Published in final edited form as:

Curr Mol Med. 2004 November ; 4(7): 787–797.

Cross-Talk Between Nitric Oxide and Transforming Growth Factor- β 1 in Malaria

Yoram Vodovotz^{*,†}, Ruben Zamora[§], Matthew J. Lieber[¶], and Shirley Luckhart[¶]

^{*}Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15213, USA

[§]Rangos Research Center, Children's Hospital of Pittsburgh, Pediatric Surgery Department, 3460 Fifth Ave., Pittsburgh, PA 15213, USA

[¶]Dept. of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Abstract

Malaria has re-emerged as a global health problem, leading to an increased focus on the cellular and molecular biology of the mosquito *Anopheles* and the parasite *Plasmodium* with the goal of identifying novel points of intervention in the parasite life cycle. Anti-parasite defenses mounted by both mammalian hosts and *Anopheles* can suppress the growth of *Plasmodium*. Nonetheless, the parasite is able to escape complete elimination *in vivo*, perhaps by thwarting or co-opting these mechanisms for its own survival, as do numerous other pathogens. Among the defense systems used by the mammalian host against *Plasmodium* is the synthesis of nitric oxide (NO), catalyzed by an inducible NO synthase (iNOS). Nitric oxide produced by the action of an inducible *Anopheles stephensi* NO synthase (AsNOS) may be central to the anti-parasitic arsenal of this mosquito. In mammals, iNOS can be modulated by members of the transforming growth factor- β (TGF- β) cytokine superfamily. Transforming growth factor- β is produced as an inactive precursor that is activated following dissociation of certain inhibitory proteins, a process that can be promoted by reaction products of NO as well as by heme. Ingestion by *Anopheles* of blood containing *Plasmodium* initiates parasite development, blood digestion which results in the accumulation of heme (hemin) in the insect midgut, and induction of both AsNOS and TGF- β -like (As60A) gene expression in the midgut epithelium. Active mammalian TGF- β 1 can be detected in the *A. stephensi* midgut up to 48h post-ingestion and latent TGF- β 1 can be activated by midgut components *in vitro*, a process that is potentiated by NO and that may involve heme. Further, mammalian TGF- β 1 is perceived as a cytokine by *A. stephensi* cells *in vitro* and can alter *Plasmodium* development *in vivo*. Bloodfeeding by *Anopheles*, therefore, results in a juxtaposition of evolutionarily conserved mosquito and mammalian TGF- β superfamily homologs that may influence transmission dynamics of *Plasmodium* in endemic regions.

Introduction

Malaria infection results in 1-2 million human deaths annually and is, hence, the focus of intense efforts to lessen morbidity and mortality. Interruption of transmission by the female *Anopheles* mosquito has also been targeted as a goal in the control of malaria, with novel strategies based on the development and release of transgenic mosquitoes that are resistant to parasite transmission [1]. In *Anopheles*, malaria parasite (*Plasmodium* spp.) development begins with the ingestion of blood containing sexual stage gametocytes. Parasite fertilization commences within minutes of ingestion. Zygotes penetrate the midgut epithelium 24-36h later

*Address correspondence to this author at the Department of Surgery, University of Pittsburgh, Montefiore Hospital, NW653, 3459 Fifth Ave. Pittsburgh, PA 15213, USA; Tel: 412-647-5609; Fax: 412-647-5959; Email: vodovotzy@msx.upmc.edu; vodovotz+@pitt.edu.

and transform into vegetative oocysts under the basal lamina in the hemolymph-filled, open circulatory system of the mosquito. As blood digestion proceeds to completion at ~48h, parasite development continues. Oocysts grow and develop for 10-12 days, then release thousands of haploid sporozoites into the hemolymph. Sporozoites invade the salivary glands, where they are released into the saliva during subsequent bloodfeeding.

In addition to parasite development, bloodfeeding stimulates the process of digestion. *Anopheles stephensi* can ingest the equivalent of 10 μ l of blood, containing ~550 μ g of protein, the majority of which is hemoglobin (Hb) [2]. The midgut environment changes predictably as digestion proceeds: proteolytic activity appears within 3h and peaks at 24h, with a decrease in protein content that reaches 80% at 36h [3]. Undigested blood cells are restricted to the central core by 24-36h. The globins are dissociated from heme and digested, while the heme groups are polymerized to insoluble hematin under oxygenated and slightly alkaline conditions [4]. Digestion is complete at ~48 hr, when the peritrophic membrane and hematin are excreted.

As we describe below, the process of bloodfeeding underlies an immunological cross-talk between the mammalian and mosquito hosts of *Plasmodium*. This cross-talk involves the free radical nitric oxide (NO) and the cytokine transforming growth factor (TGF)- β 1, both of which are involved in regulating *Plasmodium* development in mammalian hosts. Our data reveal that, in an analogous fashion, NO and TGF- β 1 are also involved in regulating malaria parasite development in the *Anopheles* mosquito.

Physiological Importance of NO

The inorganic free radical nitric oxide (NO) plays a central role in such fundamental physiological processes as nonspecific immunological cytotoxicity to microbial pathogens [5], neurotransmission [6], and vasorelaxation [7], as well as in such pathophysiological processes as cardiac arrhythmia [8,9] arthritis [10,11], atherosclerosis [12], neurodegeneration [13], and sepsis [14]. Nitric oxide, whether delivered by iNOS from activated macrophages [15,16] or from chemical NO donors [17-20], can suppress the proliferation of many cell types. This effect is due, at least in part, to suppression of mitochondrial respiration [21] reduction of DNA synthesis [22,23], and inhibition of iron metabolism [24]. Nitric oxide also stimulates cellular differentiation (generally in tumor cells) [17-19,25], blocks tumor metastasis by reducing the adhesion of tumor cells to blood vessels [26] or ischemia/reperfusion injury by blocking adhesion of neutrophils to injured blood vessels [27], and triggers apoptosis in numerous cell types [28,29]. In contrast, NO increases the proliferation of certain cell types, most notably endothelial cells [30,31], and protects hepatocytes [32] and endothelial cells [33] from apoptosis.

Nitric oxide is produced enzymatically by NO synthases (NOS). In mammals, neuronal NOS (nNOS; NOS I) and endothelial NOS (eNOS; NOS III) are expressed constitutively and produce small amounts of NO. Most mammalian cell types, including macrophages, fibroblasts, and epithelial cells can produce NO via iNOS, an isoform of NOS whose expression is induced by cytokines and bacterial products [34]. The regulation of iNOS is complex, and occurs pre- and post-transcriptionally [34,35] as well as pre- and post-translationally [35-37]. The expression and activity of iNOS are also modulated by numerous cytokines, including TGF- β 1, as well as by NO itself [38].

Regulation of iNOS by TGF- β 1

Numerous cytokines modulate the expression of iNOS in a wide variety of mammalian cells, thereby ensuring homeostasis [34,39-41]. Recent studies suggest that among this panoply of cytokines, TGF- β 1 is perhaps the most potent regulator of iNOS [39-41]. In primary cells of

the vertebrate innate immune system (natural killer cells, neutrophils, and macrophages), TGF- β 1 reduces iNOS enzyme activity [40,41], iNOS expression [35,40,41], iNOS mRNA stability and translation [35,41], and iNOS protein stability [35,41]. In contrast to this role as a negative regulator, TGF- β 1 can upregulate iNOS expression and enzyme activity in “sentinel” cells associated with tissue and innate immunity, including fibroblasts, chondrocytes, and immunocompetent retinal epithelial cells [42–44]. These latter cell types, although commonly implicated as first line defenders of the host against infection [45,46], have received less attention than those cell types more commonly associated with adaptive immunity.

The mammalian isoforms of TGF- β (TGF- β 1, - β 2, and - β 3) often display overlapping spectra of actions [47], including the regulation of NO production *in vitro* [40,41]. However, TGF- β 1 appears to be the most important isoform with regard to regulating iNOS under physiological conditions [41,48,49], and thus constitutes the focus of this review. TGF- β 1, like all isoforms of TGF- β , is produced in a latent form, which must be activated in order to exert its biological effects. The TGF- β 1 precursor homodimerizes intracellularly, and is then cleaved extracellularly to yield the active TGF- β 1 dimer as well as the remaining portion of its dimerized precursor, the latency-associated peptide (LAP). Under physiological conditions, TGF- β 1 is expressed almost completely in its latent form, which consists of the active TGF- β 1 dimer bound non-covalently to LAP. Additional proteins such as alpha 2-macroglobulin, latent TGF- β binding proteins (LTBPs), or proteoglycans (e.g. decorin) are bound to latent TGF- β 1 in what is known as the large latent complex. Latency-associated peptide and other proteins must be dissociated from latent TGF- β 1, in a process known as activation, before TGF- β 1 gains biological activity [50]. Numerous agents, including plasmin, transglutaminase, cytokines, and radiation can activate latent TGF- β 1 [50,51]. Recent data suggest that oxygen free radicals [52] and NO [53] can also activate latent TGF- β 1.

Once activated in mammalian cells, TGF- β 1 binds to a complex of three proteins, the type I, type II, and type III receptors, though other binding proteins are also expressed on the surface of some cell types. These receptors interact in a complex manner in which the type III receptor, which does not have an intracellular portion, binds TGF- β 1 through interactions with glycosaminoglycans in its extracellular portion and “presents” the bound ligand to a signaling complex made up of the type I and type II receptors [54]. Ligand-induced activation of the type II receptor, a serine/threonine kinase, results in phosphorylation of the associated type I receptor to form a fully activated complex. Once activated, the TGF- β receptor complex recruits and phosphorylates cytoplasmic proteins generally known as receptor Smads (Mothers Against Decapentaplegic Homologs) [54,55]. Phosphorylated receptor Smads are released from the receptor and form multimeric complexes with cytoplasmic co-Smads. The receptor Smad/co-Smad complex then translocates to the nucleus and interacts with other DNA-binding proteins to regulate transcription of relevant target genes [56].

Recently, it has been appreciated that crosstalk between iNOS and TGF- β 1 may extend further to mutual feedback regulation: *in vitro*, we have shown that latent TGF- β 1 can be activated by exogenous and perhaps endogenous NO [53]. Studies with macrophages from TGF- β 1^{-/-} mice — mice that exhibit spontaneously elevated expression of iNOS [48] — suggest that the presence of endogenous TGF- β 1 is necessary for the suppression of cellular iNOS activity in the presence of elevated systemic NO production [57]. In contrast, TGF- β 1 can also induce iNOS expression and continued synthesis of NO in some cell types [42–44], which in turn can induce the *de novo* synthesis of additional TGF- β 1 [58,59], yielding a cycle of positive rather than negative feedback regulation.

Modulation of *Plasmodium* Infection By NO and TGF- β 1

In the vertebrate host, innate immunity has a significant impact on *Plasmodium* infection. This innate defense is mediated, in part, by interferon- γ (IFN) produced by natural killer cells and $\gamma\delta$ T cells, which enhances circulating levels of tumor necrosis factor- α (TNF) and NO [60]. Although high levels of iNOS expression and plasma NO have been correlated with decreased severity of malaria infection [61], an unregulated response resulting in overproduction of IFN and TNF can lead to severe pathology [62]. Likewise, overproduction of NO can damage the host, as demonstrated for pediatric anemia that was correlated with parasite-induced NO synthesis in Tanzanian children [63].

The vertebrate host response to *Plasmodium*, therefore, must be controlled to optimize parasite clearance and minimize host damage. This control is mediated in part by TGF- β 1. The role of TGF- β 1 in regulating the immune response to *Plasmodium* is best understood from murine models in which timing and circulation levels influence protection of the host from overwhelming parasitemia. Early in parasite infection, low levels of TGF- β 1 appear to promote pro-inflammatory responses (e.g., induction of iNOS) that inhibit parasite growth, while later in infection, elevated levels of TGF- β 1 are anti-inflammatory and serve to limit host autoimmune pathology [64]. While the role of TGF- β 1 in altering the balance of *Plasmodium* infection in the vertebrate host is supported by the literature, the quixotic nature of this balance, as well as its regulation, are not well understood.

Plasmodium is transferred from the vertebrate to mosquito host through the act of bloodfeeding. Parasite infection in *Anopheles* modulates expression of a variety of innate immune genes in the mosquito midgut (see review by Barillas-Mury [65]) including the TGF- β superfamily homolog As60A [66,67]. AsNOS-produced NO, however, is the only known effector that is both naturally induced by parasite infection and that limits parasite development [68].

Invertebrate NO and TGF- β

Nitric oxide-based physiology has been characterized in a variety of invertebrate groups, but is found predominantly in representatives of Mollusca and Arthropoda [69]. While these groups are fairly primitive relative to vertebrates, their representatives display one or all of the known physiological functions of NO including neurotransmission, epithelial cell signaling, and inducible innate immunity [69]. Nitric oxide-dependent immunity, however, is the least studied aspect of NO physiology in these organisms. Among the half dozen or so invertebrate species in which a role for NO in immunity has been characterized, the *A. stephensi* NOS/NO response remains among the best characterized.

The only characterized genomic cognate of an invertebrate NOS encodes *A. stephensi* NOS (AsNOS). *Anopheles stephensi* NOS is a single copy gene with structural features reminiscent of vertebrate neuronal, endothelial, and inducible NOS genes [70]. Sequence analyses of the AsNOS 5'-flanking region have revealed a striking concentration of consensus transcription factor binding sites, reminiscent of cytokine-inducible iNOS promoters [70]. Genome sequencing has similarly confirmed the presence of single copies of *Drosophila* and *A. gambiae* NOS, supporting the contention that insects possess a single, multifunctional NOS gene. It has been suggested that the three vertebrate NOS genes evolved from a single ancestral gene [71]. If NOS genes evolved this way, continued study of AsNOS molecular biology and regulation will provide insight to events on the path to NOS gene duplication, divergence, structural complexity, and regulation in higher organisms.

Multiple AsNOS transcripts are generated by a complex array of alternative splicing and initiation events, with three transcripts induced in response to *Plasmodium* infection [72]. Similar transcriptional complexity has been described for human NOS I and iNOS [73,74],

suggesting that homology among vertebrate and invertebrate NOS genes extends beyond features of genomic organization into gene regulation.

At first glance, the life cycle of *Plasmodium* in *Anopheles* displays little evidence of host hostilities to parasite development. In even the most susceptible mosquito, however, parasite development does not proceed undetected or unrestricted. Research on mosquito immunity to *Plasmodium* to date has focused on three major molecular mechanisms: melanotic encapsulation of midgut oocysts mediated by a serine protease-activated prophenoloxidase cascade, the synthesis of antibacterial proteins by the midgut and fat body, and a NOS-mediated midgut and humoral defense as identified by Luckhart *et al.* [68]. The ultrastructural effects of melanization have been described for *Plasmodium*, but the toxic mechanism is not clearly known and the contribution of this response to host defense remains uncertain [75]. Endogenous defensins and cecropins, polypeptides toxic to a range of bacteria and some eukaryotic cells, have also been characterized from *A. gambiae* [76,77]. A direct role for these compounds in limiting *Plasmodium* development in *Anopheles*, however, has not been demonstrated and, in some cases, questioned altogether [78]. In *A. stephensi*, NOS expression and activity are induced by *Plasmodium* infection and this response intrinsically limits parasite development [68]. Hence, AsNOS is the only endogenous immune effector described that inhibits parasite development and is naturally induced by *Plasmodium* infection.

Induction of NOS expression is a nearly universal response of vertebrate cells following exposure to infectious agents or combinations of cytokines [34]. Within the last decade, immunological assays have demonstrated that invertebrates possess factors in their hemolymph or hemocytes similar to IL-1, -2, TNF, the IFNs, and TGF- β 1 [79-82], and that invertebrate cells are responsive to vertebrate cytokines. In one striking case, *Asterias forbesi* IL-1-like protein(s) enhanced NO synthesis by starfish immune cells [82]. Other work has clearly demonstrated that invertebrate cells are responsive to vertebrate cytokines. For example, NOS activity was induced in snail hemocytes following exposure to various human cytokines [83], TGF- β 1 altered cell shape and induced biogenic amine release in molluscan hemocytes [84, 85], and co-receptors responsive to human IFN γ were identified on *Manduca sexta* hemocytes [86,87]. Homologous signaling pathways likely mediate these extracellular signals: Barillas-Mury *et al.* [88], reported cloning of an *A. gambiae* homolog of vertebrate STAT (Signal Transducer and Activator of Transcription) factors, which are responsive to a variety of inflammatory cytokines. While some observed cytokine-like activity is apparently the result of convergent evolution [89], it is also likely that invertebrates share with vertebrates a genetic basis for cytokine functional homology. We suggest that the invertebrate TGF- β s are examples of this phenomenon.

Drosophila TGF- β superfamily homologs include an activin, myoglianin, screw (scw), maverick, decapentaplegic (dpp), and glass bottom boat-60A (gbb-60A). Dpp, gbb-60A, scw and myoglianin are most closely related to the vertebrate bone morphogenetic proteins (BMPs) 2-4, 5-7 and 11, respectively, while *Drosophila* activin is most similar to vertebrate activin β B [90]. These ligands signal through prototypical BMP and TGF- β /activin signal transduction pathways in flies [91,92]. Specifically, signals from *Drosophila* TGF- β s, like those from vertebrate activins/TGF- β s and BMPs, are transduced through heteromeric serine/threonine kinase receptors recruited by bound ligand. Six type I and type II receptors that can bind BMP and TGF- β /activin homologs have been characterized from *Drosophila* [90,92]. The first signal transducers for TGF- β were identified in *Drosophila* and include two receptor Smads, dSmad2 and Mad, a co-Smad Medea, and an anti-Smad Dad (Fig. 1) [90].

Extensive crosstalk among mammalian and *Drosophila* TGF- β homologs and the related signaling Smads has been demonstrated in numerous laboratories (Fig. 1). For example, genetic screens in *Drosophila* have revealed that gbb-60A, which is involved in the development of

the embryonic midgut, patterning of the larval cuticle, and development of fat body morphology [93], potentiates dpp signaling, perhaps by signaling through the dpp receptor complex [94], through concerted activation of Mad [95], or through formation of gbb-60A/dpp heterodimers that act through Mad and Medea [96]. Bone morphogenetic protein 4 can substitute for dpp during development of transgenic *Drosophila* [97], and dpp and gbb-60A can induce bone formation in mammalian cells [98]. Exposure of *Drosophila* cells to BMP2 results in rapid phosphorylation of Mad [99]. *Drosophila* dSmad2 can mediate TGF- β 1 signaling in mammalian cells [92]. Smad 4 and Medea, the invertebrate co-Smad partner for both Mad and dSmad2 [100,101], appear to be fully interchangeable [102,103]. Both mammalian and invertebrate Smads, in addition to mediating effects through association with interchangeable transcription factors like tinman and Nkx2-5 (Fig. 1) [104], can bind to identical consensus control elements (the “GCCG box”) in target gene promoters following nuclear translocation [105]. Finally, in a manner somewhat analogous to TGF- β 1 signaling, activation of some target genes by dpp also depends on binding of the *Drosophila* CBP co-activator homolog, dCREB B, to CRE (Cyclic Adenosine Monophosphate Responsive Element) sites [106].

Crosstalk also occurs at the level of signal termination (Fig. 1). TGF- β homolog signaling in mammalian cells is down-regulated through a gradual repression of receptor Smad expression and rapid induction of anti-Smad (Smads 6 and 7) expression [107,108]. This form of transcriptional regulation is conserved in *Drosophila*: overexpression of dpp results in increased transcription of the anti-Smad Dad [109]. Dad blocks nuclear translocation of Mad [110], shares considerable sequence homology with Smad7 [90], and can block signaling by BMP-4 in *Xenopus* cells [109]. The fact that murine and *Xenopus* Smad7 can inhibit signaling from both BMP and TGF- β /activin pathways [111,112] suggests that *Drosophila* Dad may similarly regulate both BMP and TGF- β /activin signaling pathways. The first experimental evidence of this phenomenon in *Drosophila* was recently obtained with gain-of-function mutants: *Drosophila* lines overexpressing Dad, BMP-associated human Smad 6, or TGF- β -associated human Smad 7 produced nearly identical phenotypes, suggesting extensive cross-talk by these anti-Smads in different signaling pathways (Fig. 1) [101]. BMP and TGF- β /activin signaling pathways also intersect at the level of a common receptor component: activin and the BMP OP-1 antagonize one another at the level of a shared type II receptor [113]. These observations attest to a remarkable degree of genetic and functional conservation both among and between insect and mammalian TGF- β superfamily members. While the impact of this conservation on biology of these organisms is clear, we are the first to propose that this conservation also underlies an immunological cross-talk between bloodfeeding insects and mammals. Such cross-talk, for example, may have significant implications for the regulation of the mosquito response to *Plasmodium* development. Indeed, we have proposed that since both NOS and TGF- β are so well conserved throughout evolution, so perhaps is the regulation of inducible NOS by a TGF- β -like protein [41].

We have characterized the first mosquito TGF- β superfamily homolog, *A. stephensi* 60A or As60A [66,67]. Like AsNOS, As60A is abundantly expressed in the *A. stephensi* midgut and other tissues and is responsive in a dose-dependent manner to *Plasmodium* infection [67]. If As60A functions directly in immune signaling, we would expect the As60A gene to encode consensus sequences indicative of immune regulation. In addition, we would expect mosquito cells to possess type I and type II receptor homologs, various Smads, and relevant binding sites in immune-related target genes. The following observations support the existence of at least some of these elements. We have identified putative *dorsal/Gambif1*-like [114] and *dorsal-repression element* binding sites [115] in conserved intron 2 of As60A [66]. These binding sites suggest a basis for regulation of As60A via Toll-like pathway signaling. Indeed, the architecture for immune-related *Anopheles* Toll signaling is in place. Ten Toll genes, together with associated signaling proteins, are known from the *A. gambiae* genome [116], four *A.*

gambiae Tolls have been characterized [117], and we have partially characterized two Toll genes from *A. stephensi* (unpublished data). In *Drosophila*, dorsal and other Toll pathway homologs were first known for their involvement in embryonic development. We now know that these pathways are variously utilized in adult insects to regulate anti-pathogen responses [118]. We have cloned cDNAs encoding *A. stephensi* Smad homologs of Mad and Medea; both are expressed in *A. stephensi* ASE cells *in vitro* and in the midgut epithelium (unpublished data). The predicted amino acid sequence of a putative dSmad2 encoded by the *A. gambiae* genome is 58% identical to the amino acid sequence of *Drosophila* dSmad2. In a manner similar to Toll signaling, we propose that Smad signaling, which controls dipteran development, also regulates components of the mosquito immune response to *Plasmodium*. In support of this hypothesis, the putative promoter of AsNOS [70] contains three consensus Smad binding elements (unpublished data). One site is homologous to a mammalian TGF- β 1-related Smad binding site and two are homologous to the *Drosophila* Mad binding site, suggesting that AsNOS can be regulated both by TGF- β /activin and BMP-related pathways.

Activation of TGF- β 1 by NO, Nitroxyl, and Heme

The finding that TGF- β 1 suppressed inducible NO production and iNOS expression both *in vitro* and *in vivo* [41,48,49], that TGF- β 1 could be activated by reactive oxygen species [52], and that pro-inflammatory stimuli that activate TGF- β 1 [50,119-121] suggested the novel possibility that NO could activate latent TGF- β 1. This hypothesis led to studies in which treatment of cells with the chemical NO donor diethylamine-NONOate (DEANO) did indeed lead to activation of latent TGF- β 1 [53]. This effect appears to be indirect, since treatment of recombinant latent TGF- β 1 with DEANO *in vitro* did not activate latent TGF- β 1, as assessed by a bioassay of the specific anti-proliferative effect of TGF- β 1 on mink lung epithelial cells (CCL-64) [53]. Treatment of recombinant human LAP with NO interfered with the ability of LAP to inhibit active TGF- β 1, coincident with S-nitrosation of LAP but not active TGF- β 1 [53]. More recent studies used an enzyme-linked immunosorbent assay (ELISA) specific for active TGF- β 1; only active TGF- β 1 binds to TGF- β type II receptor immobilized on a microtiter plate, and detection of bound ligand is based on TGF- β 1-specific antisera (Quantikine™, R & D Systems, Minneapolis, MN). These studies indicated that S-nitrosated LAP cannot prevent the binding of TGF- β 1 to type II TGF- β receptor, unlike untreated LAP (Fig. 2). Further, based on the ability of HgCl₂ to displace NO, it appears that all three cysteines on recombinant human LAP can be S-nitrosated (Fig. 3).

Heme, which is directly cytotoxic and which can both catalyze the synthesis of oxygen free radicals and potentiate damage mediated by superoxide and hydrogen peroxide [122,123], is released in the midgut of *A. stephensi* during Hb digestion. Heme is polymerized to hematin, but this process is not instantaneous nor does it completely abolish radical chemistry [124]. The synthesis of oxygen free radicals during blood digestion, therefore, is likely. As described above, recent studies have indicated that oxygen free radicals can activate latent TGF- β 1 *in vitro* [52], leading to the hypothesis that free heme, provided as hemin, may also augment the activation of latent TGF- β 1. Indeed, treatment of latent TGF- β 1 with hemin activated this cytokine to levels comparable to that observed with heat, a positive control [125].

Nitroxyl (HNO) and peroxynitrite, toxic pro-oxidants associated with NO synthesis [126] that are also likely mediators of oxidative and nitrosative stress in the *A. stephensi* midgut (unpublished data), had very different effects on TGF- β 1 activation. Angeli's salt, a nitroxyl donor, activated latent TGF- β 1 directly, while peroxynitrite did not activate latent TGF- β 1 [125]. Barcellos-Hoff and Dix implicated hydroxyl radical in the activation of latent TGF- β 1 by oxidizing species, while hydrogen peroxide was not capable of activating TGF- β 1 [52]. Our findings, along with other published results [52], suggest that TGF- β 1 is sensitive to specific types of oxidative stress. One possible mechanism of activation is the modification of LAP by

redox-active heme such that it could no longer bind to active TGF- β 1, as has been demonstrated for activation of latent TGF- β 1 by heat [127,128] and NO [53]. Pre-incubation of LAP with heme indeed abrogated the capacity of LAP to neutralize active TGF- β 1 [125]. These findings suggest that heme, like NO, can modify LAP. It is not clear why NO cannot activate latent TGF- β 1 directly while heme can. We propose that a free cysteine sulfhydryl is required for S-nitrosation of LAP. The high affinity of LAP for active TGF- β 1 presumably prevents LAP S-nitrosation by masking cysteine residues. However, heme may disrupt the interaction between LAP and active TGF- β 1, permitting LAP S-nitrosation by NO. We propose that S-nitrosated LAP does not re-bind and inactivate TGF- β 1. In essence, NO in the presence of heme may lead to sustained activation of latent TGF- β 1.

Activation and Persistence of TGF- β 1 in the *Anopheles* Midgut Following Bloodfeeding

The studies presented above demonstrate that TGF- β 1 can be activated *in vitro* by heme and by some reaction products of NO. Furthermore, treatment with a chemical NO donor or co-incubation with activated macrophages that produce NO activates cell-associated or intracellular mammalian TGF- β 1 [53]. Do these processes have any relevance for *A. stephensi*? Midgut lysates from *A. stephensi* treated with phosphate-buffered saline as a control appeared to contain active TGF- β 1 from 1-48h post-bloodfeeding; by 7d, active TGF- β 1 was almost undetectable [125]. Thus, *A. stephensi* midguts contain a protein antigenically similar to active TGF- β 1 post-bloodfeeding. Could free heme participate in activating TGF- β 1 at early times ($t = 1$ h) post-bloodfeeding? It has been reported that *Anopheles* trypsin is activated rapidly after feeding [129] and thus could be expected to liberate heme from Hb in quantities sufficient to activate latent TGF- β 1. This activation would be analogous to activation of latent TGF- β 1 with heme *in vitro* [125]. Latent TGF- β 1 was not activated *in vitro* by midgut lysates alone [125]. However, latent TGF- β 1 was activated after incubation with midgut lysates in the presence of the chemical NO donor S-nitroso-N-acetylpenicillamine (SNAP), which was used to simulate NO synthesis following infected bloodfeeding [68,125]. These results suggest that *A. stephensi* midguts contain substance(s) that can activate latent human TGF- β 1 in the presence of NO. Based on these *in vitro* studies, it was suggested that heme/hematin interacts with NO to activate latent TGF- β 1 in the midguts of *A. stephensi* mosquitoes post-bloodfeeding.

Transient acidification using HCl [127,128], in order to examine whether any residual latent TGF- β 1 was present in the lysates yielded no increase in detectable active TGF- β 1, suggesting that all TGF- β 1 present in *A. stephensi* midguts within 1h post-bloodfeeding was active [125]. Further, because active TGF- β 1 is not present in mouse plasma [125,130], it was proposed that *A. stephensi* ingested and rapidly activated latent TGF- β 1, a process that may have involved NO generated by AsNOS catalysis.

Mammalian TGF- β 1 is Recognized as a Cytokine by *A. Stephensi* Cells *in vitro*

Given that active mammalian TGF- β 1 is present in the *A. stephensi* midgut, we sought to determine if *A. stephensi* cells could respond to mammalian TGF- β 1. In replicated assays, *A. stephensi* MSQ43 cells treated with human TGF- β 1 showed significantly enhanced spreading compared to controls [125], a behavior consistent with actin-dependent immune responsiveness of immortalized mosquito cells [131] and other insect cells [132]. Furthermore, immortalized *A. stephensi* ASE cells treated with human TGF- β 1 exhibited reduced 3 H-thymidine incorporation, suggesting reduced proliferation of these insect cells in response to TGF- β 1 [125].

Given this sensitivity of *A. stephensi* cells to TGF- β 1, we sought to determine whether human TGF- β 1 could induce AsNOS expression in a manner analogous to its effects on iNOS expression in mammalian sentinel immune cells. To address this question, two assays were performed. In the first assay, ASE cells were treated either with human TGF- β 1 or with diluent alone as a control. In the second assay, cells were pre-treated with human recombinant TGF- β 1 in saline or with saline alone as a control. After pre-treatment, cells were exposed to heat-killed *E. coli*. Heat-killed bacteria induce AsNOS expression *in vitro* and, therefore, can be used to reveal the effects of TGF- β 1 pre-exposure on AsNOS induction. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to assess AsNOS expression in samples as described [67]. While no significant effects were observed at 6h and 48h, AsNOS expression was induced ~3.7-fold in ASE cells at 24h post-exposure to human TGF- β 1 [125]. Further, TGF- β 1 pre-treatment increased AsNOS induction in the presence of *E. coli* as an immune stimulus [125]. These results indicate that *A. stephensi* cells *in vitro* recognize human TGF- β 1 as a cytokine. Further, we propose that induction of AsNOS by TGF- β 1 is analogous to induction of iNOS in “sentinel” immune cells of mammalian tissues by TGF- β 1.

Given these striking effects of TGF- β 1 on AsNOS, we sought to determine whether human TGF- β 1 could alter *Plasmodium* development in *A. stephensi*. To address this question, separate pools of sibling *A. stephensi* were fed on identical aliquots of cultured *P. falciparum* in the presence or absence of TGF- β 1. At 7d post-feeding, 2 pg/ml human TGF- β 1 reduced the number of *P. falciparum* oocysts by 46% and 26%, respectively, compared to the controls [125]. Similarly, 200 pg/ml TGF- β 1 reduced oocyst numbers by 37% and 27%, respectively, compared to controls [125]. In contrast, the highest concentration of TGF- β 1 (2000 pg/ml) had no effect on oocyst numbers in either cohort when compared to the controls [125]. While the magnitude of the effect of TGF- β 1 appears to be dependent on parasite infection intensity, the pattern of the effect suggests that, in the range of levels detected following ingestion, human TGF- β 1 limits parasite development in *A. stephensi*. Taken together with previous observations, these results suggest that low doses of TGF- β 1 inhibit parasite development *in vivo* through induction of AsNOS in the midgut epithelium. Further, parasite inhibition at lower TGF- β 1 doses and lack of inhibition at the highest dose are consistent with the pro- and anti-inflammatory roles of TGF- β 1 in balancing parasite infection in mammalian hosts [133].

***Anopheles Stephensi* As60A: Inflammatory Cytokine-Like Behavior in the Mosquito Midgut**

While the studies presented so far suggest a novel regulatory process involving mammalian TGF- β 1 and mosquito AsNOS to control parasite development, the question arises as to whether an endogenous TGF- β carries out similar effects in *A. stephensi*. Indeed, the first mosquito TGF- β superfamily homolog, *A. stephensi* 60A (As60A) has been characterized recently [66,67]. Northern analyses detected a single abundant transcript and one minor transcript [67]. Quantitative RT-PCR demonstrated increased expression of As60A in the mosquito midgut and carcass (body minus midgut) following ingestion of blood from *P. berghei*-infected mice compared to uninfected mice [67]. These data suggest that As60A is an inflammatory cytokine in *A. stephensi* and that induction in the midgut within the first 24h of bloodfeeding could juxtapose mosquito As60A with active mammalian TGF- β 1. These findings raise an intriguing possibility: if A60A, like TGF- β 1, regulates *Plasmodium* development in the *Anopheles*, these signaling factors may activate intersecting signaling pathways, altering expression of anti-parasite effector genes *in vivo*.

Conclusions

In this article, we describe the reciprocal interactions between NO and TGF- β 1, both in mammals and mosquitoes. What do these studies suggest about *Plasmodium* transmission in the field? The impact of crosstalk on parasite transmission may be significant. If *Anopheles* were to feed on a host attempting to clear *Plasmodium* (e.g., a host with low TGF- β 1 levels relatively early in infection), parasite development may be impeded by TGF- β 1 activation of AsNOS in the mosquito. Alternate human hosts with high levels of TGF- β 1 early in parasite infection, which would be predicted to suffer from overwhelming parasitemia [64], may be available as well. If a mosquito were to feed on this type of host, parasite development may proceed unchecked by a disabled immune defense in the mosquito, ensuring survival and transmission of the parasite ingested from a dying host. The impact of mammalian immunity on parasite transmission by *Anopheles* has practical implications for malaria control. Transgene products targeted to the midgut may be influenced by mammalian factors that remain active in the midgut environment following ingestion. Given the panoply of cytokines and other signaling factors whose plasma concentrations are influenced by nutritional and diseased states of the mammalian host, prudent experimental designs would include assessments of parasite development in transgenic *Anopheles* following exposure to a variety of hosts with physiological conditions and stresses typical of endemic regions.

Acknowledgements

The authors would like to acknowledge the technical assistance of Binnie Betten (University of Pittsburgh, Department of Surgery) for the experiments on S-nitrosation of LAP.

References

1. Hoffman SL, Subramanian GM, Collins FH, Venter JC. Plasmodium, Nature 2002;415:702–709.
2. Vaughan JA, Noden BH, Beier JC. J Med Entomol 1991;28:780–786. [PubMed: 1770513]
3. Billingsley PF, Hecker H. J Med Entomol 1991;28:865–871. [PubMed: 1770523]
4. Berner R, Rudin W, Hecker H. J Ultrastruct Res 1983;83:195–204. [PubMed: 6345801]
5. Nathan CF, Hibbs JB Jr. Curr Opin Immunol 1991;3:65–70. [PubMed: 1711326]
6. Bredt DS, Snyder SH. Neuron 1992;8:3–11. [PubMed: 1370373]
7. Moncada S, Palmer RMJ, Higgs EA. Pharmacol Rev 1991;43:109–142. [PubMed: 1852778]
8. Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL. Science 1992;257:387–389. [PubMed: 1631560]
9. Roberts AB, Vodovotz Y, Roche NS, Sporn MB, Nathan CF. Mol Endocrinol 1992;6:1921–1930. [PubMed: 1282674]
10. McCartney-Francis N, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, Wahl SM. J Exp Med 1993;178:749–754. [PubMed: 7688035]
11. Ialenti A, Moncada S, Di Rosa M. Br J Pharmacol 1993;110:701–706. [PubMed: 8242242]
12. White CR, Brock TA, Chang LY, Crapo J, Briscoe J, Ku D, Bradley WA, Gianturco SH, Gore J, Freeman BA, Tarpey MM. Proc Natl Acad Sci USA 1994;91:1044–1048. [PubMed: 8302829]
13. Knowles, RG. Int Acad Biomed Drug Res. Racagni, G.; Brunello, N.; Langer, SZ., editors. Basel: 1994. p. 112–118.
14. Johnson ML, Billiar TR. World J Surg 1998;22:187–196. [PubMed: 9451935]
15. Drapier JC, Wietzerbin J, Hibbs JB. Eur J Immunol 1988;18:1587. [PubMed: 3142779]
16. Stuehr DJ, Nathan CF. J Exp Med 1989;169:1543–1555. [PubMed: 2497225]
17. Magrinat G, Mason SN, Shami PJ, Weinberg JB. Blood 1992;80:1880–1884. [PubMed: 1382708]
18. Maragos CM, Wang JM, Hrabie JA, Oppenheim JJ, Keefer LK. Cancer Res 1993;53:564–568. [PubMed: 8425188]
19. Yamazaki A, Birnboim HC. Leukemia Res 1995;19:325–335. [PubMed: 7769833]

20. Pipili-Synetos E, Papageorgiou A, Sakkoula E, Sotiropoulou G, Fotsis T, Karakiulakis G, Maragoudakis ME. *Br J Pharmacol* 1995;116:1829–1834. [PubMed: 8528567]
21. Drapier JC, Hibbs JB Jr. *J Immunol* 1988;140:2829–2838. [PubMed: 2451695]
22. Kwon NS, Stuehr DJ, Nathan CF. *J Exp Med* 1991;174:761–767. [PubMed: 1717630]
23. Lepoivre M, Fieschi F, Coves J, Thelander L, Fontecave M. *Biochem Biophys Res Commun* 1991;179:442–448. [PubMed: 1652957]
24. Drapier JC, Hirling H, Wietzerbin J, Kaldy P, Kühn LC. *EMBO J* 1993;12:3643–3649. [PubMed: 7504626]
25. Munoz-Fernandez MA, Cano E, O'Donnell CA, Doyle J, Liew FY, Fresno M. *J Neurochem* 1994;62:1330–1336. [PubMed: 7510778]
26. Xie K, Dong Z, Fidler IJ. *J Leukoc Biol* 1996;59:797–803. [PubMed: 8691063]
27. Kubes P, Suzuki M, Granger DN. *Proc Natl Acad Sci USA* 1991;88:4651–4655. [PubMed: 1675786]
28. Nicotera P, Bonfoco E, Brüne B. *Adv Neuroimmunol* 1995;5:411–420. [PubMed: 8746513]
29. Brune B, Mohr S, Messmer UK. *Rev Physiol Biochem Pharmacol* 1996;127:1–30. [PubMed: 8533007]
30. Ziche M, Morbidelli L, Masini E, Granger H, Geppetti P, Ledda F. *Biochem Biophys Res Commun* 1993;192:1198–1203. [PubMed: 8389543]
31. Guo J, Siegfried MR, Lefer AM. *Meth Find Exp Clin Pharmacol* 1994;16:347–354.
32. Wang Y, Vodovotz Y, Kim PKM, Zamora R, Billiar TR. *Ann N Y Acad Sci* 2002;962:415–422. [PubMed: 12076992]
33. Tzeng E, Kim YM, Pitt BR, Lizonova A, Kovesdi I, Billiar TR. *Surgery* 1997;122:255–263. [PubMed: 9288130]
34. Nathan C. *FASEB J* 1992;6:3051–3064. [PubMed: 1381691]
35. Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C. *J Exp Med* 1993;178:605–613. [PubMed: 7688028]
36. Griscavage JM, Rogers NE, Sherman MP, Ignarro LJ. *J Immunol* 1993;151:6329–6337. [PubMed: 7504017]
37. Vodovotz Y, Kwon NS, Popischil M, Manning J, Paik J, Nathan C. *J Immunol* 1994;152:4110–4118. [PubMed: 7511667]
38. Vodovotz, Y.; Barcellos-Hoff, MH. *Nitric Oxide and Inflammation*. Salvemini, D.; Billiar, TR.; Vodovotz, Y., editors. Birkhauser-Verlag; Basel: 2001. p. 41-58.
39. Bogdan, C.; Vodovotz, Y.; Xie, QW.; Nathan, C.; Röllinghoff, M. *Immunotherapy of Infections*. Masihi, N., editor. Marcel Dekker; New York: 1994. p. 37-54.
40. Vodovotz Y, Bogdan C. *Prog Growth Factor Res* 1994;5:341–351. [PubMed: 7540059]
41. Vodovotz Y. *Nitric Oxide Biol and Chem* 1997;1:3–17.
42. Gilbert RS, Herschmann HR. *Biochem Biophys Res Commun* 1993;195:380–384. [PubMed: 7689839]
43. Goureau O, Lepoivre M, Becquet F, Courtois Y. *Proc Natl Acad Sci USA* 1993;90:4276–4280. [PubMed: 7683432]
44. Blanco FJ, Geng Y, Lotz M. *J Immunol* 1995;154:4018–4026. [PubMed: 7535818]
45. Smith RS, Smith TJ, Blieden TM, Phipps RP. *Am J Pathol* 1997;151:317–322. [PubMed: 9250144]
46. Lo D, Feng L, Li L, Carson MJ, Crowley M, Pauza M, Nguyen A, Reilly CR. *Immunol Rev* 1999;169:225–239. [PubMed: 10450520]
47. Roberts, AB.; Sporn, MB. *The Molecular and Cellular Biology of Wound Repair*. Clark, RAF., editor. Plenum Press; New York: 1996. p. 275-308.
48. Vodovotz Y, Geiser AG, Chesler L, Letterio JJ, Campbell A, Lucia MS, Sporn MB, Roberts AB. *J Exp Med* 1996;183:2337–2342. [PubMed: 8642342]
49. Vodovotz Y, Kopp JB, Takeguchi H, Shrivastav S, Coffin D, Lucia MS, Mitchell JB, Webber R, Letterio J, Wink D, Roberts AB. *J Leukoc Biol* 1998;63:31–39. [PubMed: 9469470]
50. Flaumenhaft R, Kojima S, Abe M, Rifkin DB. *Advances in Pharmacology* 1993;24:51–76. [PubMed: 8504067]

51. Barcellos-Hoff MH, Ehrhart EJ, Kalia M, Jirtle R, Flanders K, Tsang MLS. *Am J Pathol* 1995;147:1228–1237. [PubMed: 7485387]
52. Barcellos-Hoff MH, Dix TA. *Mol Endocrinol* 1996;10:1077–1083. [PubMed: 8885242]
53. Vodovotz Y, Chesler L, Chong H, Kim SJ, Simpson JT, DeGraff W, Cox GW, Roberts AB, Wink DA, Barcellos-Hoff MH. *Cancer Res* 1999;59:2142–2149. [PubMed: 10232601]
54. Massague J. *Annu Rev Biochem* 1998;67:753–791. [PubMed: 9759503]
55. Attisano L, Wrana JL. *Curr Opin Cell Biol* 2000;12:235–243. [PubMed: 10712925]
56. Pouponnot C, Jayaraman L, Massague J. *J Biol Chem* 1998;273:22865–22868. [PubMed: 9722503]
57. Vodovotz Y, Letterio JJ, Geiser AG, Chesler L, Roberts AB, Sparrow J. *J Leukoc Biol* 1996;60:261–270. [PubMed: 8773588]
58. Kim SJ, Jeang KT, Glick AB, Sporn MB, Roberts AB. *J Biol Chem* 1989;264:7041. [PubMed: 2708352]
59. Kim SJ, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB. *Mol Cell Biol* 1990;10:1492–1497. [PubMed: 2108318]
60. Fell AH, Smith NC. *Parasitol Today* 1998;14:364–369. [PubMed: 17040818]
61. Anstey NM, Weinberg JB, Hassanali MY, Mwaikambo ED, Manyenga D, Misukonis MA, Arnelle DR, Hollis D, McDonald MI, Granger DL. *J Exp Med* 1996;184:557–567. [PubMed: 8760809]
62. Kremsner PG, Neifer S, Chaves MF, Rudolph R, Bienzle U. *Eur J Immunol* 1992;22:2873–2878. [PubMed: 1425913]
63. Anstey NM, Granger DL, Hassanali MY, Mwaikambo ED, Duffy PE, Weinberg JB. *Am J Trop Med Hyg* 1999;61:249–252. [PubMed: 10463675]
64. Omer FM, Kurtzhals JA, Riley EM. *Parasitol Today* 2000;16:18–23. [PubMed: 10637583]
65. Barillas-Mury C, Wikel B, Han YS. *Insect Biochem Mol Biol* 2000;30:429–442. [PubMed: 10802234]
66. Crampton AL, Luckhart S. *Cytokine* 2001;13:65–74. [PubMed: 11145845]
67. Crampton AL, Luckhart S. *Infection, Genetics, and Evolution* 2001;1:131–141.
68. Luckhart S, Vodovotz Y, Cui L, Rosenberg R. *Proc Natl Acad Sci USA* 1998;95:5700–5705. [PubMed: 9576947]
69. Moroz LL. *Microsc Res Tech* 2000;49:557–569. [PubMed: 10862112]
70. Luckhart S, Rosenberg R. *Gene* 1999;232:25–34. [PubMed: 10333518]
71. Hall AV, Antoniou H, Wang Y, Cheung AH, Arbus AM, Olson SL, Lu WC, Kau CL, Marsden PA. *J Biol Chem* 1994;269:33082–33090. [PubMed: 7528745]
72. Luckhart S, Li K. *Insect Biochem Mol Biol* 2001;31:249–256. [PubMed: 11167094]
73. Eissa NT, Strauss AJ, Haggerty CM, Choo EK, Chu SC, Moss J. *J Biol Chem* 1996;271:27184–27187. [PubMed: 8900212]
74. Wang Y, Newton DC, Marsden PA. *Crit Rev Neurobiol* 1999;13:21–43. [PubMed: 10223522]
75. Cui L, Luckhart S, Rosenberg R. *Insect Mol Biol* 2000;9:127–137. [PubMed: 10762420]
76. Vizioli J, Bulet P, Charlet M, Lowenberger C, Blass C, Muller HM, Dimopoulos G, Hoffmann J, Kafatos FC, Richman A. *Insect Mol Biol* 2000;9:75–84. [PubMed: 10672074]
77. Richman AM, Bulet P, Hetru C, Barillas-Mury C, Hoffmann JA, Kafatos FC. *Insect Mol Biol* 1996;5:203–210. [PubMed: 8799739]
78. Blandin S, Moita LF, Kocher T, Wilm M, Kafatos FC, Levashina EA. *EMBO Rep* 2002;3:852–856. [PubMed: 12189180]
79. Ottaviani E, Paemen LR, Cadet P, Stefano GB. *Eur J Pharmacol* 1993;248:319–324. [PubMed: 8181538]
80. Beck G, Habicht GS. *Ann N Y Acad Sci* 1994;712:206–212.
81. Franchini A, Kleitsas D, Ottaviani E. *Histochem J* 1996;28:599–605. [PubMed: 8910030]
82. Beck G, Ellis T, Zhang H, Lin W, Beauregard K, Habicht GS, Truong N. *Dev Comp Immunol* 2001;25:1–10. [PubMed: 10980315]
83. Ottaviani E, Caselgrandi E, Franceschi C. *Biochem Biophys Res Commun* 1995;207:288–292. [PubMed: 7857278]
84. Ottaviani E, Caselgrandi E, Kleitsas D. *FEBS Lett* 1998;427:255–258. [PubMed: 9607322]

85. Kletsas D, Sassi D, Franchini A, Ottaviani E. *Eur J Cell Biol* 1998;75:362–366. [PubMed: 9628322]
86. Parker MS, Ourth DD. *Comp Biochem Physiol B Biochem Mol Biol* 1999;122:155–163. [PubMed: 10327605]
87. Parker MS, Ourth DD. *Comp Biochem Physiol B Biochem Mol Biol* 2000;125:337–345. [PubMed: 10818267]
88. Barillas-Mury C, Han YS, Seeley D, Kafatos FC. *EMBO J* 1999;18:959–967. [PubMed: 10022838]
89. Beschin A, Bilej M, Brys L, Torreele E, Lucas R, Magez S, De Baetselier P. *Nature* 1999;400:627–628. [PubMed: 10458158]
90. Raftery LA, Sutherland DJ. *Dev Biol* 1999;210:251–268. [PubMed: 10357889]
91. Nakayama T, Cui Y, Christian JL. *Cell Mol Life Sci* 2000;57:943–956. [PubMed: 10950309]
92. Brummel T, Abdollah S, Haerry TE, Shimell MJ, Merriam J, Raftery L, Wrana JL, O'Connor MB. *Genes Dev* 1999;13:98–111. [PubMed: 9887103]
93. Wharton KA, Cook JM, Torres-Schumann S, de Castro K, Borod E, Phillips DA. *Genetics* 1999;152:629–640. [PubMed: 10353905]
94. Khalsa O, Yoon JW, Torres-Schumann S, Wharton KA. *Development* 1998;125:2723–2734. [PubMed: 9636086]
95. Conley CA, Silburn R, Singer MA, Ralston A, Rohwer-Nutter D, Olson DJ, Gelbart W, Blair SS. *Development* 2000;127:3947–3959. [PubMed: 10952893]
96. Chen Y, Riese MJ, Killinger MA, Hoffmann FM. *Development* 1998;125:1759–1768. [PubMed: 9521913]
97. Padgett RW, Wozney JM, Gelbart WM. *Proc Natl Acad Sci USA* 1993;90:2905–2909. [PubMed: 8464906]
98. Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM. *Proc Natl Acad Sci USA* 1993;90:6004–6008. [PubMed: 8327474]
99. Newfeld SJ, Mehra A, Singer MA, Wrana JL, Attisano L, Gelbart WM. *Development* 1997;124:3167–3176. [PubMed: 9272957]
100. Das P, Inoue H, Baker JC, Beppu H, Kawabata M, Harland RM, Miyazono K, Padgett RW. *Genes Cells* 1999;4:123–134. [PubMed: 10320478]
101. Marquez RM, Singer MA, Takaesu NT, Waldrip WR, Kraytsberg Y, Newfeld SJ. *Genetics* 2001;157:1639–1648. [PubMed: 11290719]
102. Wisotzkey RG, Mehra A, Sutherland DJ, Dobens LL, Liu X, Dohrmann C, Attisano L, Raftery LA. *Development* 1998;125:1433–1445. [PubMed: 9502724]
103. Hudson JB, Podos SD, Keith K, Simpson SL, Ferguson EL. *Development* 1998;125:1407–1420. [PubMed: 9502722]
104. Ranganayakulu G, Elliott DA, Harvey RP, Olson EN. *Development* 1998;125:3037–3048. [PubMed: 9671578]
105. Kusanagi K, Inoue H, Ishidou Y, Mishima HK, Kawabata M, Miyazono K. *Mol Biol Cell* 2000;11:555–565. [PubMed: 10679014]
106. Szuts D, Eresh S, Bienz M. *Genes Dev* 1998;12:2022–2035. [PubMed: 9649506]
107. Takase M, Imamura T, Sampath TK, Takeda K, Ichijo H, Miyazono K, Kawabata M. *Biochem Biophys Res Commun* 1998;244:26–29. [PubMed: 9514869]
108. Mori Y, Chen SJ, Varga J. *Exp Cell Res* 2000;258:374–383. [PubMed: 10896788]
109. Tsuneizumi K, Nakayama T, Kamoshida Y, Kornberg TB, Christian JL, Tabata T. *Nature* 1997;389:627–631. [PubMed: 9335506]
110. Inoue H, Imamura T, Ishidou Y, Takase M, Udagawa Y, Oka Y, Tsuneizumi K, Tabata T, Miyazono K, Kawabata M. *Mol Biol Cell* 1998;9:2145–2156. [PubMed: 9693372]
111. Casellas R, Brivanlou AH. *Dev Biol* 1998;198:1–12. [PubMed: 9640328]
112. Souchelnytskyi S, Nakayama T, Nakao A, Moren A, Heldin CH, Christian JL, Ten Dijke P. *J Biol Chem* 1998;273:25364–25370. [PubMed: 9738003]
113. Piek E, Afrakhte M, Sampath K, van Zoelen EJ, Heldin CH, Ten Dijke P. *J Cell Physiol* 1999;180:141–149. [PubMed: 10395283]

114. Barillas-Mury C, Charlesworth A, Gross I, Richman A, Hoffmann JA, Kafatos FC. EMBO J 1996;15:4691–4701. [PubMed: 8887560]
115. Sanchez-Salazar J, Pletcher MT, Bennett RL, Brown SJ, Dandamudi TJ, Dennell RE, Doctor JS. Dev Gene Evol 1996;206:237–246.
116. Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G, Hetru C, Hoa NT, Hoffmann JA, Kanzok SM, Letunic I, Levashina EA, Loukeris TG, Lycett G, Meister S, Michel K, Moita LF, Muller HM, Osta MA, Paskewitz SM, Reichhart JM, Rzhetsky A, Troxler L, Vernick KD, Vlachou D, Volz J, von Mering C, Xu J, Zheng L, Bork P, Kafatos FC. Science 2002;298:159–165. [PubMed: 12364793]
117. Luna C, Wang X, Huang Y, Zhang J, Zheng L. Insect Biochem Mol Biol 2002;32:1171–1179. [PubMed: 12213252]
118. Tauszig S, Jouanguy E, Hoffmann JA, Imler JL. Proc Natl Acad Sci USA 2000;97:10520–10525. [PubMed: 10973475]
119. Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB. Proc Natl Acad Sci USA 1987;84:6020–6024. [PubMed: 2888109]
120. Twardzik DR, Mikovits JA, Ranchalis JE, Purchio AF, Ellingsworth L, Ruscetti FW. Ann N Y Acad Sci 1990;593:276–284. [PubMed: 2115757]
121. Nunes I, Shapiro RL, Rifkin DB. J Immunol 1995;155:1450–1459. [PubMed: 7636210]
122. Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, Jacob HS. Lab Invest 1991;64:648–655. [PubMed: 2030579]
123. Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM. Proc Natl Acad Sci USA 1993;90:9285–9289. [PubMed: 8415693]
124. Dix TA, Fontana R, Panthani A, Marnett LJ. J Biol Chem 1985;260:5358–5365. [PubMed: 4039321]
125. Luckhart S, Crampton AL, Zamora R, Lieber MJ, Dos Santos PC, Peterson TML, Emmith N, Lim J, Wink DA, Vodovotz Y. Infect Immun 2003;71:3000–3009. [PubMed: 12761076]
126. Wink, DA.; Feelisch, M.; Vodovotz, Y.; Fukuto, J.; Grisham, MB. Reactive Oxygen Species in Biological Systems: An Interdisciplinary approach. Colton, CA.; Gilbert, DL., editors. Kluwer Academic / Plenum Publishing; New York: 1999. p. 245-291.
127. Wakefield LM, Smith DM, Broz S, Jackson M, Levinson AD, Sporn MB. Growth Factors 1989;1:203–218. [PubMed: 2629880]
128. Brown PD, Wakefield LM, Levinson AD, Sporn MB. Growth Factors 1990;3:35–43. [PubMed: 2200452]
129. Muller HM, Catteruccia F, Vizioli J, della TA, Crisanti A. Exp Parasitol 1995;81:371–385. [PubMed: 7498434]
130. Sanderson N, Factor V, Nagy P, Kopp J, Kondaiah P, Wakefield L, Roberts AB, Sporn MB, Thorgeirsson SS. Proc Natl Acad Sci USA 1995;92:2572–2576. [PubMed: 7708687]
131. Levashina EA, Moita LF, Blandin S, Vriend G, Lagueux M, Kafatos FC. Cell 2001;104:709–718. [PubMed: 11257225]
132. Lavine M, Strand M. Insect Biochem Mol Biol 2002;32:1295–1309. [PubMed: 12225920]
133. Wahl SM. J Exp Med 1994;180:1587–1590. [PubMed: 7964446]

Abbreviations

As60A

Anopheles stephensi 60A

AsNOS

Anopheles stephensi nitric oxide synthase

BMP

Bone morphogenetic protein

CRE

Cyclic Adenosine Monophosphate Responsive Element

DEANO	Diethylamine-NONOate
dpp	Decapentaplegic
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
gbb-60A	Glass bottom boat-60A
Hb	Hemoglobin
iNOS	Inducible nitric oxide synthase
IFN	Interferon- γ
LAP	Transforming growth factor- β latency-associated peptide
LTBP	Latent transforming growth factor- β binding protein
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
RT-PCR	Reverse transcription polymerase chain reaction
scw	Screw
Smad	Mothers Against Decapentaplegic Homolog
SNAP	S-nitroso-N-acetylpenicillamine
STAT	Signal Transducer And Activator of Transcription
TGF-β	Transforming growth factor- β
TNF	Tumor necrosis factor- α

Figure 1.

TGF-β superfamily cross-species functionality. Components of the *Drosophila* 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 *Drosophila* protein functioned properly in mammalian cells and/or the mammalian protein functioned properly in *Drosophila* cells. Medea and Dad are boxed to indicate that their activities are likely relevant to both BMP and TGF-β/activin signaling pathways. Signal terminators for *Drosophila* gbb-60A and activin are unknown. See text for references.

Figure 2.

Inhibition of LAP bioactivity by NO. The ability of LAP to neutralize active TGF-β1 was assessed using an ELISA specific for active TGF-β1. Binding of bioactive recombinant human LAP to recombinant active TGF-β1 reduces the binding of active TGF-β1 to the capture reagent (type II TGF-β receptor). In contrast, LAP that has been S-nitrosated cannot bind to active TGF-β1. Treatment of active TGF-β1 with the chemical NO donor SNAP does not affect binding of active TGF-β1 to the type II receptor.

Figure 3.

S-nitrosothiols are present on LAP following reaction with NO. The S-nitrosothiol content of LAP was assessed by characteristic absorbance at 335 nm following incubation with SNAP. Furthermore, S-nitrosothiols were displaced from S-nitroso-LAP by reaction with HgCl₂ at various molar ratios of LAP:Hg²⁺. Decreases in O.D.₃₃₅ were observed up to a LAP:Hg²⁺ of 1:3, suggesting that three cysteine residues might be S-nitrosated on LAP.