Immunological Crosstalk between Human Transforming Growth Factor-β1 and the Malaria Vector *Anopheles stephensi*

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Abstract

The emergence of pesticide-resistant mosquitoes and drug-resistant parasites in the last twenty years has made control of malaria more difficult. One novel strategy to better control malaria is the development and release of transgenic mosquitoes whose enhanced immunity prevents transmission of the parasite to the mammalian host. One candidate effector gene is *Anopheles stephensi* nitric oxide synthase (AsNOS), whose inducible expression and subsequent synthesis of nitric oxide (NO) limits *Plasmodium* development in *A. stephensi*.

In mammals, one of the most potent physiological regulators of NOS gene expression and catalytic activity is transforming growth factor-β (TGF-β). Moreover, human TGF-β can activate *Drosophila melanogaster* Smads, the proteins responsible for TGF-β signal transduction. We have determined that following a bloodmeal, active human TGF-β1 (hTGF-β1) persists in the midgut of *A. stephensi* for up to 48 hours. My data demonstrate that the midgut epithelium recognizes hTGF-β1 as an immunomodulatory cytokine. Specifically, induction of *AsNOS* by hTGF-β1 occurs in the midgut within minutes of bloodfeeding. Moreover, hTGF-β1 limits development of the human malaria parasite *Plasmodium falciparum* in the midgut. In other experiments, provision of the AsNOS catalytic inhibitor L-NAME partially reverses the effect of hTGF-β1 on *Plasmodium* development. These results suggest that AsNOS is a target of hTGF-β1 signaling and additional effectors that impact parasite development may be regulated by hTGF-β1 as well.

The fact that hTGF-β1 signals mosquito cells to limit malaria parasite development suggests that there is an endogenous TGF-β signaling network in place. An analysis of the *A. gambiae* genome database revealed the presence of six TGF-β ligands, including gene duplication in the *60A* gene, the first evidence of ligand gene duplication outside of chordates. In addition to five receptors, three Smads were identified in the *A. gambiae* genome predicted to support TGF-β/Activin- and BMP-like signaling. Midgut epithelial cells and an immunocompetent *A. stephensi* cell line express all three Smads, confirming that a signaling pathway is in place to support signaling by divergent exogenous and endogenous TGF-β superfamily proteins.

The results presented here provide the first evidence of immunological crosstalk between divergent free living hosts of a single parasite. Further, these results imply that the interface between mammals and the mosquitoes that feed on them provide a unique opportunity for circulating molecules in the blood, including TGF-β and other cytokines, to alter the mosquito immune response

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Abbreviations

Act	Activin
Ag	*Anopheles gambiae*
Ag60A	Ag Gbb-60A
Ag60A2	Ag Gbb-60A2
AgAct	Ag Activin
AgBabo	Ag Baboon
AgBrk	Ag Brinker
AgMav	Ag Maverick
AgMyo	Ag Myoglianin
AgPut	Ag Punt
AgSax	Ag Saxophone
AgTkv	Ag Thickveins
AgWit	Ag Wishful thinking
As	*Anopheles stephensi*
As60A	As Gbb-60A
As60A2	As Gbb-60A2
Babo	Baboon
b.f.	bloodfeeding
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
cDNA	complementary DNA
CeDaf3	*Caenorhabditis elegans* Dauer formation 3 protein

CeDaf4	*Caenorhabditis elegans* Dauer formation 4 protein
Dad	Daughters against dpp
DDT	1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane
Dm	*Drosophila melanogaster*
Dm60A	Dm Gbb-60A
DmAct	Dm Activin
DmALP23B	Dm Activin-like protein at 23B
DmBabo	Dm Baboon
DmMav	Dm Maverick
DmMyo	Dm Myoglianin
DmSax	Dm Saxophone
DmScw	Dm Screw
DmTkv	Dm Thickveins
DmWit	Dm Wishful thinking
Dpp	Decapentaplegic
DvDpp	*Drosophila virilis* Dpp
E5	Minimum essential medium supplemented with 5% Fetal Calf Serum
ECM	Extracellular matrix
ED_{50}	Effective dose to produce an effect in 50% of the population
ELISA	Enzyme linked immunosorbent assay
FAD	Flavin adenine dinucleotide
FKBP12	12 kDa FK506-binding protein
FMN	Flavin mononucleotide

G	Glycine-Serine rich domain
Gbb-60A	Glass bottom boat at chromosome region 60A1-3
GDNF	Glial cell-line derived neurotrophic factor
GgMyo	*Gallus gallus* Myoglianin
GgSki	*Gallus gallus* Ski
GPI	Glycosylphosphatidylinositol
HDAC	Histone deacetylase
HEG	Homing endonuclease
Hgs/Hrs	Hepatic growth factor-regulated tyrosine kinase substrate
Hs	*Homo sapiens*
HsActIIR	Hs Activin type II receptor
HsALK2R	Hs Activin-receptor like kinase 2
HsBMP1BR	Hs BMP type IB receptor
HsBMPIIR	Hs BMP type II receptor
HsGDF8	Hs Growth and differentiation factor-8
HsTGFbIR	Hs TGF-β type I receptor
HsTGFb3	Hs TGF-β3
ICR	Institute of Cancer Research
IFN-γ	Interferon-γ
IL	Interleukin
LAP	Latency associated peptide
LLC	Large latent complex
LPS	Lipopolysaccharide

LTBP	Latent TGF-β binding protein
Mad	Mothers against dpp
Mav	Maverick
MAPK	Mitogen activated protein kinase
MH1	Mad Homology 1 domain
MH2	Mad Homology 2 domain
MIS	Müllerian inhibiting substance
Mm	*Mus musculus*
MmActAR	Mm Activin A type I receptor
MmActIIB4	Mm Activin type II receptor isoform B4
MmBMPIIR	Mm BMP type II receptor
MmBMP1BR	Mm BMP type IB receptor
MmGDF8	Mm Growth and differentiation factor-8
MmIbA	Mm Inhibin βA
MmTGFbIR	Mm TGF-β type I receptor
MmTGFb3	Mm TGF-β3
MuLV	Murine leukemia virus
Myo	Myoglianin
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
D-NAME	Nω-nitro-D-arginine methyl ester
L-NAME	Nω-nitro-L-arginine methyl ester
NO	Nitric oxide
NO_2^-	Nitrite

NO_3^-	Nitrate
NOS	Nitric oxide synthase
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
O_2^-	Superoxide
$OONO^-$	Peroxynitrite
PBS	Phosphate-buffered saline
PLA2	Phospholipase A2
Put	Punt
Pro-prot	Pro-protein
Py17X	*Plasmodium yoelii* non-lethal strain 17X
Py17XL	*Plasmodium yoelii* lethal strain 17XL
RLM-RACE	RNA-ligase mediated rapid amplification of cDNA ends
RPE	Retinal pigment epithelium
RT-PCR	Reverse transciptase-polymerase chain reaction
qRT-PCR	quantitative RT-PCR
SARA	Smad anchor for receptor activation
Sax	Saxophone
SBE	Smad binding element
Scw	Screw
SE	Standard error
Ski	Sloan Kettering Institute

Co-Smad	Common-mediator Smad
I-Smad	Inhibitory Smad
R-Smad	Receptor-Smad
SmSmad1	*Schistosoma mansoni* Smad1
SmSmad2	*Schistosoma mansoni* Smad2
Sno	Ski-related novel gene
STK	Serine-threonine kinase domain
TAK1	TGF-β activated kinase 1
TBD	TGF-β-like domain
TcDpp	*Tribolium castaneum* Dpp
TE	Transposable element
TGF-β	Transforming growth factor-β
hTGF-β1	human TGF-β1
TGF-βRI	TGF-β type I receptor
TGF-βRII	TGF-β type II receptor
TGIF	TG-interacting factor
Tkv	Thickveins
TNF-α	Tumor necrosis factor-α
Wit	Wishful thinking
Wnt	Wingless pathway
Xl	*Xenopus laevis*
XlActbB	Xl Activin βB
XlALK2R	Xl Activin-receptor like kinase 2

XlBMPR	Xl BMP receptor
XlBMPIIR	Xl BMP type II receptor
XlOP1	Xl Osteogenic protein 1
XlSTKIR	Xl Serine-threonine kinase type I receptor

Chapter 1. Introduction

1.1. The importance of malaria

Malaria has been described in humans for more than 4000 years, and it has and continues to influence human populations and history (CDC, 2004). Today, malaria is the most prevalent tropical disease in the world, with 40% of the world's population at risk for infection (Figure 1) and over 500 million clinical cases annually, 70% of which occur in sub-Saharan Africa (Snow *et al*. 2005). This devastating disease is responsible for over one million deaths each year, mostly among children (WHO, 2004). Even in the United States, where malaria was eradicated in the 1950's, over 1300 clinical cases and 8 deaths were reported in 2002 (CDC, 2004). Finally, the number of people potentially at risk for infection is expected to increase to almost 9 billion by the year 2080 (Arnell *et al*., 2002).

The parasites responsible for malaria are members of the protozoan genus *Plasmodium*. Four species are capable of infecting humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. The malaria parasite has an indirect two-host life cycle, consisting of a definitive vertebrate host and an insect vector (Figure 2). Within 2 hours after ingestion of an infected bloodmeal, micro- and macrogametes fuse to form a zygote. From 24-36 hours the transformed zygote, now an ookinete, begins invading the midgut epithelium. The ookinete stops at the basal lamina of the midgut epithelium where it develops into an oocyst. From 9-11 days, inside the oocyst, meiotic division produces thousands of haploid sporozoites. At 12-14 days, the oocyst wall breaks down, releasing the sporozoites into the open circulatory system of the mosquito, where they spread to every tissue, including the salivary glands. During

subsequent bloodfeeding, sporozoites are released into the vertebrate host. Sporozoites invade hepatocytes and undergo several rounds of replication before releasing merozoites into the circulatory system. Merozoites invade erythrocytes and can undergo asexual reproduction and development to become trophozoites, which then become schizonts and rupture the erythrocytic membrane to release merozoites, thereby perpetuating the blood stages of the life cycle. In the vertebrate host, merozoites replicate inside parasitized erythrocytes resulting in a deformation of the cell from flexible and biconcave to rigid and spherical with an irregular surface covered with small protrusions (Sherman, 1998). The severity of the disease commonly associated with *P. falciparum* malaria is largely due to cytoadherence of these irregular erythrocytes to the vascular endothelium (Sherman, 1998). This results in sequestration of red blood cells from circulation, particularly in the brain, heart, liver, kidney, intestines and adipose tissue, leading to severe anemia, reduced oxygen and substrate supply, and ultimately potential organ failure (Sherman, 1998). Mortality in Africa south of the Sahara is largely due to *P. falciparum*.

Dr. Ronald Ross discovered that female mosquitoes of the genus *Aedes* transmit the avian parasites responsible for malaria, for which he was awarded the Nobel Prize in 1902 (Sherman, 1998). Female mosquitoes of the genus *Anopheles* transmit the mammalian species of *Plasmodium*. Upon ingestion of an infective bloodmeal, sexual stage gametocytes fuse into a zygote that transforms into a mobile ookinete, which penetrates the midgut epithelium of the mosquito (Figure 2). The midgut consists of a narrow tube-like anterior region through which blood passes, and a flask-shaped posterior region that retains the blood meal (Clements, 1992). In the midgut, digestion of the

bloodmeal begins immediately with the secretion of proteolytic enzymes and the formation of the peritrophic membrane (Clements, 1992). During *Plasmodium* infection in the midgut, several physiological events occur that could impact parasite development including erythrocyte hemolysis, changes in pH, and blood digestion (Chege and Beier, 1998). In *Anopheles gambiae*, cell free hemoglobin per total hemoglobin levels average ~10% within 20 minutes of blood feeding (Chege and Beier, 1998), creating an environment rich with free heme (Pascoa *et al*., 2002). Since the midgut epithelium is the first tissue that bloodmeal-ingested materials encounter, the midgut is a first line of defense against blood-borne pathogens.

1.2. Control Strategies

The aim of a malaria control strategy is to reduce the impact of malaria infection on a population. Since female *Anopheles* transmit malaria, considerable efforts have been focused on control of biting mosquitoes. However, insecticide resistance among mosquitoes has increased from two DDT-resistant species in 1946 to more than 50 species being resistant to one or more insecticides 50 years later (Phillips, 2001). Additionally, resistance of *Plasmodium* to anti-malarial drugs has been recorded for the last 50 years (Phillips, 2001). The development of new therapies is focused on derivatives of artemisinin, the active anti-malarial compound isolated in 1972 from the leaves of the wormwood plant (Phillips, 2001). In addition to novel therapeutics, a very basic and effective method of malaria control is based on the use of insecticide-treated bed nets (Curtis *et al*., 2003). Efforts to develop an effective vaccine are currently focused on three areas: a pre-erythrocytic stage vaccine targeting sporozoites, an asexual

stage vaccine targeting merozoites, and a transmission-blocking vaccine targeting gametocytes (Ballou *et al*., 2004). The latter strategy blocks transmission from the mammalian host to the vector mosquito.

Another vector-targeted strategy is based on the development and release of transgenic mosquitoes that are refractory to malaria transmission. This approach relies on (a) identification of effector gene products that impede parasite development, (b) development of genetic transformation protocols to introduce the gene(s) into the mosquito genome, and (c) development of a gene drive system to facilitate gene spread through a population without reducing the mosquito fitness (Phillips, 2001).

Studies have identified only a handful of gene products capable of significantly reducing *Plasmodium* development. Phospholipase A2 (PLA2) significantly inhibited ookinete invasion of the midgut epithelium when mixed with an infected blood meal provided to *Aedes aegypti* (Zieler *et al*., 2001) and to *Anopheles stephensi* (Moreira *et al*., 2002). The antimicrobial peptides gambicin and defensin have been shown to reduce parasite load in *A. gambiae* (Vizioli *et al*., 2001) and *Ae. aegypti* (Shahabuddin *et al*., 1998), respectively. The synthetic SM1 peptide that binds to the midgut and salivary gland epithelia has been shown to impair oocyst development by 82% (Ito *et al*., 2002). The complement-like thioester-containing protein (TEP1) has been shown to promote phagocytosis of gram-negative bacteria in cultured *A. gambiae* cells (Levashina *et al*., 2001). My research focuses on the endogenous effector gene product *A. stephensi* nitric oxide synthase (AsNOS). In *A. stephensi*, levels of inducible nitric oxide synthase (NO) expression and production of NO by the midgut epithelium limit development of the malaria parasites *Plasmodium berghei* (Luckhart *et al*., 1998) and *P. falciparum*

(unpublished) in *A. stephensi*. Further, dietary provision of the AsNOS catalytic inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) enhances parasite burden in *A. stephensi* while provision of the NOS substrate L-arginine decreases parasite burden in *A. stephensi* (Luckhart *et al*., 1998).

During parasite development, three main compartments are infected: the midgut lumen, hemocoel, and salivary glands. Therefore, it is highly desirable to direct to those compartments expression of effector genes and secretion of anti-parasite gene products linked to tissue-specific promoters. Several such promoters have been identified which target expression to the previously mentioned compartments. Recent studies have demonstrated that the promoter for carboxypeptidase, a gene encoding a digestive enzyme, is activated in response to a blood meal (Riehle *et al*., 2003). The midgut-specific promoter of the gene ApAper1 drives protein synthesis into secretory vesicles of midgut epithelial cells prior to a blood meal and is released immediately after blood ingestion (Riehle *et al*., 2003). The vitellogenin promoter and signal sequences were shown to drive strong gene expression in the fat body and secretion into the hemocoel (Kokoza *et al*., 2000). Salivary gland-specific promoters have been identified (Coates *et al*., 1999), although expression from these promoters was rather weak relative to controls (Riehle *et al*., 2003).

Methods to produce transiently transformed *A. stephensi* and *A. gambiae* mosquitoes are now available (Catteruccia *et al*., 2000; Grossman *et al*., 2001). One system to drive effector genes into the mosquito genome is transposable elements (TEs) due to their efficiency at spreading through populations (Riehle *et al*., 2003). Elements such as *piggybac*, *minos*, *hobo*, and *hermes* have been used to successfully transform

mosquitoes (Tu and Coates, 2004). However, there is also a fitness cost associated with the spread of TEs through a population. If the TE spreads too rapidly, it could lead to fatal mutations in the genome. Alternatively, if the TE spreads too slowly, the population will not be sufficiently transformed (Riehle *et al*., 2003). One alternative system to drive effector genes into the mosquito genome are 'selfish genes', which drive themselves through a population by using the host cell DNA repair machinery (Riehle *et al*., 2003). One example of a selfish gene is homing endonuclease gene (HEG), which encodes an enzyme that recognizes and cleaves a 20-30 bp sequence found on chromosomes not containing a copy of the HEG (Burt, 2003). The HEG is then inserted in the middle of its own recognition sequence, and so chromosomes carrying the HEG are protected from insertion (Burt, 2003).

A final alternative for malaria control is based on the development of transgenic mosquitoes to block parasite transmission in the field. Successful reproduction would require that the transgene(s) do not reduce the fitness of the transgenic mosquito relative to the wild type mosquito. Moreira *et al*. (2002) showed that when heterozygosity of transgenic mosquitoes is maintained by continuous crossing to wild-type mosquitoes, the fitness load is minimal.

1.3. Specific Aims

My area of research focuses on the response of *A. stephensi* to human transforming growth factor-β1 (hTGF-β1). We have discovered that active mammalian TGF-β1 persists in the *A. stephensi* midgut for up to 48 hr after blood feeding (Luckhart *et al*., 2003). Further, human TGF-β can activate *Drosophila melanogaster* TGF-β

signaling pathways (Padgett *et al*., 1993; Sampath *et al*., 1993; Jang *et al*., 1994; Newfeld *et al*., 1997; Secombes, 1998; Brummel *et al*., 1999; Beall *et al*., 2000). Based on these observations, I hypothesized that hTGF-β1 induces AsNOS gene expression and catalytic activity to regulate *Plasmodium* development in *A. stephensi*. To test these hypotheses, I proposed the following *Specific Aims*:

Aim #1: To determine whether hTGF-β1 regulates *Plasmodium* development in *A. stephensi* via an effect on AsNOS expression and activity.

1. Can *A. stephensi* cells recognize hTGF-β1?
2. Is *AsNOS* expression altered in ASE cells and the midgut epithelium following a blood meal supplemented with hTGF-β1?
3. Does hTGF-β1 regulate *Plasmodium* development?
4. Does inhibition of AsNOS activity limit the effect of hTGF-β1 on *Plasmodium* development?

Further, I proposed that exogenous TGF-β1 would signal through endogenous mosquito Smad signaling pathways, perhaps interfering with or synergizing with signaling by endogenous TGF-β ligands. Although Aim #2 does not test this hypothesis, it lays the groundwork for understanding the effects of hTGF-β1 on *A. stephensi* cells in Aim #1.

Aim #2: To characterize the family of TGF-β ligands, receptors, and Smad proteins in *Anopheles*.

1. Does the *A. gambiae* genome encode TGF-β ligands, receptors, and Smad proteins?
2. What are the phylogenetic relationships between these proteins and their respective homologs from other organisms?
3. Are these genes expressed in *A. gambiae* cells, in *A. stephensi* cells, and in the midgut epithelium of *A. stephensi*?

1.4. Potential impact on malaria control

My research helps to understand the AsNOS-mediated defense against *Plasmodium*. *AsNOS* is a transcriptionally complex gene with expression of 22 distinct transcripts, only three of which are inducible by parasite infection (Luckhart and Li, 2001). This feature of *AsNOS* transcription would likely make direct manipulation of *AsNOS* very difficult. Therefore, understanding the regulation of *AsNOS* may provide an alternative strategy to manipulating the AsNOS-mediated defense, which could be used to block ookinete invasion of the midgut and hence, parasite development in the mosquito.

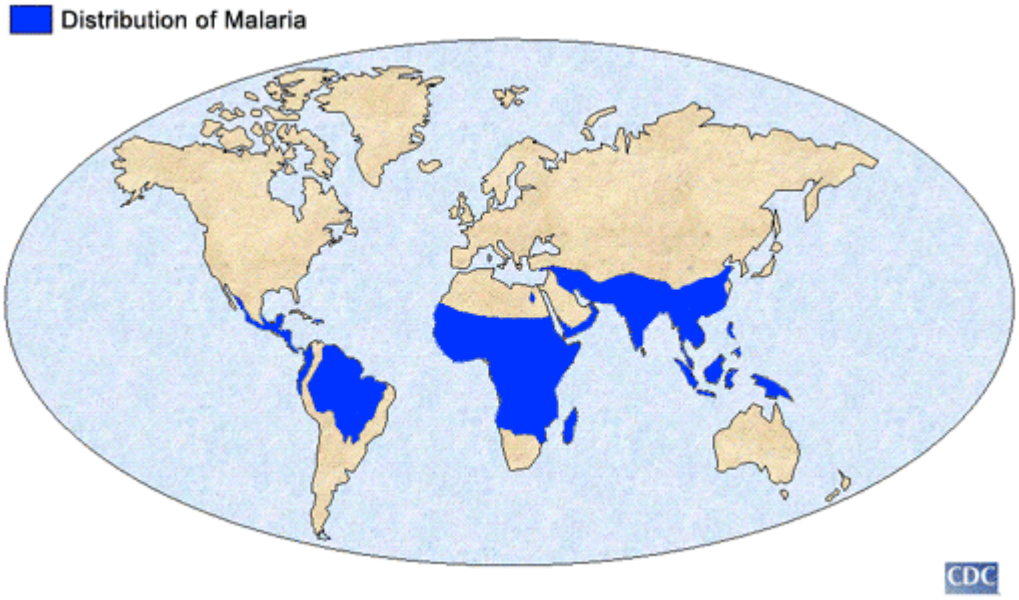

Figure 1. Geographic distribution of the malaria burden (CDC, 2004).

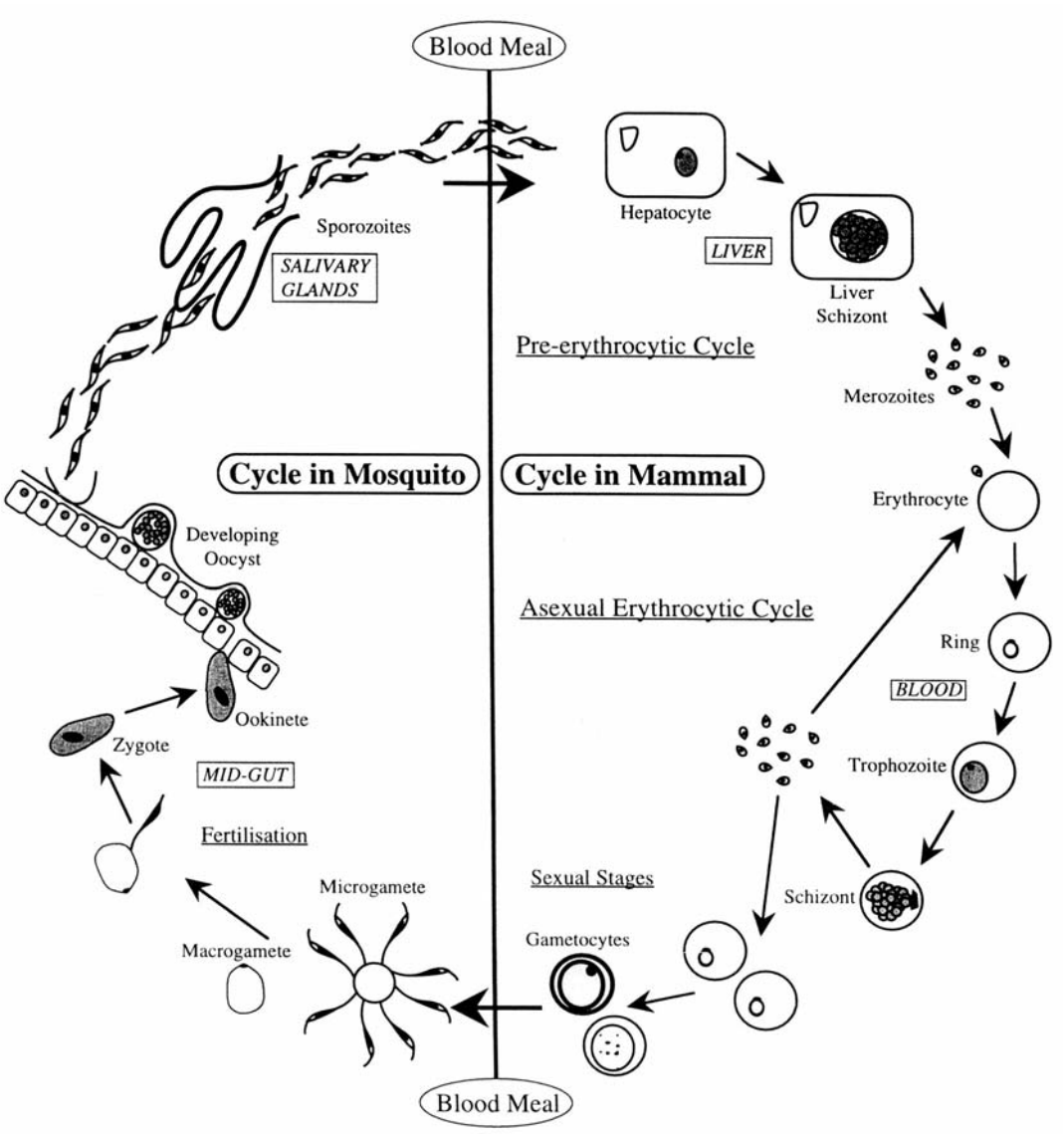

Figure 2. Life cycle of *Plasmodium* (Phillips, 2001).

Chapter 2 Literature Review

2.1. TGF-β1 signaling

Transforming growth factor-β1 (TGF-β1) is a prototypic member of a superfamily of cytokines consisting of the TGF-βs, Activins/Inhibins, bone morphogenetic proteins (BMPs), and Müllerian inhibiting substance (MIS). These cytokines are grouped according to their structural and functional relationships. Most of the sequence similarity between family members is in the C-terminal domain of the protein (Massague, 1990). Genes encoding TGF-β-like proteins are found in nearly all eukaryotes from nematodes (*Caenorhabditis elegans*) to humans (*Homo sapiens*). Phylogenetic analysis suggests that the family evolved from a common ancestral gene that predates the divergence of arthropods and chordates (Burt and Paton, 1992). TGF-βs are characterized, for the most part, by the presence of a structural feature called the cysteine knot, which consists of at least seven cysteine residues arranged in three intrachain disulfide bonds and one intermolecular cysteine bridge responsible for dimerization (Derynck and Choy, 1998).

TGF-β1 has multiple biological actions including induction of cell proliferation, tissue repair, cell migration, cell differentiation, and immune responses. TGF-β1 signaling inhibits cell proliferation in human melanoma A375 cells (Nakai *et al*., 1988), reduces proliferation of human erythroleukaemia TF-1 cells (Mire-Sluis *et al*., 1996) and reduces proliferation of murine HT-2 cells (Tsang *et al*., 1995). Cell migration, homing, and settlement during tissue formation, repair, tumor invasion, and metastasis are guided by a complex set of adhesive interactions between cells and extracellular matrices. Mollusk hemocytes respond to hTGF-β1 by forming lamellopodia or cytoplasmic extensions that facilitate immunocyte migration in a chemotactic gradient (Ottaviani *et*

al., 1997). TGF-β1 also influences cell differentiation processes and the expression of differentiated functions. For example, differentiation of mouse 3T3-L1 preadipocytes and rat L_6E_9 skeletal muscle myoblasts into mature adipocytes and multinucleated myotubes, respectively, can be blocked in the presence of picomolar concentrations of TGF-β1 (Massague, 1990). TGF-β1 is also thought to favor chondrogenesis and osteogenesis (Massague, 1990). High levels of TGF-β1 in platelets or in activated monocytes and macrophages can be physiologically delivered to sites of wound healing or inflammation (Massague, 1990). TGF-β1 also regulates inducible nitric oxide synthase (iNOS) expression and activity (Vodovotz, 1997) in mammalian cells associated with immunity (Gilbert and Herschman, 1993; Blanco *et al*., 1995).

In general, before TGF-βs can bind to their respective receptors, post-translational processing must occur to produce active protein conformations. The translated protein consists of the active dimer at the C-terminus and the latency associated peptide (LAP) at the N-terminus. After translation, TGF-βs are secreted as homodimers usually associated with several other proteins in what is called the large latent complex (LLC; Annes *et al*., 2003). This complex maintains the TGF-β dimer in an inactive state until it reaches the target cell. Cells that secrete TGF-β superfamily members are often neighbors of target cells, but these proteins can travel several cell lengths before binding to relevant target receptors (Gumienny and Padgett, 2002). Once the target cell releases the TGF-β proprotein homodimer, proteolytic cleavage of the N-terminus releases the biologically active C-terminal homodimer (Figure 3; Annes *et al*., 2003). In the extracellular milieu active and latent TGF-β1 can interact with other proteins, which can further regulate activity and availability of TGF-β1. One protein with a high affinity for active TGF-β1 is

α_2-macroglobulin, which sequesters the cytokine into an inactive form that is unable to bind to the TGF-β receptors (Derynck and Choy, 1998). In fact, nearly all plasma TGF-β1 is sequestered in an α_2-macroglobulin complex (Derynck and Choy, 1998).

After activation, the mature ligand can bind to its cognate receptor. Three major TGF-β receptors were initially identified by chemical cross linking of ^{125}I-labeled TGF-β1 to cell surface proteins. These receptors are named type I, II, and III on the basis of their decreasing electrophoretic mobility (Derynck and Choy, 1998). TGF-β receptors are transmembrane proteins with serine/threonine kinase activity which bind TGF-β superfamily ligands on the outside of the cell and activate signaling pathways inside the cell. Structural features of the receptors include an extracellular domain, a glycine-serine rich (G) domain, and a kinase domain. A significant substrate for the TGF-β type II receptor kinase (TGF-βRII) is the TGF-β type I receptor (TGF-βRI). Serine 165 in the G domain of the type I receptor is phosphorylated by the type II receptor in a ligand-dependent manner (Massague, 1998).

Signal transduction by TGF-β ligands occurs through cytoplasmic proteins called Smads. Three classes of Smads have been identified: receptor-Smads (R-Smads), common-mediator Smads (Co-Smads), and inhibitory Smads (I-Smads). Smads are characterized by the presence of two conserved domains and one non-conserved linker domain. The conserved Mad Homology 1 (MH1) domain is located in the Smad N-terminus and has DNA-binding activity (Raftery and Sutherland, 1999). The conserved Mad Homology 2 (MH2) domain is located in the C-terminus and functions in forming multi-Smad complexes (Raftery and Sutherland, 1999). Transduction begins with ligand binding to the TGF-βRII resulting in auto-phosphorylation. The TGF-βRI is then

phosphorylated by the type II receptor. An R-Smad then binds to the type I receptor and is phosphorylated in its C-terminal SSXS motif. The phosphorylated form of R-Smad then complexes with Co-Smad and this Smad complex translocates into the nucleus, where it regulates transcription either through association with DNA via the MH1 domain or by association with other transcription factors (Figure 4). One accessory protein is Smad anchor for receptor activation (SARA). This scaffold protein can anchor non-activated R-Smads to the cell membrane through cooperation with Hgs/Hrs (hepatic growth factor-regulated tyrosine kinase substrate), bridge the Smads with the receptor complex, and assist in R-Smad activation (Figure 4; Zwijsen *et al*., 2003).

Inhibition of TGF-β signaling can occur through activation of I-Smad or Ski (Sloan-Kettering Institute) and Sno (Ski-related Novel gene) oncoproteins (Figure 4). The I-Smads inhibit signaling through interaction with TGF-βRI to prevent phosphorylation of R-Smad (Inoue *et al*., 1998) or by interacting with specific E3-ubiquitin ligases that target Smads for degradation at the proteosome (Figure 4; Ebisawa *et al*., 2001). Ski and Sno are capable of binding to R-Smads or Co-Smads, preventing promoter activation through recruitment of histone deacetylase activity (Figure 4; Akiyoshi *et al*., 1999). Several other Smad inhibitory proteins have been identified including the immunophilin FK506 binding protein-12 (FKBP12; Wang *et al*., 1996), which binds to the TGF-β type I receptor preventing R-Smad binding, and the transcriptional co-repressor brinker (Kirkpatrick *et al*., 2001), which binds to TGF-β response elements in *D. melanogaster* preventing Smad complex binding to the DNA.

All TGF-β signaling pathways share the co-Smad and inhibitory Smad signaling proteins. However, the R-Smads are divided into two types: TGF-β/Activin-dependent

and BMP-dependent R-Smads (Nakao *et al*., 1997). In *D. melanogaster*, TGF-β and Activin ligands activate the R-Smad dSmad2 (Brummel *et al*., 1999), while BMP signaling is mediated by activation of Mad (Liu *et al*., 1996; Hoodless *et al*., 1996).

TGF-β signaling is also regulated at the level of gene expression. For example, TGF-β1 signaling is down regulated through a gradual repression of R-Smad expression and rapid induction of I-Smad expression (Takase *et al*., 1998; Mori *et al*., 2000). Human TGF-β1 can also induce SnoN expression, which results in termination of Smad-mediated transactivation (Stroschein *et al*., 1999).

2.2. TGF-β1 cross-talk

The TGF-β superfamily is a group of structurally related proteins with a high level of sequence conservation in the mature C-terminus of the protein. This conservation predicts that function of these proteins is conserved across a variety of species. Indeed, it has been known for several years that mammalian TGF-β1 can stimulate signaling in fish leukocytes (Secombes, 1998) and is able to reduce the respiratory burst activity in activated trout macrophages (Jang *et al*., 1994). Further, human BMP-4 can substitute for decapentaplegic (dpp) in transgenic *D. melanogaster* (Padgett *et al*., 1993). BMP-2 exposure of *D. melanogaster* cells results in rapid phosphorylation of the BMP-dependent R-Smad Mad (Figure 5; Newfeld *et al*., 1997). The trematode parasite *Schistosoma mansoni* genome encodes a type I receptor serine/threonine kinase that can be activated by hTGF-β1 (Beall *et al*., 2000).

Mammalian cells are similarly responsive to non-mammalian TGF-β ligands and Smad proteins. The *D. melanogaster* TGF-β homologs dpp and glass bottom boat-60A

(gbb-60A) can induce bone forming activity as assessed by cartilage formation and calcium content in subcutaneous rat implants in mammalian cells (Sampath *et al*., 1993). *D. melanogaster* dSmad2, a TGF-β/Activin-dependent R-Smad, can mediate signaling in mammalian cells (Brummel *et al*., 1999). The *S. mansoni* Smads, SmSmad1 and SmSmad2, undergo nuclear translocation in response to ligand in monkey kidney fibroblast cells (Beall *et al*., 2000).

In addition to transduction of TGF-β signals, Smad activation can impact other signaling pathways in eukaryotic cells. Multiple interactions between TGF-β superfamily pathways and mitogen activated protein kinase (MAPK) pathways have been described. For example, Smad1 is phosphorylated in its linker region by MAPKs and this phosphorylation appears to be inhibitory (Mehra and Wrana, 2002). In other studies, Smad2 and Smad3 phosphorylation downstream of activated Ras also leads to inhibition of TGF-β signaling (Mehra and Wrana, 2002). The activity of TGF-β associated kinase 1 (TAK1), a MAPK kinase kinase, is up-regulated by TGF-β and BMP and it modifies TGF-β transcriptional responses (Mehra and Wrana, 2002). In *Xenopus laevis*, activated TAK1 can mimic overexpression of BMP signaling in the early embryo (Shibuya *et al*., 1998). Stimulation of a human chondrosarcoma cell line, JJ012, with collagen results in increased Smad2 and Smad3 phosphorylation (Schneiderbauer *et al*., 2004). Further, stimulation of JJ012 cells with both TGF-β1 and collagen leads to a synergistic increase in phosphorylation of Smad2 in the cytoplasm over time as compared with treatments with TGF-β1 or collagen alone (Schneiderbauer *et al*., 2004).

Along with TGF-β superfamily pathways, the wingless (Wnt) pathway also plays a crucial role in embryonic development and tumor progression (Mehra and Wrana,

2002). Data from a variety of organisms have revealed that TGF-β and Wnt signaling proteins interact directly. A TGF-β-dependent interaction between Smad3 and Lef1 regulates synergistic induction of Wnt target genes (Mehra and Wrana, 2002), while phosphorylation of Smad3 by TGF-β receptors is facilitated by Smad3 association with axin, the inhibitory Wnt pathway protein (Furuhashi *et al*., 2001). Further, axin mutants, which cannot bind to Smad3, block Smad3 phosphorylation, suggesting that Smad3 association with axin is required for signaling (Mehra and Wrana, 2002).

2.3. TGF-β1, inflammation, and NOS

Mammalian TGF-β1 is an immunomodulatory cytokine that functions as a potent differentiation modulating and immunosuppressive agent (Derynck and Choy, 1998). The effects of TGF-β1 on immune cell types such as T cells, B cells, monocytes, and macrophages are concentration-dependent. For example, low concentrations of TGF-β1 elicit a sequence of inflammatory cell recruitment, fibroblast accumulation and vascular growth, similar to the normal inflammatory response to injury (Derynck and Choy, 1998). In contrast to these pro-inflammatory activities, high concentrations of TGF-β1 inhibit a variety of macrophage activities, resulting in suppression of macrophage function and resolution of the inflammatory response (Derynck and Choy, 1998). A major target of TGF-β1 regulation of development and resolution of the inflammatory response is nitric oxide synthase (NOS).

Nitric oxide synthase catalyzes the synthesis of NO, one of the major toxic mediators of the inflammatory response (Singh and Evans, 1997). In general, NOS proteins catalyze the five-electron oxidation of L-arginine to NO and L-citrulline,

requiring also FAD, FMN, NADPH, tetrahydrobiopterin, a source of thiol, and the accessory protein calmodulin (Vodovotz, 1997). In mammals, three major isoforms of NOS have been identified: endothelial NOS (eNOS or NOS III) and neuronal NOS (nNOS or NOS I) which are constitutively expressed, and iNOS (or NOS II) which is not constitutively expressed but is induced following stimulation of cells with inflammatory agents (Singh and Evans, 1997). *Anopheles stephensi NOS* (*AsNOS*) is a single copy gene that shares significant structural homology with the three mammalian *NOS* genes (Luckhart and Rosenberg, 1999) and is inducibly expressed in *Plasmodium*-infected mosquitoes (Luckhart *et al*., 1998).

Due to its ability to participate in a variety of chemical reactions, NO mediates a tremendous array of processes, with both positive and negative effects on cell viability (Vodovotz, 1997). Nitric oxide is a free radical, thus making it highly reactive with other radicals, such as the superoxide anion (O_2^-). The half-life of NO at physiological concentrations is measured in milliseconds, and the radical decomposes in aqueous solutions to nitrite (NO_2^-) and nitrate (NO_3^-), a reaction that is catalyzed by transition metals (Figure 6). In the presence of O_2^-, NO combines with this radical three times faster than the rate of superoxide dismutation, leading to formation of peroxynitrite ($OONO^-$; Figure 6). Peroxynitrite is an unstable, strong pro-oxidant with toxic effects on many cellular targets, including nucleic acids, lipids, and proteins (Singh and Evans, 1997).

Mammalian TGF-β1 regulation of NO synthesis is dependent on the cell type. In mouse macrophages, TGF-β1 suppresses NO synthesis *in vitro* in response to interferon-γ (IFN-γ; Vodovotz, 1997). TGF-β1 suppresses synthesis of NO in other cell types,

including mesangial cells, smooth muscle cells, glial cells, endothelial cells, hepatocytes, keratinocytes, rat fibroblasts, and osteoblasts (Vodovotz, 1997). In addition to its role as a negative regulator, TGF-β1 can enhance NO synthesis in some cell types. In retinal pigment epithelial (RPE) cells, TGF-β1 treatment increases NO synthesis in the presence of lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α; Goureau *et al*., 1993). Pre-incubation of human chondrocytes with TGF-β1 and interleukin-1β (IL-1β) for 48 hr produces a synergistic increase in *iNOS* mRNA (Blanco *et al*., 1995). TGF-β1 substantially augments LPS induction of *iNOS* gene expression in Swiss 3T3 fibroblast cells (Gilbert and Herschman, 1993) and induces *iNOS* expression in myocytes (Chen *et al*. 2001). TGF-β1 also enhances NO synthesis in mouse fibroblasts, human chondrocytes, bovine epithelial cells, and leukocytes (Vodovotz, 1997; De Servi *et al*., 2002). These results suggest a vital role for TGF-β1 in regulating the bioavailability of NO (Vodovotz, 1997), and that NO production in cells in response to TGF-β1 is cell-type dependent.

The mechanism through which TGF-β1 suppresses NO synthesis has been intensively studied. RPE cells and macrophages from TGF-β1 null mice (TGF-$\beta1^{-/-}$) produce 40% more NO in response to IFN-γ and LPS than wild-type mice (TGF-$\beta1^{+/+}$; Vodovotz *et al*., 1996). Exogenous treatment of TGF-$\beta1^{-/-}$ mice with TGF-β1 decreases both iNOS protein and NO synthesis (Vodovotz *et al*., 1996). In macrophages, TGF-β1 does not affect transcription of *iNOS*, but does reduce the stability and rate of translation of *iNOS* mRNA and increases the rate of degradation of iNOS protein (Vodovotz, 1997). Subsequent studies in smooth muscle cells suggested that TGF-β1 can regulate the expression of *iNOS* (Perrella *et al*., 1994). TGF-β1 can also affect the catalytic activity of

iNOS through effects on the availability of substrates and co-factors required for catalysis (Vodovotz, 1997). For example, TGF-β1 potentiates arginase activity in macrophages thereby limiting the bioavailability of L-arginine (Boutard *et al*., 1995). TGF-β1 can also suppress the production of tetrahydrobiopterin in cultured smooth muscle cells (Vodovotz, 1997).

2.4. TGF-β1 and malaria

During malaria parasite infection, TGF-β1 appears to maintain an immunological balance between parasite killing and host auto-pathology (Omer *et al*., 2000). Levels of TGF-β1 are inversely correlated with the severity of malaria infection in mice. In malaria-resistant mice (C57BL/10), administration of recombinant TGF-β1 resulted in 100% mortality by 12 days after infection (Tsutsui and Kamiyama, 1999). In malaria-susceptible mice (BALB/c), monoclonal antibody to TGF-β1 resulted in greater survival rates to *Plasmodium chabaudi chabaudi* infection (Tsutsui and Kamiyama, 1999). In *P. berghei*-infected mice, the decline of TGF-β1 production is inversely related to parasitemia (Omer and Riley, 1998). Infection with the lethal rodent malaria parasite *Plasmodium yoelii* 17XL (Py17XL) leads to a very early rise in TGF-β1 production that is associated with suppression of IFN-γ and TNF-α synthesis and a rapid increase in parasite replication, leading to death from overwhelming parasitemia. In contrast, mice infected with the non-lethal strain of *P. yoelii* (Py17X) exhibit a delayed and more moderate TGF-β1 response, which leads to controlled parasite growth and survival from infection (Omer *et al*., 2003b). Interestingly, treatment of Py17XL-infected mice with neutralizing antibodies to TGF-β1 resulted in increased levels of the anti-inflammatory

cytokine interleukin-10 (IL-10) compared to control-treated mice (Omer *et al*., 2003b). These data suggest that in the absence of TGF-β1, there is a compensatory rise in IL-10 that can down-regulate the pro-inflammatory response (Omer *et al*., 2003b) and lead to death from overwhelming parasitemia. Finally, treatment of Py17XL-infected mice with neutralizing antibodies to TGF-β1 and IL-10 controlled parasitemia and increased survivorship (Omer *et al*., 2003b).

In children with severe *P. falciparum* malaria, circulating levels of active TGF-β1 are higher than normal (28 pg/ml vs. 6 pg/ml; Musumeci *et al*., 2003). Plasma levels of TGF-β1 in malaria patients may be high because *P. falciparum*-infected erythrocytes can activate recombinant latent TGF-β1 *in vitro* (Omer *et al*., 2003a). In contrast, some studies have found that total TGF-β1 plasma levels in malaria patients are reduced relative to controls, indicating that the type of TGF-β1 assay used can significantly influence the results and their interpretation (Chaiyaroj *et al*., 2004; Perkins *et al*., 2000). An early production of TGF-β1 in malaria infection activates monocytes/macrophages to induce phagocytosis of parasitized red blood cells and killing of ingested parasites (Malaguarnera and Musumeci, 2002). In contrast, too much TGF-β1 too early prevents Th1-cell-mediated immunity, through inhibition of IFN-γ and TNF-α, from repressing a rapid escalation of parasitemia (Malaguarnera and Musumeci, 2002)

In summary, at low concentrations, TGF-β1 appears to promote Th1-mediated mechanisms that control parasite growth and at high concentrations, TGF-β1 down-regulates Th1-like responses to limit inflammation-associated host pathology (Omer *et al*., 2000).

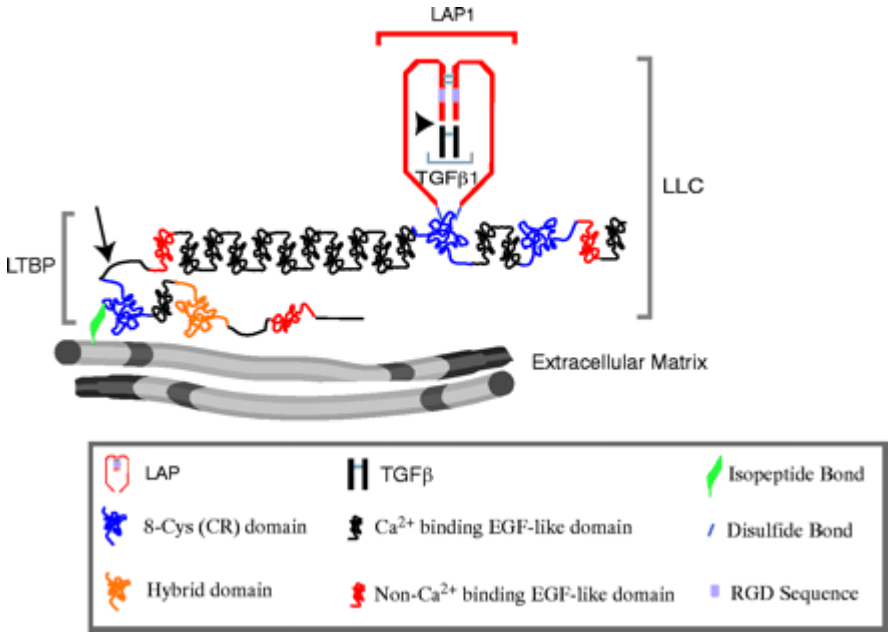

Figure 3. The TGF-β1 large latent complex (LLC). The LLC comprises the C-terminal dimer (black), the N-terminal latency associated peptide (LAP; red) and latent TGF-β binding protein (LTBP). TGF-β1 and LAP are proteolytically cleaved at the site indicated by the arrowhead. After processing, TGF-β1 remains non-covalently associated with LAP. LAP and LTBP are joined by disulfide bonds (light blue lines). The LLC is covalently linked to the extracellular matrix (ECM) through an isopeptide bond (green) between the N-terminus of LTBP (between the EGF2 and the hinge domain) and a currently unidentified matrix protein. The hinge domain (arrow) of LTBP is a protease-sensitive region that allows LLC to be proteolytically released from the ECM (Annes *et al.*, 2003).

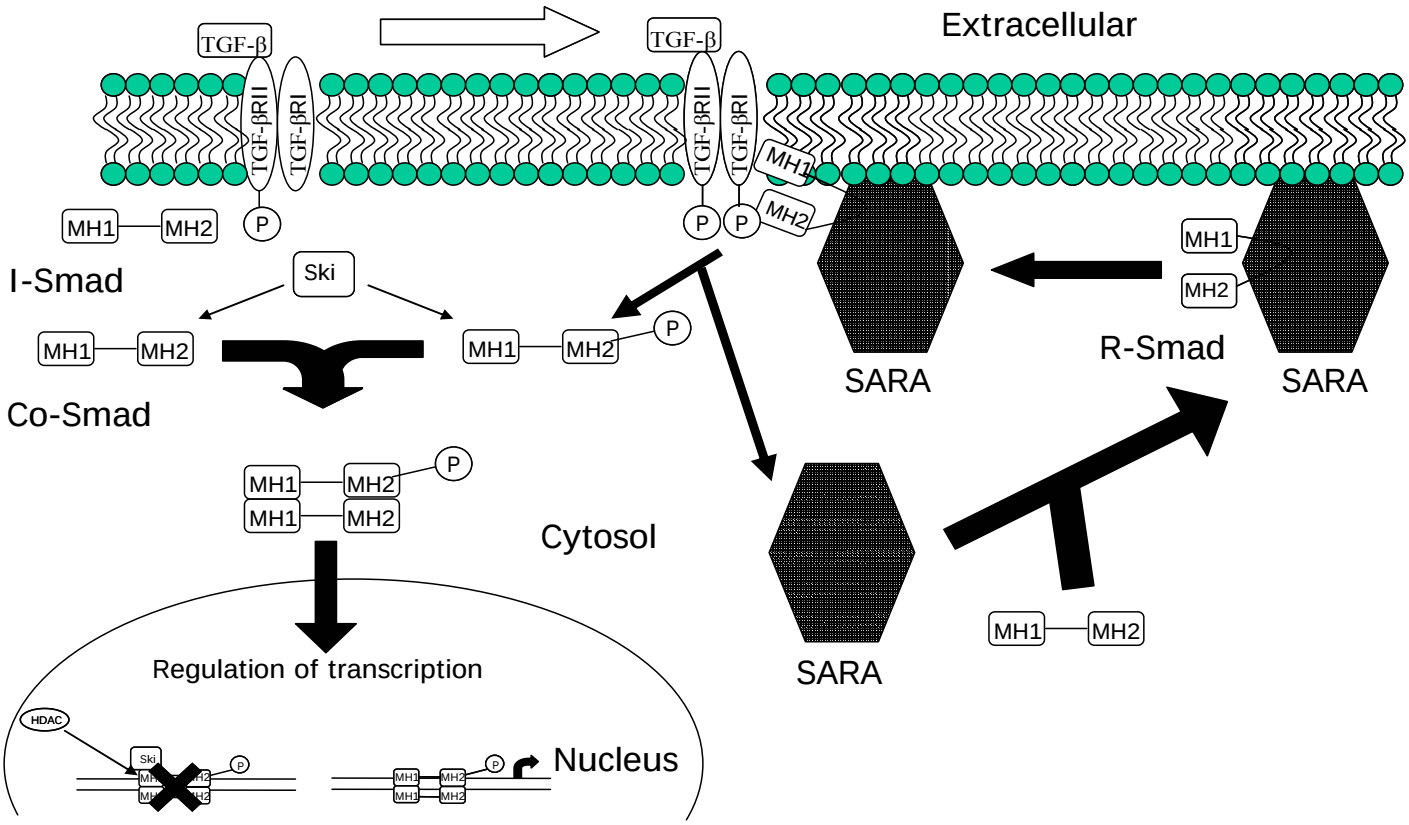

Figure 4. TGF-β signal transduction. Upon ligand binding, the type II receptor is phosphorylated, and then phosphorylates the type I receptor. An R-Smad is then recruited to the cell membrane where it is phosphorylated in the MH2 domain by the type I receptor. Phosphorylation of R-Smad results in release from SARA. Phosphorylated R-Smad can then complex with co-Smad, and this complex translocates to the nucleus, where the Smads interact with other transcriptional co-regulators to regulate expression of target genes. Smad signaling can be blocked by the activities of I-Smad or Ski. I-Smad can block Smad signaling by competing with R-Smad for TGF-βRI phosphorylation (Inoue *et al*., 1998) or by interaction with E3-ubiquitin ligases which results in Smad protein degradation (Ebisawa *et al*., 2001). Ski can block Smad signaling by recruiting histone deacetylase (HDAC) activity to Smad target promoters (Akiyoshi *et al*., 1999).

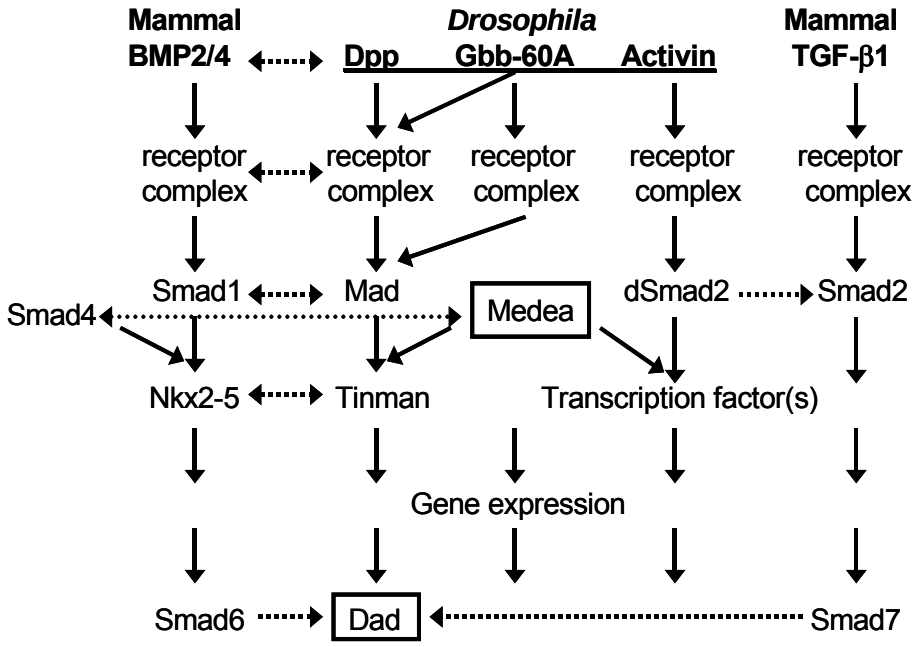

Figure 5. TGF-β superfamily cross-species functionality. Components of the *D. melanogaster* and mammalian BMP and TGF-β1/activin signaling pathways. Downward arrows indicate the movement of inductive signals and the point at which each component participates in signaling. Horizontal dashed arrows indicate that cross-species experiments have been performed in the direction(s) indicated. In each case, the *D. melanogaster* protein functioned properly in mammalian cells and/or the mammalian protein functioned properly in *D. melanogaster* cells. Medea and Dad are boxed to indicate that their activities are likely relevant to BMP and TGF-β/Activin signaling pathways. Signal terminators for *D. melanogaster* gbb-60A and Activin are unknown. See text for references.

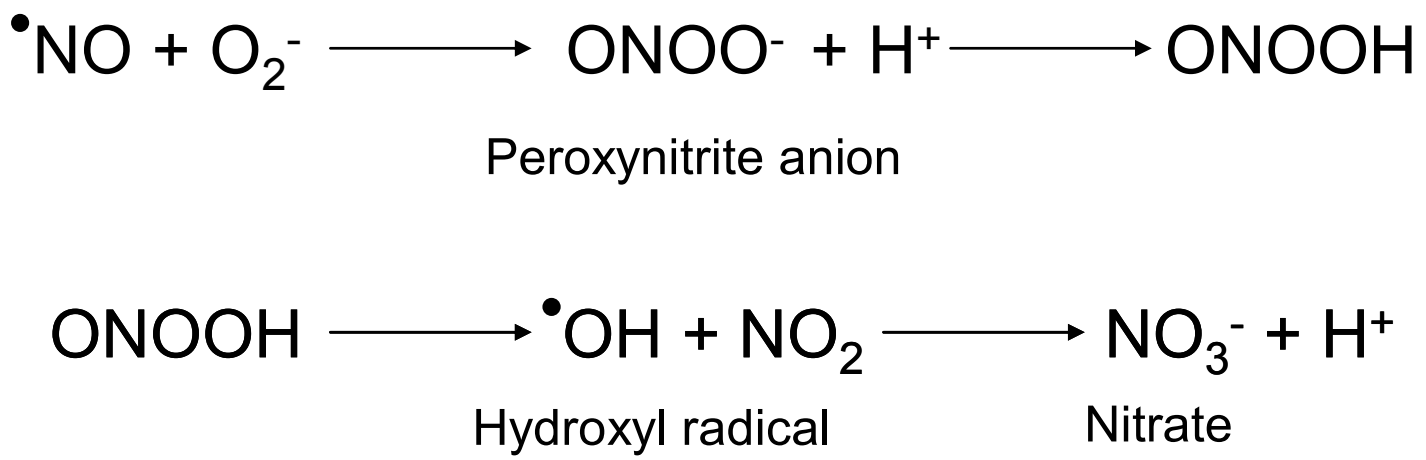

Figure 6. Interactions of nitric oxide with reactive oxygen species. •NO: nitric oxide; O_2^-: superoxide anion; NO_2: nitrogen dioxide (adapted from Singh and Evans, 1997).

Chapter 3 Human TGF-β1 regulates *Plasmodium* development via an effect on AsNOS expression and activity

3.1. Abstract

The mosquito *Anopheles stephensi* limits malaria parasite (*Plasmodium* spp.) development with inducible synthesis of nitric oxide (NO) catalyzed by *A. stephensi* NO synthase (AsNOS). Transforming growth factor-β1 (TGF-β1), a prototypic member of the TGF-β superfamily, regulates mammalian iNOS activity and immunity to *Plasmodium*. Active mammalian TGF-β1 is present in the mosquito midgut to 48h post-bloodfeeding (b.f.). Cytoplasmic proteins known as Smads mediate signaling by TGF-β. Because human TGF-β can activate *D. melanogaster* Smads, I hypothesized that *Anopheles* cells could respond to human TGF-β1 (hTGF-β1) and that this response could influence *AsNOS* expression and *Plasmodium falciparum* development in *A. stephensi*.

After treatment with hTGF-β1, *A. stephensi* cells were characterized by a change in cell morphology from round to spread and lower rates of DNA synthesis. Human TGF-β1 also induced *AsNOS* expression in *A. stephensi* cells *in vitro* and in midgut epithelial cells. In other experiments, *A. stephensi* were fed on *P. falciparum*-infected blood supplemented with hTGF-β1 or PBS as a control. My data showed that, within the concentration range detected in the midgut, hTGF-β1 significantly reduced oocyst numbers in treated mosquitoes compared to controls. Provision of the AsNOS catalytic inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) in a hTGF-β1-supplemented bloodmeal partially reverses of the effect of hTGF-β1 on *P. falciparum* development, suggesting that AsNOS is a target of hTGF-β1 in the mosquito midgut. Together, these

results provide the first evidence for immunological cross-talk between divergent free living hosts of a single parasite.

The cell morphology experiments in this chapter were performed in collaboration with Nicole Emmith in the lab. I performed the [^{3}H]-thymidine incorporation assays and all of the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays. Dr. Shirley Luckhart, Dr. Andrea Crampton, Dr. Junghwa Lim, Tina Peterson, and I performed the infection experiments. The cell morphology data (Figure 8), ASE cell qRT-PCR data (Figure 11), and effect of hTGF-β1 on *P. falciparum* data (Figure 13) have been published in Luckhart S, Crampton AL, Zamora R, Lieber MJ, Dos Santos PC, Peterson TM, Emmith N, Lim J, Wink DA, Vodovotz Y. (2003). Mammalian Transforming Growth Factor β1 Activated after Ingestion by Anopheles stephensi Modulates Mosquito Immunity. Infection and Immunity, Vol. 71, pp. 3000-3009.

3.2. Introduction

Nitric oxide (NO), a free radical gas synthesized by nitric oxide synthase (NOS), limits *Plasmodium* development in the midgut of the mosquito *Anopheles stephensi* (Luckhart *et al*., 1998). One of the most important physiological regulators of NOS activity and expression in mammals is transforming growth factor-β1 (TGF-β1; Vodovotz, 1997). Further, ingested mammalian TGF-β1 remains active in the midgut up to 48 hrs post-b.f. (Figure 7; Luckhart *et al*., 2003).

Based on these observations, I hypothesized that human TGF-β1 (hTGF-β1) regulates *P. falciparum* development through an effect on *A. stephensi* NOS (AsNOS) expression and activity. To address this hypothesis, I asked the following questions: Do mosquito cells recognize hTGF-β1? Does hTGF-β1 regulate *AsNOS* expression in cultured *A. stephensi* cells and in the midgut epithelium? Does hTGF-β1 regulate *P. falciparum* development in the mosquito midgut? Does inhibition of AsNOS catalytic activity block the effect of hTGF-β1 on *P. falciparum* development in the mosquito midgut?

3.3. Methods and Materials

1. Infection of *A. stephensi* with *P. falciparum*

For infection with *P. falciparum* (NF54 strain), 4- to 7-day-old female *A. stephensi* were allowed to feed through a 37°C water-jacketed membrane on identical aliquots of a mixture of parasites cultured in human erythrocytes with added uninfected erythrocytes (washed twice with RPMI 1640 medium) and human serum. Human serum used for mosquito feeding was stored at -70°C, thawed, and then stored at 4°C for approximately 1 week during experimental feedings. For analysis of the effects of human recombinant TGF-β1 on parasite development, equivalent volumes of sterile phosphate-buffered saline (PBS; Mediatech Incorporated, Herndon, VA) or hTGF-β1 (R & D Systems, Minneapolis, MN) to a final concentration of 2, 200, or 2,000 pg/ml were added to the infected blood immediately before membrane feeding. Infected mosquitoes were kept at 28°C after blood feeding (b.f.). At 7 days post-infection, *A. stephensi* midguts were dissected to count mature *P. falciparum* oocysts on the midgut epithelium. Two separate cohorts of *A. stephensi* were used for these assays; oocysts from 60 to 75 mosquitoes from each treatment group and the PBS controls were counted. In separate assays, equivalent volumes of sterile PBS, hTGF-β1 to a final concentration of 2 or 200 pg/ml, L-NAME (Sigma Chemical Company, St. Louis, MO) to a final concentration of 1 mg/ml, or a combination of hTGF-β1 and L-NAME were added to the infected blood immediately before membrane feeding. Oocyst counts were performed as described in the previous experiment. Differences between each treatment group and the PBS control group were analyzed by using the Student *t* test or the Kruskal-Wallis test.

2. Analyses of *A. stephensi* responses to hTGF-β1 *in vitro* and *in vivo*

Immortalized *A. stephensi* cell lines (MSQ43 and ASE) were maintained in modified Eagle minimum essential medium supplemented with 5% heat-inactivated fetal calf serum as described previously (E5; Fallon and Stollar 1987).

2.1. Analyses of MSQ43 cell morphology

For these assays, MSQ43 cells were plated in E0 medium (no serum) and allowed to recover overnight. Duplicate plates of cells were treated with diluent (4 mM HCl, 1 mg/ml BSA in PBS) or hTGF-β1 in diluent at 0.6, 6.0, or 30 pg/ml for 20 min or 60 min. Cells were then fixed and cover slipped for microscopic examination. At least 100 cells per treatment were scored as "round" or "spread"; the lengths (in centimeters) of the spread cells were measured on enlarged photomicrographs along the axis perpendicular to the cell body. Differences among percentages of round cells from assay replicates were analyzed by χ^2 analysis with commercially available software (SPSS), while differences between mean cell lengths were analyzed by using the Student *t* test.

2.2. Analyses of ASE cell DNA synthesis

For these assays, duplicate plates of ASE cells were treated with PBS or 0.08, 0.8, 8, 80, or 800 pg/ml hTGF-β1 in PBS for 48 hrs. [^{3}H]-thymidine was added to each plate to 5 μCi/ml for 30 min at room temperature. After incubation, DNA was precipitated from washed cells with 10% trichloroacetic acid and incorporated counts were measured by liquid scintillation as described previously (Stein *et al*. 1994). Data from three separate assays were analyzed by using the Student *t* test.

3. Analysis of *AsNOS* upstream sequence

The putative promoter region of the *AsNOS* gene (Luckhart and Rosenberg, 1999) was analyzed for consensus transcription factor binding sites using MatInspector (Quandt *et al*., 1995). This program utilizes a library of matrix descriptions for transcription factor binding sites to locate matches in sequences of unlimited length. Matches with greater than 75% identity to known transcription factor binding sites were labeled as putative transcription factor binding sites in the promoter region of *AsNOS*.

4. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) of AsNOS expression

For analysis of *AsNOS* expression in cultured cells, duplicate plates of ASE cells were treated with PBS or 6 pg of hTGF-β1/ml for 6, 24, or 48 h. For analysis of *AsNOS* expression in midgut epithelial cells, 4- to 7-day old female *A. stephensi* were subdivided for membrane feeding on uninfected human blood to which equivalent volumes of the following were added: (a) PBS, (b) 2 pg/ml hTGF-β1, (c) 200 pg/ml hTGF-β1 or (c) 2000 pg/ml hTGF-β1 immediately prior to feeding. To assess whether hTGF-β1 can modulate *AsNOS* expression *in vivo*, *A. stephensi* midguts were dissected at 0, 1, 6, 12, 24, 36, and 48 hr after feeding. Total RNA was isolated from cells or midguts post-assay using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Quantitative RT-PCR of *AsNOS* expression was performed as described previously (Crampton and Luckhart 2001a). Data from replicated assays were analyzed by using the Student *t* test or the Kruskal-Wallis test.

3.4. Results

1. Human TGF-β1 alters the morphology of *A. stephensi* cells *in vitro*

Previous work has shown that marine mollusk hemocytes respond to hTGF-β1 by forming lamellopodia or cytoplasmic extensions that facilitate immunocyte migration in a chemotactic gradient. This response was dose-dependent (0.05-5 pg/ml) with high doses (5 pg/ml) resulting in more lamellopodia (Ottaviani *et al*., 1997). To assess whether hTGF-β1 could alter *A. stephensi* cell morphology, I treated *A. stephensi* cells (MSQ43) with hTGF-β1 at 0.6-30 pg/ml. At 20 min and 60 min after treatment, MSQ43 cells respond to hTGF-β1 with a decrease in the percentage of round cells and an increase in mean cell length of spread cells (Figure 8; Luckhart *et al*., 2003). The reported effective dose (ED_{50}) of hTGF-β1 to inhibit [^{3}H]-thymidine incorporation of human cells is 20-60 pg/ml (Tsang *et al*., 1995), suggesting that *A. stephensi* cells respond to hTGF-β1 at biologically relevant doses.

2. Human TGF-β1 reduces cell DNA synthesis in *A. stephensi* cells *in vitro*

Reported effects of TGF-β1 signaling include inhibition of cell proliferation in human melanoma A375 cells (Nakai *et al*., 1988), reduced proliferation of human erythroleukaemia TF-1 cells at concentrations of 0.5-10 ng/ml (Mire-Sluis *et al*., 1996) and reduced proliferation of murine HT-2 cells at concentrations of 20-60 pg/ml (Tsang *et al*., 1995). Cell proliferation can be measured directly by quantifying DNA synthesis (Stein *et al*., 1994). To assess whether hTGF-β1 could alter *A. stephensi* cell DNA synthesis rates, I treated *A. stephensi* cells (ASE) with hTGF-β1 at 0.08-800 pg/ml. In response to 8, 80, and 800 pg/ml hTGF-β1, ASE cell DNA synthesis was decreased by ~20% relative to diluent-treated controls at 24 hr after treatment (Figure 9; Luckhart *et*

al. 2003). There was no significant inhibition of ASE cell DNA synthesis by 0.08 or 0.8 pg/ml hTGF-β1. These results suggest that 8 pg/ml is the threshold concentration for a hTGF-β1-mediated decrease in *A. stephensi* cell DNA synthesis. While a 20% decrease in DNA synthesis is modest, similar levels of inhibition have been observed in human fibroblasts treated with TGF-β1 (Agocha *et al*., 1997).

3. Human TGF-β1 regulates *AsNOS* expression

Given that TGF-β1 regulates inducible NOS (Vodovotz, 1997), I hypothesized that hTGF-β1 could regulate AsNOS. In support of this hypothesis, analysis of the putative promoter of AsNOS (Luckhart and Rosenberg, 1999) revealed the presence of one vertebrate TGF-β/Activin Smad binding element (Figure 10; Zawel *et al*., 1998) and two consensus GCCG *D. melanogaster* Mad binding sites (Figure 10; Kim *et al*., 1997). Based on these observations, I developed assays to determine whether hTGF-β1 regulates AsNOS in *A. stephensi*.

3.1. *In vitro* analysis of an *A. stephensi* cell line

To assess whether hTGF-β1 could alter *AsNOS* expression *in vitro*, I treated ASE cells with hTGF-β1 at 6 pg/ml. Relative to PBS-treated control cells, significant induction of expression occurred at 24 hr in response to 6 pg/ml hTGF-β1, while no significant induction of expression occurred at 6 hr and 48 hr (Figure 11; Luckhart *et al.* 2003). These results suggest that AsNOS is a target of hTGF-β1 regulation in *Anopheles* cells *in vitro*.

3.2. *In vivo* analysis of the *A. stephensi* midgut epithelium.

I determined that 6 pg/ml hTGF-β1 induces *AsNOS* expression in ASE cells (Figure 11; Luckhart *et al.* 2003). However, the physiology of the mosquito midgut may

differ from that of ASE cells. Therefore, I fed *A. stephensi* on human blood supplemented with hTGF-β1 at various doses to determine the optimal conditions for induction of *AsNOS* by hTGF-β1 in the mosquito midgut. In response to 2 pg/ml and 200 pg/ml hTGF-β1, significant changes in *AsNOS* expression were induced immediately after and at 36 hr after feeding (Figure 12); while inductions >2-fold were noted at other times, these results were not statistically significant. These results suggest that the optimal dose range for hTGF-β1 induction of *AsNOS* expression is 2 - 200 pg/ml. The >5-fold induction of *AsNOS* at 0 hr and 36 hr, together with the trend toward significant induction at 6 hr (Figure 12), is reminiscent of the biphasic induction of *AsNOS* expression that has been observed in *A. stephensi* midgut epithelial cells in response to *P. berghei* (Luckhart *et al*., 2003).

4. Human TGF-β1 regulates *Plasmodium* development in *A. stephensi* via an effect on AsNOS activity

TGF-β1 has been shown to regulate *Plasmodium* development in mammals (Omer *et al*., 2000). Luckhart *et al*. (1998) showed that inducible AsNOS expression and activity inhibits *Plasmodium* development in *A. stephensi*. Moreover, dietary provision of the NOS catalytic inhibitor L-NAME enhances parasite development, while provision of the NOS substrate L-Arg limits *Plasmodium* development (Luckhart *et al*., 1998). Based on my observations that hTGF-β1 can induce AsNOS *in vitro* (Figure 11; Luckhart *et al.* 2003) and *in vivo* (Figure 12) and that active TGF-β1 is present in the *A. stephensi* midgut post-b.f. for 48 hr (Figure 7; Luckhart *et al*., 2003), I hypothesized that ingested TGF-β1 could alter parasite development, perhaps through induction of AsNOS in the midgut.

4.1. Treatment of *A. stephensi* with hTGF-β1

To address whether hTGF-β1 alters *P. falciparum* development in *A. stephensi*, two separate cohorts of mosquitoes were fed on *P. falciparum*-infected blood supplemented with PBS or hTGF-β1 at 2 pg/ml, 200 pg/ml, or 2000 pg/ml. Parasite intensity of infection was determined by counting oocysts at 7 days post-b.f. In both cohorts, significantly fewer oocysts were observed in the 2 pg/ml and 200 pg/ml treated mosquitoes relative to control mosquitoes, but not in the 2000 pg/ml treated mosquitoes (Figure 13; Luckhart *et al.* 2003). In murine malaria parasite infection, low concentrations of TGF-β1 are pro-inflammatory to control parasite replication, while high concentrations of TGF-β1 are anti-inflammatory to limit host cell pathology (Omer *et al*., 2000). My results with *A. stephensi* suggest that TGF-β1 is pro- and anti-inflammatory in mosquitoes as well.

4.2. Treatment of *A. stephensi* with hTGF-β1 and L-NAME

To address whether ingested hTGF-β1 regulates *P. falciparum* development via an effect on AsNOS, a single cohort of mosquitoes were subdivided for membrane feeding on *P. falciparum*-infected human blood to which equal volumes of the following were added: (a) PBS, (b) 2 pg/ml hTGF-β1, (c) 2 pg/ml hTGF-β1 and 1 mg/ml L-NAME, (d) 2 pg/ml hTGF-β1 and 1 mg/ml D-NAME (Sigma Chemical Company, St. Louis, MO), (e) 200 pg/ml hTGF-β1, (f) 200 pg/ml hTGF-β1 and 1 mg/ml L-NAME, and (g) 200 pg/ml hTGF-β1 and 1 mg/ml D-NAME (h) 1 mg/ml L-NAME, and (i) 1 mg/ml D-NAME. Intensity of parasite infection was determined by counting oocysts at 7 days post-blood feeding. Infection intensity was lowest in hTGF-β1 treated samples, and highest in L-NAME treated samples (Figure 14). At both doses of hTGF-β1, L-NAME

partially reversed the effect of hTGF-β1 on *P. falciparum* (Figure 14). D-NAME, the inactive isomer of L-NAME, had no effect on parasite development either alone or in the presence of hTGF-β1 (Figure 14). Taken together, these results suggest that AsNOS is a target of hTGF-β1 in the regulation of *P. falciparum* development. Further analysis revealed that the effect of hTGF-β1 on parasite development was dose-dependent (Figure 15), and that L-NAME eliminated this dose-dependence. These results suggest that an effect of hTGF-β1 on AsNOS catalytic activity accounts for the dose-dependent effects of hTGF-β1 on parasite development.

3.5. Discussion and Future Directions

My results provide the first evidence for immunological cross-talk between divergent free living hosts of a single parasite. Specifically my data demonstrate that (i) human TGF-β1 is recognized by mosquito cells as an immunomodulatory cytokine, and (ii) human TGF-β1 regulates *P. falciparum* development via an effect on AsNOS gene expression and catalytic activity. Further, hTGF-β1 regulates AsNOS gene expression in cultured cells as well as the midgut epithelium (Figures 11 and 12). These results suggest that hTGF-β1 activates an *A. stephensi* signaling pathway. In support of this conclusion, TGF-β ligands, receptors and Smad proteins have been identified in *A. stephensi* (Lieber and Luckhart, 2004) and are discussed in Chapter 4.

Feeding of *A. stephensi* on hTGF-β1-supplemented human blood induces *AsNOS* expression in the midgut epithelium soon after feeding and again at 36 hours post-b.f. (Figure 12). Similar biphasic induction of *AsNOS* expression has been observed in *A. stephensi* midgut epithelial cells in response to *P. berghei* (Luckhart *et al*., 2003) and *P. falciparum* glycosylphosphatidylinositols (GPIs; Lim *et al*., 2005). Induction of *AsNOS* expression immediately after b.f. could be a result of a large influx of foreign material into the midgut, given that this tissue is a first-line defense against ingested materials. The induction of *AsNOS* expression at 36 hours after b.f. could be due to lipopolysaccharide (LPS) synthesized by rapidly growing bacteria. LPS synthesized during bacterial growth could enhance *AsNOS* expression post-b.f., whereas parasite invasion and *P. berghei* metabolites further stimulate expression in the highly reactive midgut (Luckhart *et al*., 1998).

During malaria parasite infection in mammals, TGF-β1 is both pro-inflammatory, enhancing the immune response to assist in parasite clearance early in infection, and anti-inflammatory, down-regulating the immune response to limit host pathology later in infection (Omer *et al*., 2000). Omer *et al*. (1998) showed that levels of TGF-β1 are inversely related to murine malaria parasitemia. Treatment of *P. falciparum*-infected mosquitoes with 2 or 200 pg/ml hTGF-β1 reduced oocyst numbers relative to control *P. falciparum*-infected mosquitoes (Figure 13). However, this effect disappeared at a dose of 2000 pg/ml (Figure 13). *AsNOS* gene expression is induced at 36 hours post-b.f. in the midgut epithelium of mosquitoes treated with 2 or 200 pg/ml hTGF-β1, while there is no change in *AsNOS* expression in mosquitoes treated with 2000 pg/ml hTGF-β1 (Figure 12). Therefore, the effect of hTGF-β1 on *P. falciparum* development may be due to regulation of *AsNOS* gene expression. I propose that the role of TGF-β1 as both a pro- and anti-inflammatory cytokine during malaria infection is functionally conserved in mosquitoes.

I hypothesized that hTGF-β1 regulates *P. falciparum* development through an effect on AsNOS gene expression and catalytic activity. To test this hypothesis, I used a NOS catalytic inhibitor, L-NAME, which has been shown to inhibit AsNOS activity *in vivo* (Luckhart *et al*., 1998). Mosquitoes that received a human bloodmeal supplemented with both hTGF-β1 and L-NAME had average oocyst counts that were significantly higher than hTGF-β1-treated mosquitoes and significantly lower than L-NAME-treated mosquitoes (Figure 14). These results suggest that AsNOS activity is at least partially responsible for the effect of hTGF-β1 on *P. falciparum* development. However, these results also suggest that hTGF-β1 activates other effectors in the mosquito that may

regulate *P. falciparum* development. In mammals, TGF-β1 is suspected to regulate transcription of as many as 4000 different genes (Zavadil *et al*., 2001). To help identify additional effectors activated by hTGF-β1 in *Anopheles* we will assess the global transcriptome in *Anopheles* cells treated with hTGF-β1. This work will serve as a foundation for future efforts to determine whether other hTGF-β1-induced effectors regulate *Plasmodium* development in *A. stephensi*.

My results also imply that the immune state of the infected mammalian host may influence mosquito immunity towards the parasite. Specifically, bloodmeals taken from a mammal in the early stages of infection are more likely to have low levels of circulating active TGF-β1 which could lead to a stronger immune response in the mosquito. Conversely, bloodmeals from a mammal in the latter stages of infection are more likely to have high levels of circulating active TGF-β1 which could lead to a repression of the immune response in the mosquito. The impact of mammalian immunity on the ability of the mosquito to transmit parasites could have serious consequences on global malaria control. Current efforts are directed toward development of transgenic mosquitoes that are refractory for malaria transmission (Phillips, 2001). This strategy is based on the concept of enhancing endogenous anti-parasite genes or introducing transgenes capable of limiting parasite transmission. The results presented here imply that the interface between mammals and the mosquitoes that feed on them establishes an opportunity for molecules such as TGF-β1 to alter the mosquito immune response. For example, TNF-α, IFN-γ, IL-4, IL-10, IL-12, and IL-18 produce both control and pathology during the mammalian immune response to *P. falciparum* (Malaguarnera and Musucemi, 2002). We must understand the regulation of the anti-*Plasmodium* defenses in *Anopheles* in

order to create a strategy for malaria control based on the enhancement of these immune responses.

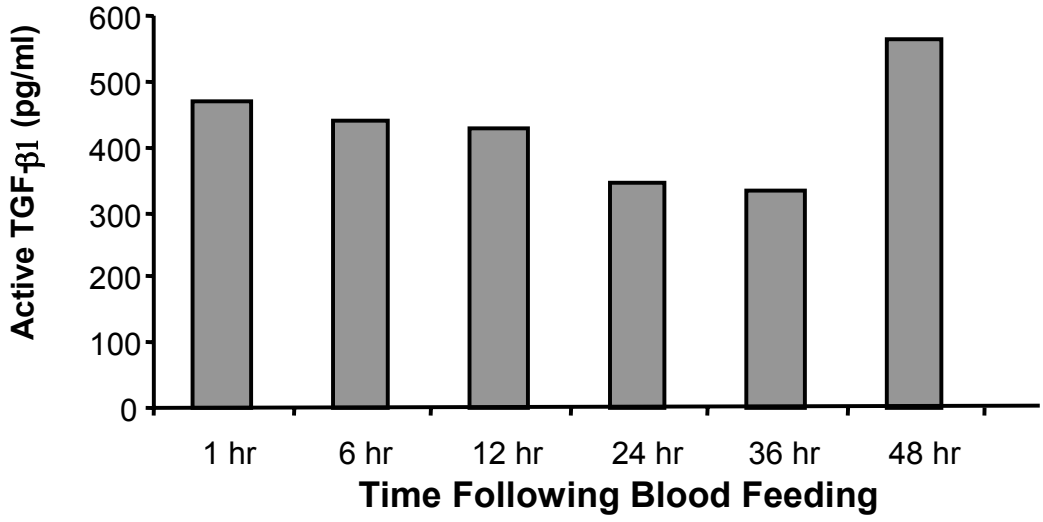

Figure 7. Ingested hTGF-β1 remains active up to 48 hours after blood feeding. At various times after blood feeding on ICR mice, *A. stephensi* midguts were dissected, sonicated, and analyzed by a receptor-based Quantikine™ ELISA (R & D Systems, Minneapolis, MN). From 1 hr – 48hr after feeding, active TGF-β1 is present at 300-500 pg/ml. In ICR mice, circulating levels of total TGF-β1 are ~5 ng/ml, 100% of which is present in the latent form (data not shown). In addition, all samples were transiently acidified to activate any remaining TGF-β1. No additional TGF-β1 was detected, indicating all of the ingested TGF-β1 was activated in the midgut (Luckhart *et al*., 2003).

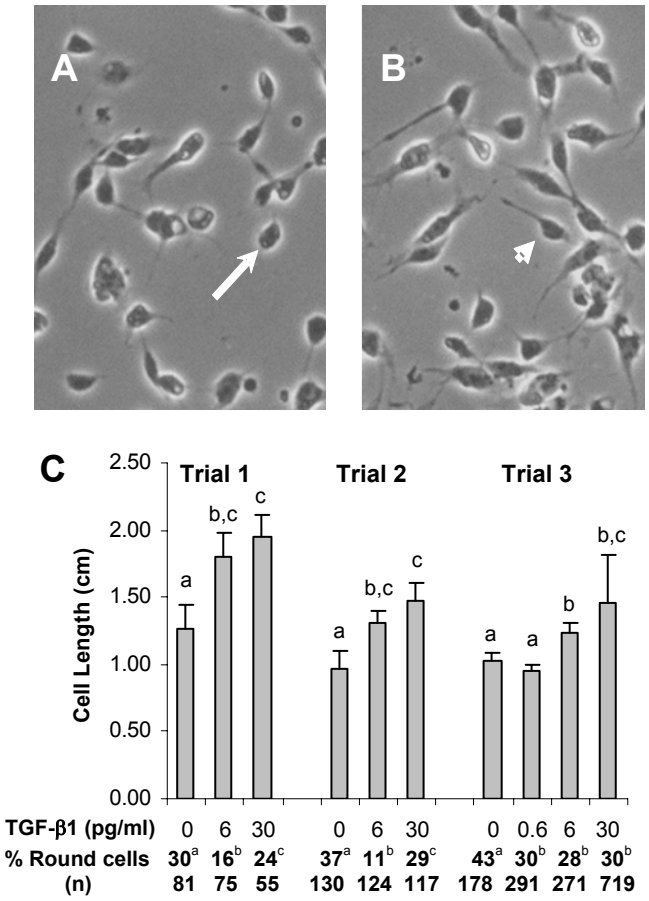

Figure 8. Human TGF-β1 alters the morphology of *A. stephensi* MSQ43 cells *in vitro*. (A) Typical morphology of MSQ43 cells treated with diluent. Note the predominance of rounded cells (arrow) compared to spread cells. (B) Typical morphology of MSQ43 cells treated with 6 or 30 pg of hTGF-β1. Note the predominance of spread cells with filopodia (arrowhead) compared to rounded cells. (C) Lengths of spread MSQ43 cells and percentages of rounded cells (n) were determined from photomicrographs of treated and control cells from three trials. Significant differences among treatments within a trial are indicated by different lower case letters (Luckhart *et al*., 2003)

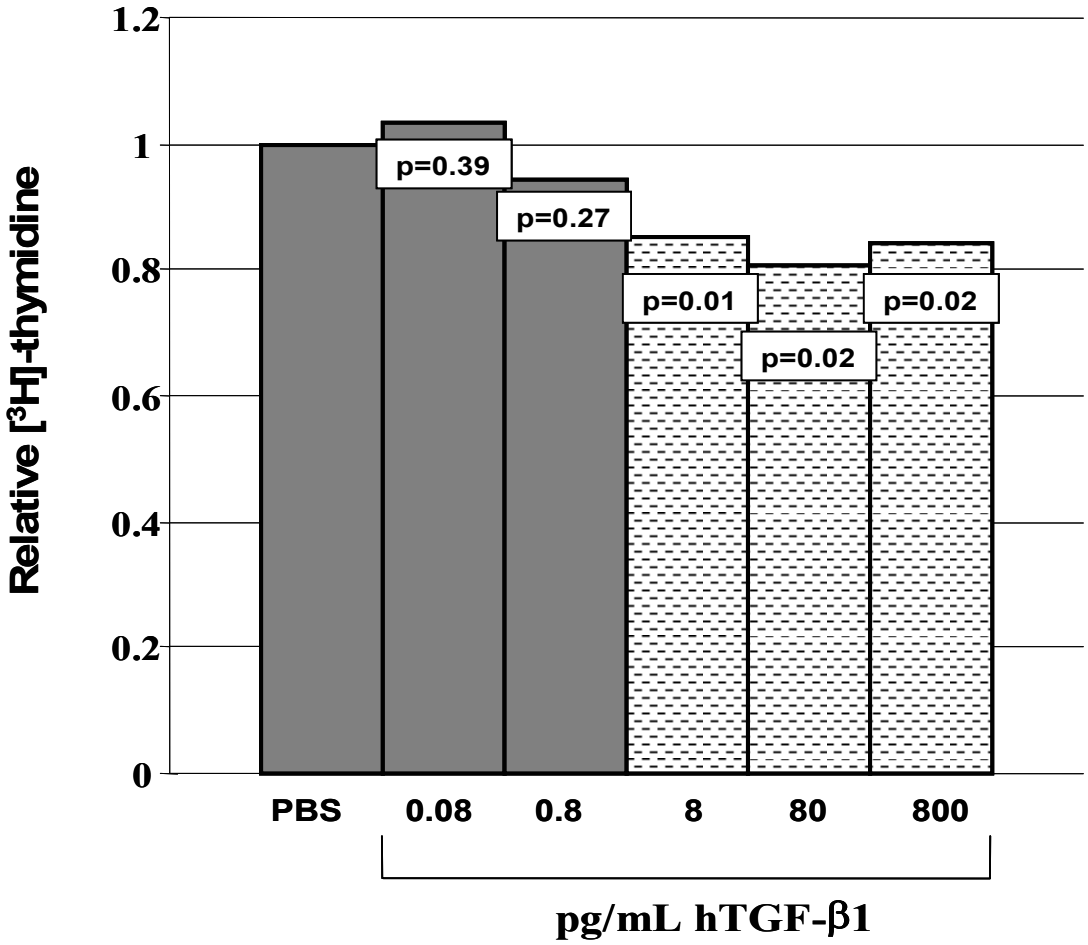

Figure 9. Human TGF-β1 reduces cell DNA synthesis in *A. stephensi* ASE cells. Inhibition of cell proliferation can be measured directly by quantifying DNA synthesis (Stein *et al*. 1994). The amount of [^{3}H]-thymidine incorporation into ASE cells treated with hTGF-β1 was measured as described by Stein *et al.* (1994). Data were analyzed using Student's *t* test; p-values are shown for treatments relative to PBS controls. In response to 8, 80, and 800 pg/ml hTGF-β1 (dashed bars), ASE cell DNA synthesis was decreased by ~20%. There was no significant inhibition of ASE cell DNA synthesis at 0.08 and 0.8 pg/ml (gray bars). Results shown are averages from 5 independent experiments.

Figure 10. Three Smad binding elements are present in the upstream sequence of *AsNOS*. Approximately 3 kb of the putative promoter for *AsNOS* (Luckhart and Rosenberg, 1999) was analyzed for consensus sequences for Smad binding elements (SBE) using MatInspector (Quandt *et al*., 1995). Dashed underlined text indicates consensus *D. melanogaster* Mad binding elements (Kim *et al*., 1997). Dotted underlined text indicates consensus vertebrate TGF-β/activin SBE's (Zawel *et al*., 1998).

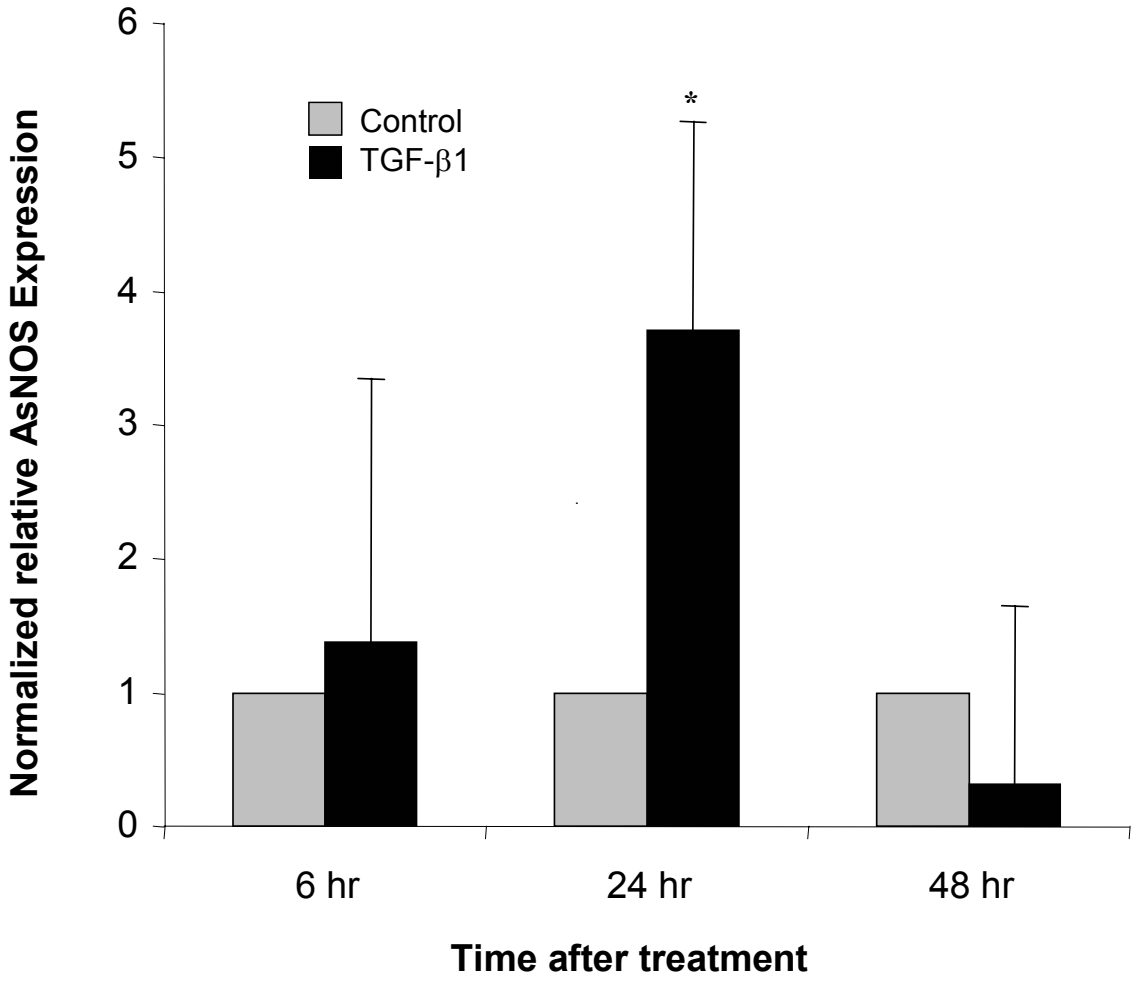

Figure 11. Human TGF-β1 induces AsNOS gene expression *in vitro*. ASE cells were treated with PBS or hTGF-β1 at 6 pg/ml for 6, 24, or 48 hours. Total RNA was extracted from cells with TRIzol™ reagent. Quantitative RT-PCR was used to analyze AsNOS expression in samples as described (Crampton and Luckhart, 2001a). Relative AsNOS expression was normalized against ribosomal S7 protein gene expression. Data were analyzed using Student's *t* test (α=0.05). Relative to PBS-treated control cells significant induction of expression occurred at 24 hours, while no significant expression occurred at 6 and 48 hours (Luckhart *et al*., 2003). Results shown are averages from 3 independent experiments.

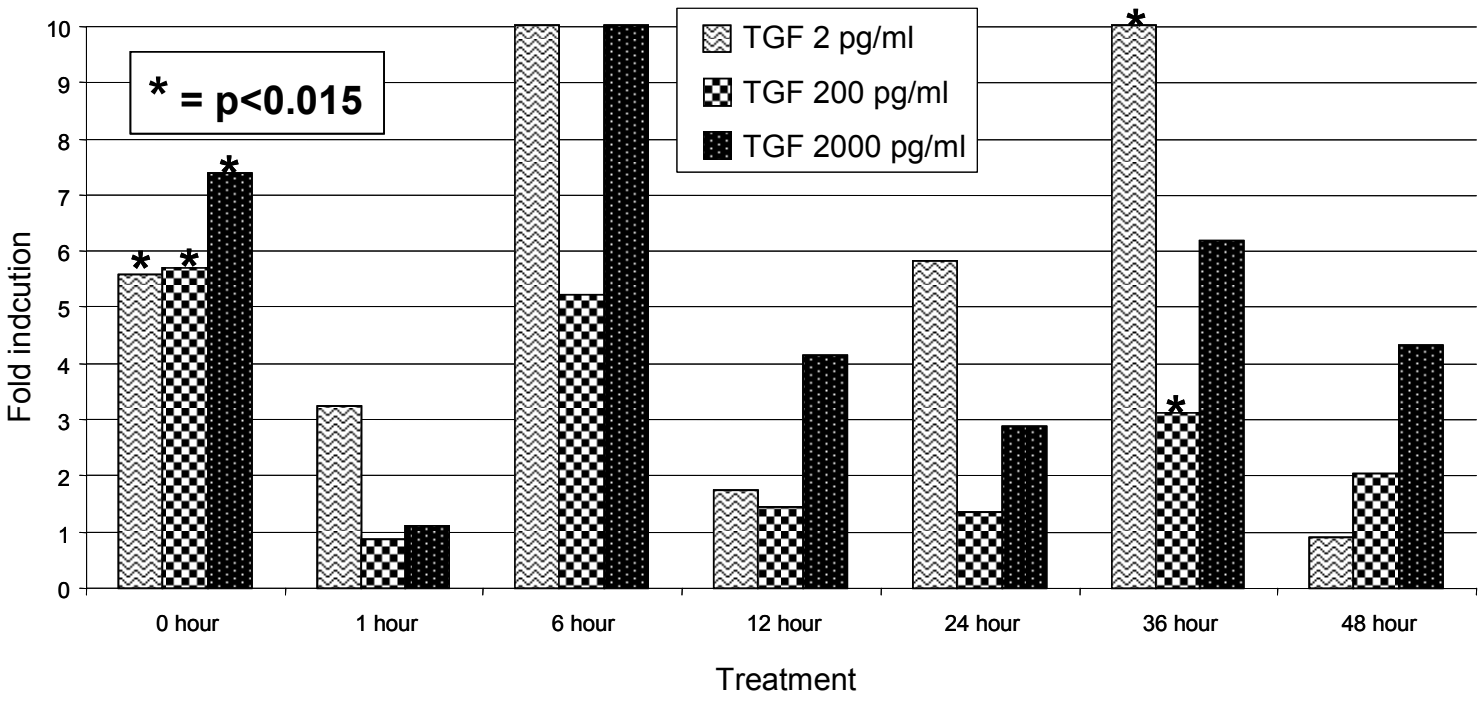

Figure 12. Human TGF-β1 induces AsNOS gene expression in the midgut epithelium. To assess whether hTGF-β1 could enhance AsNOS expression in the midgut epithelium mosquitoes were provided human blood supplemented with PBS or hTGF-β1 at 2, 200 or 2000 pg/ml. Quantitative RT-PCR was used to analyze AsNOS gene expression in RNA from dissected midguts at various times post-blood meal as described (Crampton and Luckhart, 2001a). AsNOS expression relative to PBS-treated controls was normalized against ribosomal S7 protein gene expression. Differences between TGF-β1 treated and PBS-treated samples were analyzed using a Kruskal-Wallis Test. Induction levels ≥ 2-fold with p-values ≤ 0.1 are shown. Results shown are averages from 5 independent experiments.

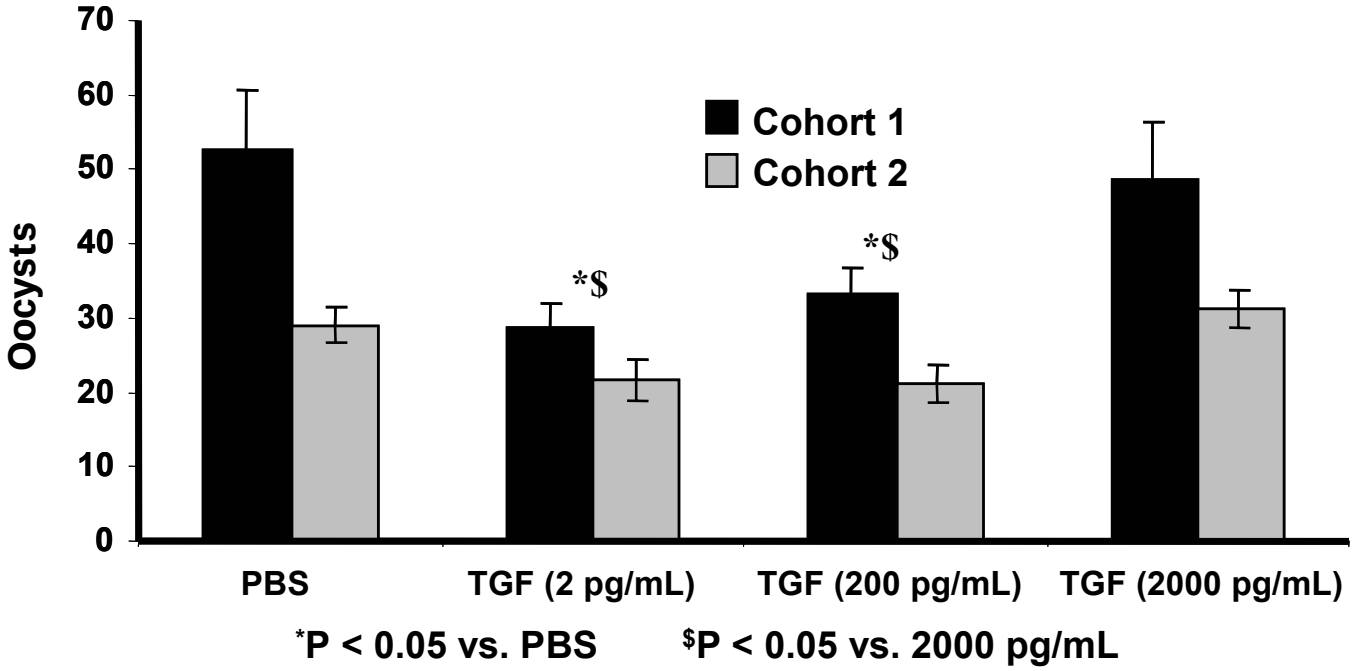

Figure 13. Human TGF-β1 attenuates *P. falciparum* development in the mosquito midgut. Two cohorts of mosquitoes were fed *P. falciparum*-infected blood with PBS or TGF-β1 at 2 pg/ml, 200 pg/ml or 2,000 pg/ml in PBS. To determine intensity of infection, parasite oocysts were counted from 60-75 mosquitoes per group; data from cohorts 1 and 2 were not combined because mean infections were not equivalent. Data were analyzed by Student's *t* test; values are means ± SEs. For cohorts 1 and 2, hTGF-β1 at 2 pg/ml reduced the number of *P. falciparum* oocysts by 46% and 26%, respectively, compared to the controls. Similarly, 200 pg/ml TGF-β1 reduced oocyst numbers by 37% and 27%, respectively, compared to controls. In contrast, the highest concentration of hTGF-β1 (2,000 pg/ml) had no effect on oocyst numbers in either cohort when compared to the controls (Luckhart *et al*., 2003).

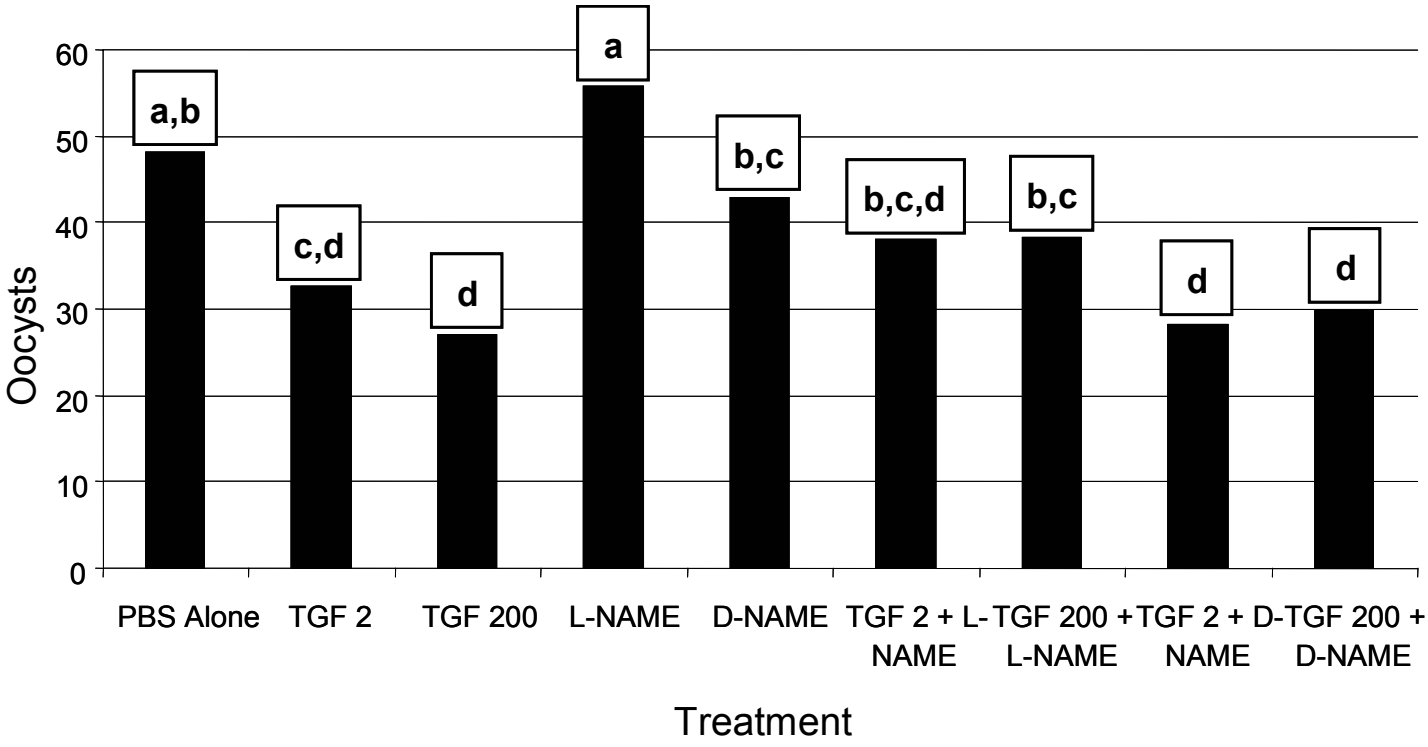

Figure 14. Provision of L-NAME partially blocks the effect of hTGF-β1 on parasite development. To assess whether the effect of hTGF-β1 on *Plasmodium* development was due to an effect on AsNOS activity, mosquitoes were fed on *P. falciparum*-infected human blood supplemented with PBS, 2 pg/ml hTGF-β1, 200 pg/ml hTGF-β1, the AsNOS catalytic inhibitor L-NAME, the inactive isomer D-NAME, hTGF-β1 and L-NAME, or hTGF-β1 and D-NAME. Parasite intensity of infection was determined by counting oocysts after seven days. Approximately 75 mosquitoes per treatment were analyzed. Differences between TGF-β1, L-NAME, D-NAME, and control-treated samples were analyzed using Kruskal-Wallis test. Different lowercase letters represent significant differences between treatments.

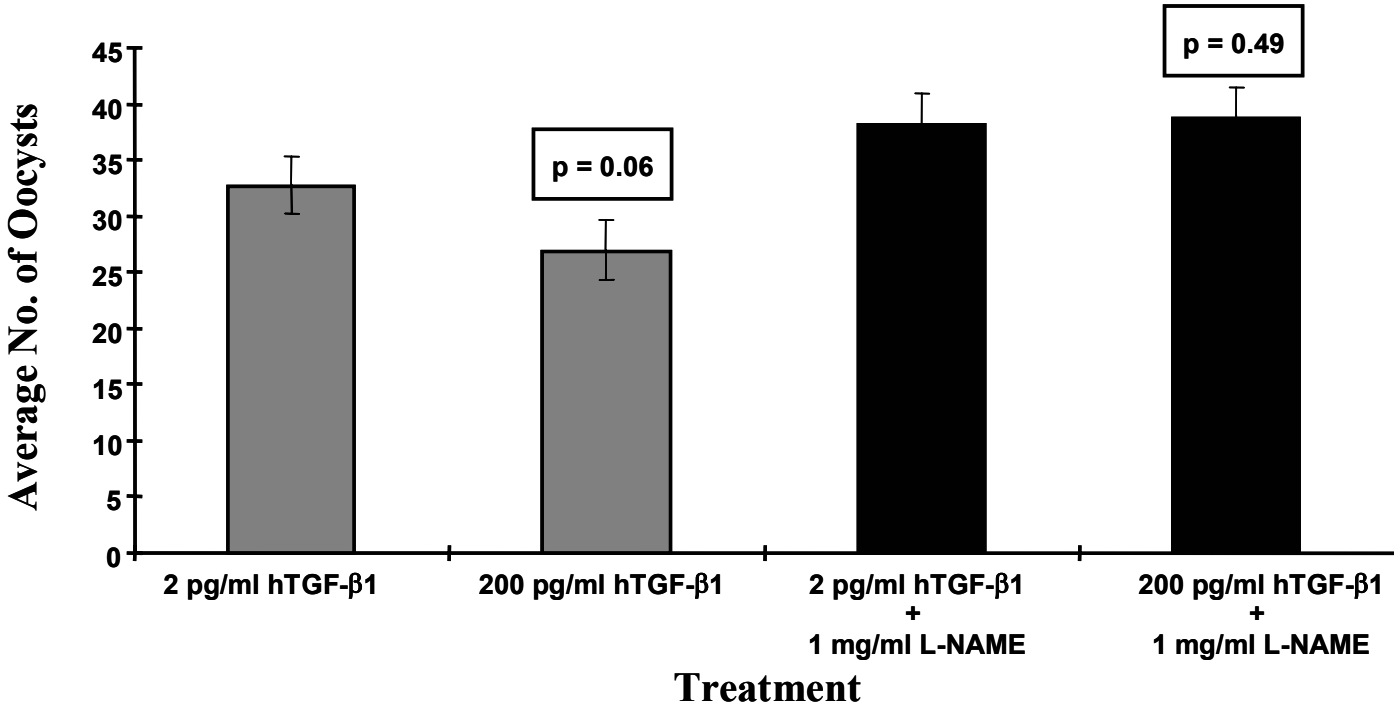

Figure 15. The effect of hTGF-β1 on *Plasmodium* development is dose-dependent, and this dose-dependence is eliminated by L-NAME. To show more clearly that the effect of hTGF-β1 in Figure 16 was dose-dependent, the data presented here are repeated from Figure 14. Representative p-values are derived by comparing bars of the same color using Student's *t* test; values are means ± SEs. In this experiment the hTGF-β1-treated mosquitoes exhibited a dose-dependent effect on *P. falciparum* development (gray bars). In the presence of L-NAME (black bars), this dose-dependence is abolished, suggesting that the dose-dependent effect of hTGF-β1 on *P. falciparum* development may be due to an effect on AsNOS catalytic activity.

Chapter 4 Transforming growth factor-βs and related gene products in mosquito vectors of human malaria parasites

4.1. Abstract

The participation of a divergent mosquito transforming growth factor-β (TGF-β) and mammalian TGF-β1 in the *Anopheles stephensi* response to malaria parasite development suggests that a network of *Anopheles* TGF-β ligands and signaling pathways figure prominently in immune defense of this important vector group. To provide a basis for identifying the roles of these proteins in *Anopheles* innate immunity, I identified six predicted TGF-β ligand-encoding genes in the *Anopheles gambiae* genome, including two expressed, diverged copies of 60A, the first evidence of ligand gene duplication outside of chordates. In addition to five predicted type I and II receptors, I identified three Smad genes in the *A. gambiae* genome that would be predicted to support both TGF-β/Activin- and bone morphogenetic protein (BMP)-like signaling. All three Smad genes are expressed in an immunocompetent *A. stephensi* cell line and in the *A. stephensi* midgut epithelium, confirming that a conserved signaling architecture is in place to support signaling by divergent exogenous and endogenous TGF-β superfamily proteins.

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4.2. Introduction

Mosquito As60A and mammalian TGF-β1 are divergent members of the TGF-β superfamily, with As60A in the Decapentaplegic (Dpp)/bone morphogenetic protein (BMP) subfamily (Crampton and Luckhart, 2001b) and TGF-β1 in the TGF-β/Activin subfamily. In *D. melanogaster*, BMP-like signals are transduced by Mad (a receptor- or R-Smad) and Medea (a Co-Smad), while TGF-β/Activin-like signals are transduced by dSmad2 (an R-Smad) and Medea (Raftery and Sutherland, 1999). Signaling through both pathways appears to be terminated by the inhibitory Smad (I-Smad) Dad (Marquez *et al*., 2001).

Based on these observations, I asked the following questions: Does the *A. gambiae* genome encode TGF-β ligands, receptors, or Smad proteins? What are the phylogenetic relationships among these proteins and their respective homologs from other organisms? Are these genes expressed in *A. gambiae* cells, in *A. stephensi* cells, and in the midgut epithelium of *A. stephensi*?

4.3. Methods and Materials

1. Maintenance and bloodfeeding of *A. stephensi* and maintenance of *A. stephensi* and *A. gambiae* immortalized cell lines

Anopheles stephensi Liston were reared at 27 °C and 75% relative humidity. Non-bloodfed midgut samples were prepared by dissecting 5–10 female mosquito midguts into TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) for RNA isolation. For preparation of bloodfed midgut RNA samples, 4- to 7-day-old female *A. stephensi* were allowed to feed to repletion on either anesthetized naïve Institute of Cancer Research (ICR) mice or human erythrocytes and serum. For the latter, 1.5 ml anonymous donor type A^+ serum was mixed with 0.5 ml anonymous donor Type O^+ packed red blood cells (Continental Services Group, Miami, FL) and added to a 37 °C water-jacketed glass membrane feeder (Kontes Glass, Vineland, NJ) with a Parafilm membrane (Fisher Scientific, Pittsburgh, PA). At 1 h, 3 h, 12 h, and 24 h post-bloodfeeding (b.f.), 5–10 mosquito midguts were dissected into TRIzol reagent for RNA isolation. Aliquots of isolated total RNA were used in reverse transcriptase-polymerase chain reaction (RT-PCR) assays as described below. Immortalized *A. stephensi* (ASE) cells were grown and passaged in modified Minimal Essential Medium (Mediatech Incorporated, Herndon, VA) containing 5% heat-inactivated fetal bovine serum at 28 °C under 5% CO_2. Immortalized *A. gambiae* Sua-1B cells (generously provided by Hans-Michael Müller, EMBL) were grown and passaged in Schneider's medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum at 28 °C.

2. Sequence analyses

The *A. gambiae* genome database was searched using TBLASTN (Altschul *et al*., 1997) with default parameters and all known *D. melanogaster* TGF-β ligand, receptor, and Smad protein sequences as queries (Raftery and Sutherland, 1999; Newfeld *et al*., 1999; Adams *et al*., 2000). The *D. melanogaster* sequences, indicated with accession numbers, included: Activin (Act; AAL51005), Dpp (AAC47554), Glass bottom boat-60A (Gbb-60A; AAA28307), Maverick (Mav; AAF99658), Myoglianin (Myo; AAD24472), Screw (Scw; AAA56872), Punt (Put; AAC41566), Baboon (Babo; NP_476999), Saxophone (Sax; AAA28878), Thickveins (Tkv; NP_787989), Wishful thinking (Wit; AAF60175), Mad (AAB60230), dSmad2 (AAD22443), Medea (AAC38971), and Dad (BAA22841). In addition, *Gallus gallus* Ski (GgSki; AAA48730) was used as a query sequence. Nucleotide sequences corresponding to the most significant hits to *A. gambiae* scaffolds were verified as TGF-β ligand-, TGF-β receptor-, Smad- or Ski-encoding sequences using BLASTN (Altschul *et al*., 1997) analyses of the GenBank, EMBL, DDBJ and PDB nucleotide databases (formerly the NCBI non-redundant nucleotide database). Putative nucleotide matches were translated in all reading frames to identify complete open reading frames, which were then verified as genes of interest using TBLASTN analyses (Altschul *et al*., 1997) of all non-redundant GenBank CDS translations and PDB, SwissProt, PIR and PRF peptide databases. Sequences of predicted proteins were analyzed using the NCBI Conserved Domain Architecture Retrieval Tool to identify relevant conserved regions, including the TGF-β-like domain for putative ligands, the Type I and Type II receptor domain, the GS-rich domain, the

serine/threonine kinase catalytic domain for putative receptors, and the Mad Homology 1 (MH1) and MH2 domains for putative Smads.

DNA sequence corresponding to *A. stephensi 60A2* (*As60A2*) was obtained using degenerate primers designed against *A. gambiae 60A2* sequence in RT-PCR assays of cDNA derived from the *A. stephensi* cell line ASE. Additional 3′ and 5′ gene fragments were amplified using the RNA ligase-mediated rapid amplification of cDNA ends kit (RLM-RACE; Ambion Incorporated, Austin, TX) according to manufacturer's instructions. Multiple cloned amplimers were sequenced in both orientations and aligned to obtain a consensus sequence for *As60A2*. Predicted amino acid sequences for putative *A. gambiae* TGF-β ligands, from the first of seven conserved cysteines to the C-terminus of the mature receptor-binding domain (108 characters), were aligned with corresponding regions from 25 ligand sequences (Accession numbers for ligands are as follows: MmBMP7 (P23359), HsBMP7 (P18075), XlOP1 (AAD09399), MmBMP5 (P49003), HsBMP5 (P22003), As60A (AAG13400), Dm60A (P27091), DmScw (NP_524863), DmDpp (P07713), DvDpp (AAC47555), XlBMP2I (P25703), MmBMP2 (S45355), HsBMP4 (P12644), TcDpp (AAB38392), DmAct (AAL51005), MmIbA (Q04998), XlActbB (I51199), MmTGFb3 (AAH05513), HsTGFb3 (CAA33024), DmALP23B (NP_523461), HsGDF8 (NP_005250), MmGDF8 (O08689), GgMyo (AAK18000), DmMyo (NP_524627), DmMav (NP_524626), HsGDNF (Q07731)), including As60A (Crampton and Luckhart, 2001b) and As60A2 using CLUSTAL X version 1.83 and default parameters (Thompson *et al*., 1997). Predicted amino acid sequences for putative *A. gambiae* TGF-β receptors, from the first conserved glycine to the terminal conserved arginine of the cytoplasmic serine/threonine kinase domain (305 characters), were

aligned with corresponding regions from 19 receptor sequences as described for ligand sequences. Accession numbers for receptors are as follows: DmPunt (AAC41566), HsActIIR (I37134), MmActIIB4 (284695), DmWit (AAF60175), HsBMPIIR (AAA86519), MmBMPIIR (XP_129719), XlBMPIIR (1764144), DmTkv (XP_079689), MmBMP1BR (NP_031586), HsBMP1BR (NP_001194), XlBMPR (A54985), DmSax (NP_523652), MmActAR (NP_031420), HsALK2R (CAA80256), XlALK2R (AAB88259), DmBabo (NP_477000), MmTGFbIR (JC2062), HsTGFbIR (547777), XlSTKIR (841310), CeDaf4 (P50488).

DNA sequences corresponding to *A. stephensi Mad* (*AsMad*), *Medea* (*AsMedea*) and *dSmad2* (*AsdSmad2*) were obtained using degenerate primers designed against relevant *A. gambiae* sequences in RT-PCR assays of cDNA derived from ASE cells. Additional 3′ and 5′ gene fragments were amplified using the RLM-RACE kit according to manufacturer's instructions. Multiple cloned amplimers were sequenced in both orientations and aligned to obtain consensus sequences. Predicted amino acid sequences for putative *A. gambiae* Smad genes, from the first conserved tryptophan of the MH2 domain to the C-terminus (230 characters), were aligned with corresponding regions from 35 Smads, including AsMad, AsdSmad2, and AsMedea.

Phylogenetic trees for all predicted protein sequences were constructed using PAUP*4.0b10 (Swofford, 2001) with sequences of human Glial cell line-derived neurotrophic factor (GDNF; AAD43139), *Caenorhabditis elegans* Daf4 (AAC02726.1), and *C. elegans* Daf3 (AAB61748) as outgroups for the ligand, receptor, and Smad data sets, respectively. Distance and parsimony trees were generated by neighbor joining or heuristic searches with bootstrap analyses for both data sets performed with 1000

replications. Accession numbers for Smads are as follows DmMad (AAB60230), DmdSmad2 (AAD11458), DmMedea (AAC38971), DmDad (BAA22841), HsSmad1 (Q15797), HsSmad2 (Q15796), HsSmad3 (Q92940), HsSmad4 (Q13485), HsSmad5 (Q99717), HsSmad6 (O43541), HsSmad7 (O15105), HsSmad9 (O15198), MmSmad1 (P70340), MmSmad2 (Q62432), MmSmad3 (Q92940), MmSmad4 (P97471), MmSmad5 (P97454), MmSmad6 (O35182), MmSmad7 (O35253), MmSmad9 (Q9JIW5), XlMad1 (AAB39738), XlMad2 (AAB39329), XlSmad6 (AAB94138), XlSmad7 (AAB39329), XlSmad8a (AAL86772), XlSmad8b (AAL86773), XlSmad8c (AAL86774), XlSmad10 (AAD16879), and CeDaf3 (AAB61748).

3. RT-PCR analyses of gene expression in *A. gambiae* cells and in *A. stephensi* cells and midgut tissue

Total RNA was extracted from *A. gambiae* Sua-1B and *A. stephensi* ASE cells and from dissected *A. stephensi* midgut tissue using TRIzol reagent. Reverse transcription was carried out using 5 μg total RNA and 50 units MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). An *Ag60A2* fragment was amplified from Sua-1B cell cDNA with primers Ag60A2SF2 (GCGAAATCCGTTCCTGG) and Ag60A2SR2 (ATCGGATTCAGCTTGGTCG) with the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 63.5 °C for 30 s, and 72 °C for 30 s. An *As60A2* fragment was amplified from ASE cell cDNA and from midgut cDNA with primers As60A2SF2 (TTGGAGTACATTGGGGAACAGTC) and As60A2SR2 (GGTGATTGATGCGGTTGGT) with the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. An

AsMad amplimer was generated with primers AsMadF1 (CTGTCCCAGTCCGTCAACC) and AsMadR1 (AGCACCTTGTCCAGCCACT) and the following cycling conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s. An *AsdSmad2* amplimer was generated with primers AsdSmad2F1 (GATTTGTCACGCATGTCACC) and AsdSmad2R1 (GGTGGCAAATTCCTGATTGT) using the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. An *AsMedea* fragment was amplified with primers AsMedeaF1 (GCACAATACTGGCCCCACAG) and AsMedeaR1 (TCACTGAGACACCGCAACCA) using the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. All Smad fragments were amplified from ASE cell cDNA and from midgut cDNA. An *AgSki* amplimer was generated from Sua-1B cell cDNA with primers AgSkiSF1 (GACTTCAGCGTGGAGCAGAT) and AgSkiSR2 (AACTTCTGGGGCGAGAACA) using the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. An *AsSki* amplimer was generated from ASE cell cDNA and from midgut cDNA using primers AsSkiSF1 (AGTGACGAAGATGTCCAGCCA), AsSkiSR1 (CGAGCAGAAAGCACCCGAT), and the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. All analyses of gene expression were performed with controls lacking RT to demonstrate lack of contamination and amplification from genomic DNA.

4.4. Results

1. *Anopheles* TGF-β ligands and expression of duplicated, divergent 60A genes

My analyses of the *A. gambiae* genome database yielded highly significant translated sequence matches to the mature domains (TGF-β-like domains or TBDs) of *D. melanogaster* Act, Dpp, Gbb-60A, Mav, and Myo (Table 1; AgAct, AY578797; AgDpp, AY578800; Ag60A, AY578796; AgMav, AY578804; AgMyo, AY578806). Similar to the *D. melanogaster* sequences, the chromosomal locations of the *A. gambiae* ligand sequences are not linked (Table 1). Predicted N-termini of the *A. gambiae* proteins were not resolvable from the database sequences, likely because of divergence from the *D. melanogaster* queries. Intriguingly, exhaustive searches of the database did not yield a significant *A. gambiae* hit to *D. melanogaster* Scw, a ligand that groups phylogenetically with Gbb-60A (Burt and Law, 1994) and that functions with Dpp to specify pattern elements in the dorsal embryonic epidermis of *D. melanogaster* (Neul and Ferguson, 1998).

Two *A. gambiae* sequence matches to the single copy *D. melanogaster* Gbb-60A were identified (Table 1). The predicted protein sequences, which I have named Ag60A and Ag60A2 (AY578795), are encoded by different *A. gambiae* chromosomes and show significant identity across their predicted C-termini (Figure 16). However, Ag60A is 90% identical to the mature domain of As60A (Crampton and Luckhart, 2001b), but only 65% identical to Ag60A2 (Figure 16); this homology is reflected in the relationships among these how predicted proteins in both distance and parsimony trees (Figure 17A and B). Similarly, As60A2 shares greater sequence homology with Ag60A2 than with

As60A (Figure 16), resulting in a phylogenetic grouping for 60A2 that is distinct from that for 60A (Figure 17A and B). A comparison of the genomic structures of *Ag60A* and *Ag60A2* suggests this duplication event may have occurred. The number and positions of predicted introns of *Ag60A* are precisely conserved with those of *As60A* (not shown). However, based on sequence homology and predicted GT/AG intron boundaries, the genomic sequence of *Ag60A2* appears to encode a single intron, which is precisely conserved with intron 1 of the *Ag60A* and *As60A* genes (Figure 18). In intron-poor eukaryotic genomes, intron position is biased toward the 5′ end of genes, suggesting a consistent pattern of intron loss based on comparisons with homologous genes from intron-rich genomes (Mourier and Jeffares, 2003). The mechanism of 3′ intron loss is probably best explained by homologous recombination between a 5′-truncated, reverse-transcribed cDNA copy and the original gene copy in a process known as RNA-mediated gene conversion, as has been experimentally demonstrated in *Saccharomyces cerevisiae* (Derr, 1998). Based on these observations, I suggest that *Ag60A2* and *As60A2* were derived from a gene duplication event followed by a double crossover with a truncated cDNA copy. Further, I suggest that the duplication event occurred prior to the separation of taxa in the genus *Anopheles*. The identification of a fragment of an *A. stephensi* paralog of As60A, which we have named As60A2 (AY578815) and which groups with Ag60A2 (Figure 17A and B), confirms that ligand gene duplication is a conserved feature of the genomes of *Anopheles* spp. The lack of ligand gene duplication in the *D. melanogaster* genome, which is generally viewed as a compact genome characterized by large-scale deletions (Petrov, 2002), may or may not represent an exception among

insects. As such, additional support for this assertion will depend on sequence analyses of other completed insect genome sequences.

While the preservation of diverged 60A2 genes in the *A. gambiae* and A. *stephensi* genomes suggests that these genes may be functional, further support of this hypothesis is provided by our observation that *Ag60A2* is expressed in the immunocompetent *A. gambiae* cell line Sua-1B (Figure 19A). Similarly, *As60A2* is expressed in the immunocompetent *A. stephensi* cell line ASE (Figure 19B) and in the *A. stephensi* midgut epithelium (Figure 19B), which participates in innate immune defense against malaria parasite infection (Luckhart *et al*., 1998; Crampton and Luckhart, 2001a; Luckhart *et al*., 2003).

2. *Anopheles gambiae* TGF-β receptors

My analyses of the *A. gambiae* genome database yielded highly significant translated sequence matches to the *D. melanogaster* Type I receptors Babo, Sax, and Tkv and to the Type II receptors Put and Wit (Table 2; AgBabo, AY578798; AgSax, AY578808; AgTkv, AY578811; AgPut, AY578807; AgWit, AY578812). Like the *D. melanogaster* receptor sequences, the *A. gambiae* sequences are not linked to each other or to the ligand sequences (Table 2). Predicted N-termini of *A. gambiae* Babo and Put were not resolvable from the database sequences, likely due to divergence from the *D. melanogaster* queries. However, in good agreement with their homologies to either Type I or Type II receptors, predicted *A. gambiae* Sax, Tkv, and Wit share conserved domain architectures with their *D. melanogaster* counterparts. Specifically, the Type I orthologs (Sax, Tkv) encode a Type I/Type II receptor domain (I, II), a GS-rich domain (G), and a

serine/threonine kinase catalytic domain (STK), while the Type II ortholog (Wit) lacks a GS-rich domain (Table 2).

3. Identification and expression of *Anopheles* Smads and a Smad co-repressor

My analyses of the *A. gambiae* genome database yielded highly significant translated sequence matches to *D. melanogaster* Mad, Medea, and dSmad2 (*AgMad*, AY578803; *AgMedea*, AY578805; *AgdSmad2*, AY578801). Homologous genes from *A. stephensi* (*AsMad*, AY578813; *AsMedea*, AY578816; *AsdSmad2*, AY532652), identified using degenerate primers designed against *A. gambiae* sequences, are expressed in ASE cells and in the *A. stephensi* midgut epithelium (Figure 19B). Expression of both Dpp/BMP-related (Mad, Medea) and TGF-β1/Activin-related (dSmad2, Medea) Smads in the *A. stephensi* midgut during blood digestion indicates that cellular machinery is available for signaling by both endogenous and exogenous (e.g., mammalian TGF-β1) proteins from this large superfamily of cytokines and growth factors.

Exhaustive searches for a Dad homolog in the *A. gambiae* genome database were performed using TEpipe (Biedler and Tu, 2003), a computer program available from these authors. TEpipe rapidly performs TBLASTN analysis with a query and then analyzes all of the BLAST hits by BLASTN analysis to find similar sequences. For these analyses, I used full-length *D. melanogaster* Dad and sequences corresponding to the Dad N-terminus, linker, and MH2 domains as queries. A significant *A. gambiae* hit to *D. melanogaster* Dad was not evident in the output: all hits were manually identified as *AgMad*-, *AgMedea*-, or *AgdSmad2*-encoding sequences. The lack of an *A. gambiae* Dad-encoding sequence is perhaps not surprising given the observed divergence of Dad from

other known and predicted antagonistic Smads (Newfeld *et al*., 1999). Among the known I-Smads, *D. melanogaster* Dad lacks any semblance of an MH1 domain and groups very weakly with the related human proteins, Smad6 and Smad7 (Newfeld *et al*., 1999). Additional searches with human and mouse Smad6 (HsSmad6, O43541; MmSmad6, O35182) and Smad7 (HsSmad7, O15105; MmSmad7, O35253), and *Xenopus laevis* Smad6 (AAB94138), Smad7 (CAA05893) and Smad10 (CAA04708) sequences as queries were also unproductive. While a match for an antagonistic Smad gene is not apparent in the *A. gambiae* genome database, we have identified genes encoding putative homologs of *D. melanogaster* Smurf, brinker and the 12 kDa FK506-binding protein (FKBP12; *AgSmurf*, AY578810; *AgBrk*, AY578799; *AgFKBP12*, AY578802), three gene products that can negatively regulate Smad signaling (Wang *et al*., 1996; Arora and Warrior, 2001; Kirkpatrick *et al*., 2001). In addition, I have identified a putative *A. gambiae* homolog of the related oncoproteins Ski and SnoN, which have not previously been identified in insects and are known to act as co-repressors to antagonize Smad signaling in mammalian cells (Liu *et al*., 2001). I have named this homolog AgSki (AY578809) and have determined that, like Ag60A2, it is expressed in immunocompetent *A. gambiae* Sua-1B cells (Figure 19A). The related *A. stephensi* ski gene or *AsSki* (AY578814) identified using degenerate primers designed against the *A. gambiae* sequence, is expressed in ASE cells and in the midgut epithelium of *A. stephensi* during blood digestion (Figure 19B). I suggest that AsSki may play a role in regulating Smad signaling in *A. stephensi*, but the more global role of Ski in regulating cellular transcription in mammalian cells (Liu *et al*., 2001) suggests that AsSki may also be essential for growth and differentiation of mosquito cells.

4. Phylogenetic analyses of *Anopheles* TGF-β ligand, receptor, and Smad sequences

As expected from homology analyses (Table 1), both parsimony and distance analyses revealed that the predicted *A. gambiae* and *A. stephensi* TGF-β ligands were more commonly associated with the corresponding *D. melanogaster* orthologs (Figure 17A and B) than with orthologs from other species. In general, predicted proteins in the TGF-β ligand trees were divided into the two recognized subfamilies of the TGF-β superfamily: the Dpp/BMP and TGF-β/Activin subfamilies (Figure 17A and B; Burt and Law, 1994; Newfeld *et al*., 1999). Within the Dpp/BMP subfamily, predicted proteins clustered into 60A/BMP5-7 and Dpp/BMP2-4 subgroups, as observed by (Newfeld *et al*., 1999; Nguyen *et al*., 2000; Herpin *et al*., 2004). In contrast to stable clustering of *A. gambiae* and *A. stephensi* 60A and 60A2 with *D. melanogaster* 60A in both distance and parsimony analyses, however, mosquito Dpp clustering was unstable. In distance analyses, *A. gambiae* Dpp was grouped with vertebrate orthologs rather than with *D. melanogaster* Dpp (64% bootstrap support; Figure 17A), while in the more conservative parsimony analyses, *A. gambiae* Dpp was grouped with *D. melanogaster* Dpp (51% bootstrap support; Figure 17B).

In parsimony analyses, *D. melanogaster* and *A. gambiae* Mav were assigned to the juncture of the Dpp/BMP and TGF-β/Activin subfamilies, in agreement with the observations of (Nguyen *et al*., 2000) for *D. melanogaster* Mav. The predicted *A. gambiae* Mav, like *D. melanogaster* Mav, encodes nine conserved cysteines typical of TGF-β/Activin subfamily proteins (Daopin *et al*., 1992), but yet shows the highest sequence identities to orthologs of the Dpp/BMP subfamily (not shown). The Mav

orthologs, therefore, represent a subfamily of invertebrate TGF-βs that share sequence homologies with both Dpp/BMP and TGF-β/Activin subfamily proteins.

Parsimony analysis showed a clustering of the prototypical TGF-βs with *D. melanogaster* Activin subfamily separate from the Myo and GDF8 proteins (Figure 17B). Nguyen *et al*. (2000) reported a phylogram showing a clustering of *D. melanogaster* Myoglianin with human GDF8/GDF11 and of *D. melanogaster* Activin with vertebrate Activins A–C (relationships supported by the presence of nine conserved cysteines typical of TGF-β/Activin subfamily proteins in Myoglianin and GDF8/GDF11). However, the authors did not report bootstrap support for these associations. In contrast to my parsimony analysis, distance analysis grouped the insect Myoglianins (AgMyo, DmMyo) and vertebrate Myostatin (GgMyo) and Growth and differentiation factor 8 (GDF8) within the TGF-β/Activin subfamily (Figure 17). Herpin *et al*. (2004) also reported clustering of *D. melanogaster* Myoglianin and Mav and a molluscan GDF with prototypical vertebrate TGF-βs (39% bootstrap support), the first time such a phylogenetic relationship had been reported for protostome sequences. The alignment used by Herpin *et al*. (2004) was unique in that it was based on complete precursor protein sequences, which are more divergent than the commonly used ligand domain sequences. Identification and phylogenetic analysis of the *A. gambiae* complete precursor sequences may help to clarify the relationships of these unusual insect proteins with their vertebrate counterparts.

The lack of a gene predicted to encode an *A. gambiae* ortholog of the ligand Scw is intriguing. The bootstrap values for the relationship of Scw to other ligands are similar in both parsimony and distance trees (Figure 17A and B), indicating reasonable support

for the placement of *D. melanogaster* Scw. The depth of the branch root in both trees suggests that the gene duplication that gave rise to Scw is relatively ancient, predating the separation of insects and chordates. A Scw ortholog may have been lost from the *A. gambiae* genome or this gene may have evolved more quickly in *D. melanogaster* and, thus, may have diverged beyond recognition as a conserved ligand within the Diptera. The lack of authentic Scw orthologs from the mouse and human genomes, however, appears to support the hypothesis that Scw has been lost from a variety of lineages.

As expected from homology analyses (Table 2), both parsimony and distance analyses revealed that predicted *A. gambiae* receptors were strongly associated with the corresponding *D. melanogaster* orthologs, grouping into Type I (Tkv, Babo, and Sax) or Type II receptors (Punt, Wit) and, within receptor type, according to ligand association (Figure 20A and B). In general, parsimony and distance tree topologies were also largely congruent with trees based on full-length sequences presented by Newfeld *et al*. (1999) and by Herpin *et al*. (2004). My trees, however, provide stronger support for the assertion of Newfeld *et al*. (1999) that Type II receptors represent the ancestral state among divergent organisms. For the two dipterans represented here, the ancestral Type II receptor appears to be Punt.

In constructing the Smad phylogenies, we utilized an alignment of MH2 domains, which are responsible for the formation of multi-Smad complexes and interactions with Type I receptors and which allowed the inclusion of Dad because it lacks an MH1 domain. As expected from homology analyses (not shown), both parsimony and distance analyses revealed that predicted *A. gambiae* and *A. stephensi* Smads were strongly associated with the corresponding *D. melanogaster* orthologs, grouping into the

polyphyletic Dpp/BMP and TGF-β/Activin R-Smads and the ligand-independent Co-Smads and I-Smads (Figure 21A and B); similar topologies were reported by Newfeld *et al*. (1999) and Herpin *et al*. (2004). Studies to date of *D. melanogaster* Mad and dSmad2 indicate that these signaling proteins do not transduce signals outside of their related ligand subclasses, suggesting that like *D. melanogaster*, signaling from the diversity of ligands in *A. gambiae* and *A. stephensi* likely depends on combinatorial flexibility of receptors and/or additional signaling co-activators or repressors.

4.5. Discussion and Future Directions

Identification of sequences predicted to encode both Dpp/BMP and TGF-β/Activin ligands, receptors, and Smads in the *A. gambiae* genome indicates that this medically important insect possesses the known range of TGF-β superfamily signal transduction pathways. With the addition of *A. stephensi* to my studies, I provide novel insights into these functionally related gene products. For example, homology and expression analyses of 60A2 in *A. gambiae* and *A. stephensi* indicate that duplication of functional TGF-β ligand genes predates the separation of insects and chordates. Further, expression of both Dpp/BMP- and TGF-β/Activin-related Smads in the *A. stephensi* midgut confirms that a signaling architecture is in place for observed cross-talk with mammalian TGF-β1 (Luckhart *et al*., 2003) and for transducing signals from As60A and other mosquito TGF-βs that may be involved in innate immunity to *Plasmodium*.

The role of As60A in the midgut inflammatory response to *Plasmodium* and the temporal similarity of *As60A2* expression (Figure 19B; Crampton and Luckhart, 2001a) suggest that As60A and As60A2 may play similar roles in this tissue. If As60A2 participates, however, it is unlikely to duplicate the role of As60A. Specifically, if the genomic structures of *Ag60A2* and *As60A2* are identical, *As60A2* would be predicted to lack the putative, conserved regulatory sequences identified in the second intron of *As60A* (Crampton and Luckhart, 2001b). These regulatory sequences, which are also present in conserved second introns of *Ag60A* (not shown), *D. melanogaster* Dpp and *Tribolium castaneum* Dpp (Sanchez-Salazar *et al*., 1996), include binding sites for the Rel family member Dorsal and for co-repressors of Dorsal, key elements in insect immune response signaling (Cramer *et al*., 1999). Further, the 5′ flanking sequences

(~3.3 kb) of *Ag60A* and *Ag60A2* are only 26% identical (not shown), suggesting that putative upstream regulatory sequences of *As60A* and *As60A2* differ significantly. Future efforts will be focused on identifying the impact of these differences on the biological role of As60A2 in the midgut.

Human TGF-β1 limits *Plasmodium* development in the midgut epithelium (Figure 13; Luckhart *et al*., 2003) through an effect on AsNOS activity (Figure 14). Responsiveness to hTGF-β1 suggests that *A. stephensi* TGF-β/Activin homolog(s) signal endogenously through shared or identical pathway components. Although no prototypical TGF-β homologs are known from invertebrates, an invertebrate activin-like ligand has been described, *D. melanogaster* activin (DmAct; Kutty *et al*., 1998). The *A. gambiae* genome contains one homolog of *D. melanogaster* activin (AgAct) with 48% sequence identity and 54% amino acid identity to the mature domain of DmAct (Table 1). Phylogenetic analysis of AgAct confirmed that it is an activin/TGF-β-like homolog (Figure 17). Based on these observations, I suggest that the *A. stephensi* genome encodes a homolog of AgAct and that, in a manner similar to the response of *A. stephensi* to *P. falciparum* observed with hTGF-β1, this Activin homolog regulates *Plasmodium* development. Future efforts will focus on testing this hypothesis in a model of *A. stephensi* infection with *P. falciparum*.

The biological need for multiple TGF-β/Activin-like receptors (Punt, Sax, Babo) in insects may be explained by the recognition that Myoglianin and Mav are more closely related to TGF-β *sensu stricto* than to other ligands in this large superfamily (Figure 17A; Herpin *et al*., 2004). On the other hand, Punt, for example, can bind both Activin (Letsou *et al*., 1995) and Dpp (Simin *et al*., 1998) and the newly described *D. melanogaster* co-

activator TGIF physically interacts with both dSmad2 and Mad (Hyman *et al*., 2003). These observations suggest that, in contrast to the case in mammals, the cellular machinery of *A. gambiae* and *A. stephensi*, like that of *D. melanogaster*, permits a flexible signaling cross-talk between and among Dpp/BMP and TGF-β/Activin ligands and receptors which may or may not mirror phylogenetic relationships.

Extensive crosstalk is also well documented among *D. melanogaster* and mammalian TGF-βs and related signaling proteins. For example, human BMP4 can substitute for *D. melanogaster* Dpp during development of transgenic flies (Padgett *et al*., 1993), and *D. melanogaster* Dpp and Gbb-60A can induce bone formation in mammalian cells (Sampath *et al*., 1993). Further, *D. melanogaster* Dad and dSmad2 can mediate BMP-4 and TGF-β1 signaling in mammalian cells, respectively, (Tsuneizumi *et al*., 1997; Brummel *et al*., 1999), while human Smad4 and the Co-Smad Medea appear to be fully interchangeable in cells from both hosts (Wisotzkey *et al*., 1998; Hudson *et al*., 1998). These facts suggest that, as has been observed for *D. melanogaster*, TGF-β ligands and signaling pathways in *A. gambiae* and *A. stephensi* are functionally conserved with those of mammals on a much larger scale than is currently appreciated.

What is the significance of this functional conservation? At the interface between human blood and midgut cells of *A. stephensi* during feeding, I have determined that crosstalk by hTGF-β1 appears to impact a well-defined response to *P. falciparum* development (Figures 15 and 16; Luckhart *et al*., 2003). This observation suggests that, like in mammals, TGF-β plays a major role as a mediator of the immune response (Omer *et al*., 2000), which may be conserved among *Anopheles* TGF-β ligands. Future efforts will focus on defining the functions of the TGF-β ligands identified here, specifically in

the context of malaria infection. However, TGF-β1 regulates a wide range of biological activities including cell growth and death, cell differentiation, cell morphology, and extracellular matrix synthesis which, in turn, influences the development and physiology of nearly every major tissue and organ system in mammals (Massague *et al*., 2000). Given this panoply of effects, I suggest that hTGF-β1, through crosstalk with a variety of endogenous, conserved mosquito TGF-β signaling pathways, may alter multiple aspects of physiology and the response to parasite development in *Anopheles* spp., providing a new approach to identifying novel effectors that limit parasite development. For example, the effects of hTGF-β1 on DNA synthesis and morphology of *A. stephensi* cells (Luckhart *et al*., 2003) parallel the effects of TGF-β1 on some human cell types (Agocha *et al*., 1997; Evans *et al*., 2003). Given that growth factor-induced changes in cell morphology are required for the early development of *Plasmodium* within mammalian hepatocytes (Carrolo *et al*., 2003) and that parasite invasion of the mosquito midgut epithelium produces profound changes in the actin cytoskeleton (Han *et al*., 2000), I suggest that mammalian TGF-β1 and perhaps endogenous mosquito TGF-βs also play a role in signaling cell shape changes associated with parasite infection in the mosquito. Validation of these and other hypotheses will reveal for the first time that conserved mammalian and insect TGF-β ligands can regulate analogous physiological processes under biologically relevant, naturally occurring conditions and may yield novel targets for the development of transgenically modified mosquitoes that are refractory to malaria parasite development.

```
Ag60A     RNKRSQPARKRKSSKTD-HR-----HPF--QYHPTYDQHKSCRIQQLYVSFKDLQWHEWI
As60A     RNKRSQPSRKRKPGKTE-HR-----HPF--QYHQPYDQHKSCRIQQLYVSFKDLQWHEWI
Ag60A2    RSKRS--VRRKAGTGTG-KRSDRVRNPFLERFGGGGERHKSCQIQTLYVSFRDLNWQDWI
As60A2    RSKRS--ARRGNKGGTGGKRSERVRNPFLERFGAASERQKSCQIQTLYVSFRDLNWQDWI

Ag60A     IAPEGYGAYYCSGECNFPLNAHMNATNHAIVQTLVHLNHPTKVPKPCCAPTKLIPISVLY
As60A     IAPEGYGAYYCSGECNFPLNAHMNATNHAIVQTLVHLNHPTKVPKPCCAPTKLIPISVLY
Ag60A2    IAPDGFGAFFCFGECNFPLNTHMNATNHALIQTLVHLMHPTRVPKPCCAPTKLNPISVLY
As60A2    IAPDGFGAFFCFGECNFPLNSHMNATSHALIQTLVHLMHPTRVPKPCCAPTKLNPISVLY

Ag60A     HIDESNVNLKKYKNMVVKSCGCH
As60A     HIDEANVNLKKYKNMVVKSCGCH
Ag60A2    HIDDANINLKKYKNMVVKSCGCL
As60A2    HIDDANINLKKYKNMVVKSCGCL
```

Figure 16. Alignment of the C-terminal mature domains of predicted *A. gambiae* 60A (Ag60A), *A. stephensi* 60A (As60A), As60A2, and Ag60A2. Conserved residues are highlighted in bold text.

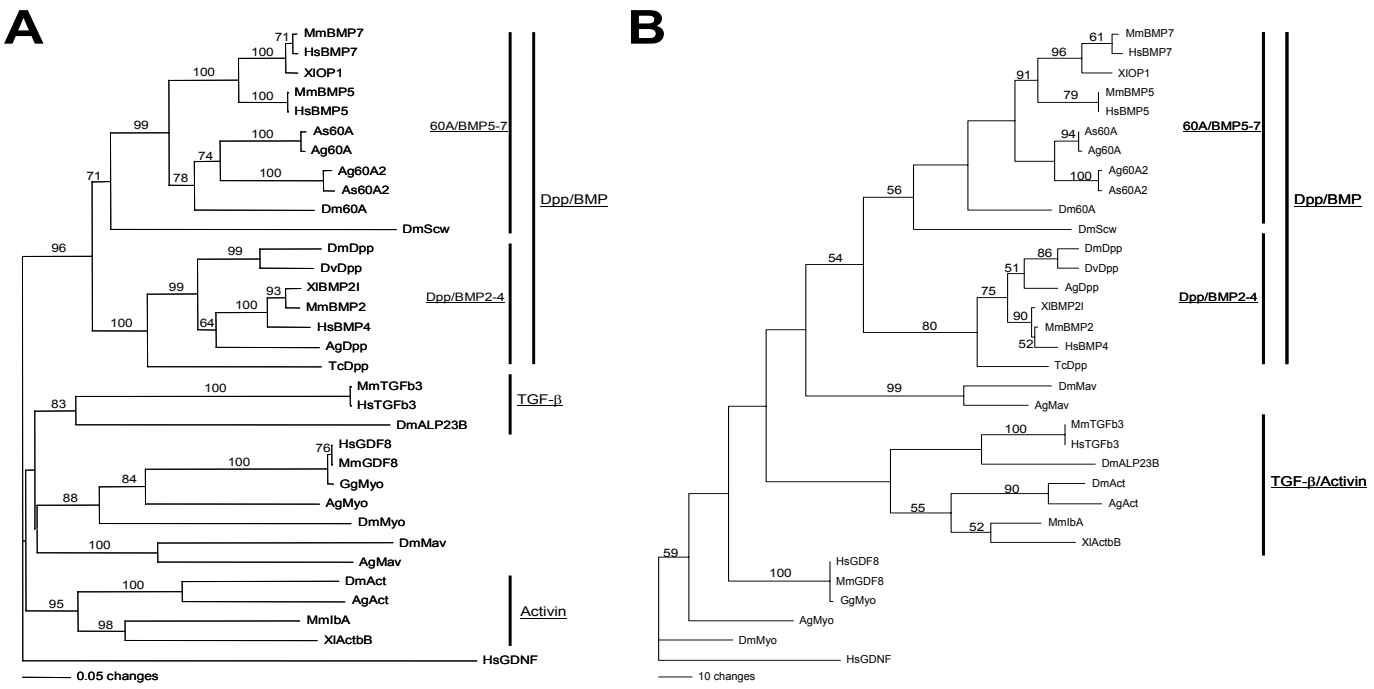

Figure 17. Phylogeny of predicted TGF-β ligands. Predicted amino acid sequences of *A. gambiae* and *A. stephensi* ligands, from the first of seven conserved cysteines to the C-terminus (108 characters), were aligned with corresponding regions from 24 ligand sequences from a variety of species using CLUSTAL X, version 1.83, and default parameters (Thompson *et al*. 1997). All characters were of equal weight and unordered. Trees were constructed using PAUP*4.0b10 (Swofford 2001) with human Glial cell line-derived neurotrophic factor (HsGDNF) as an outgroup. Bootstrap values from 1000 replications are shown; only groupings that scored higher than 50% are marked. Vertical lines to the right of each tree demarcate members of the TGF-β subfamilies (Dpp/BMP and TGF-β/Activin) and subgroups of the Dpp/BMP subfamily (60A/BMP5-7 and Dpp/BMP2-4). **A**, Distance tree generated by neighbor joining. **B**, Parsimony tree generated by heuristic search. The heuristic search was conducted using the tree bisection-reconnection branch-swapping algorithm.

As60A

```
301 gttcgaggagcagcaggagatcgagaatgaaattttgaacctgctcgggcttccgggacc 360
55    F  E  E  Q  Q  E  I  E  N  E  I  L  N  L  L  G  L  P  G  P 74
361 acggccggccgtgcgtcatctacattcctccgtcggGTGAGTAGTGTGCGTGTGTGTGTG 420
75    R  P  A  V  R  H  L  H  S  S  V  G                       86
421 TGTGCAGAGAGACCCATACGCACCCCAGGAAGACCAGGATGTCTCTGGGGAAGAAATCGC 480
481 CCATACAACATTAAACCCAATCTGGCGTTATTCTTTTTCCCCCTATGTGTGTGTGTGTGT 540
541 GTATGTGTGATAATATCTAGcaaatctgcgcctcaatttttgctcaatgtctacgatcaa 600
87                        K  S  A  P  Q  F  L  L  N  V  Y  D  Q  99
601 ctgcagcaggaggagaacgagtccggtggtggggcacggatacgaaaggttcgtagtaca 659
100  L  Q  Q  E  E  N  E  S  G  G  G  A  R  I  R  K  V  R  S  T 120
```

Ag60A

```
181 atcgagaacgaaatattgaacctgctcggacttccggggccgcggccggccgtccgccat 240
61   I  E  N  E  I  L  N  L  L  G  L  P  G  P  R  P  A  V  R  H  80
241 ctacattcctcggtcggGTAGGTGTACTGGCCGCGCGTTTGGTTGCATCGGTGCAAACGC 300
81   L  H  S  S  V  G                                          86
301 TCCTGTGCGACTGTCTCACTCTCTCTCTCTCTGTCTCTCCGTGCCGTTCTGTGCTGATAT 360
361 GTTCGGTTTTTGTCTTCCTCCAGcaaatctgcgcctcaatttctgctcaatgtctacgat 420
87                           K  S  A  P  Q  F  L  L  N  V  Y  D  98
421 CAACTGCAGCAGGAGGAGACCGACGCGCCAGCCGGGGCTGGCCGGATACGAAAGGTTCGT 480
99   Q  L  Q  Q  E  E  T  D  A  P  A  G  A  G  R  I  R  K  V  R 118
```

Ag60A2

```
361 ctcgaactgctgggtcttcccgatcgtcccaacaagcaacatgtgcatccttccttgagG 420
121  L  E  L  L  G  L  P  D  R  P  N  K  Q  H  V  H  P  S  L  R 140
421 TAGAGCGCGGATACTCCTTACAACCTAAATGTGATATTTAAAAATGGAATGGAAATTAAT 480
481 AGCAATTTTTATTGTATTTCTTCTTTCCTCACAGaaaatcagcaccccagttcctgctca 540
141                                  K  S  A  P  Q  F  L  L   148
541 acatctaccacaagtttacggaggaaatgaacggtggccggcggcggaaacggtgcgcg  600
149 N  I  Y  H  K  F  T  E  E  M  N  G  G  R  R  R  K  R  Y  A  168
```

Figure 18. Conserved positions of intron 1 in *Ag60A2*, *As60A*, and *Ag60A*. Underlined, bold letters represent splice acceptor and donor sites.

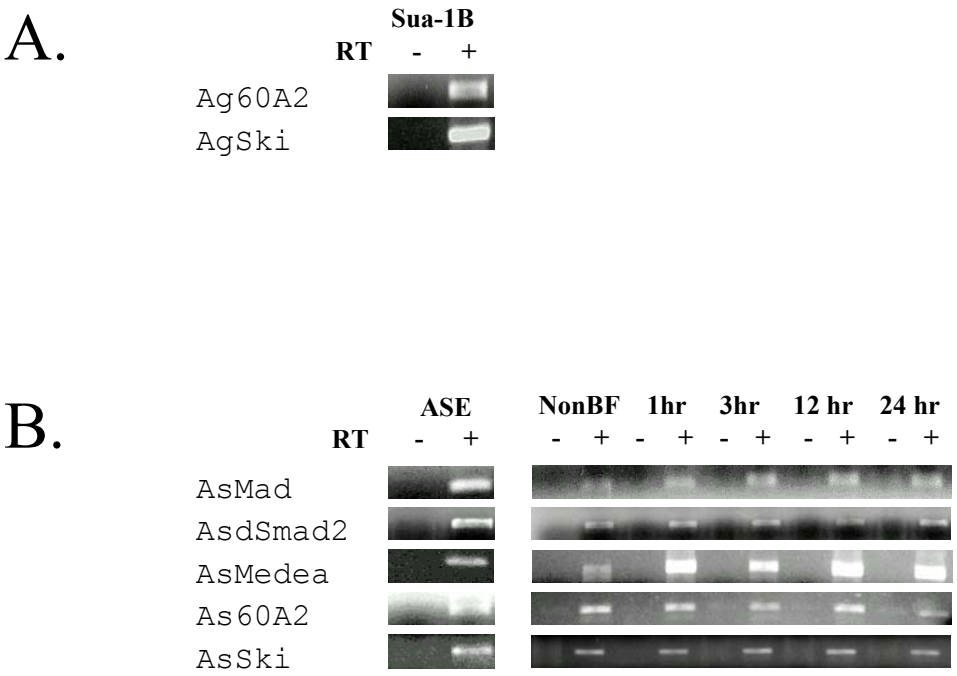

Figure 19. RT-PCR detection of transcripts from *A. gambiae* and *A. stephensi.* **A,** Expression of *Ag60A2* and *AgSki* in *A. gambiae* Sua-1B cells. **B,** Expression of *AsMad*, *AsdSmad2*, *AsMedea*, *As60A2*, and *AsSki* in *A. stephensi* ASE cells and in the *A. stephensi* midgut epithelium in the absence of a bloodmeal (NonBF) and at 1, 3, 12, and 24hr after a bloodmeal. RT-PCR conditions and primers can be found in Materials and Methods. Lack of amplification from genomic DNA was confirmed with control reactions lacking reverse transcriptase (RT-).

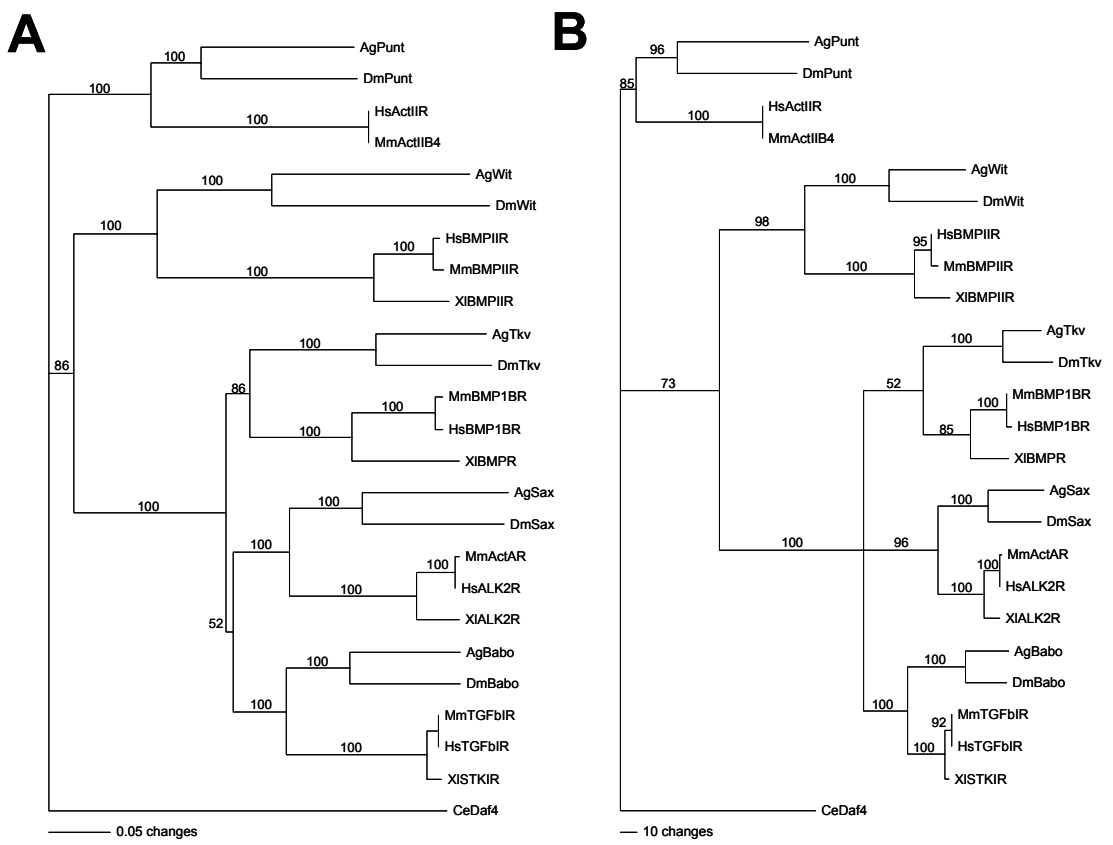

Figure 20. Phylogeny of predicted TGF-β receptors. Predicted amino acid sequences for the putative *A. gambiae* TGF-β receptors, from the first conserved glycine to the terminal conserved arginine residue of the serine/threonine kinase domain (305 characters), were aligned with corresponding regions from 19 receptor sequences from a variety of species using CLUSTAL X, version 1.83, and default parameters (Thompson *et al*. 1997). All characters were of equal weight and unordered. Trees were constructed using PAUP*4.0b10 (Swofford 2001) with *C. elegans* Daf4 as the outgroup. Bootstrap values from 1000 replications are shown; only groupings that scored higher than 50% are marked. **A,** Distance tree generated by neighbor joining. **B,** Parsimony tree generated by heuristic search. The heuristic search was conducted using the tree bisection-reconnection branch-swapping algorithm.

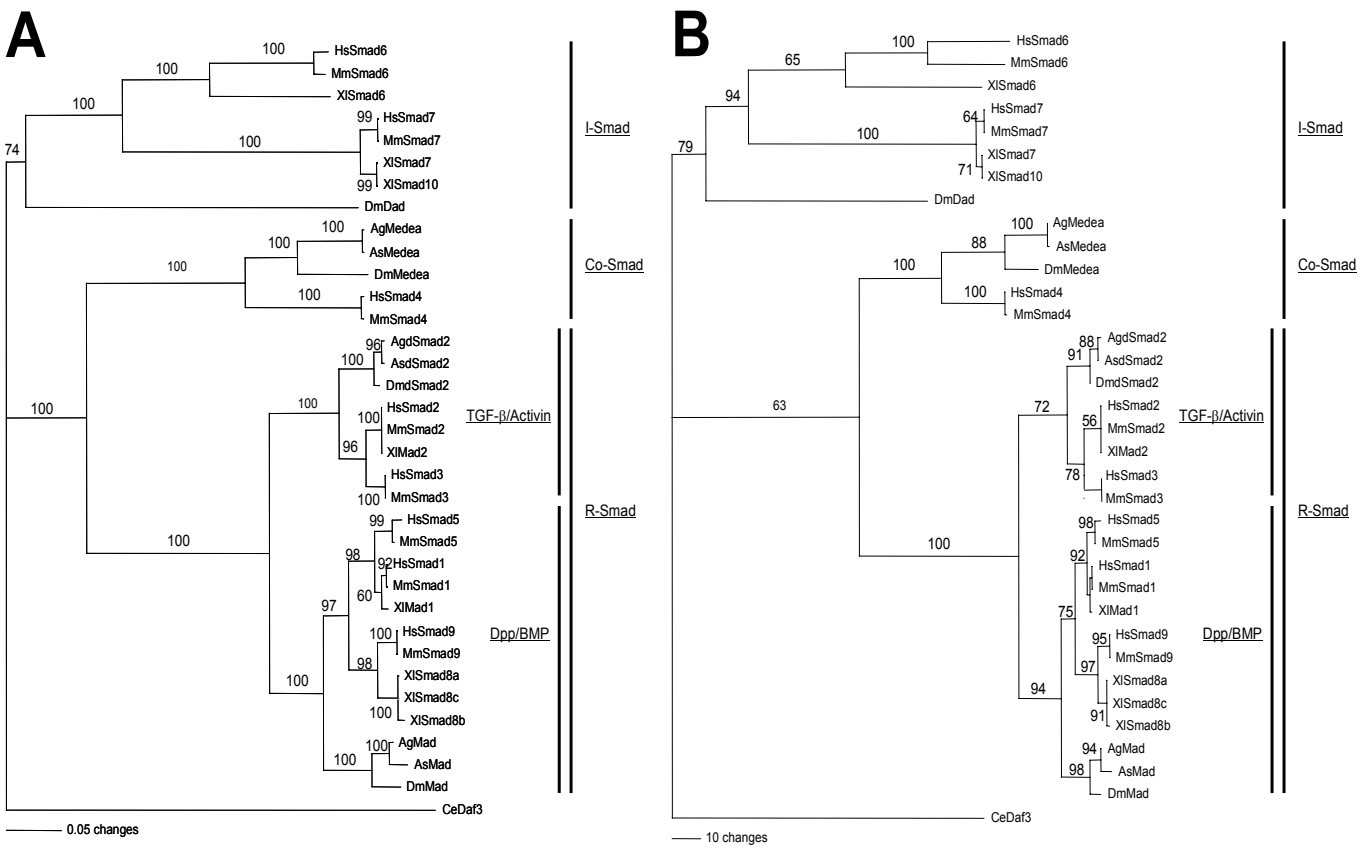

Figure 21. Phylogeny of predicted Smads. Predicted amino acid sequences for the putative *A. gambiae* and *A. stephensi* Smads, from the first conserved tryptophan of the MH2 domain to the C-terminus of the MH2 domain (249 characters), were aligned with corresponding regions from 28 Smad sequences from a variety of species using CLUSTAL X, version 1.83, and default parameters (Thompson *et al*. 1997). All characters were of equal weight and unordered. Trees were constructed using PAUP*4.0b10 (Swofford 2001) with *C. elegans* Daf3 as the outgroup. Bootstrap values from 1000 replications are shown; only groupings that scored higher than 50% are marked. **A,** Distance tree generated by neighbor joining. **B,** Parsimony tree generated by heuristic search. The heuristic search was conducted using the tree bisection-reconnection branch-swapping algorithm.

Table 1. Comparison of predicted TGF-β ligands encoded by the *A. gambiae* and *D. melanogaster* genomes.

Ligand	Structure[a]	Length	Chromosome[b] (Homology[c])	Ligand	Structure[a]	Length[d]	Chromosome[b]
A. gambiae Activin	TBD	100 aa	X; 9.5 x 10^6 (50%, 58%)	*D. melanogaster* Activin	Pro-prot TBD	373 aa 118 aa	4; 8.7 x 10^5
A. gambiae Decapentaplegic	TBD	111 aa	3; 3.8 x 10^6 (75%, 86%)	*D. melanogaster* Decapentaplegic	Pro-prot TBD	588 aa 120 aa	2L; 2.4 x 10^6
A. gambiae Glass Bottom Boat (Gbb)-60A	TBD	135 aa	X; 16.2 x 10^6 (78%, 89%)	*D. melanogaster* Gbb-60A	Pro-prot TBD	455 aa 134 aa	2R; 18.8 x 10^6
A. gambiae Gbb-60A2	TBD	140 aa	3; 6.7 x 10^6 (72%, 88%)				
A. gambiae Maverick	TBD	133 aa	3; 4.5 x 10^6 (55%, 75%)	*D. melanogaster* Maverick	Pro-prot TBD	701 aa 116 aa	4; 5.9 x 10^5
A. gambiae Myoglianin	TBD	110 aa	Unmapped (55%, 77%)	*D. melanogaster* Myoglianin	Pro-prot TBD	598 aa 110 aa	4; 6.5 x 10^5

[a]TBD, TGF-β-like domain; Pro-prot, pro-protein.

[b]Chromosome and approximate location as per *A. gambiae* Genome View (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search?chr=agambiae.inf) and Flybase (http://www.flybase.org).

[c]Percent identity and similarity of *A. gambiae* predicted amino acid sequence to corresponding region of *D. melanogaster* ortholog.

[d]Length (amino acids, aa) of total predicted protein (including Pro-prot and TBD) or of predicted TBD only.

Table 2. Comparison of predicted TGF-β receptors encoded by the *A. gambiae* and *D. melanogaster* genomes.

Receptor	Structure[a]	Length	Chromosome[b] (Homology[c])	Receptor	Structure[a]	Length[d]	Chromosome[a]
A. gambiae Baboon	STK	291 aa	3; 7.8 x 10^6 3; 13.8 x 10^6 (79%, 88%)	*D. melanogaster* Baboon	I,II G STK	601 aa 290 aa	2R; 3.7 x 10^6
A. gambiae Saxophone	I,II G STK	496 aa 288 aa	2; 135.4 x 10^6 (76%, 89%)	*D. melanogaster* Saxophone	I,II G STK	570 aa 290 aa	2R; 2.8 x 10^6
A. gambiae Thickveins	I,II G STK	565 aa 288 aa	3; 38.9 x 10^6 (81%, 87%)	*D. melanogaster* Thickveins	I,II G STK	563 aa 296 aa	2L; 5.1 x 10^6
A. gambiae Punt	STK	287 aa	X; 2.5 x 10^6 (74%, 85%)	*D. melanogaster* Punt	I,II STK	516 aa 288 aa	3R; 10.4 x 10^6
A. gambiae Wishful thinking	I,II STK	933 aa 288 aa	2; 103 x 10^6 (65%, 82%)	*D. melanogaster* Wishful thinking	I,II STK	903 aa 304 aa	3L; 4.0 x 10^6

[a]STK, Serine-threonine kinase domain; I, II, Type I, Type II receptor domain; G, Glycine-serine-rich domain.

[b]Chromosome and approximate location as per *A. gambiae* Genome View (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search?chr=agambiae.inf) and Flybase (http://www.flybase.org).

[c]Percent identity and similarity of *A. gambiae* predicted amino acid sequence to corresponding region of *D. melanogaster* ortholog.

[d]Length (amino acids, aa) of total predicted protein (including I, II; G; STK) or of predicted STK only.

Chapter 5. Summary

One novel approach to better control malaria is the development of a transgenic mosquito whose enhanced immune response would prevent parasite transmission. This strategy is based on the identification of effector molecules capable of killing the parasite. One such effector molecule is nitric oxide (NO), whose inducible synthesis is catalyzed by *Anopheles stephensi* NO synthase (AsNOS; Luckhart *et al*., 1998). However, the transcriptional complexity of the *AsNOS* gene suggests that direct manipulation of this gene may be difficult (Luckhart and Li, 2001). As such we proposed to elucidate the regulation of AsNOS as a means to identify novel ways to manipulate expression. In mammals, transforming growth factor-β1 (TGF-β1) is one of the most potent physiological regulators of NOS expression and activity (Vodovotz, 1997). Further, TGF-β1 is known to be functionally conserved across a wide variety of species (Padgett *et al*., 1993; Sampath *et al*., 1993; Jang *et al*., 1994; Newfeld *et al*., 1997; Secombes, 1998; Brummel *et al*., 1999; Beall *et al*., 2000). Finally, TGF-β1 appears to maintain an immunological balance between parasite killing and limiting host pathology during malaria infection (Omer *et al*. 2000). Based on these observations, I proposed two questions: (1) Does human TGF-β1 (hTGF-β1) regulate *Plasmodium* development via an effect on AsNOS expression and activity? (2) Does the *A. gambiae* genome encode a TGF-β signaling pathway and are these genes expressed in *A. stephensi* cells?

To address the first question, I examined the response of *A. stephensi* cells in culture and in the midgut epithelium to hTGF-β1. My data demonstrate that *A. stephensi* cells recognize hTGF-β1 as an immunomodulatory cytokine and limit *P. falciparum* development. Specifically, hTGF-β1 induces *AsNOS* expression in cultured cells and the

mosquito midgut epithelium. Further, hTGF-β1 limits *P. falciparum* development, but only at low concentrations (2-200 pg/ml hTGF-β1). These results suggest that, in a manner akin to mammals (Omer *et al*., 2000), hTGF-β1 is pro- and anti-inflammatory in the mosquito. In other experiments, dietary provision of the AsNOS catalytic inhibitor L-NAME partially reverses the effect of hTGF-β1 on *P. falciparum* development, suggesting that AsNOS is a target of hTGF-β1 signaling and that hTGF-β1 may regulate additional effector genes in *A. stephensi*. Future experiments will focus on the identification of these additional effectors. Taken together, these results predict the following situation: Ingested hTGF-β1 binds to target receptors on midgut epithelial cells, where *AsNOS* expression is induced. Induction of *AsNOS* leads to higher AsNOS catalytic activity, which limits *P. falciparum* development within the midgut. Human TGF-β1 also induces other effector genes, which limit *P. falciparum* development (Figure 22).

To address the second question, I queried the *A. gambiae* genome database with *D. melanogaster* TGF-β ligand, receptor, and Smad sequences. From these analyses, I identified six ligands, five receptors and three Smads in the *A. gambiae* genome and three Smads in the *A. stephensi* genome. Not surprisingly, phylogenetic analysis revealed that the *A. gambiae* ligands (AgAct, Ag60A, AgDpp, AgMav, and AgMyo), receptors (AgWit, AgPunt, AgBabo, AgTkv, and AgSax), and *A. gambiae* and *A. stephensi* Smads (AgMad/AsMad, AgdSmad2/AsdSmad2, and AgMedea/AsMedea) all grouped with their *D. melanogaster* orthologs. I also discovered a second Gbb-60A gene (*Ag60A2*), the first evidence of TGF-β ligand gene duplication outside of chordates. This gene duplication was also found in *A. stephensi* (*As60A2*); *As60A* was described previously in our lab

(Crampton and Luckhart, 2001a; Crampton and Luckhart, 2001b). These results suggest that ligand gene duplication is a conserved feature of the genomes of *Anopheles* spp. To validate that *A. stephensi* Smads are present to transmit TGF-β signals, RT-PCR analyses of ASE cells and the midgut epithelium revealed that *A. stephensi* Smads are expressed in these immune competent cells. These results suggest that the signaling architecture necessary to transduce exogenous and endogenous TGF-β signals are in place.

Ingested hTGF-β1 remains active in the midgut of *A. stephensi* up to 48 hours post-bloodfeeding (Luckhart *et al*., 2003). This suggests that the presence of hTGF-β1 could coincide with the presence of endogenous TGF-βs in the midgut. Because the *A. gambiae* genome encodes only one TGF-β/Activin-like ligand (AgAct), I hypothesize that the *A. stephensi* genome also encodes a single TGF-β/Activin-like ligand. Further, I suggest that this endogenous Activin ligand may be expressed in the midgut when hTGF-β1 is present and that both ligands may activate a dSmad2-mediated signaling pathway (Figure 23). Future efforts will focus on elucidating the potential synergy or interference of an endogenous mosquito Activin on the hTGF-β1-regulated immune response of *Anopheles* to *Plasmodium* spp.

Current strategies to better control malaria include development of transgenic mosquitoes refractory for *Plasmodium* transmission (Phillips, 2001). These strategies are based on the identification and development of exogenous and endogenous effector gene products capable of limiting parasite development. However, without a better understanding of how the mosquito regulates its immune response to *Plasmodium* could impede the effectiveness of this strategy. My research suggests that human immunomodulatory factors, such as TGF-β1, may profoundly impact the response of *A.*

stephensi to *P. falciparum* infection. Future efforts will help elucidate the potential impact of mosquito TGF-βs and the milieu of proteins that circulate in the blood on the immune system of *Anopheles*.

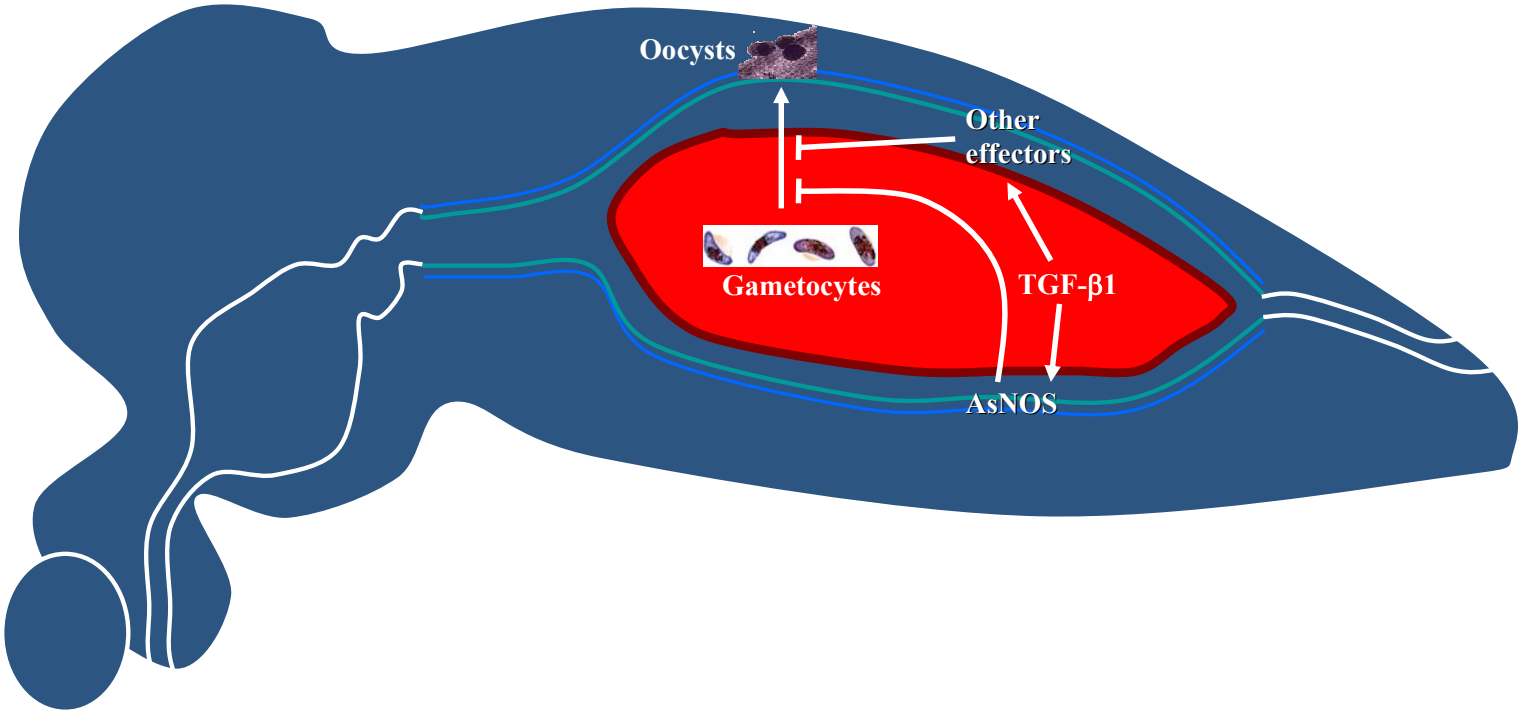

Figure 22. Predicted model for anti-parasitic activity of hTGF-β1 in the midgut of *A. stephensi*. Human TGF-β1, ingested in the bloodmeal, binds to target receptors on the midgut epithelium. These cells respond with induction of *AsNOS* and other effectors gene expression. These gene products can then limit *Plasmodium* development between ingested gametocytes in the midgut and the development of oocysts on the basal lamina.

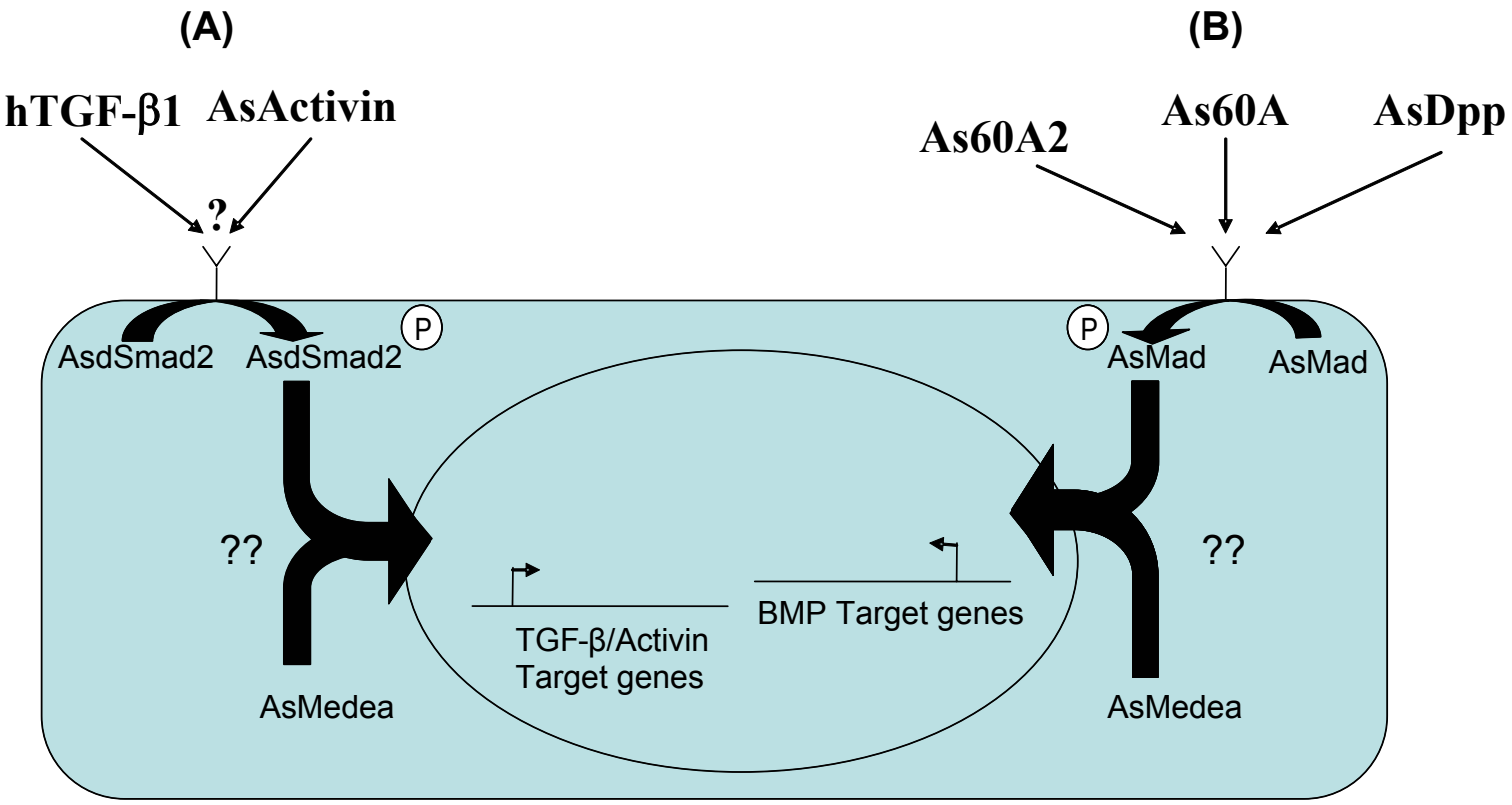

Figure 23. Model for potential exogenous and endogenous TGF-β activity in *A. stephensi* cells. (A) *Anopheles stephensi* TGF-β/Activin-like ligands and hTGF-β1 bind to target receptors, and activate an AsdSmad2-mediated Smad pathway. Activated AsdSmad2 would complex with AsMedea, and translocate to the cell nucleus, where TGF-β/Activin target genes are regulated. (B) *Anopheles stephensi* BMP-like ligands bind to target receptors, and activate an AsMad-mediated Smad pathway. Activated AsMad would complex with AsMedea, and translocate to the cell nucleus, where BMP target genes are regulated.

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F.*, et al.* (2000). The genome sequence of *Drosophila melanogaster*. Science *287*, 2185-2195.

Agocha, A., Lee, H. W., and Eghbali-Webb, M. (1997). Hypoxia regulates basal and induced DNA synthesis and collagen type I production in human cardiac fibroblasts: effects of transforming growth factor-β1, thyroid hormone, angiotensin II and basic fibroblast growth factor. J Mol Cell Cardiol *29*, 2233-2244.

Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K., and Kawabata, M. (1999). c-Ski acts as a transcriptional co-repressor in transforming growth factor-β signaling through interaction with smads. J Biol Chem *274*, 35269-35277.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res *25*, 3389-3402.

Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003). Making sense of latent TGF-β activation. J Cell Sci *116*, 217-224.

Arnell, N. W., Cannell, M. G. R., Hulme, M., Kovats, R. S., Mitchell, J. F. B., Nicholls, R. J., Parry, M. L., Livermore, M. T. J., and White, A. (2002). The Consequences of CO_2 Stabilisation for the Impacts of Climate Change. Climatic Change *53*, 413-446.

Arora, K., and Warrior, R. (2001). A new Smurf in the village. Dev Cell *1*, 441-442.

Ballou, W. R., Arevalo-Herrera, M., Carucci, D., Richie, T. L., Corradin, G., Diggs, C., Druilhe, P., Giersing, B. K., Saul, A., Heppner, D. G.*, et al.* (2004). Update on the

clinical development of candidate malaria vaccines. Am J Trop Med Hyg *71*, 239-247.

Beall, M. J., McGonigle, S., and Pearce, E. J. (2000a). Functional conservation of *Schistosoma mansoni* Smads in TGF-β signaling. Mol Biochem Parasitol *111*, 131-142.

Biedler, J., and Tu, Z. (2003). Non-LTR retrotransposons in the African malaria mosquito, *Anopheles gambiae*: unprecedented diversity and evidence of recent activity. Mol Biol Evol *20*, 1811-1825.

Blanco, F. J., Geng, Y., and Lotz, M. (1995). Differentiation-dependent effects of IL-1 and TGF-β on human articular chondrocyte proliferation are related to inducible nitric oxide synthase expression. J Immunol *154*, 4018-4026.

Boutard, V., Havouis, R., Fouqueray, B., Philippe, C., Moulinoux, J. P., and Baud, L. (1995). Transforming growth factor-β stimulates arginase activity in macrophages: Implications for the regulation of macrophage cytotoxicity. J. Immuno *155*, 2077-2084.

Brummel, T., Abdollah, S., Haerry, T. E., Shimell, M. J., Merriam, J., Raftery, L., Wrana, J. L., and O'Connor, M. B. (1999). The *Drosophila* activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development. Genes Dev *13*, 98-111.

Burt, A. (2003). Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc R Soc Lond B Biol Sci *270*, 921-928.

Burt, D. W., and Law, A. S. (1994). Evolution of the transforming growth factor-β superfamily. Prog Growth Factor Res *5*, 99-118.

Burt, D. W., and Paton, I. R. (1992). Evolutionary origins of the transforming growth factor-β gene family. DNA Cell Biol *11*, 497-510.

Carrolo, M., Giordano, S., Cabrita-Santos, L., Corso, S., Vigario, A. M., Silva, S., Leiriao, P., Carapau, D., Armas-Portela, R., Comoglio, P. M.*, et al.* (2003). Hepatocyte growth factor and its receptor are required for malaria infection. Nat Med *9*, 1363-1369.

Catteruccia, F., Nolan, T., Loukeris, T. G., Blass, C., Savakis, C., Kafatos, F. C., and Crisanti, A. (2000). Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature *405*, 959-962.

CDC (2004). Centers for Disease Control.

Chaiyaroj, S. C., Rutta, A. S., Muenthaisong, K., Watkins, P., Na Ubol, M., and Looareesuwan, S. (2004). Reduced levels of transforming growth factor-β1, interleukin-12 and increased migration inhibitory factor are associated with severe malaria. Acta Trop *89*, 319-327.

Chege, G. M., and Beier, J. C. (1998). Blood acquisition and processing by three *Anopheles* (Diptera: Culicidae) species with different innate susceptibilities to *Plasmodium falciparum*. J Med Entomol *35*, 319-323.

Chen, H., Li, D., Saldeen, T., and Mehta, J. L. (2001). TGF-β1 modulates NOS expression and phosphorylation of Akt/PKB in rat myocytes exposed to hypoxia-reoxygenation. Am J Physiol Heart Circ Physiol *281*, H1035-1039.

Clements, A. N. (1992). The biology of mosquitoes, 1st edn (London ; New York, Chapman & Hall).

Coates, C. J., Jasinskiene, N., Pott, G. B., and James, A. A. (1999). Promoter-directed expression of recombinant fire-fly luciferase in the salivary glands of Hermes-transformed *Aedes aegypti*. Gene *226*, 317-325.

Cramer, P., Varrot, A., Barillas-Mury, C., Kafatos, F. C., and Muller, C. W. (1999). Structure of the specificity domain of the Dorsal homologue Gambif1 bound to DNA. Structure Fold Des *7*, 841-852.

Crampton, A., and Luckhart, S. (2001a). The role of As60A, a TGF-β homolog, in *Anopheles stephensi* innate immunity and defense against *Plasmodium berghei* infection. Infection, Genetics, and Evolution *1*, 131-141.

Crampton, A. L., and Luckhart, S. (2001b). Isolation and characterization of As60A, a transforming growth factor-β gene, from the malaria vector *Anopheles stephensi*. Cytokine *13*, 65-74.

Curtis, C. F., Jana-Kara, B., and Maxwell, C. A. (2003). Insecticide treated nets: impact on vector populations and relevance of initial intensity of transmission and pyrethroid resistance. J Vector Borne Dis *40*, 1-8.

Daopin, S., Piez, K. A., Ogawa, Y., and Davies, D. R. (1992). Crystal structure of transforming growth factor-β2: an unusual fold for the superfamily. Science *257*, 369-373.

De Servi, B., La Porta, C. A., Bontempelli, M., and Comolli, R. (2002). Decrease of TGF-β1 plasma levels and increase of nitric oxide synthase activity in leukocytes as potential biomarkers of Alzheimer's disease. Exp Gerontol *37*, 813-821.

Derr, L. K. (1998). The involvement of cellular recombination and repair genes in RNA-mediated recombination in *Saccharomyces cerevisiae*. Genetics *148*, 937-945.

Derynck, R., and Choy, L. (1998). Transforming Growth Factor-β and its Receptors. In The Cytokine Handbook, A. Thomson, ed. (London, Academic Press).

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem *276*, 12477-12480.

Evans, R. A., Tian, Y. C., Steadman, R., and Phillips, A. O. (2003). TGF-β1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins. Exp Cell Res *282*, 90-100.

Fallon, A.M. and Stollar, V. (1997) The biochemistry and genetics of mosquito cells in culture. *Advances in Cell Culture*, **5**, 97-137.

Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K., and Kato, M. (2001). Axin facilitates Smad3 activation in the transforming growth factor-β signaling pathway. Mol Cell Biol *21*, 5132-5141.

Gilbert, R. S., and Herschman, H. R. (1993). Transforming growth factor-β differentially modulates the inducible nitric oxide synthase gene in distinct cell types. Biochem Biophys Res Commun *195*, 380-384.

Goureau, O., Lepoivre, M., Becquet, F., and Courtois, Y. (1993). Differential regulation of inducible nitric oxide synthase by fibroblast growth factors and transforming growth factor-β in bovine retinal pigmented epithelial cells: inverse correlation with cellular proliferation. Proc Natl Acad Sci U S A *90*, 4276-4280.

Grossman, G. L., Rafferty, C. S., Clayton, J. R., Stevens, T. K., Mukabayire, O., and Benedict, M. Q. (2001). Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. Insect Mol Biol *10*, 597-604.

Gumienny, T. L., and Padgett, R. W. (2002). The other side of TGF-β superfamily signal regulation: thinking outside the cell. Trends Endocrinol Metab *13*, 295-299.

Han, Y. S., Thompson, J., Kafatos, F. C., and Barillas-Mury, C. (2000). Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. Embo J *19*, 6030-6040.

Herpin, A., Lelong, C., and Favrel, P. (2004). Transforming growth factor-β-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. Dev Comp Immunol *28*, 461-485.

Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M. B., Attisano, L., and Wrana, J. L. (1996). MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell *85*, 489-500.

Hudson, J. B., Podos, S. D., Keith, K., Simpson, S. L., and Ferguson, E. L. (1998). The *Drosophila* Medea gene is required downstream of dpp and encodes a functional homolog of human Smad4. Development *125*, 1407-1420.

Hyman, C. A., Bartholin, L., Newfeld, S. J., and Wotton, D. (2003). *Drosophila* TGIF proteins are transcriptional activators. Mol Cell Biol *23*, 9262-9274.

Inoue, H., Imamura, T., Ishidou, Y., Takase, M., Udagawa, Y., Oka, Y., Tsuneizumi, K., Tabata, T., Miyazono, K., and Kawabata, M. (1998). Interplay of signal mediators of decapentaplegic (Dpp): molecular characterization of mothers against dpp, Medea, and daughters against dpp. Mol Biol Cell *9*, 2145-2156.

Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A., and Jacobs-Lorena, M. (2002). Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature *417*, 452-455.

Jang, S. I., Hardie, L. J., and Secombes, C. J. (1994). Effects of transforming growth factor-β1 on rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst activity. Dev Comp Immunol *18*, 315-323.

Kim, J., Johnson, K., Chen, H. J., Carroll, S., and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. Nature *388*, 304-308.

Kirkpatrick, H., Johnson, K., and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. J Biol Chem *276*, 18216-18222.

Kokoza, V., Ahmed, A., Cho, W. L., Jasinskiene, N., James, A. A., and Raikhel, A. (2000). Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci U S A *97*, 9144-9149.

Kutty, G., Kutty, R. K., Samuel, W., Duncan, T., Jaworski, C., and Wiggert, B. (1998). Identification of a new member of transforming growth factor-β superfamily in *Drosophila*: the first invertebrate activin gene. Biochem Biophys Res Commun *246*, 644-649.

Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., Massague, J., and O'Connor, M B. (1995). *Drosophila* Dpp signaling is mediated by the punt gene product: a dual ligand-binding type II receptor of the TGF-β receptor family. Cell *80*, 899-908.

Levasina, E. L., Moita L. F., Blandin, S., Vriend, G., Lagueux, M., and Kafatos, F. C. (2001). Conserved role of a Complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. Cell *104*, 709-718.

Lieber, M. J., and Luckhart, S. (2004). Transforming growth factor-βs and related gene products in mosquito vectors of human malaria parasites: signaling architecture for immunological crosstalk. Mol Immunol *41*, 965-977.

Lim, J., Gowda, D C., Krishnegowda, G., and Luckhart, S. Induction of nitric oxide synthesis in *Anopheles stephensi* by *Plasmodium falciparum*: mechanism of signaling and the role of parasite glycosylphosphatidylinositols. Infect Immun (*In Press*)

Liu, F., Hata, A., Baker, J. C., Doody, J., Carcamo, J., Harland, R. M., and Massague, J. (1996). A human Mad protein acting as a BMP-regulated transcriptional activator. Nature *381*, 620-623.

Liu, X., Sun, Y., Weinberg, R. A., and Lodish, H. F. (2001). Ski/Sno and TGF-β signaling. Cytokine Growth Factor Rev *12*, 1-8.

Luckhart, S., Crampton, A. L., Zamora, R., Lieber, M. J., Dos Santos, P. C., Peterson, T. M., Emmith, N., Lim, J., Wink, D. A., and Vodovotz, Y. (2003). Mammalian transforming growth factor-β1 activated after ingestion by *Anopheles stephensi* modulates mosquito immunity. Infect Immun *71*, 3000-3009.

Luckhart, S., and Li, K. (2001). Transcriptional complexity of the *Anopheles stephensi* nitric oxide synthase gene. Insect Biochem Mol Biol *31*, 249-256.

Luckhart, S., and Rosenberg, R. (1999). Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. Gene *232*, 25-34.

Luckhart, S., Vodovotz, Y., Cui, L., and Rosenberg, R. (1998). The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proc Natl Acad Sci U S A *95*, 5700-5705.

Marquez, R. M., Singer, M. A., Takaesu, N. T., Waldrip, W. R., Kraytsberg, Y., and Newfeld, S. J. (2001). Transgenic analysis of the Smad family of TGF-β signal transducers in *Drosophila melanogaster* suggests new roles and new interactions between family members. Genetics *157*, 1639-1648.

Massague, J. (1990). The transforming growth factor-β family. Annu Rev Cell Biol *6*, 597-641.

Massague, J. (1998). TGF-β signal transduction. Annu Rev Biochem *67*, 753-791.

Massague, J., Blain, S. W., and Lo, R. S. (2000). TGF-β signaling in growth control, cancer, and heritable disorders. Cell *103*, 295-309.

Mehra, A., and Wrana, J. L. (2002). TGF-β and the Smad signal transduction pathway. Biochem Cell Biol *80*, 605-622.

Mire-Sluis, A. R., Page, L. A., Meager, A., Igaki, J., Lee, J., Lyons, S., and Thorpe, R. (1996). An anti-cytokine bioactivity assay for interferons-α, -β and -ω. J Immunol Methods *195*, 55-61.

Mittal, P. K. (2003). Biolarvicides in vector control: challenges and prospects. J Vector Borne Dis *40*, 20-32.

Moreira, L. A., Ito, J., Ghosh, A., Devenport, M., Zieler, H., Abraham, E. G., Crisanti, A., Nolan, T., Catteruccia, F., and Jacobs-Lorena, M. (2002). Bee venom

phospholipase inhibits malaria parasite development in transgenic mosquitoes. J Biol Chem *277*, 40839-40843.

Mori, Y., Chen, S. J., and Varga, J. (2000). Modulation of endogenous Smad expression in normal skin fibroblasts by transforming growth factor-β. Exp Cell Res *258*, 374-383.

Mourier, T., and Jeffares, D. C. (2003). Eukaryotic intron loss. Science *300*, 1393.

Musumeci, M., Malaguarnera, L., Simpore, J., Messina, A., and Musumeci, S. (2003). Modulation of immune response in *Plasmodium falciparum* malaria: role of IL-12, IL-18 and TGF-β. Cytokine *21*, 172-178.

Nakai, S., Mizuno, K., Kaneta, M., and Hirai, Y. (1988). A simple, sensitive bioassay for the detection of interleukin-1 using human melanoma A375 cell line. Biochem Biophys Res Commun *154*, 1189-1196.

Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997). TGF-β receptor-mediated signaling through Smad2, Smad3 and Smad4. Embo J *16*, 5353-5362.

Neul, J. L., and Ferguson, E. L. (1998). Spatially restricted activation of the SAX receptor by SCW modulates DPP/TKV signaling in *Drosophila* dorsal-ventral patterning. Cell *95*, 483-494.

Newfeld, S. J., Mehra, A., Singer, M. A., Wrana, J. L., Attisano, L., and Gelbart, W. M. (1997). Mothers against dpp participates in a DDP/TGF-β responsive serine-threonine kinase signal transduction cascade. Development *124*, 3167-3176.

Newfeld, S. J., Wisotzkey, R. G., and Kumar, S. (1999). Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-β family ligands, receptors and Smad signal transducers. Genetics *152*, 783-795.

Nguyen, M., Parker, L., and Arora, K. (2000). Identification of maverick, a novel member of the TGF-β superfamily in *Drosophila*. Mech Dev *95*, 201-206.

Omer, F. M., de Souza, J. B., Corran, P. H., Sultan, A. A., and Riley, E. M. (2003a). Activation of transforming growth factor-β by malaria parasite-derived metalloproteinases and a thrombospondin-like molecule. J Exp Med *198*, 1817-1827.

Omer, F. M., de Souza, J. B., and Riley, E. M. (2003b). Differential induction of TGF-β regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal *Plasmodium yoelii* infections. J Immunol *171*, 5430-5436.

Omer, F. M., Kurtzhals, J. A., and Riley, E. M. (2000). Maintaining the immunological balance in parasitic infections: a role for TGF-β? Parasitol Today *16*, 18-23.

Omer, F. M., and Riley, E. M. (1998). Transforming growth factor-β production is inversely correlated with severity of murine malaria infection. J Exp Med *188*, 39-48.

Ottaviani, E., Sassi, D., and Kletsas, D. (1997). PDGF- and TGF-β-induced changes in cell shape of invertebrate immunocytes: effect of calcium entry blockers. Eur J Cell Biol *74*, 336-341.

Padgett, R. W., Wozney, J. M., and Gelbart, W. M. (1993). Human BMP sequences can confer normal dorsal-ventral patterning in the *Drosophila* embryo. Proc Natl Acad Sci U S A *90*, 2905-2909.

Pascoa, V., Oliveira, P. L., Dansa-Petretski, M., Silva, J. R., Alvarenga, P. H., Jacobs-Lorena, M., and Lemos, F. J. (2002). *Aedes aegypti* peritrophic matrix and its interaction with heme during blood digestion. Insect Biochem Mol Biol *32*, 517-523.

Perkins, D. J., Weinberg, J. B., and Kremsner, P. G. (2000). Reduced interleukin-12 and transforming growth factor-β1 in severe childhood malaria: relationship of cytokine balance with disease severity. J Infect Dis *182*, 988-992.

Perrella, M. A., Yoshizumi, M., Fen, Z., Tsai, J. C., Hsieh, C. M., Kourembanas, S., and Lee, M. E. (1994). Transforming growth factor-β1, but not dexamethasone, down-regulates nitric oxide synthase mRNA after its induction by interleukin-1β in rat smooth muscle cells. J Biol Chem *269*, 14595-14600.

Petrov, D. A. (2002). DNA loss and evolution of genome size in *Drosophila*. Genetica *115*, 81-91.

Phillips, R. S. (2001). Current status of malaria and potential for control. Clin Microbiol Rev *14*, 208-226.

Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res *23*, 4878-4884.

Raftery, L. A., and Sutherland, D. J. (1999). TGF-β family signal transduction in *Drosophila* development: from Mad to Smads. Dev Biol *210*, 251-268.

Riehle, M. A., Srinivasan, P., Moreira, C. K., and Jacobs-Lorena, M. (2003). Towards genetic manipulation of wild mosquito populations to combat malaria: advances and challenges. J Exp Biol *206*, 3809-3816.

Sampath, T. K., Rashka, K. E., Doctor, J. S., Tucker, R. F., and Hoffmann, F. M. (1993). Drosophila transforming growth factor-β superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci U S A *90*, 6004-6008.

Sanchez-Salazar, J., Pletcher, M. T., Bennett, R. L., Brown, S. J., Dandamudi, T. J., Denell, R. E., and Doctor, J. S. (1996). The *Tribolium* decapentaplegic gene is similar in sequence, structure, and expression to the *Drosophila* dpp gene. Development, Genes, and Evolution *206*, 237-246.

Schneiderbauer, M. M., Dutton, C. M., and Scully, S. P. (2004). Signaling "cross-talk" between TGF-β1 and ECM signals in chondrocytic cells. Cell Signal *16*, 1133-1140.

Secombes, C. (1998). The Phylogeny of Cytokines. In The Cytokine Handbook, A. Thomson, ed. (London, Academic Press).

Shahabuddin, M., Fields, I., Bulet, P., Hoffmann, J. A., and Miller, L. H. (1998). *Plasmodium gallinaceum*: differential killing of some mosquito stages of the parasite by insect defensin. Exp Parasitol *89*, 103-112.

Sherman, I. W., ed. (1998). Malaria: parasite biology, pathogenesis, and protection (Washington, DC, ASM Press).

Shibuya, H., Iwata, H., Masuyama, N., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E., and Ueno, N. (1998). Role of TAK1 and TAB1 in BMP signaling in early *Xenopus* development. Embo J *17*, 1019-1028.

Simin, K., Bates, E. A., Horner, M. A., and Letsou, A. (1998). Genetic analysis of punt, a type II Dpp receptor that functions throughout the *Drosophila melanogaster* life cycle. Genetics *148*, 801-813.

Singh, S., and Evans, T. W. (1997). Nitric oxide, the biological mediator of the decade: fact or fiction? Eur Respir J *10*, 699-707.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., and Hay, S. I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature *434*, 214-217

Stein, G., Stein, J., Lian, J., Last, T., Owen, T., and McCabe, L. (1994). Synchronization of Normal Diploid and Transformed Mammalian Cells. In Cell Biology: A Laboratory Handbook, J. Celis, ed. (London, Academic Press).

Stroschein, S. L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999). Negative feedback regulation of TGF-β signaling by the SnoN oncoprotein. Science *286*, 771-774.

Swofford, D. L. (2001). PAUP* Version 4.0b10 ((A commercial test version; completed version 4.0 to be distributed by Sinauer, Sunderland, Mass.)).

Takase, M., Imamura, T., Sampath, T. K., Takeda, K., Ichijo, H., Miyazono, K., and Kawabata, M. (1998). Induction of Smad6 mRNA by bone morphogenetic proteins. Biochem Biophys Res Commun *244*, 26-29.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res *25*, 4876-4882.

Tsang, M. L., Zhou, L., Zheng, B. L., Wenker, J., Fransen, G., Humphrey, J., Smith, J. M., O'Connor-McCourt, M., Lucas, R., and Weatherbee, J. A. (1995). Characterization of recombinant soluble human transforming growth factor-β receptor type II (rhTGF-βsRII). Cytokine *7*, 389-397.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T. B., Christian, J. L., and Tabata, T. (1997). Daughters against dpp modulates dpp organizing activity in *Drosophila* wing development. Nature *389*, 627-631.

Tsutsui, N., and Kamiyama, T. (1999). Transforming growth factor β-induced failure of resistance to infection with blood-stage *Plasmodium chabaudi* in mice. Infect Immun *67*, 2306-2311.

Tu, Z., and Coates, C. (2004). Mosquito transposable elements. Insect Biochem Mol Biol *34*, 631-644.

Vizioli, J., Bulet, P., Hoffmann, J. A., Kafatos, F. C., Muller, H. M., and Dimopoulos, G. (2001). Gambicin: A novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci U S A *16*, 16.

Vodovotz, Y. (1997). Control of nitric oxide production by transforming growth factor-β1: mechanistic insights and potential relevance to human disease. Nitric Oxide *1*, 3-17.

Vodovotz, Y., Letterio, J. J., Geiser, A. G., Chesler, L., Roberts, A. B., and Sparrow, J. (1996). Control of nitric oxide production by endogenous TGF-β1 and systemic nitric oxide in retinal pigment epithelial cells and peritoneal macrophages. J Leukoc Biol *60*, 261-270.

Wang, T., Li, B. Y., Danielson, P. D., Shah, P. C., Rockwell, S., Lechleider, R. J., Martin, J., Manganaro, T., and Donahoe, P. K. (1996). The immunophilin FKBP12 functions as a common inhibitor of the TGF-β family type I receptors. Cell *86*, 435-444.

WHO (2004). World Health Organization.

Wisotzkey, R. G., Mehra, A., Sutherland, D. J., Dobens, L. L., Liu, X., Dohrmann, C., Attisano, L., and Raftery, L. A. (1998). Medea is a *Drosophila* Smad4 homolog that is differentially required to potentiate DPP responses. Development *125*, 1433-1445.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y. C., Massimi, A., Kneitz, S., Piek, E., and Bottinger, E. P. (2001). Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. Proc Natl Acad Sci U S A *98*, 6686-6691.

Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell *1*, 611-617.

Zieler, H., Keister, D. B., Dvorak, J. A., and Ribeiro, J. M. (2001). A snake venom phospholipase A2 blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. J Exp Biol *204*, 4157-4167.

Zwijsen, A., Verschueren, K., and Huylebroeck, D. (2003). New intracellular components of bone morphogenetic protein/Smad signaling cascades. FEBS Lett *546*, 133-139.

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EDUCATION

2000-2005 M.S., Biochemistry, Virginia Polytechnic Institute and State University Blacksburg, VA
GPA: 3.575, Expected Graduation: May 2005
Advisor: Dr. Shirley Luckhart.
Thesis title: "Human Transforming Growth Factor-β signaling cross-talk in the malaria vector *Anopheles stephensi*"

1996-2000 B.S., Environmental and Forest Biology, State University of New York - College of Environmental Science and Forestry, Syracuse, NY GPA: 3.2

WORK EXPERIENCE

1. Laboratory Assistant I
   Department of Medical Microbiology and Immunology
   University of California at Davis, Davis, CA
   Sept 2004 – June 2005
   - Analyzed immune responses of *Anopheles* mosquitoes to malaria parasites
   - Ordered lab supplies and reagents
   - Assisted in the establishment and maintenance of mosquito colonies
   - Organized the lab and trained new personnel after moving from Virginia Tech to UC Davis

2. Graduate Research Assistant
   Department of Biochemistry
   Virginia Polytechnic Institute and State University, Blacksburg, VA
   Aug 2000 – Aug 2004
   Project: "Crosstalk among mosquito and mammalian immune factors"
   - Investigated the molecular response of *Anopheles* cells in culture and *in vivo* to human TGF-β1 and *Plasmodium* infection
   - Discovered genes encoding members of the TGF-β superfamily in the *Anopheles gambiae* genomic database

   a) Graduate Teaching Assistant "BCHM 2024: Concepts in Biochemistry"
      Department of Biochemistry
      Virginia Polytechnic Institute and State University, Blacksburg VA
      Jan 2000 - May 2000
      - Organized handouts and graded test material
      - Held review sessions and office hours

3. Undergraduate Research Assistant
   Department of Environmental and Forest Biology
   State University of New York – College of Environmental Science and Forest Biology, Syracuse, NY
   Sept 1998 – May 2000
   - Determined the mutagenic properties of natural pepper extracts in mice
   - Performed lab maintenance duties

OTHER EXPERIENCE

1. Graduate Student Representative
   Graduate Student Assembly
   Virginia Polytechnic Institute and State University, Blacksburg, VA
   Aug 2002 – Aug 2004
   - Selected to represent the Biochemistry department as a delegate to the Graduate Student Assembly
   - Served as the graduate student representative to the campus-wide Commission on Student Affairs, which consisted of faculty, staff, graduate and undergraduate students
2. Peer reviewer
   Graduate Student Assembly Travel Fund Program Selection Committee
   Virginia Polytechnic Institute and State University, Blacksburg, VA
   Nov 2002 – April 2003
   - Reviewed science graduate students' applications for funding
3. Database manager
   Department of Development and Outreach
   Human Rights Watch, New York, NY
   Summer 1999/2000, Winter 1999
   - Managed donor database
   - Transferred donor database onto new platform
   - Entered direct mail donations into database
   - Analyzed budget reports for Director of Development
   - Informed consumers about donation process

PUBLICATIONS

1. Vodovotz Y, Zamora R, **Lieber MJ**, Luckhart S. 2004. Cross-talk between nitric oxide and transforming growth factor-β1 in malaria. Curr Molec. Med. 4(7):787-97.
2. **Lieber MJ**, Luckhart S. 2004. Transforming growth factor-βs and related gene products in mosquito vectors of human malaria parasites: signaling architecture for immunological crosstalk. Molec. Immunol. 41(10):965-77.
3. Luckhart S, Crampton AL, Zamora R, **Lieber MJ**, Dos Santos PC, Peterson TM, Emmith N, Lim J, Wink DA, Vodovotz Y. 2003. Mammalian transforming growth factor beta1 activated after ingestion by *Anopheles stephensi* modulates mosquito immunity. Infect. Immun. 71(6):3000-9.

SELECTED CONFERENCE ABSTRACTS (out of 10)

1. **Lieber MJ**, Dos Santos P, Emmith N, Luckhart S. "Human transforming growth factor-β signaling cross-talk in the malaria vector *Anopheles stephensi*" Experimental Biology 2004 (2004) (Invited Oral Presentation)
2. Luckhart S, Vodovotz Y, Crampton AL, Gowda DC, **Lieber MJ**, Lim J, Peterson TML, Zamora R, Gowdahalli K. "Immunological cross-talk and conservation of anti-parasite resistance between *Anopheles* and mammals" Entomological Society of America Annual Meeting (2004)
3. **Lieber MJ** and Luckhart S. "An analysis of transforming growth factor-βs and related gene products in *Anopheles* spp." 52nd Annual Meeting of the American Society of Tropical Medicine and Hygiene (2003)

4. Luckhart S, Crampton AL, Zamora R, **Lieber MJ**, Dos Santos P, Peterson TML, Emmith N, Lim, J, Wink D, Vodovotz Y. "Mammalian transforming growth factor-β1, activated after ingestion by *Anopheles*, modulates mosquito immunity" Gordon Research Conference on Nitric Oxide (2003)
5. **Lieber MJ**, Dos Santos P, Emmith N, and Luckhart S. "Human transforming growth factor-β signaling cross-talk in the malaria vector *Anopheles stephensi*" 51st Annual Meeting of the American Society of Tropical Medicine and Hygiene (2002)

HONORS

1. Graduate Student Assembly Community Service Award (2004)
2. Graduate Student Assembly Travel Fund Award (2003)
3. Graduate Student Assembly Travel Fund Award (2002)
4. James F. Eheart Travel Scholarship (2002)
5. President's Scholarship (1996-1997)
6. President's Honor List (1996-1997)

SKILLS

Biochemical

SDS-PAGE, recombinant protein purification (purification of 6x His-tagged proteins expressed in insect cells)

Molecular Biology

DNA/RNA extraction, PCR (RT-PCR, 5'-RACE, 3'-RACE), plasmid cloning, library screening of *A. stephensi* phage library, western blot, NOSdetect assay™ (Stratagene, La Jolla, CA; measures catalytic activity of nitric oxide synthase), fluorescence microscopy, light microscopy, use of radioisotopes ([^{32}P]-dATP, [^{3}H]-thymidine, [^{3}H]-arginine), real-time quantitative RT-PCR (using Taqman™ technology and SYBR-Green), phylogenetic analysis (using the software programs PAUP and Clustal X), automated sample preparation for microarray analysis

Cell culture

Maintenance of immortalized insect cell lines, morphological analyses of cell lines in response to experimental treatments, molecular analyses of cell lines in response to experimental treatments, cell transfection

In vivo techniques

Maintenance of a murine model for malaria infection, morphological and molecular analyses of *Plasmodium*-infected mosquitoes

Computer

Operating systems: Windows, Mac OS

Programs: DNAman, MatInspector, use of genomic databases, 1D Image software for the Kodak DC290 Digital Imaging System, photoshop, MS Office, Endnote

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science (AAAS)
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