the midgut epithelium. Parasite glycosylphosphatidyinositol (GPIs), which are elaborated during death of asexual stages in the blood mass and also during parasite invasion of the midgut (Han et al., 2000), contribute to the biphasic pattern of *AsNOS* expression (Lim et al., 2005). However, blood components, including TGF- β 1 and insulin, can also function as signals to mosquito cells and alter mosquito physiology *in vivo* after ingestion (Luckhart et al., 2003, Lim et al., 2005 and reviewed in Akman-Anderson et al., 2007). In the work reported herein, we have expanded our understanding of TGF- β 1 regulation of *AsNOS* and parasite development *in vivo* and, in the process, provided unique insights into the role of TGF- β 1 in maintaining an immunological balance during parasite infection.

To determine whether TGF- β 1 regulation of *AsNOS* expression was time- and dose-dependent, each of five separate cohorts of *A. stephensi* were divided into four treatment groups receiving artificial bloodmeals supplemented with 2, 200 or 2000 pg/ml TGF- β 1, in accord with previous studies (Luckhart et al., 2003), or with an equivalent volume of PBS. Relative to the PBS-treated control group, provision of human TGF- β 1 at 2 pg/ml or 2000 pg/ml resulted in significant ($p < 0.05$) increases in *AsNOS* expression in the midgut epithelium immediately following the 30 min feeding period (0 h; Fig. 1A). In addition, *AsNOS* induction by 2000 pg/ml TGF- β 1 was significantly higher than induction by 2 pg/ml TGF- β 1 immediately after feeding indicating a sensitivity of *AsNOS* induction to TGF- β 1 dose ($p < 0.05$; Fig. 1A). Although the increase in *AsNOS* expression at 0 h in response to 200 pg/ml TGF- β 1 was consistent with the overall trend of dose dependence, induction levels at this dose and time did not achieve statistical significance relative to the control.

The rapidity of *AsNOS* induction at first glance may be unexpected, but the fact that some insects feed very quickly after access to blood is provided suggests that the response developed within 30 min. At 6 h and less so at 12 h after feeding, *AsNOS* expression levels in the TGF- β 1-treated groups were also induced, but these levels were not significantly different from the PBS-treated control groups at those timepoints (Fig. 1A). By 24 h, little to no induction of *AsNOS* expression was observed in the treatment groups relative to the control group. In contrast, at 36 h after feeding, significant induction of *AsNOS* expression ($p < 0.05$) was observed in the groups treated with either 2 pg/ml or 2000 pg/ml TGF- β 1 relative to the PBS-treated control, indicating that TGF- β 1 upregulation of *AsNOS* expression is temporally biphasic (Fig. 1A). As observed at 0 h, *AsNOS* expression was induced at 36 h by treatment with 200 pg/ml TGF- β 1 relative to the control group, but high variation precluded statistical significance at this dose (Fig. 1A). Although the inductions at 2 pg/ml and 2000 pg/ml were not significantly different from each other at 36 h, patterns of induction at 0 h and 36 h were nonetheless suggestive of an inverse pattern of biphasic induction (Fig. 1A). Specifically, the highest increase in *AsNOS* expression at 0 h was observed at 2000 pg/ml, while the highest mean increase at 36 h was observed in the group treated with 2 pg/ml TGF- β 1 (Fig. 1A).

Based on the fact that NO regulates expression and activity of NOS in mammalian cells (Griscavage et al., 1995 and Grumbach et al., 2005), we hypothesized that high induction of *AsNOS* by 2000 pg/ml TGF- β 1 at 0 h resulted in early NO synthesis at levels that reduced *AsNOS* expression in the midgut at 36 h after feeding. We reported previously that the isoform non-specific NOS inhibitor L-NAME directly inhibited induced but not basal *AsNOS* activity in mosquito lysates (Luckhart et al., 1998). These findings suggested that L-NAME could also

be used to inhibit TGF- β 1-inducible AsNOS activity. Accordingly, we designed an artificial feeding assay with L-NAME and D-NAME to test the hypothesis that AsNOS-derived NO feeds back on AsNOS expression following early induction by TGF- β 1. Each of five separate cohorts of *A. stephensi* were subdivided into treatment groups that received artificial bloodmeals supplemented with 2000 pg/ml TGF- β 1 with or without 1 mg/ml L-NAME or D-NAME. Controls were provided with an identical meal supplemented with an equivalent volume of PBS. Mosquitoes were allowed to feed for 30 min, and then dissected at 15 min after feeding as described above.

As expected 2000 pg/ml TGF- β 1 induced AsNOS expression relative to the controls (Fig. 1B); the level of induction was comparable to that observed immediately (0 h) after feeding in the previous assay (Fig. 1A). In contrast, AsNOS induction levels in mosquitoes provided an artificial meal containing TGF- β 1 and L-NAME were significantly ($p < 0.05$) higher than AsNOS levels in the groups treated with TGF- β 1 alone or with TGF- β 1 and D-NAME or with PBS (Fig. 1B). These results supported our hypothesis that NO produced following treatment with a high dose of TGF- β 1 is involved in negative feedback of AsNOS expression.

In previous studies, we demonstrated that TGF- β 1 at 2 pg/ml and 200 pg/ml significantly reduced parasite load in *P. falciparum*-infected mosquitoes relative to insects provided with either 2000 pg/ml TGF- β 1 or with the infectious meal only (Luckhart et al., 2003). Our data suggested that this effect was due to TGF- β 1 regulation of not only AsNOS expression (Fig. 1A) but also AsNOS catalytic activity (Fig. 1B). To test this hypothesis, treatment groups of 50 mosquitoes each were provided artificial bloodmeals containing *P. falciparum* supplemented with 2 pg/ml or 200 pg/ml TGF- β 1, 1 mg/ml L-NAME or D-NAME, combinations of these treatments, or an equivalent volume of PBS as a control.

In agreement with our previous studies (Luckhart et al., 2003), treatment with TGF- β 1 only at 2 pg/ml and 200 pg/ml resulted in reduced parasite loads relative to controls treated with PBS ($p < 0.05$; Fig. 2). Treatment with L-NAME increased parasite loads ($p < 0.10$), while D-NAME had no effect relative to PBS-treated controls (Fig. 2), again in accord with previous observations (Luckhart et al., 1998). The addition of L-NAME together with TGF- β 1, however, partially reversed the TGF- β 1-induced decrease on parasite load. Specifically, the average numbers of oocysts in both groups treated with TGF- β 1 + L-NAME were significantly lower than numbers observed in the PBS controls ($p < 0.05$), but intermediate to those in the groups treated only with TGF- β 1 or with L-NAME (Fig. 2). The average numbers of oocysts in the TGF- β 1 + D-NAME treated groups were significantly lower than numbers observed in the PBS controls and not significantly different from those in the groups treated with TGF- β 1 only, suggesting that D-NAME had no effect on the TGF- β 1-induced reduction in parasite load relative to the controls (Fig. 2). Taken together, these findings confirmed that the reduction of parasite development observed previously following provision of 2 pg/ml or 200 pg/ml of TGF- β 1 (Luckhart et al., 2003) depends in part on regulation of AsNOS catalytic activity.

In sum, our findings indicate that negative feedback of increasing concentrations of TGF- β 1 does not directly limit AsNOS induction as has been observed for TGF- β 1 induction of NOS in mammalian cells. Rather, AsNOS expression was increased even at the highest dose of TGF- β 1 tested (Fig. 1A), with negative feedback in our system occurring at the level of NO synthesis. This feedback is relieved at the highest dose of TGF- β 1 tested by simultaneous provision of the NOS inhibitor L-NAME (Fig. 1B). These findings suggest that concentrations of TGF- β 1 exceeding 200 pg/ml ultimately shut down NO synthesis in mosquito and mammalian cells, allowing parasitemia to develop unabated in both hosts.

Most studies to date have associated inducible NO production in the mammalian host with anti-parasitic effects (Rockett et al., 1991, Naotunne et al., 1993, Mellouk et al., 1994, Anstey

et al., 1996, Pino et al., 2004, Sharma et al., 2004) as well as with increased inflammatory pathology to the host (Clark and Cowden, 2003 and Riley et al., 2006). In contrast, two studies have concluded that higher levels of NO in humans (Hobbs et al., 2002) and in mice (Gramaglia et al., 2006) were associated with less severe malaria but no differences in parasitemia levels. The cause of these discrepancies is unclear at this point, but may be due to differences in experimental protocol, dietary and retained levels of nitrite/nitrate, specific outcomes examined, or the anatomical compartment (e.g., blood versus brain) under investigation.

In *A. stephensi*, we have examined within a single anatomical compartment – the midgut – parasite induction of *AsNOS* (Luckhart et al., 1998 and Luckhart et al., 2003), the synthesis of parasite-induced reactive nitric oxide metabolites (Peterson et al., 2007), TGF- β 1 regulation of parasite infection (Luckhart et al., 2003) and, in the present study, TGF- β 1 induction of *AsNOS* expression and catalytic activity as well as the direct combined effects of these events on malaria parasite development. All of our data are consistent with the prevailing hypothesis that TGF- β 1 maintains an immunological balance during malaria parasite infection. The mosquito host depends only on innate immune responses to regulate parasite infection. This fact, together with the ability to focus in the mosquito on a single critical tissue for parasite development, can be used to significant advantage in studying host control of parasite infection. Further, our findings extend the hypothesis of TGF- β 1-associated immunological balance to the invertebrate host of *Plasmodium* spp., suggesting that these phenomena have been conserved through some 600 million years of evolution. Nonetheless, to unravel the complexity of interactions among the three organisms that contribute to the final outcome in malaria – *Plasmodium*, *Anopheles*, and the mammalian host (reviewed in Akman-Anderson 2007) – will likely require more global, systems-based approaches such as those being applied to the inflammatory response in other contexts (Vodovotz, 2006). As in other systems, we expect that a systems-based approach may lead to non-intuitive hypotheses and novel insights regarding the interactions of host and parasite in malaria.

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Abbreviations

AsNOS	<i>Anopheles stephensi</i> nitric oxide synthase
TGF-β1	transforming growth factor- β 1
NO	nitric oxide
L-NAME	<i>N</i> ^ω -nitro-L-arginine methyl ester
D-NAME	<i>N</i> ^ω -nitro-D-arginine methyl ester

References

- Akman-Anderson, L.; Vodovotz, Y.; Zamora, R.; Luckhart, S. Bloodfeeding as an interface of mammalian and arthropod immunity. In: Beckage, N., editor. *Insect Immunology*. San Diego: Elsevier; 2007.
- Anstey NM, Weinberg JB, Hassanali MY, Mwaikambo ED, Manyenga D, Misukonis MA, Arnelle DR, Hollis D, McDonald MI, Granger DL. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *Journal of Experimental Medicine* 1996;184:557–567. [PubMed: 8760809]
- Clark IA, Cowden WB. The pathophysiology of falciparum malaria. *Pharmacology and Therapeutics* 2003;99:221–260. [PubMed: 12888113]
- Crampton A, Luckhart S. The role of As60A, a TGF-beta homolog, in *Anopheles stephensi* innate immunity and defense against *Plasmodium* infection. *Infection, Genetics and Evolution* 2001;1:131–141.
- Gramaglia I, Sobolewski P, Meays D, Contreras R, Nolan JP, Frangos JA, Intaglietta M, van der Heyde HC. Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. *Nature Medicine* 2006;12:1417–1422.
- Griscavage JM, Wilk S, Ignarro LJ. Serine and cysteine proteinase inhibitors prevent nitric oxide production by activated macrophages by interfering with transcription of the inducible NO synthase gene. *Biochemical and Biophysical Research Communications* 1995;215:721–729. [PubMed: 7488014]
- Grumbach IM, Chen W, Mertens SA, Harrison DG. A negative feedback mechanism involving nitric oxide and nuclear factor kappa-B modulates endothelial nitric oxide synthase transcription. *Journal of Molecular and Cellular Cardiology* 2005;39:595–603. [PubMed: 16099468]
- Han YS, Thompson J, Kafatos FC, Barillas-Mury C. Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *The EMBO Journal* 2000;19:6030–6040. [PubMed: 11080150]
- Hobbs MR, Udhayakumar V, Levesque MC, Booth J, Roberts JM, Tkachuk AN, Pole A, Coon H, Kariuki S, Nahlen BL, Mwaikambo ED, Lal AL, Granger DL, Anstey NM, Weinberg JB. A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children. *Lancet* 2002;360:1468–1475. [PubMed: 12433515]
- Lim J, Gowda DC, Krishnegowda G, Luckhart S. Induction of nitric oxide synthase in *Anopheles stephensi* by *Plasmodium falciparum*: mechanism of signaling and the role of parasite glycosylphosphatidylinositols. *Infection and Immunology* 2005;73:2778–2789.
- Luckhart S, Crampton AL, Zamora R, Lieber MJ, Dos Santos PC, Peterson TM, Emmith N, Lim J, Wink DA, Vodovotz Y. Mammalian transforming growth factor beta1 activated after ingestion by *Anopheles stephensi* modulates mosquito immunity. *Infection and Immunity* 2003;71:3000–3009. [PubMed: 12761076]
- Luckhart S, Li K. Transcriptional complexity of the *Anopheles stephensi* nitric oxide synthase gene. *Insect Biochemistry and Molecular Biology* 2001;31:249–256. [PubMed: 11167094]
- Luckhart S, Rosenberg R. Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. *Gene* 1999;232:25–34. [PubMed: 10333518]
- Luckhart S, Vodovotz Y, Cui L, Rosenberg R. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:5700–5705.
- Mellouk S, Hoffman SL, Liu ZZ, de la Vega P, Billiar TR, Nussler AK. Nitric oxide-mediated antiparasitic activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. *Infection and Immunity* 1994;62:4043–4046. [PubMed: 8063424]
- Naotunne TS, Karunaweera ND, Mendis KN, Carter R. Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates. *Immunology* 1993;78:555–562. [PubMed: 8495973]
- Omer FM, Kurtzals JA, Riley EM. Maintaining the immunological balance in parasitic infections: a role for TGF-beta? *Parasitology Today* 2000;16:18–23. [PubMed: 10637583]

- Omer FM, Riley EM. Transforming growth factor beta production is inversely correlated with severity of murine malaria infection. *Journal of Experimental Medicine* 1998;188:39–48. [PubMed: 9653082]
- Perkins DJ, Weinberg JB, Kremsner PG. Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: relationship of cytokine balance with disease severity. *Journal of Infectious Diseases* 2000;182:988–992. [PubMed: 10950804]
- Peterson TM, Gow AJ, Luckhart S. Nitric oxide metabolites induced in *Anopheles stephensi* control malaria parasite infection. *Free Radical Biology and Medicine* 2007;42:132–142. [PubMed: 17157200]
- Pino P, Vouldoukis I, Dugas N, Conti M, Nitcheu J, Traore B, Danis M, Dugas B, Mazier D. Induction of the CD23/nitric oxide pathway in endothelial cells downregulates ICAM-1 expression and decreases cytoadherence of *Plasmodium falciparum*-infected erythrocytes. *Cellular Microbiology* 2004;6:839–848. [PubMed: 15272865]
- Riley EM, Wahl S, Perkins DJ, Schofield L. Regulating immunity to malaria. *Parasite Immunology* 2006;28:35–49. [PubMed: 16438675]
- Rockett KA, Awburn MM, Cowden WB, Clark IA. Killing of *Plasmodium falciparum* *in vitro* by nitric oxide derivatives. *Infection and Immunity* 1991;59:3280–3283. [PubMed: 1879941]
- Sharma A, Eapen A, Subbarao SK. Parasite killing in *Plasmodium vivax* malaria by nitric oxide: implication of aspartic protease inhibition. *The Journal of Biochemistry (Tokyo)* 2004;136:329–334.
- Tsutsui N, Kamiyama T. Transforming growth factor beta-induced failure of resistance to infection with blood-stage *Plasmodium chabaudi* in mice. *Infection and Immunity* 1999;67:2306–2311. [PubMed: 10225888]
- Vodovotz Y. Control of nitric oxide production by transforming growth factor-beta1: mechanistic insights and potential relevance to human disease. *Nitric Oxide* 1997;1:3–17. [PubMed: 9701040]
- Vodovotz Y. Deciphering the complexity of acute inflammation using mathematical models. *Immunology Research* 2006;36:237–245.
- Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. *Journal of Experimental Medicine* 1993;178:605–613. [PubMed: 7688028]
- Vodovotz Y, Zamora R, Lieber MJ, Luckhart S. Cross-talk between nitric oxide and transforming growth factor-beta1 in malaria. *Current Molecular Medicine* 2004;4:787–797. [PubMed: 15579025]
- Walther M, Tongren JE, Andrews L, Korbel D, King E, Fletcher H, Andersen RF, Bejon P, Thompson F, Dunachie SJ, Edele F, de Souza JB, Sinden RE, Gilbert SC, Riley EM, Hill AV. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 2005;23:287–296. [PubMed: 16169501]
- Walther M, Woodruff J, Edele F, Jeffries D, Tongren JE, King E, Andrews L, Bejon P, Gilbert SC, De Souza JB, Sinden R, Hill AV, Riley EM. Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage *Plasmodium falciparum* correlate with parasitological and clinical outcomes. *Journal of Immunology* 2006;177:5736–5745.
- Wenisch C, Parschalk B, Burgmann H, Looareesuwan S, Graninger W. Decreased serum levels of TGF-beta in patients with acute *Plasmodium falciparum* malaria. *Journal of Clinical Immunology* 1995;15:69–73. [PubMed: 7559910]

Figure 1A

Figure 1.

Human TGF- β 1 regulation of *AsNOS* expression in the *A. stephensi* midgut epithelium is dose- and time-dependent. *Anopheles stephensi* were provided an artificial bloodmeal supplemented with recombinant human TGF- β 1 alone (A) or in combination with L-NAME or D-NAME (B). **A:** *AsNOS* expression was monitored using real-time PCR in *A. stephensi* midguts dissected immediately after (0 h) to 36 h following feeding of 2, 200, or 2000 pg/ml recombinant human TGF- β 1. Data from five replicates are reported as mean normalized $\Delta C_T \pm$ SEM. **B:** *AsNOS* expression was monitored using real-time PCR in *A. stephensi* midguts dissected at 15 min following feeding of 2000 pg/ml recombinant human TGF- β 1 +/– 1mg/ml L-NAME or D-NAME. Matched controls received an identical bloodmeal supplemented with an equivalent volume of PBS. Data from five replicates are reported as

mean normalized $\Delta C_T \pm$ SEM. Legend: * p-value < 0.05 versus PBS control, \$ p-value < 0.05 versus 2 pg/ml TGF- β 1.

Figure 2.

Human TGF- β 1 reduces *P. falciparum* infection in *A. stephensi* through an effect on AsNOS catalytic activity. Three- to five-day-old female *A. stephensi* from a single cohort were divided into treatment groups (n = 50 per group) and provided artificial bloodmeals containing *P. falciparum* NF54 gametocytes supplemented with 2 pg/ml recombinant human TGF- β 1, 200 pg/ml TGF- β 1, 1 mg/ml (3.7 mM) L-NAME, 1 mg/ml (3.7 mM) D-NAME, or combinations of TGF- β 1 with L-NAME or D-NAME. Mosquito midguts were dissected at 7 d post-infection to count *P. falciparum* oocysts. Data are represented as mean oocysts per midgut $\pm$ SEM. Legend: * p-value versus PBS < 0.05, # p-value versus 2 pg/ml TGF- β 1 < 0.10, \$ p-value versus PBS < 0.10, § p-value versus 200 pg/ml TGF- β 1 < 0.05.