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Rickettsial pathogen uses arthropod tryptophan pathway metabolites to evade reactive oxygen species in tick cells

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Abstract

Reactive oxygen species (ROS) that are induced upon pathogen infection plays an important role in host defense. The rickettsial pathogen *Anaplasma phagocytophilum*, which is primarily transmitted by *Ixodes scapularis* ticks in the United States, has evolved many strategies to escape ROS and survive in mammalian cells. However, little is known on the role of ROS in *A. phagocytophilum* infection in ticks. Our results show that *A. phagocytophilum* and hemin induces activation of L-tryptophan pathway in tick cells. Xanthurenic acid (XA), a tryptophan metabolite, supports *A. phagocytophilum* growth in tick cells through inhibition of tryptophan dioxygenase (TDO) activity leading to reduced L-kynurenine levels that subsequently affects buildup of ROS. However, hemin supports *A. phagocytophilum* growth in tick cells by inducing TDO activity leading to increased L-kynurenine levels and ROS production. Our data reveal that XA and Kynurenic acid (KA) chelate hemin. Furthermore, treatment of tick cells with 3-hydroxyl L-kynurenine limits *A. phagocytophilum* growth in tick cells. RNAi-mediated knockdown of kynurenine aminotransferase expression results in increased ROS production and reduced *A. phagocytophilum* burden in tick cells. Collectively, these results suggest that L-tryptophan pathway metabolites influence *A. phagocytophilum* survival by affecting build of ROS levels in tick cells.

Keywords

Anaplasma phagocytophilum; *Ixodes scapularis*; *Rickettsia*; Xanthurenic acid (XA); Kynurenic acid (KA); hemin; tryptophan metabolites; reactive oxygen species (ROS)

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Conflict of interest

Authors declare no conflicts of interests.

Introduction

Ticks are important vectors that transmit several pathogens to humans and wide range of animals (Dumler *et al.*, 2005; Foley *et al.*, 2011). In the United States, *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis (HGA), originally termed as human granulocytic ehrlichiosis (HGE), is primarily transmitted by *Ixodes persulcatus* complex ticks including *Ixodes scapularis* in the Eastern region and *Ixodes pacificus* in the Western region (Dumler *et al.*, 2005; Foley *et al.*, 2011). Within ticks, *A. phagocytophilum* is not transovarially transmitted but can be maintained transstadially in different developmental stages (de la Fuente *et al.*, 2010; Rar and Golovljova, 2011). Upon entry into ticks through a blood-meal, *A. phagocytophilum* modulates several intracellular molecular signaling pathways in the vector host (Sultana *et al.*, 2010; Khanal *et al.*, 2017; Taank *et al.*, 2017; Alberdi *et al.*, 2019; Turck *et al.*, 2019). Some of the molecular pathways affected includes manipulation of immune response, remodeling of the cytoskeleton, metabolism, inhibition of apoptosis, and redox pathways (Rikihisa, 2010; Alberdi *et al.*, 2019). In response to the pathogen infection, tick cells may exhibit many strategies to fight against bacterial propagation. One of the defense mechanisms from host cells in response to bacterial pathogen infection is the production of Reactive Oxygen Species (ROS) (Sena and Chandel, 2012). The role of ROS in host defense is mainly mediated by Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) in phagosomes that lead to direct killing of pathogens (Sena and Chandel, 2012), (Holzerová and Prokisch, 2015).

Anaplasma phagocytophilum has evolved strategies to prevent NOX-mediated ROS production by inhibiting superoxide synthesis in both human and murine neutrophils (Rikihisa, 2010). Recently, it was reported that the redox system in ticks acts to limit *A. phagocytophilum* infection (Alberdi *et al.*, 2019). ROS also acts as a generic molecule that could bring together other active and reactive partially reduced O₂ metabolites including oxygen and hydroxyl radicals (O₂ •- and •OH, respectively) (Alberdi *et al.*, 2019). These later molecules can be defined as true free radicals that can undergo redox reactions by interacting with surrounding molecules forming stable non-radical conditions (Alberdi *et al.*, 2019). Whereas, hydrogen peroxide (H₂O₂) is more a pro-oxidant non-radical agent (Novo and Parola, 2008). The process on how *A. phagocytophilum* interacts with other free radicals is poorly understood.

The regulation of ROS is critical for its ability to participate in physiological process of the cell (Sena and Chandel, 2012). These regulatory events for ROS are controlled at several levels including negative feed-back mechanisms, mitochondrial localization, and function of antioxidant enzymes (Sena and Chandel, 2012). A number of active metabolites from kynurenine pathway was shown to act as peroxyl radical scavengers and antioxidants (Badawy, 2017). The kynurenine pathway is the primary route for tryptophan degradation (Badawy, 2017). The metabolites in the kynurenine pathway have different anti- and pro-oxidant properties that are essential in the maintenance of cell homeostasis (Han *et al.*, 2007). Among the kynurenine pathway metabolites, Xanthurenic acid (XA) was shown to act as an antioxidant in *Aedes aegypti* midgut during digestion of blood meal (Lima *et al.*, 2012) and Kynurenine was shown to prevent *Toxoplasma gondii* growth through induction of apoptosis (Majumdar *et al.*, 2019). The 3 hydroxyl- L-Kynurenine (3-HK), another

tryptophan metabolite, is shown to stimulate ROS production (Han *et al.*, 2007; Amaral and Outeiro, 2013). However, in another study, authors suggested that 3HK has powerful antioxidant property that lead to scavenging of peroxy radicals (Christen *et al.*, 1990). The 3-HK can also initiate the Nicotinamide metabolic pathway leading to the production of many biologically active metabolites (Badawy, 2017). Kynurenic acid (KA) is also shown to act as a potential endogenous antioxidant through its role on scavenging hydroxyl radicals, superoxide anion, and peroxynitrite (Campesan *et al.*, 2011; Breda *et al.*, 2016; Fujigaki *et al.*, 2017). Furthermore, Tryptophan 2,3 dioxygenase (TDO) and indolamine 2,3 dioxygenase (IDO), the two enzymes that initiate L-tryptophan catabolic pathways uses superoxide anion as a cofactor (Xu *et al.*, 2018). TDO and IDO can efficiently clear free radicals much more than that of superoxide dismutase enzyme (Xu *et al.*, 2018).

Our previous study reported involvement of tryptophan pathway in the establishment of *A. phagocytophilum* infection in ticks mainly through XA and kynurenine aminotransferase (KAT), an enzyme important in the production of XA and KA (Taank *et al.*, 2017). The purpose of the present study is not only to investigate the effect of other tryptophan pathway metabolites on *A. phagocytophilum* infection in tick ISE6 cells but also to establish the consequence of redox state and ROS production on bacterial growth in these cells.

Methods

Bacterial isolates, Ticks and tick cell line.

A. phagocytophilum isolate NCH-1 was used throughout this study and referred as *Aph* in figures. *Escherichia coli* JM109 strain was used as a cloning host for generating different pGEM-T plasmids. Ticks used in this study were obtained from continuously maintained colony at the Department of Entomology, Connecticut Agricultural Experiment Station (New Haven, CT). The *I. scapularis* tick cell line *ISE6* was maintained as previously described (Taank *et al.*, 2017). Tick rearing was conducted in an incubator at $23 \pm 2^\circ\text{C}$ with 95% relative humidity and a 14/10-hour light/dark photoperiod regiment.

Mice and Ethics Statements.

C3H/HeN mice (4–6 weeks, females, CharlesRiver Laboratories, USA) were used in this study. Uninfected or *A. phagocytophilum*-infected unfed nymphs were generated by feeding larvae on uninfected or *A. phagocytophilum*-infected mice and then molted to nymphs as previously described (Taank *et al.*, 2017). All animal studies were carried out following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The animal protocol (permit number: 16–017 and 19–009) approved by the Old Dominion University Institutional Animal Care and Use Committee (Animal Welfare Assurance Number: A3172–01) was used in this study. Acepromazine was used as a tranquilizer that was administered into mice prior to handling.

RNA or DNA extractions and quantitative Real-time PCR (QRT-PCR) Analysis.

Total RNA or DNA extraction from ISE6 tick cells or ticks, and QRT-PCR were performed as previously reported (Taank *et al.*, 2017) using Aurum Total RNA mini kit (Bio-Rad, USA) and Qiagen DNeasy blood and tissue kit (Qiagen, USA). The cDNA was prepared from

RNA using BioRAD cDNA synthesis kit (BioRAD, USA). QRT-PCR reactions were performed using iQ-SYBR Green Supermix (BioRad, USA) as described (Khanal *et al.*, 2017; Taank *et al.*, 2017). Oligonucleotides used in the study are mentioned in Supplementary Table 1. As an internal control and to normalize the amount of template, *I. scapularis* beta-actin was quantified using oligonucleotides as mentioned in Supplementary Table 1. Total DNA or known amounts of actin was also used as an internal control to normalize amount of template used in each reaction. In QRT-PCR reactions, the standard curve was generated using 6-fold serial dilutions starting from 10^{-2} to 10^{-7} ng of known quantities of respective fragments.

dsRNA synthesis:

The dsRNA against *kat* transcript was generated as previously described (Taank *et al.*, 2017). *kynu 1* dsRNA was first generated by amplifying the gene sequence from cDNA obtained from ticks using primers listed in Supplementary Table 1. PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA), according to the manufacturer's instructions. The recombinant plasmid was transformed and propagated in *E. coli* JM109, and these plasmids were sequenced at Eurofins USA and used as templates to amplify the corresponding gene fragments with primers that contain T7 promoter sequence overhangs (Supplementary Table 1). The dsRNA for each gene was synthesized using the MEGAscript RNAi Kit (Ambion Inc.) following manufacturer's instruction.

Reagent and stock solution preparations.

XA, KA, 3-HK and Ro-61–8048 was obtained from Sigma Aldrich, USA. Hemin was purchased from MP Biomedicals (Fisher Scientific, USA). L-kynurenine was obtained from Thermoscientific, USA. Stocks (10 mM) of XA, KA, hemin or Ro-61–8048 were made by dissolving corresponding weight in 1ml 0.5 N NaOH solutions. The volume was then increased to 10 ml by adding 1x PBS. The solution was then filtered through 0.45 μ m filter. Serial dilution of 1:10 was made in 1x PBS to obtain smaller concentrations. The highest concentration of the vehicle (NaOH) used in the experiment was 0.005N contained in 1mM solution of each compound. Mock solution was prepared to contain 0.005N of NaOH and used in all experiments as control. L-kynurenine stock solution of 50 mM was made by dissolving required weight in 1ml 0.5M HCL. The volume was then increased to 10 ml by adding 1x PBS. The solution was filtered through 0.45 μ m filter. Two serial dilutions of 1:10 and 1:5 were made in 1x PBS to obtain 1 mM solution of L-kynurenine (with 0.001 M HCL final concentration). Mock solutions that contained 0.005N NaOH and 0.001M HCL were made and used in experiment with L-kynurenine. The other stock solution of L-kynurenine was made from 1:10 dilution in 1x PBS. Mock solution was first tested on ISE6 cells. Treatment revealed no change in cell morphology and media color at 24 and 48h post-treatments (Supplementary Fig. 2). Finally, the 10 mM stock solution of 3HK was dissolved in 1xPBS, and lower concentration solutions were made by serial dilution of 1:10.

Tick cell line experiments and reactive oxygen species measurement.

Strain *A. phagocytophilum* NCH-1 was maintained in human promyelocytic cell line (HL-60, American Type Culture Collection, USA), and cell-free bacteria isolated from these cells were used for *in vitro* infection of ticks cells as described (Thomas and Fikrig, 2007).

Tick cells (3×10^5) were seeded onto 12 well plates and incubated for 24 h. In experiment involving only XA or KA, tick cells were treated with different concentrations of XA or KA (1, 10, 100 μ M). Equal volumes of mock solution (corresponding to 100 μ M volume) were added to control cells. Where hemin is involved, cells were seeded into two groups with or without hemin. For the group without hemin, cells were treated with 100 μ M of XA or KA and equal volumes of mock solution (corresponding to 100 μ M volume) as control. For the groups with hemin treatment, cells were treated as above, then, 100 μ M final concentration of hemin was added to each well. The mock group was also treated with 100 μ M final concentration of hemin and an equal volume of mock solution (corresponding to the volume for 100 μ M concentration stocks). To allow the comparison between the two groups with or without hemin, equal volume of mock solution (corresponding to 100 μ M volume) was added to the cells without hemin. After 4 h of treatment, tick cells were infected with *A. phagocytophilum* and incubated for 24 h. Cells were then harvested and divided in two equal portions, one was subjected to ROS quantification and the other portion for RNA or DNA extractions to measure mRNA transcripts of tick genes or *A. phagocytophilum* load, respectively. Similar methodology was used when Ro-61–8048, L-kynurenine, or 3HK was analyzed in the experiments with or without XA or KA. ROS quantification was measured using DCFDA/H2DCFDA-Cellular ROS Assay Kit (Abcam, USA) following the manufacturer's recommendations. Fluorescence was measured using fluorimeter (Tecan, USA) with excitation at 495nm and emission at 529nm.

Tick cell line silencing experiments and microscopy.

dsRNA knockdown was performed as previously described (Taank *et al.*, 2017). Briefly, gene expression silencing experiments in tick cells were performed with Lipofectamine transfection reagent (ThermoFisher Scientific, USA). 4×10^5 tick cells were plated in L-15B300 medium on 12 well plates and incubated for 24 h followed by addition of dsRNA mixed with Lipofectamine reagent. 2x L15-B300 medium was added after 6 hours and plates were further incubated for 16 h. Cell free *A. phagocytophilum* isolated from HL60 cells was added after 24 h post transfection and tick cells were incubated for additional 24 h and processed for ROS quantification or for analysis of bacterial loads. Microscopic images of tick cells upon treatment with different metabolites were collected with EVOS FL Microscope. Representative images are shown in supplementary figure 2. Silencing efficiency and bacterial burden were measured as described (Khanal *et al.*, 2017; Taank *et al.*, 2017).

TDO assay and L-Kynurenine assay.

The enzymatic assay was modified from a method previously described study (Gibney *et al.*, 2014). Briefly, tick cells were seeded onto 12 well plates at a density of 2×10^5 cells/well. Treatment of the tick cells with different metabolites was performed as described above. Following the infection, uninfected and *A. phagocytophilum*-infected tick cells were harvested at 24h pi. Tick cells were homogenized in 1x PBS, and 10 μ l 30% (v/v) trichloroacetic acid was added to each well. Cells were incubated for 30 min at 50° C to analyze hydrolysis of N-formylkynurenine to kynurenine. After incubation, cells were centrifuged at $3000 \times g$ for 10 min, and 100uL of the supernatant was transferred to 96-well flat-bottom plate and mixed with equal volume of Ehrlich's reagent (in glacial acetic acid)

and absorbance was measured at 480 nm. A standard curve was generated with defined concentrations of L-kynurenine (0–400 μ M; Thermofisher, USA). N-formylkynurenine can be hydrolysed to L-kynurenine by heating. This reaction represents TDO activity. Kynurenine concentrations in the supernatants were determined by comparing to the standard curve. TDO activity in the supernatants is presented as μ M of L-kynurenine. For the L-kynurenine assay, cells were processed as above but without hydrolyzing the N-formylkynurenine to kynurenine.

Hemin titration.

Heme titrations were performed by difference spectroscopy at room temperature and aerobically using TECAN spectrophotometer as described (Soto *et al.*, 2012). Briefly, we used 5 μ M of XA or KA and hemin in 50 mM Tris buffer pH 8.0. Hemin was dissolved in 5% DMSO to minimize its oligomerization. A baseline was selected by scanning samples from 300 to 700 nm. The samples used were 50 mM Tris buffer pH 8.0 in the reference cuvette and 50 mM Tris buffer pH 8.0 with 5 μ M of XA or KA in the sample cuvette. Once the baseline was set, hemin (at equal concentration) was added to both reference and sample cuvettes. For this assay, we used low concentration of XA or KA (5 μ M) and increasing concentrations of hemin (5–90 μ M). We first scanned for XA or KA in the sample cuvettes and compared the readings from the reference cuvette containing buffer only (50 mM Tris pH:8.0) in the wavelength range from 300–700 nm. This number was considered as a baseline reading. Furthermore, hemin (with increasing concentrations) was added to both the samples and to the reference cuvettes. The obtained readings reflect only the binding of XA or KA with hemin and not the absorbance of the free hemin. The data obtained from the heme-XA or heme-KA titrations were plotted in Microsoft Excel 2016 and fit to an equation ($Y = B_{max} * X / (K_d + X)$) that describes a single binding site.

Statistics.

Statistical significance in the data sets was analyzed using Microsoft Excel 2016. Data was presented as mean and standard deviation. Lines on the top of histogram represent standard deviation. For data to compare two means, the non-paired Student t-test was performed. P values of < 0.05 were considered significant in all analysis. Wherever necessary, statistical test and P values used are shown.

Results

***Ixodes scapularis* and *I. ricinus* have conserved pathway for NAD(P)⁺ biosynthesis from the L-tryptophan pathway:**

Nicotinamide adenine dinucleotide (NAD) exists either as NAD⁺ (oxidized form) and NADH (reduced form). The NAD⁺ is produced by salvage pathway by recycling preformed components such as nicotinamide to NAD⁺ or by de novo synthesis from L-Tryptophan (Magni *et al.*, 2004). We searched tryptophan catabolic pathway gene orthologs in phylum Arthropoda using three steps. First, we retrieved nucleotide and annotated protein sequences of *I. scapularis* genes involved in the tryptophan pathway from the information available on KEGG database (Fig. 1A). Each amino acid sequence was used as a query to search orthologs from other arthropods in National Center for Biotechnology Information (NCBI)

and VectorBase databases. Secondly, we have used nucleotide sequence for each ortholog as a query in BLASTn search at NCBI against genomes of other arthropods. Finally, each annotated amino acid sequence retrieved from the first two searches was blasted against NCBI conserved domain to predict conserved domain(s). The results that were retrieved from only complete genomes were considered in the analyses. We have used the sequences from Chordata, and Mollusca, as an out-group, to allow the comparison with upper eukaryotic species (Fig. 1B). In mammals, the kynurenine pathway is initiated via the catalysis of L-tryptophan to N-formylkynurenine by tryptophan 2,3-dioxygenase (TDO, KEGG enzyme entry number EC: 1.13.11.11) and Indolamine di-oxygenase (IDO, EC: 1.13.11.52). The organisms in phylum Arthropoda have lost IDO and this enzyme seems to be conserved only in upper eukaryotes and mammals, making Arthropoda depending on TDO to catalyze the first reaction. Duplication of TDO is seen in some species including Homoptera *Acyrtosiphon pisum*, and the Lepidoptera *Plutella xylostella* and *Helicoverpa armigera* (Fig. 1B). Arylformamidase (KYF) (EC: 3.5.1.9) is a hydrolase that catalyzes the transformation of N-formylkynurenine to L-Kynurenine (L-Kyn). This enzyme is also involved in the glyoxylate and dicarboxylate metabolism pathway making it as not specific to the tryptophan metabolism. Arylformamidase is lost in Arachnida *Tetranychus urticae*, *Dermatophagoides pteronyssinus*, *Sarcoptes scabiei* and *Leptotrombidium deliense* (Fig. 1B). Arylformamidase is *duplicated* in the Hymenoptera *Pseudomyrmex gracilis*, and the Coleoptera *Tribolium castaneum* and *Dendroctonus ponderosae* (Fig. 1B).

Through the L-Kynurenine catabolic pathway, 3 enzymes use L-Kynurenine as a substrate to initiate 3 different pathways (Fig. 1A). Kynurenine aminotransferase (KAT, EC: 2.6.1.7) initiates the synthesis of Kynurenate. Kynureninase (KNYU, EC: 3.7.1.3) initiates Anthranilate pathway, and kynurenine 3-monooxygenase (KMO, EC:1.14.13.9) catalyzes the hydroxylation of L-kynurenine to 3-hydroxy-L-kynurenine (3-HK) (Fig. 1A). KAT can then use 3-HK as a substrate to initiate the Xanthurenate pathway (Fig. 1A). KMO seems to be conserved in all arthropods (Fig. 1B). Lepidoptera species like *Danaus plexippus*, *Trichoplusia ni* and *Plutella xylostella* have duplicate copies of KMO (Fig. 1B). KAT seems to be under duplication in some arthropods (Fig. 1B). However, the number of *kat* gene copies varied among arthropods. Some arthropods (eg. Ixodid and *Phlebotomus papatasi*) have two copies and others (eg. Arachnida, *Leptotrombidium deliense*) have up to 10 copies of *kat* genes. However, most of the organisms belonging to order Diptera and Sarcophagales have only one copy of *kat* gene (Fig 1B). KNYU also acts upstream to L-kynurenine and catalyzes the cleavage of N-formylkynurenine to Formylanthranilate (Fig. 1A). Arylformamidase can then catalyze the transformation of the substrate to Anthranilate (Fig. 1A). With the available genome information, we were able to observe that KNYU is found only among Acari species including *I. scapularis* and *I. ricinus*, which seem to be under duplication (two copies). KNYU is also the key enzyme that links the catabolism of L-tryptophan pathway to Nicotinamide metabolism and the synthesis of NAD⁺ and NADP⁺ co-factors through the catabolism of 3-hydroxy-L-kynurenine and the initiation of Quinolinate pathway (Badawy, 2017). Absence of KNYU in a large group of arthropods suggests that the synthesis of NAD⁺ and NADP⁺ co-factors in these organisms occurs possibly from nicotinic acid (salvage pathway) rather than from quinolinic acid (main pathway).

Bioinformatic analysis of proteins that participate in Nicotinamide pathway (Supplementary Fig. 1) suggests that large number of arthropods use different strategies to synthesize NAD⁺ and NADP⁺. *Ixodes scapularis* and *I. ricinus* are able to initiate the synthesis from nicotinamide, niacin or quinolinate pathways (Badawy, 2017). The other arthropod species are undergoing either loss or gain of genes involved in the Nicotinamide pathway, including duplication events for some genes. As an example, *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Lutzomyia longipalpis*, and *Phlebotomus papatasi* are only able to use niacin to initiate complete NAD⁺ and NADP⁺ synthesis (Supplementary Fig. 1). *Aedes albopictus*, *Anopheles darlingi*, and *Glossina morsitans* are able to use the complete pathway from both nicotinamide and/or niacin (Supplementary Fig. 1). In summary, bioinformatics analysis indicates that *I. scapularis* and *I. ricinus* have complete enzymatic panel that may result in rapid scavenging and cleavage of L-tryptophan metabolites leading to the maintenance of Kynurenate, Xanthurenate and NAD(P)⁺ pool. Our analysis also indicated that large number of arthropods lacks KNYU that could result in increased accumulation of Kynurenate, Xanthurenate from L-tryptophan pathway. We believe that blood feeding arthropods that lack KYNH may depend on the acquisition of vitamin B3 group for NAD(P)⁺ biosynthesis.

Tryptophan catabolism pathway genes are upregulated in *I. scapularis* ticks and ISE6 tick cells upon *A. phagocytophilum* infection.

Our previous studies showed that *A. phagocytophilum* up-regulates expression of *kat*, a gene critical in L-tryptophan catabolism pathway (Khanal *et al.*, 2017; Taank *et al.*, 2017). In this study, we investigated whether *A. phagocytophilum* modulates expression of other genes in L-tryptophan pathway. QRT-PCR was performed with RNA samples extracted from uninfected and *A. phagocytophilum*-infected unfed nymphs (Fig. 2A–D) or tick cells (Fig. 2E–H). QRT-PCR analysis revealed significant ($P < 0.05$) upregulation of *tdo* (Fig. 2A and E), *kmo* (Fig. 2C and G) and *knyu* (Fig. 2D and H) gene transcripts in both *A. phagocytophilum*-infected unfed ticks (Fig. 2A–D) and tick cells (Fig. 2E–H) in comparison to respective uninfected controls. However, no significant ($p > 0.05$) difference in the expression of arylformamidase transcripts was noted between *A. phagocytophilum*-infected unfed ticks (Fig. 2B) or tick cells (Fig. 2F) in comparison to their respective uninfected controls (Fig. 2B and F). Taken together, results from this study and from our previous study (Taank *et al.*, 2017) indicates that most of the L-tryptophan catabolism pathway genes are upregulated upon *A. phagocytophilum* infection of ticks or tick cells.

Xanthurenic acid and Kynurenic acid acts differently on *A. phagocytophilum* growth in tick cells.

An antioxidant property for arthropod XA has been previously reported in mosquitoes (Lima *et al.*, 2012). In addition, our previous study showed that exogenous addition of XA increases *A. phagocytophilum* loads in tick salivary glands and ISE6 cells (Taank *et al.*, 2017). The observation of induced gene expression of many genes involved in the L-tryptophan catabolism pathway upon *A. phagocytophilum* infection of ticks or tick cells (Fig. 2), prompted us to investigate the effects of XA and KA on bacterial growth and on the production of Reactive Oxygen Species (ROS). Treatment of ISE6 tick cells with different concentrations of XA or KA (0.1–100 μ M) revealed no changes in cell morphology at 4 or

24 h post infection (p.i.) in comparison to mock-treated cells (Supplementary Fig. 2). After 24 h p.i., tick cells were harvested and split into two equal portions, one was subjected to ROS quantification and the other portion for DNA extraction. In mock-treated group, significantly ($p < 0.05$) increased ROS levels were noted in *A. phagocytophilum*-infected tick cells in comparison to the levels noted in uninfected control (Fig. 3A). However, upon 100 μ M XA treatment, significantly ($P < 0.05$) reduced ROS levels were observed in *A. phagocytophilum*-infected tick cells in comparison to levels observed in uninfected control (Fig. 3A). No differences in the ROS levels were noted between *A. phagocytophilum*-infected XA-treated and mock-treated groups (Fig. 3A). Treatment of tick cells with 0.1, 1 or 10 μ M concentration of XA showed no significant ($P > 0.05$) differences in the ROS levels between uninfected and *A. phagocytophilum*-infected groups (Supplementary Fig. 3A). QRT-PCR analysis revealed significantly ($P < 0.05$) increased *A. phagocytophilum* burden in tick cells upon treatment with 0.1, 1 or 100 μ M concentration of XA in comparison to mock-treated control (Fig. 3B and Supplementary Fig. 3B). Upon 100 μ M KA treatment, significantly ($P < 0.05$) reduced ROS levels were observed in *A. phagocytophilum*-infected tick cells in comparison to levels observed in KA-treated uninfected control (Fig. 3C). As like the observations noted with mock treatment, significant ($P < 0.05$) difference in ROS levels were noted upon treatment with lower concentrations (0.1, 1 μ M) of KA in uninfected or *A. phagocytophilum*-infected cells (Supplementary Fig. 3C). Significant ($P < 0.05$) increase in bacterial burden was noted in 0.1 μ M KA-treated *A. phagocytophilum*-infected cells in comparison to mock-treated control (Supplementary Fig. 3D). However, treatment of tick cells with other concentrations of KA (1, 10, 100 μ M) had no significant ($P > 0.05$) effect on bacterial growth in comparison to mock-treated control (Fig. 3D and Supplementary Fig. 3D). These results indicate that XA supports multiplication of *A. phagocytophilum* associated with reduction in the ROS level and the antioxidant effects from KA do not support bacterial multiplication in tick cells. In addition, these results suggest that the effect of XA and KA on *A. phagocytophilum* growth is concentration dependent and independent of ROS level.

Hemin supports *A. phagocytophilum* multiplication in tick cells.

Ixodes scapularis lacks the pathway to synthesize heme (Perner *et al.*, 2016). Therefore, these ticks are depend on the hemoglobin in the host blood meal to acquire heme (Narasimhan *et al.*, 2014). Hemin is an organic compound that can be formed from processed red blood cells in the blood meal. Previous report shows that upon *A. phagocytophilum* infection, expression of genes involved in tick hemoglobinolytic and heme transport pathways were upregulated (Villar *et al.*, 2016). A study has shown that heme has both pro-oxidant and cytotoxic effects (Yachie *et al.*, 2019). We therefore investigated whether exogenous treatment of hemin has any effect on *A. phagocytophilum* growth and ROS production. The ISE6 tick cells were treated with 100 μ M of hemin for 4 hours. Additionally, tick cells were treated with same concentrations of XA or KA along with hemin (100 μ M). *Anaplasma phagocytophilum*-infected mock-treated cells were used as controls. Cells were infected 4 h after treatment and harvested at 24 h p.i. to evaluate ROS levels and bacterial burden. Significantly ($P < 0.05$) high levels of ROS were noted in hemin-treated *A. phagocytophilum*-infected tick cells in comparison to the levels noted in *A. phagocytophilum*-infected mock-treated control (Fig. 3 E and Supplementary Fig. 3E).

Anaplasma phagocytophilum-infected cells treated with hemin along with XA or KA also showed significant ($P < 0.05$) higher levels of ROS in comparison to the levels noted in *A. phagocytophilum*-infected mock-treated control (Fig. 3E). Furthermore, significantly ($P < 0.05$) increased levels of ROS was observed in *A. phagocytophilum*-infected tick cells treated with both hemin and XA in comparison to the levels noted in *A. phagocytophilum*-infected tick cells treated with hemin only (Fig. 3E). Interestingly, a significant ($P < 0.05$) increase in *A. phagocytophilum* loads were noted in hemin-treated *A. phagocytophilum*-infected tick cells in comparison to the loads noted in mock-treated *A. phagocytophilum*-infected cells (Fig. 3F). However, significantly ($P < 0.05$) reduced bacterial loads were evident in *A. phagocytophilum*-infected tick cells treated with hemin along with XA or KA in comparison to the loads noted in *A. phagocytophilum*-infected cells treated only with hemin (Fig 3F). These results indicate that hemin alone, like XA, supports *A. phagocytophilum* growth in tick cells but in combination with XA or KA affects bacterial growth.

XA and KA chelate hemin.

The observation of reduced *A. phagocytophilum* loads in tick cells upon treatment with hemin along with XA or KA (Fig. 3F) suggested that the later metabolites could chelate hemin. We monitored the hemin titration and binding to XA or KA in separate experiments by UV-visible spectroscopy as reported (Soto *et al.*, 2012). Hemin exhibits absorbance in the UV-Vis spectrum due to a Soret band near 400 nm (Comer and Zhang, 2018). When 5 μM of XA was titrated with incremental concentration of hemin, we observed the Soret band shifting to 364 nm with a significant increase in its amplitude (Fig. 3G). The difference spectra at pH 8.0 (Absorbance) between hemin and hemin-binding to XA revealed a Soret band at 334, 372, 398, 611, and 657 nm (Fig. 3 G & H). The Absorbance at 334 nm showed a B_{max} value at 55 μM for hemin followed by saturation suggesting specific binding between hemin and XA (Fig. 3I). We have observed that XA binds hemin with good affinity (equilibrium dissociation constant, $K_D = 0.05 \pm 0.009 \mu\text{M}$). Furthermore, we measured the absorbance from free XA at 350 nm. The results showed reduction in the B_{max} value for XA with increase in concentration of hemin suggesting binding between both molecules and availability of less free XA (Fig. 3 J). We also monitored KA binding to hemin. We observed a shift in the hemin absorbance to 343 upon KA binding (Fig. 3K). The difference in the spectra at pH 8.0 (Absorbance) between hemin and hemin binding KA revealed a Soret band at 355 and 387 nm (Fig 3K & L). The Absorbance at 387 nm showed a B_{max} value at 55 μM for hemin then saturation was reached suggesting specific binding of this molecule to KA (Fig. 3 M). Furthermore, our results revealed that KA binds hemin with good affinity ($K_D = 0.04 \pm 0.009 \mu\text{M}$). The absorbance from free KA was monitored at 334 nm. The results showed reduction in the B_{max} value for KA as the concentration of hemin increased, suggesting binding between these molecules and availability of less free KA (Fig. 3 N). These results indicate that both XA and KA are potent hemin chelators.

Hemin, XA and KA modulate tryptophan dioxygenase activity.

Tryptophan dioxygenase (TDO) is the first rate-limiting enzyme in L-tryptophan pathway (Badawy, 2017). We therefore determined if hemin, XA or KA modulate TDO activity in tick cells. The enzymatic assay to assess the level of TDO activation was performed using

colorimetric method as previously reported (Gibney *et al.*, 2014). Cells were harvested at 24 h p.i. and processed for this assay. Significantly ($P<0.05$) increased TDO activity was observed in *A. phagocytophilum*-infected cells in comparison to the levels noted in uninfected control (Fig. 4A and Supplementary Fig. 4A). Upon hemin treatment (100 μ M), significantly ($P<0.05$) increase in TDO activity was observed in both uninfected and *A. phagocytophilum*-infected tick cells in comparison to respective control groups without treatment (Fig. 4A and Supplementary Fig. 4A and B). Furthermore, significantly ($P<0.05$) reduction in TDO activity was observed in *A. phagocytophilum*-infected cells upon treatment with XA (100 μ M) in comparison to the activity noted in *A. phagocytophilum*-infected mock-treated cells (Fig. 4B). In contrast, significantly increased TDO activity was noted in *A. phagocytophilum*-infected cells upon treatment with KA (100 μ M) in comparison to the activity noted in mock-treated cells (Fig. 4B). However, in the presence of hemin, significantly reduced TDO activity was noted in *A. phagocytophilum*-infected cells upon KA treatment in comparison to activity noted in *A. phagocytophilum*-infected cells treated with hemin alone (Fig. 4B). Even though not significant ($P>0.05$), reduced TDO activity was observed in hemin+XA-treated *A. phagocytophilum*-infected cells in comparison to hemin-alone treated cells (Fig. 4B). Collectively, these results not only indicate that infection or hemin treatment induces TDO activity but also reveal that XA and KA inhibit hemin-mediated impact on TDO activity.

Hemin, XA and KA modulate L-kynurenine levels in tick cells.

We then investigated the impact of TDO activity on the pool of L-Kynurenine in tick cells. We performed the experiment similar to the assay used to assess TDO activity, and L-Kynurenine level was measured without hydrolyzing N-formyl kynurenine to L-Kynurenine (Alegre *et al.*, 2005). This enzymatic assay gives the pool of L-Kynurenine accumulated in cells at 24 h p.i. Similar to the observation noted in TDO assay, the L-Kynurenine assay shows that *A. phagocytophilum* infection and hemin treatment significantly ($P<0.05$) increases the level of L-kynurenine in tick cells (Fig. 4C and Supplementary Fig. 4C). In both uninfected and *A. phagocytophilum*-infected hemin-treated cells, a significant ($P<0.05$) increase in the amount of L-kynurenine was noted compared to the levels observed in the respective mock-treated cells (Fig 4C and Supplementary Fig. 4D). Upon XA treatment, significantly ($P<0.05$) reduced L-kynurenine levels were noted in *A. phagocytophilum*-infected tick cells in comparison to the levels noted in mock-treated control (Fig. 4D). However, upon KA treatment, no significant ($P>0.05$) differences in the L-kynurenine levels were noted between KA-treated *A. phagocytophilum*-infected tick cells in comparison to mock-treated *A. phagocytophilum*-infected tick cells control (Fig. 4D). A significantly reduced L-kynurenine levels were noted in hemin + KA group in comparison to the levels noted in hemin-alone-treated *A. phagocytophilum*-infected tick cells (Fig. 4D). However, no significant differences in the L-kynurenine levels were observed between hemin-alone-treated and hemin+XA-treated *A. phagocytophilum*-infected tick cells (Fig. 4D). Taken together, these results indicate that infection or hemin treatment induces L-kynurenine levels, whereas, the presence of KA inhibits hemin-mediated effect on L-kynurenine levels.

Exogenous addition of L-kynurenine affects XA-mediated *A. phagocytophilum* growth in tick cells.

L-Kynurenine is the substrate used by KMO to synthesize the 3-hydroxy-L-kynurenine (3HK) (Fig. 1A). It has been reported that 3HK can scavenge peroxyl radicals (Thomas *et al.*, 1996). However, it is also reported that auto-oxidation of 3HK leads to the hyper production of ROS that damages cellular lipids, proteins, and DNA (Zhuravlev *et al.*, 2016). Recently, it was shown that overexpression of KMO creates a metabolic environment during which the cell exhibits increased tolerance for exogenous 3HK-mediated cellular injury (Wilson *et al.*, 2016). Using the selective KMO-inhibitor (Ro-61-8048), we checked the effect of KMO inhibition on ROS production and *A. phagocytophilum* growth in tick cells. Our results show that in the absence of hemin, treatment of *A. phagocytophilum*-infected cells with Ro-61-8048 (100 μ M) had no significant ($P < 0.05$) impact on ROS (Fig. 4E) or bacterial levels (Fig. 4F) compared to their respective controls. Whereas, in the presence of hemin, treatment of *A. phagocytophilum*-infected cells with Ro-61-8048 showed significant reduction in ROS level (Fig. 4E) and significant increase in bacterial loads (Fig. 4F) compared to their respective control groups. These results suggest that hemin-mediated ROS induction is due to the cleavage of L-Kynurenine to 3-HK by KMO. Furthermore, in the absence of hemin, treatment of *A. phagocytophilum*-infected tick cells with different doses of L-kynurenine (1, 10, 100 μ M) along with XA resulted in significantly ($P < 0.05$) reduced bacterial burden in comparison to levels noted upon treatment with XA-alone (Fig. 4G). These results support the conclusion that XA mediates *A. phagocytophilum* growth by reducing TDO activity that leads to the decreased accumulation of L-kynurenine in tick cells. The overall data suggest that the level of conversion of L-Kynurenine to 3-HK impacts *A. phagocytophilum* multiplication in tick cells.

Exogenous treatment with 3 hydroxyl L-Kynurenine affects *A. phagocytophilum* growth in tick cells.

Next, we investigated if the accumulation of 3HK that results from the increased flux in the kynurenine pathway could influence bacterial growth. We treated *A. phagocytophilum*-infected tick cells with two different concentrations of 3HK (10 and 100 μ M) in the absence or presence of hemin. The results showed dose-dependent response in ROS production (Fig. 5A) and effect on bacterial burden (Fig. 5B). In hemin non-treated cells, we noted significantly ($p < 0.05$) increase in ROS production in *A. phagocytophilum*-infected tick cells upon treatment with 100 μ M of 3HK compared to the levels noted with 10 μ M of 3HK or mock treatment (Fig. 5A). In contrast, significantly ($P < 0.05$) reduced bacterial burden was noted upon treatment with 100 μ M of 3HK in comparison to the burden noted upon treatment with 10 μ M of 3HK or mock control (Fig. 5B). In the presence of hemin, treatment with 100 μ M 3HK significantly ($P < 0.05$) reduced ROS production (Fig. 5A) and bacterial burden (Fig. 5B) in *A. phagocytophilum*-infected tick cells in comparison to their respective infected mock-treated controls. Even though ROS levels were significantly low (Fig. 5A), no significant difference in the bacterial burden (Fig. 5B) was noted upon treatment with 10 μ M 3HK. The observation of reduced bacterial loads, even when ROS levels were low at 24 h p.i., suggests that 3HK could induce ROS at earlier time point resulting in clearance of bacteria from tick cell. To test if 3HK induces ROS at earlier time point, we performed an experiment with uninfected tick cells and measured ROS production after 3 h addition of

3HK. In the absence of hemin, no effect of 3HK on ROS production was noted in uninfected tick cells at both tested (10, 100 μ M) doses (Fig. 5C). However, in the presence of hemin, significantly ($P<0.05$) reduced ROS levels were noted upon treatment with 3HK at both tested doses (10, 100 μ M) in comparison to the untreated control (Fig. 5C). The overall results indicate that hemin could lead to rapid elimination of 3HK leading to scavenging of ROS. However, in the absence of hemin, accumulation of 3HK could lead to its autooxidation and thereby increases ROS levels.

Knockdown of kynurenine aminotransferase (KAT) and kynureninase lead to the inhibition of *A. phagocytophilum* growth.

Our previous study showed that knockdown of *kat* gene expression by RNAi leads to significantly reduced bacterial burden in tick cells (Taank *et al.*, 2017). In this study, we determined whether knockdown of *kat* has any impact on ROS production. In addition, the observation of reduced *A. phagocytophilum* burden upon 3HK treatment suggested testing the effect of kynureninase knockdown on ROS production and bacterial burden in tick cells. Knockdown efficiency for each targeted gene is shown in Figures 6A and B. In the absence of hemin, significantly ($P<0.05$) increased ROS levels were noted in *kat*-dsRNA-treated *A. phagocytophilum*-infected cells in comparison to the levels noted in mock-treated *A. phagocytophilum*-infected cells (Fig. 6C). However, no significant differences in ROS levels were noted between *knyu*-dsRNA-treated *A. phagocytophilum*-infected cells and mock-treated *A. phagocytophilum*-infected cells (Fig. 6C). However, double knock-down of both *kat* and *knyu* gene expression resulted in significantly increased ROS levels in *kat+knyu*-dsRNA treated *A. phagocytophilum*-infected cells in comparison to the mock-treated control (Fig. 6C). In the presence of hemin, knockdown of gene expression for *kat* or *knyu* or both resulted in significantly ($P<0.05$) reduced ROS levels in comparison to the mock-treated control (Fig. 6C). QRT-PCR analysis revealed significantly reduced bacterial burden upon knockdown of gene expression for *kat* or *knyu* or both in comparison to mock-control in presence or absence of hemin (Fig. 6D). These results suggest that metabolism of 3HK by KAT and KYNU is critical for *A. phagocytophilum* survival in tick cells.

Discussion

Over the last decade, several studies have implicated the role of tryptophan metabolism in many physiological pathways (Badawy, 2017; Davis and Liu, 2017). Almost 99% of the ingested tryptophan not used for protein synthesis is catabolized in the kynurenine pathway (Davis and Liu, 2017). Initial studies were focused on the characterization of kynurenine pathway in the biogenesis of nicotinamide adenine dinucleotide (NAD) (Badawy, 2017). However, recent studies link kynurenine pathway with neurodegenerative diseases, tumor proliferation, inflammation, immunity, and depression (Zhuravlev *et al.*, 2016; Badawy, 2017; Davis and Liu, 2017; Höglund *et al.*, 2019). Furthermore, L-tryptophan pathway was also reported to have a role during infections caused by virus like HIV (Lyons *et al.*, 2014) and bacteria like *Rickettsia conorii* (Feng and Walker, 2000) and *A. phagocytophilum* (Taank *et al.*, 2017). The L-tryptophan pathway leads to accumulation of many active metabolites (Sas and Szab, 2018). KA and quinolinic acid are most studied due to their neuroactive roles in brain. TDO, IDO, 3-HK, XA and 3-hydroxyanthranilic acid have roles

in redox system (Reyes Ocampo *et al.*, 2014). Even though most of the experiments in this study were performed with tick cells and might not reflect events in live ticks, we believe that this study provides important evidence that indicates the impact of L-tryptophan pathway metabolites on the survival of *A. phagocytophilum* in its vector host.

Bioinformatic analysis of the Arthropoda genes involved in L-tryptophan and nicotinamide pathways showed that *I. scapularis* and *I. ricinus* have conserved the link between the kynurenine and nicotinamide pathways through duplication of KNYU enzyme. We believe that this would facilitate the two tick species to synthesize NAD(P)⁺ from the accumulation of bioactive kynurenine metabolite. The presence of complete enzymatic panel of genes in *I. scapularis* and *I. ricinus* ticks suggests that L-tryptophan is rapidly cleaved to other downstream metabolites including KA, XA and NAD(P)⁺ in these ticks. KNYU enzyme seems to be lost in many arthropods including blood-feeding arthropods suggesting that these species depend on niacin and nicotinamide to insure the pool of NAD⁺ and NADP⁺ co-factors. The loss of KNYU in many arthropods suggests increased levels of L-kyn and 3HK that subsequently may lead to more accumulation of XA in them. This notion supports previous study that reported observation of abundant amounts of XA in *Aedes aegypti* mosquito (Lima *et al.*, 2012). We noted that arthropods have lost IDO and retained TDO in them. Even though TDO has low affinity to L-tryptophan compared to IDO, it can efficiently catabolize this metabolite (Badawy, 2017). IDO, unlike that of TDO, can be inhibited at high L-tryptophan concentration (Badawy, 2017). L-tryptophan is an essential amino acid. Therefore, acquisition of this amino acid is completely dependent on the diet (Lindseth *et al.*, 2015). It is reasonable to hypothesize that substrate inhibition of IDO by L-Tryptophan might have led to the loss of this gene in arthropods.

Anaplasma phagocytophilum is transstadially maintained in different developmental stages of ticks. During molting, ticks may elicit ROS response to control bacterial replication in unfed stage. We therefore used unfed ticks to analyze the expression of genes involved in L-tryptophan pathway. The observation of increased expression of all arthropod genes involved in L-tryptophan pathway suggests that *A. phagocytophilum* infection activates this pathway in both ticks and tick cells. We monitored the production of ROS in each experiment due to the reason that the metabolites from the kynurenine pathway are associated with the redox state of the cell (Sas and Szab, 2018). Our data indicated that XA and KA metabolites act differently on ROS production and bacterial growth. XA allows bacterial growth and protect cells by not allowing buildup of ROS upon *A. phagocytophilum* infection. At high concentration, KA reduces ROS levels without having an impact on *A. phagocytophilum* growth. However, at low concentration, KA induces both ROS and bacterial growth. Previous studies have shown that XA and KA acts as antioxidants, removing reactive-oxygen and -nitrogen and enhance cell survival against free radical damage (Xu *et al.*, 2018). KA is a competitive inhibitor of the glycine co-agonist site of the N-methyl-D-aspartate (NMDA) receptor (Campesan *et al.*, 2011; Castellano-gonzalez *et al.*, 2019). KA inhibits the release of glutamate in a dose-dependent manner (Campesan *et al.*, 2011). Recent study reported that ROS production by activated murine neutrophils upon bacterial infection is mediated by glutamate release through the action on NMDA receptor. The release of neurotoxic quantities of the neurotransmitter glutamate is shown to activate neuronal NADPH oxidase leading to the injurious production of superoxide (del Arroyo *et al.*, 2019).

The inhibition of NMDA receptor leads to the reduction of ROS production (del Arroyo *et al.*, 2019). *Anaplasma phagocytophilum* is totally dependent on exogenous acquisition of L-glutamine or L-glutamate (Huang *et al.*, 2007). From our study, it seems that at high concentration, KA may act on NMDA receptor for the retention of L-glutamate. This hypothesis is also supported from previous observation that reported decrease in L-glutamate in *A. phagocytophilum*-infected ISE6 cells (Cabezas-Cruz *et al.*, 2017).

The observation of increased ROS levels and reduced bacterial growth upon treatment of tick cells with hemin in combination with XA or KA suggests that tryptophan metabolites are controlling the action of hemin in cells. Our study also showed that hemin is chelated by XA or KA. This binding could block the functional activity of XA or hemin in promoting bacterial growth in tick cells. We found that *A. phagocytophilum* infection, hemin and KA induce TDO activity and XA inhibits TDO activity. The reduced TDO activity noted upon treatment of tick cells with hemin in combination XA or KA could be due to the chelating property of tryptophan metabolites with hemin. TDO is synthesized as an apoenzyme that interacts with heme to become heme-containing holoenzyme. Hemin is also used by TDO as a cofactor (Badawy, 2017). Ticks lack complete pathway to synthesize heme (Perner *et al.*, 2016). Therefore, observation of increased TDO activity upon hemin treatment of tick cells is not surprising. Collectively, our data suggests that hemin, XA and KA have synergistic effect on L-tryptophan pathway to modulate bacterial growth and ROS levels.

Our data suggests that hemin-mediated influence on *A. phagocytophilum* growth is associated with increased levels of L-kynurenine. L-kynurenine is cleaved by 3 enzymes including KAT, KMO and KMO (Badawy, 2017). However, KMO is considered as the major route for the kynurenine pathway because of its high affinity for L-kynurenine than other enzymes (Fujigaki *et al.*, 2017; Castellano-gonzalez *et al.*, 2019). Kynurenine oxidized by KMO results in the formation of 3-HK, a metabolite that can stimulate production of reactive oxygen species (Han *et al.*, 2007; Campesan *et al.*, 2011). The observation of reduced ROS levels and increased bacterial burden upon KMO inhibition in the presence of hemin could be due to low 3-HK levels. This data indicates that hemin-mediated induction of ROS production could be due to conversion of L-kynurenine to 3-HK. The observation of reduced TDO activity upon XA treatment and reduced bacterial burden upon L-kynurenine treatment suggests that XA supports *A. phagocytophilum* growth by reducing amounts of L-kynurenine through inhibition of TDO activity upstream of the L-tryptophan pathway.

A study has reported that 3-HK has both pro-oxidant and antioxidant properties (Reyes Ocampo *et al.*, 2014). Micromolar concentrations as low as 1 μ M are noted to be toxic to cultured striatal neurons (Okuda *et al.*, 1996). However, we noted that ISE6 cells were resistant to 3-HK at different concentrations (Supplementary Fig. 2). The resistance of tick cells to toxicity induced by 3HK suggests importance of this molecule in several physiological aspects of these cells. In the presence of hemin, 3-HK seems more likely to protect tick cells from cytotoxicity possibly by reducing ROS levels. 3-HK was reported as a scavenger of superoxide anion in insects and during infection and inflammatory disease in human derived cells (Goshima N, Wadano A and K., 1986; Christen *et al.*, 1990). The auto oxidation of 3HK leads to the production of unstable xanthommatin that is rapidly oxidized to hydroxanthommatin (Atherton *et al.*, 1993; Reyes Ocampo *et al.*, 2014).

Hydroxanthommatin is more stable and can react with superoxide anion (Atherton *et al.*, 1993; Reyes Ocampo *et al.*, 2014). It is also reported that hemoglobin and hematin (hemin) mediate auto oxidation of 3-HK to hydroxanthommatin (Ishii *et al.*, 1992). Therefore, it is reasonable to hypothesize that the observed inhibition in ROS levels upon 3-HK treatment of tick cells could be due to auto oxidation of this metabolite mediated by hemin.

In mosquitoes, oxidation of 3HK leading to xanthommatin production is important for eye pigmentation (Li *et al.*, 1999). However, similar role for 3-HK in ticks is not known. The observation of reduced bacterial loads in tick cells upon 3-HK treatment in the absence of hemin could be due to increased ROS levels that might be deleterious for bacterial growth. Our results suggest that the metabolism of 3-HK is important for *A. phagocytophilum* survival in tick cells. Higher levels of ROS observed in *kat*-dsRNA treated cells but not in *kynu*-dsRNA-treated cells in the absence of hemin suggests that KAT is more efficient to metabolize 3-HK than KNYU. The observation of reduced bacterial burden in *kat*-dsRNA-treated cells or *kynu*-dsRNA-treated cells or in cells treated with both dsRNAs in the presence or absence of hemin suggests that KAT and/or KNYU are important for *A. phagocytophilum* survival in tick cells. The influence of KAT and KNYU on bacterial growth seems to be independent of ROS levels. We previously reported that KAT (Taank *et al.*, 2017) is critical for *A. phagocytophilum* survival in tick cells. In this study, we show that KNYU activity is also essential for *A. phagocytophilum* survival in tick cells. Moreover, these findings suggest importance of 3-HK metabolism in the survival of *A. phagocytophilum* in tick cells.

The metabolism of 3-HK is associated with the tricarboxylic acid cycle (TCA cycle). KAT and KNYU cleaves L-kynurenine resulting in the formation of L-glutamate and L-alanine, respectively. Genome analysis of many Rickettsial pathogens have reported absence of alanine and glutamate synthesis pathways (Dunning Hotopp *et al.*, 2006). However, in *I. scapularis*, all genes required for synthesis of glutamate and alanine from alpha-ketoglutarate and pyruvate, respectively are present (Cabezas-Cruz *et al.*, 2017). Studies have shown that *A. phagocytophilum* decreases glutamate and alanine levels upon infection in ticks (Cabezas-Cruz *et al.*, 2017; Villar *et al.*, 2018). The observation of reduced bacterial burden upon knockdown of KAT and/or KNYU suggests that production and the catabolism of 3-HK and other metabolites in L-tryptophan pathways are critical for the supply of glutamate and alanine to *A. phagocytophilum* metabolism. Glutamate is a glucogenic amino acid that can be transformed to alpha-ketoglutarate that aids in bacterial TCA cycle (Cabezas-cruz *et al.*, 2017). Glutamate is also the precursor of arginine (Cabezas-Cruz *et al.*, 2017), another amino acid that cannot be synthesized by *A. phagocytophilum* (Dunning Hotopp *et al.*, 2006). As *A. phagocytophilum* was reported to have incomplete TCA pathway, exogenous supplementation of L-glutamate and glutamine from cells is essential for the bacterial growth (Huang *et al.*, 2007). Interestingly, it was reported that *A. phagocytophilum* inhibits tick TCA cycle (Cabezas-cruz *et al.*, 2017). Authors have suggested that TCA cycle inhibition allows *A. phagocytophilum* to prevent the entry of glutamine and glutamate to the TCA cycle via alpha-ketoglutarate (Cabezas-cruz *et al.*, 2017). Our study suggests that L-tryptophan and kynurenine pathways appear as an alternative route for *A. phagocytophilum* to get glutamate and alanine for its TCA cycle. Taken together, based on the results from current and previous studies, we propose a

mechanistic model on the role of L-tryptophan metabolites and hemin in influencing redox state of tick cells during *A. phagocytophilum* infection (Fig. 7).

In summary, *A. phagocytophilum* and hemin activates L-tryptophan pathway in ticks that leads to accumulation of bioactive metabolites. Even though metabolites from L-tryptophan pathway play different roles in influencing the redox state of the cells, XA seems to allow *A. phagocytophilum* survival through inhibition of TDO activity that subsequently affects L-kynurenine levels and buildup of ROS. 3-HK metabolism seems to be essential for *A. phagocytophilum* survival in tick cells. This study not only highlights the role of L-tryptophan pathway and hemin in *A. phagocytophilum* survival in tick cells but also provides a comprehensive view on the molecular changes in the redox state of the cells during vector-pathogen interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Putative orthologs of genes involved in L-Tryptophan catabolic pathway in arthropods. **A)** Putative L-tryptophan metabolism pathway in *I. scapularis* ticks is shown. The schematic from KEGG pathway for *I. scapularis* tryptophan metabolism was modified to a simpler format. Enzymes are shown in dashed-line boxes. L-Tryptophan metabolites are shown in solid line boxes. XA, KA and Nicotinamide metabolism are shown in thick solid-line boxes. **B)** L-Tryptophan catabolic pathway orthologs genes shared between Arthropoda, Chordata, and Mollusca genomes. Genes those are present as a single copy (dark gray) or duplicated copies (light gray) or more than two copies (white) are shown in different key colors. Genes that are absent/lost in the genomes are indicated with filled black box. Order, genus and species names for each organism are also indicated. Only complete genome sequences were considered in this analysis.

Figure 2. *A. phagocytophilum* up-regulate genes involved in tick L-Tryptophan metabolic pathway in ticks and tick cells.

The mRNA transcript levels of tryptophan 2,3-dioxygenase (TDO) (A), Arylformamidase (B), kynurenine 3-monooxygenase (KMO) (C) and kynureninase (KYNU) (D) in uninfected (open bar) or *A. phagocytophilum*-infected unfed nymphal ticks (A–D) (n=8) or ISE6 tick cell line (E–H) (n=6) is shown. The levels of mRNA transcripts of L-tryptophan metabolic genes were normalized to tick β -actin levels. Statistical analysis was performed using Student's t test and asterisk indicates significance ($P < 0.05$).

Figure 3. Exogenous treatment of the ISE6 tick cells with XA or KA or hemin modulates ROS production and *A. phagocytophilum* growth.

ROS levels in uninfected (open bars) or *A. phagocytophilum*-infected (grey bars) ($n=6$) upon treatment with 100 μ M XA (A) or KA (C) is shown. QRT-PCR analysis showing *A. phagocytophilum* loads in mock-treated or XA-treated (B) or KA-treated (D) tick cells ($n=6$). E) ROS levels in *A. phagocytophilum*-infected tick cells treated with 100 μ M of hemin alone or in combination with 100 μ M XA or KA are shown. F) QRT-PCR analysis showing *A. phagocytophilum* loads in tick cells ($n=6$) treated with 100 μ M of hemin alone or in combination with 100 μ M XA or KA. Tick cells treated with mock solution are used as controls in both E and F panels. Shift in the hemin (20 μ M) absorbance Soret when hemin is incubated with XA (5 μ M) (G) or KA (5 μ M) (K) is shown. Absorption spectra (H and L) and titration curves (I and M) for XA (5 μ M) (H and I) or KA (5 μ M) (L and M) with increasing concentrations of hemin (0–55 μ M) was generated using an equation describing single binding site ($Y = [B_{max} \cdot X] / [K_d + X]$) and absorbance value at 334nm. Level of free XA (J) or KA (N) in heme titration with XA or KA is shown. Aph indicates *A. phagocytophilum*. The levels of P44 were normalized to total DNA. Statistical analysis was performed using Student's t test and asterisk indicates $P < 0.05$ (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Fig. 4: XA, KA and Hemin impacts TDO activity and kynurenine levels in tick cells.

A) TDO activity in uninfected (UI) or *A. phagocytophilum*-infected (I) tick cells (n=12) in the presence or absence of hemin is shown. **B)** TDO activity in *A. phagocytophilum*-infected tick cells (n=12) in the presence of 100 μ M hemin alone or in combination with 100 μ M of XA or KA is shown. **C)** Levels of L-kynurenine in μ M concentration in uninfected (UI) or *A. phagocytophilum*-infected (I) tick cells (n=12) in presence or absence of hemin is shown. **D)** Levels of kynurenine in μ M concentration (n=12) in *A. phagocytophilum*-infected tick cells in the presence of 100 μ M hemin alone or in combination with 100 μ M XA or KA is shown. **E)** ROS levels in *A. phagocytophilum*-infected tick cells in the presence or absence of hemin with or without Ro-61–80498 (KMO inhibitor) is shown. **F)** QRT-PCR analysis showing levels of *A. phagocytophilum*-loads in tick cells in the presence or absence of hemin with or without Ro-61–80498 treatment is shown. **G)** QRT-PCR analysis showing *A. phagocytophilum* loads in the presence or absence of XA with or without L-kynurenine is shown. Statistical analysis was performed using Student's t test and asterisk indicates $P < 0.05$ (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Figure 5. Treatment with 3HK inhibits *A. phagocytophilum* growth in tick cells. ROS levels (A, C) or bacterial burden (B) in *A. phagocytophilum*-infected tick cells (A, B) or uninfected cells (C) in the presence or absence of hemin (100 μ M) with or without 3HK (10, 100 μ M) is shown. Grey bar indicates *A. phagocytophilum*-infected cells and open bars indicate uninfected cells. Statistical analysis was performed using Student's t test and asterisk indicates $P < 0.05$ (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Figure 6: RNAi-mediated knockdown of *kat* and *kynu* gene expression affects *A. phagocytophilum* growth and ROS levels.

QRT-PCR analysis showing levels of *kat* or *kynu* transcripts in *kat*-dsRNA-treated (A) or *kynu*-dsRNA-treated (B) *A. phagocytophilum*-infected tick cells is shown. ROS levels (C) or bacterial loads (D) in *A. phagocytophilum*-infected tick cells in the presence or absence of hemin with *kat*-dsRNA or *kynu*-dsRNA or both are shown. The levels of *kat* or *kynu* transcripts were normalized to tick actin. Percentages for knockdown efficiency for *kat* or *kynu* gene expression are indicated. Statistical analysis was performed using Student's t test and asterisk indicates $P < 0.05$ (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Figure 7: Proposed model for the role of L-tryptophan metabolites and hemin in influencing *Anaplasma phagocytophilum* growth and redox state of tick cells.

Anaplasma phagocytophilum (*A.ph* shown as grey circle) and hemin (H in black circle) increases the activity of TDO that leads to increased accumulation of L-kynurenine. Hemin and bacterial-mediated TDO activity and L-kynurenine accumulation scavenge and inhibit ROS resulting in favorable condition for *A. phagocytophilum* growth and survival. L-kynurenine in the presence of hemin allows rapid bacterial growth. However, conversion of L-kynurenine to 3-HK by KMO produces hydrogen peroxide (H_2O_2) that probably controls the bacterial infection. The conversion of L-kynurenine to KA inhibits ROS production probably through its agonist action on NMDR. This could lead to the block of L-glutamate release and activation of NADPH oxidase. At low concentration, KA may support *A. phagocytophilum* growth probably through inducing L-glutamate release that prevents NADPH oxidase activation. Hemin could induce auto oxidation of 3-HK to hydroxanthommatin that can scavenge ROS through its action on superoxide anion. The metabolism of L-kynurenine and 3-HK could improve the level of L-glutamate release in the cells through direct participation of KAT reaction or after transamination of alpha-ketoglutarate by alanine transaminase (ALT) in the presence of L-alanine from KNYU reaction. L-glutamate can directly participate in *A. phagocytophilum* TCA cycle (*A.ph* TCA in grey circles) or can be used to synthesize arginine to facilitate *A. phagocytophilum* survival. Finally, in the absence of hemin, KAT can prevent the accumulation of 3-HK by its conversion to XA, thereby facilitating *A. phagocytophilum* growth in tick cells.