Transcription factors Aryl hydrocarbon receptor and TGF-inducible early gene are involved in an axis modulating immune response in mosquitoes

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Abstract

Immune homeostasis balances effective defense against pathogens with avoiding the adverse effect of immune overactivation. AhR is a ligand activated transcription factor that transduces chemical signals into transcription of a variety of target genes. In this study, we demonstrate that mosquito AhR and TIEG mediate a transcriptional axis modulating immune response negatively. When AhR was activated by diet delivery of agonist kynurenine, the anti-bacterial immunity was compromised with a reduced survival at 24hr post bacterial challenge. In contrast, when AhR was inhibited by antagonists, CH223191 or SR1, the immunity was enhanced with increased survival. The observed immune enhancement via AhR antagonist was corroborated by *AhR* gene silencing via RNAi, which resulted in increased survival upon the infection. Exploration of transcriptomes following AhR inactivation, either pharmacologically or genetically, highlighted a set of genes that are infection inducible and AhR dependent, including genes that may mediate immune suppressive functions. One of the genes, *TIEG* (TGF-β inducible early gene), a member of the Krüppel -like factor family of transcription factors, was further studied. TIEG is required for AhR mediated immune suppression. Silencing *TIEG* increased the survival and reversed the immune suppression mediated by kynurenine activation of AhR. Moreover, the mosquito cohorts with *AhR* or *TIEG* knockdown demonstrated similar transcriptomic responses upon infection. There were co-expression patterns shared between the respective cohorts treated with AhR antagonist or *AhR* and *TIEG* silencing. In the naïve mosquitoes where the IMD pathway was overactivated by silencing the inhibitor gene *Caspar*, co-silencing *Caspar* with *AhR* or *TIEG* resulted in a reduced survival, indicating the AhR-TIEG axis is required to prevent the adverse effect of overactivation of IMD without an infection. Together, AhR and TIEG are involved in a transcriptional axis that is critical for maintaining immune homeostasis.

Keywords *Anopheles gambiae*, mosquito, AhR, Krüppel like factor, TIEG, innate immunity, immune homeostasis

Introduction

The mosquitoes, like many insects, have evolved an efficient innate immune system consisting of Toll, IMD, JAK-STAT and RNAi pathways to defend against infection with bacteria, fungi, viruses and parasites [1-6]. To maintain immune homeostasis various regulatory circuits have evolved in insects to avoid deleterious effects of overaggressive immune responses and immunopathogenesis [7-9]. It has been well documented that intrinsic negative regulators Cactus [10], Caspar [11] and SOCS [12] for the Toll, IMD and Jak-STAT pathways, respectively, delicately tether the immune pathways. Besides, chemical defense mechanisms play important roles for homeostasis. Xenobiotic sensors are located at the interface with the chemicals originated from associated microbiota or environment. These sensors recognize ligands and initiate responses to coordinate context dependent xenobiotic metabolism and immune defenses [13]. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, which was initially identified in the 1970s as a xenobiotic sensor recognizing the compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice [14]. Through studies over the past 5 decades, it is now widely acknowledged that AhR can recognize various endogenous and exogenous ligands [15] and participate in multiple physiological processes including immune regulation as well as xenobiotic metabolism [15-19]. AhR is an ancient protein with an ortholog identified in the placozoan *Trichoplax* and conserved up to humans [18, 19]. According to the studies in the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and the cnidarian *Nematostella vectensis*, the ancestral functions of AhR are related to the development of sensory structures as well as neural systems (reviewed in [18]). These ancestral functions are likely mediated by endogenous ligands. During evolution, AhR has further acquired the capability to recognize foreign chemicals as an add-on function [18, 19]. In vertebrates, AhR has been recognized as a critical player in coordinating transcriptional circuits for immune regulation upon recognition of certain xenobiotics [16, 20, 21]. The connections between a xenobiotic sensor and immune regulation unveil the role of chemical sensing systems in transducing non-self signals into proper responses to execute effective immune defense while maintaining homeostasis. In contrast to the rich studies about diverse functions of AhR in vertebrates, very little is known about the AhR in insects except for the roles in development. Recently, AhR in fruit fly and nematode has been shown to be able to recognize indoles derived from symbiotic bacteria and lead to the transcription of a gene set contributing to healthy life span [22]. In this study, we dissect the role of AhR in mosquito immunity. Our data demonstrate that AhR and Krüppel like factor TIEG are connected to a transcriptional axis mediating immune suppression, suggesting that at insect level AhR has acquired the function to modulate innate immune responses.

Materials and Methods

Mosquitoes

Anopheles gambiae G3 strain was used for this study. The G3 mosquitoes were reared in an insectary with 28ºC and 80% humidity, a light/dark cycling of 14:10 hours. Larvae were cultured in water pans with food (1:1 mix of ground pellet of cat food and brewer yeast), and adults were maintained with 10% sucrose sugar meal, and fed on mice to acquire blood to produce eggs.

Phylogenetic inference using AhR protein sequences

The AhR protein sequences of representative organisms from invertebrates to mammals (Table S1) were used for inferring phylogenetic relationship. The sequences were aligned using the MUSCLE algorithm. A phylogenetic tree was made by Neighbor-Joining method using Jones-Taylor-Thornton (JTT) model, with 500 bootstrap replications. A similar tree topology was generated by Maximum Likelihood method using JTT model with 500 bootstrap replications. Only the NJ tree is shown in Fig. S1. The domains were visualized in Simple Module Architecture Research Toll (SMART, http://smart.embl-heidelberg.de/) , and the peptide sequences of domains bHLH, PAS A and PAS B were extracted and protein sequence identity between these organisms were compared.

Pharmacological manipulation of AhR activity in mosquitoes

AhR antagonists and agonists were used to manipulate AhR activity in mosquitoes. In each case, chemicals were fed to newly emerged adult mosquitoes, 50-60 females per cohort, in 10% sucrose solution for three days and then challenged with *Serratia fonticola* S1 (see below). Kynurenine (Kyn) is an endogenous ligand of AhR [23]. Kyn is generated during the oxidation of tryptophan, a reaction catalyzed by tryptopan-2,3-dioxygenase (TDO) in mosquitoes [24]. Kyn at 30µM (Sigma-Aldrich, K8625) was used as agonist to enhance AhR activity. We used two AhR antagonists CH223191 at 90 µM (Sigma-Aldrich, C8124) and Stem Regenin (SR1) at 3µM (Selleckchem, S2858). Additionally, we used 680C91 (20µM) (Sigma-Aldrich, SML0287), a TDO inhibitor, to reduce endogenous Kyn production. These concentrations of the chemicals were chosen empirically based on resultant phenotypes.

Bacterial infection

Bacterium *Serratia fonticola* S1 was isolated from a wild caught specimen of *Aedes albopictus*, collected in Florida in July 2015. Bacteria were transformed with a plasmid expressing GFP as described previously [25]. The bacteria were grown overnight in Luria Bertani broth containing ampicillin (100µg/ml) at 29°C. Bacterial culture was normalize to OD_{600nm}=1 and diluted 1000 times with sterile H_2O, which yielded approximately 1000 colony forming unit (CFU) /µl, and approximately 100 nl of this bacterial solution was injected intrathoracically into the hemocoel of *An. gambiae* at day 3 after AhR activity was manipulated as described above. Survival at 24 hours post infection was used to assess the anti-bacterial immunity. The single thorax of dead and surviving mosquitoes was homogenized in 50 µl sterile water, and 30 µl homogenates were spread to a LB plate with Ampicillin and cultured at 28 °C overnight. The colonies on the plate were examined under UV light to visualize GFP tagged bacteria. In *Serratia* injected mosquitoes, GFP tagged bacteria were recovered, while in sterile water injected mosquitoes no GFP tagged bacteria were detected. The data were generated from three experimental replicates. For each treatment, the survival rates across replicates were not statistically different (Fisher's exact test, P>0.05), therefore the replicate data were pooled to compare the survival between the treatment and vehicle control using a Chi square test.

RT-PCR

Mosquito RNA was extracted from 15 females using Trizol reagent (Invitrogen, Cat# 15596026). Genomic DNA contamination was removed by DNaseI treatment using TURBO DNA-free Kit (Invitrogen, AM1906). cDNA synthesis was carried out using NEB ProtoScript II Reverse

Transcriptase (NEB, M0368S). The cDNA was used as template for RT-PCR to determine transcript abundance for various genes. The primers used were present in Table S2. No reverse transcriptase (NRT) and no template control (NTC) served as negative controls.

RNAi mediated gene knockdown in mosquitoes

RNAi mediated *AhR*, *TIEG* and *Caspar* knockdowns were performed. For dsRNA preparation, a given target gene fragment was PCR amplified using gene specific primers with the T7 promoter sequence at the 5' end. The PCR products were used to synthesize dsRNA using a T7 RNA Polymerase Kit (Sigma-Aldrich RPOLT7-RO ROCHE). Generated dsRNAs were treated with TURBO DNA-free Kit (Invitrogen, AM1906) to remove DNA. The dsRNA of a GFP fragment was used as control dsRNA. For single gene silencing, *AhR* and *TIEG*, 0.5µg/µl dsRNA was used for injection. To co-silence *Caspar* along with either *AhR* or *TIEG*, respective dsRNA , each at 1.0µg/µl, were mixed for injection. Newly emerged *An. gambiae* female mosquitoes, 60 females per cohort, were subjected to intrathoracic injections with ~100nl of dsRNA. Treated mosquitoes were maintained on 10% sucrose solution for three days. For bacterial challenge, cohorts of dsGFP control, dsAhR and dsTIEG were injected with *Serratisa* at day 4 post dsRNA treatment as described above. The gene knockdown efficacy was verified by RT-PCR with primers in Table S2.

AhR Western blot

Rabbit Polyclonal antibody against AhR was made at GenScript (New Jersey). The antigen was a peptide fragment in the PAS B domain of AhR protein (aa 208-394). For Western blot, mosquito proteins were extracted using Insect Cell-PE LB™ (G Biosciences, 786□411) at three days post dsRNA injection. The protein samples (20 µg/well) were separated by SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed by the AhR antibody (1:1000). GAPDH antibody (GeneTex, GTX100118) was used as loading control. HRP labelled secondary antibody was used to detect signal using KPL LumiGLO kit following manufacturer's instruction. The blot was scanned to visualize the signal using the IMAGE STUDIO, Version 5.x, LI-COR.

Transcriptome analysis

RNA-sequencing (RNAseq) was used to compare transcriptomes between samples with AhR manipulation. To examine the effect of pharmacological AhR inhibition, three cohorts of females were used. Mosquitoes were fed with AhR antagonist SR1 or vehicle control for three days, and then were challenged with *Serratia* infection, as described above. An injury control (in which mosquitoes were injected with sterile H_2O) was included to control the effect of damage associated with injection. To examine the effect of gene knockdown, three cohorts of newly emerged females were used; each was treated with dsAhR, dsTieg, or dsGFP control, for 3 days, and then challenged with *Serratia* infection. Total RNA from 15 live mosquitoes at 24hr post challenge was isolated using Trizol reagent, and then DNaseI treatment was followed to remove DNA contamination. Triplicate RNA samples were prepared for each treatment. The RNA samples were further processed at Genewiz, NJ, where the cDNA libraries were prepared and sequenced using Illumina Hiseq, 2 x 150 bp paired-end chemistry. At least 25M clean reads were generated from each RNA sample, which provided a sequencing depth sufficient for transcriptome analysis. The reads were mapped against transcripts of *An. gambiae* (NCBI),

implemented by using Array Star v.12 (DNAstar). Read counts were normalized using the median of ratios method [26] using DESeq2 software [27]. In determining normalized read counts, this method accounts for sequencing depth and RNA composition by calculating normalization factors for each sample in comparison to a pseudo-reference sample. After determining normalized read counts, an independent filter was utilized which removed transcripts with normalized counts less than 5. This resulted in a dataset of 10,714 transcripts. The clustering of all samples revealed that the replicate 2 of dsAhR/*Serratia* infection was not consistent with the other two replicates, likely due to a quality issue associated the replicate, therefore, this replicated was removed from analysis. Differentially expressed genes were identified using a negative binomial generalized linear model (GLM) available through DESeq2 [27]. Likelihood ratio tests were conducted to identify transcripts that exhibited differential expression between all groups. Pairwise differential expression comparisons were made and statistical significance was determined by computing q-values that preserve the False Discovery Rate (FDR) [28, 29] [26]. For example, concluding that a transcript was differentially expressed between two groups with a q-value of 0.05 would imply that there was a 5% chance (expected) that this conclusion was a false positive. To determine a lower dimensional representation of the transcriptomic data, principal components analysis (PCA) was conducted using regularized log-transformed (rlog) data. PCA seeks to find a small set of "principal components" that capture a large proportion of the variance in the original data [30]. The rlog data was determined using DESeq2, while the "prcomp" function in R [31] was utilized to determine the PCA. The proportion of the variance captured by each of the principal components was determined. Weighted gene co-expression network analysis (WGCNA) [32] was conducted to identify modules (or sets) of transcripts that are co-expressed. This analysis was conducted as follows. First, the topological overlap between transcripts in a signed and soft-thresholded correlation network determined from the rlog data was determined using the R package WGCNA [33]. From this, transcript modules were determined utilizing hierarchical clustering with an "average" link function and utilizing the hybrid version of the "Dynamic Tree Cut" algorithm [34]. To validate expression patterns revealed by RNAseq, a selected set of genes was measured using quantitative RT- PCR. Primers were presented in Table S2. The submission of the RNAseq reads to NCBI is in the process.

Results

Manipulation of AhR activity affects immunity against bacterial infection in mosquitoes

AhR is conserved from invertebrates to mammals. AhRs from mosquitoes *An. gambiae* and *Aedes aegypti* were clustered with the AhRs from fruit fly *Drosophila melanogaster* and nematode *Caenorhabditis elegans* in the same clade, while AhRs from zebrafish *Danio rerio* and mouse *Mus musculus* and human *Homo sapiens* were grouped together (Fig. S1A). The domains of bHLH and PAS A are involved in DNA binding and dimerization with cofactors, which are more conserved compared to PAS B that is involved in ligand binding (Fig. S1B). The higher level of divergence in PAS B suggests a diversity in ligand recognition. *AhR* transcripts were detected in the whole body of mosquito larvae, pupae, and adults (Fig. S1 C), indicating that *AhR* is constitutively expressed in all life forms of the mosquito. To investigate the immune regulatory role of AhR, we pharmacologically manipulated AhR activity and then examined the survival of mosquitoes upon a bacterial infection. Kynurenine (Kyn) is known as an endogenous AhR ligand [23, 35], which is an intermediate metabolite of tryptophan oxidation catalyzed by

tryptopan-2,3-dioxygenase (TDO) in mosquitoes [24]. TDO can be inhibited by 680C91[36, 37], which leads to the reduction of endogenous Kyn production. CH233191 [38, 39] and SR1 [36] are known as AhR antagonists. After AhR activity was manipulated pharmacologically, the mosquitoes were challenged with bacteria by injecting *Serratia* into the hemocoel, and survival rate was recorded at 24h post injection. As shown in Fig. 1A, the DMSO vehicle control mosquitoes with basal AhR activity had 58.6% survival. When mosquitoes were fed with Kyn to boost AhR activity, the survival was reduced to 39.9%. In contrast, when mosquitoes were fed with AhR antagonists CH233191, SR1 or 680C91, the survival was significantly increased to 78.7-85.4%. Increased survival This phenotypic pattern was also observed in *AhR* gene silencing using RNAi. When the *AhR* gene was silenced by dsAhR via RNA interference, an increased survival rate (82.4%) was observed in comparison with 57.8% of survival in the control mosquitoes treated with dsGFP (Fig.1B) mirroring results observed when AhR antagonists were given.

Transcriptomic alterations upon AhR manipulation

To identify genes that are regulated by AhR, we conducted RNAseq to interrogate *An. gambiae* transcriptomes. AhR activity was experimentally reduced by AhR antagonist SR1, and then the treated mosquitoes were challenged with *Serratia* infection. RNA samples were collected from surviving mosquitoes at 24hr post bacterial challenge and subjected to RNAseq. Transcriptomes were analyzed using normalized read counts, and genes that were differentially expressed were identified by DESeq2. In comparison of *Serratia* infection and injury control, the expression of 2102 genes was differentially altered (using a cutoff of q-values <0.05, Table S3), which we defined as infection responsive genes. Among these genes, 265 were upregulated and 145 were downregulated with at least 2-fold. Infection inducible gene set includes typical immune genes encoding such as immune pathway components (spaetzle1, spaetzle3, PGRPLB, Rel1 and Rel2), immune effectors (DEFs, CECs, TEPs and LRIMs, CLIP serine proteases, fibrinogens) and immune regulators (Serpins, suppressor of cytokine signaling (SOCS)). The AhR antagonist treatment affected the expression of 696 infection responsive genes. In comparison to infection only, the AhR antagonist resulted in 66 upregulated and 39 downregulated genes with at least 2-fold differences. The major immune genes are not affected by the AhR antagonist, but potential immune suppressive genes, such as IAP4, SRPN6 and SRPN10 and SOCS require the presence of AhR for the response to infection. Expression patterns of *Def1*, *Tep15*, SRPN10 and *TIEG* were validated by qRT-PCR (Fig. S2).

AhR - TIEG axis mediates a negative immune modulation

One of the AhR dependent genes, AGAP009889, encodes a transcription factor in the Sp1-like/Krüppel-like family [40], which is a ortholog of the vertebrate TGF-β-inducible early-response gene (TIEG) [41]. The orthologous gene in *Drosophila* is *Cabut*, which is involved in development, metabolism and growth control [42-45]. In vertebrates, AhR and TGF-β/Smad signaling are connected to mediate immune suppression to maintain immune homeostasis [46], and TIEG is involved in TGF-β/Smad signaling [47-49]. Based on this background information, we tested the hypothesis that TIEG is downstream of AhR, mediating the transcription of genes responsible for immune modulation. First, we examined the effect of *TIEG* silencing on the *Serratia* infection outcome in *An. gambiae*. As expected, silencing *TIEG* resulted in a survival (90.1%), which was higher than that in the dsGFP control (55.7%, Chi-square, P<0.01) (Fig.

2A).

Furthermore, we conducted RNAseq to compare the transcriptomes in response to the dsAhR and dsTIEG and AhR antagonist. Clustering analysis separated transcriptomes into two clusters (Fig. 2B). Injury control was separated from Serratia infection and antagonist/infection. In the other cluster, the dsAhR/infection and dsTIEG/infection were distinct from dsGFP. PCA analysis revealed that principal component 1 (PC1) and PC2 explained 61% and 11.2% of the variance, respectively, the transcriptome replicates of each treatment were closely clustered and different treatments separated their transcriptomes clearly (Fig. 3). Replicates of dsAhR and dsTIEG were located closely, indicating a similarity in their transcriptomic response. However, the replicates of dsGFP/*Serratia* infection were distinct from the replicates of *Serratia* infection, suggesting that the dsGFP treatment posed other impacts. In addition, there was an evident separation between AhR antagonist and gene silencing, which suggests that two approaches for AhR manipulation each may have impacts other than merely affecting the AhR activity. The AhR antagonist was administrated via diet, and gene silencing were achieved through injections, an invasive method. In mosquitoes, dsRNA triggers RNAi defense against virus [50], the dsGFP injection may have an unknown influence to the response to the following bacterial challenge. These confounding effects may contribute to the patterns revealed by PC1 and PC2. The PC3 and PC4 captured 7.3% and 4.1% of the variance, which may represent the effect of AhR manipulation more genuinely, the clusters of AhR antagonist, dsAhR and dsTIEG were closer and separated from the clusters of *Serratia* infection and dsGFP/infection (Fig. 3). This pattern evident in the PCA represented the transcriptomic responses that were driven by the AhR-TIEG signaling axis. This pattern was further supported by co-expression module analysis. The weighted gene correlation network analysis (WGCNA) was implemented to identify phenotype associated modules of co-expressed genes. WGCNA identified 35 modules from the entire dataset, each contains 23-1382 genes. The infection, AhR antagonist and *AhR* or *TIEG* silencing resulted in significant transcriptional alteration in 2102, 1631, 3285 and 3260 genes (defined by q-value <0.05, Table S2), respectively. There genes fall into different modules. There were 3 categories in which modules are affected by these treatments. The category I includes 9 modules that represent 1305/2102 (62.1%) genes in the infection sample, the gene number in these modules were reduced to 498/1631 (30.5%) genes in the AhR antagonist sample and 1007/3285(30.6%) genes in the dsAhR and 888/3260 (27.2%) genes in the dsTIEG sample, suggesting that these genes in the 9 modules are AhR dependent. The category II includes 5 modules that includes 170/2102 (8.1%) genes in the infection sample. These modules were enriched with more genes in the antagonist (429/1631, 26.3%) and the dsAhR (1223/3285, 37.2%) and the dsTIEG (1185/3260, 36.4%) sample. The category III represents 7 modules that were affected differently by the antagonist and gene silencing (Fig. S3). Together, the transcriptome comparisons indicate that the two manipulation approaches had resulted in broad impacts on transcriptomes, the PCA and WGCNA module analysis extracted effectively transcriptomic patterns caused by the AhR manipulation. The transcriptomic pattern was validated by qRT-PCR, the transcription of *SRPN10, TEP15, SOCS* was down regulated by *AhR* and *TIEG* silencing in response to the bacterial challenge (Fig. 2C). Next, we reasoned that the immune suppression resulting from AhR activation would be attenuated by *TIEG* silencing. Indeed, the survival reduction by Kyn feeding (demonstrated in Fig.1 above) was by reversed by dsTIEG (Chi-square, P<0.01, Fig. 2D). This result suggests that the TIEG acts downstream of the AhR.

AhR-TIEG axis maintains an immune balance when IMD pathway was overactivated

From the observations above, we conclude that AhR and TIEG are involved in a signaling axis to control negative immune modulation. This axis may help to maintain immune homeostasis by preventing immune overactivation. To test this hypothesis, we created an over-activated immune state by silencing *Caspar*, the negative regulator of IMD signaling pathway [51], and examined the impact of *AhR* or *TIEG* silencing on the life span in *An. gambiae*. As shown in Fig. 2E, the cohorts of dsGFP, dsAhR/dsGFP, dsTIEG/dsGFP and dsCaspar/dsGFP remained ~90% survival at day 3 post respective injections. However, when *Caspar* was co-silenced with either *AhR* or *TIEG*, the survival was reduced to 70%. The results show that the activation of IMD by depleting suppressor *Caspar* in the absence of an immune insult displays a normal survival only when AhR and TIEG are present, suggesting that the potential adverse effect of IMD over-activation is prevented by the AhR-TIEG signaling axis.

Discussion

AhR is conserved from invertebrate to vertebrate [18]. During evolution, in parallel to its ancestral role in development of sensory structure and neural systems, the AhR has diversified into a chemical sensor with binding affinity for broad intrinsic and/or extrinsic ligands derived from environment or associated microbiota [52]. This type of recognition transduces chemical signals into responses in various contexts, such as xenobiotic detoxification and immune regulation in vertebrates. At the invertebrate level, *C. elegans* and *D. melanogaster* have shown the capability of recognizing indoles from gut bacteria and mediating processes for a healthy life span [22]. In this study, we demonstrate the evidence that mosquito AhR and TIEG are involved in an axis of transcriptional cascade that participate in immune homeostasis. We used a bacterial infection model to examine the role of AhR function in regulating defense against the infection. When the AhR activity was activated by agonist Kyn, we observed a reduced survival upon bacterial challenge, which suggests that the anti-bacterial immunity was suppressed by AhR. On the contrary, when the AhR was inactivated by antagonists or TDO inhibitor, the immunity was enhanced, resulting in an increased survival upon the challenge (Fig. 1A). In line with the pharmacological manipulation, *AhR* gene silencing increased mosquito survival upon the bacterial challenge (Fig. 1B). Next, AhR dependent genes in response to infection challenge were screened by RNAseq in mosquitoes in which AhR was inhibited by either antagonist or gene silencing (Table S3). The AhR manipulation did not affect transcriptionally the canonical immune effector genes, such as Defensin and Cecropin, suggesting that AhR pathway is functioning distinct from IMD and TOLL pathways. A set of genes were identified as bacterial challenge inducible and AhR dependent, which include genes with possible immune suppressive functions, such as *SRPN10* and *SOCS*. This implied that AhR activation in the infection context may mediate transcriptional regulations of immune suppressive genes to maintain an immune balance. In line with this function, we further characterized the gene *TIEG*, encoding a C2H2 transcription factor [42, 45], the homolog of Krüppel like factor, which is conserved from invertebrate to vertebrates [41]. TIEG has functional connections with TGFβ1 pathway in Drosophila and vertebrates [45, 49]. Recently, AhR-TGFβ1/Smad signaling has been shown to mediate immune suppression in scurfy mice, a mouse autoimmune model [46]. We hypothesize that AhR and TIEG form a transcriptional axis to mediate immune regulation and supported this hypothesis with three lines of evidence. First, mosquitoes with silenced *TIEG* showed enhanced resistance to bacterial challenge with increased survival (Fig. 2A); second, the immune

repressive effect induced by the AhR activation via Kyn was reversed by *AhR* and *TIEG* silencing (Fig. 2D); third, silencing *AhR* and silencing *TIEG* resulted in similar transcriptional profiles by RNAseq (Fig. 3, Fig. S3). The immune suppressive function of AhR-TIEG axis is beneficial in maintain immune homeostasis. It has been shown that silencing IMD suppressor *Caspar* enhances mosquito immunity to fight against malaria parasites *Plasmodium berghei* and *P. falciparum* [11]. But in naïve conditions without an insult, immune machinery is leashed by negative regulators to prevent deleterious consequences, such as excessive inflammation, from happening [7]. The AhR-TIEG axis appears to act as a distinct immune regulatory loop that keeps the adverse effect of *Caspar* knockdown under control, as co-silencing *Caspar* and the *AhR-TIEG* axis, the survival was compromised (Fig.2E).

Taken together, this study identified AhR and TIEG as components of a transcriptional axis which is involved in maintaining immune homeostasis in mosquitoes. Considering a recent finding that the AhR in nematode and fruit fly mediates healthy life span upon recognition of bacterial indole, it would be interesting to recognize and characterize indigenous and exogenous AhR ligands in mosquito immune contexts. Furthermore, to functionally elucidate the transcriptional axis, further work is required to characterize the role of TIEG in the cascade. *Drosophila* TIEG is the ancestor of Krüppel like factor 10 and 11 in the family of Sp1/Krüppel-like zinc finger transcription factors [41]. *Drosophila* TIEG plays a role as a transcriptional repressor in S2 cells [42] and positively modulates TGF-β signaling as well as JAK/STAT pathway in the patterning and cell proliferation during wing development [45]. The KLF10 and 11 in vertebrates mediate TGF-β signaling [53]. TGF-β signal pathway plays critical roles in immune regulations in both invertebrates and vertebrates [54-56]. There are cross talks between AhR and TGF-β pathway in anti-inflammatory processes demonstrated in mammals [57-59]. A direct evidence of AhR/TGF-β/Smad pathway in an anti-inflammation context was demonstrated lately in scurfy mice, a mouse model of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, in which ligand activated AhR-TGF-β/Smad signaling mediates autoimmune suppression [40, 46]. The current findings that AhR signaling connects chemical sensing and immune regulation in mosquitoes warrant further study to elucidate the cross talk between xenobiotic defense and immune defense in insect level.

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Author contributions

Conceived and designed the study: JX and AK. Performed the experiments: AK, AP, WY, PK, JX. Analyzed the data: AK, JX, PT, SC. Wrote the paper: JX, AK.

Conflict of interest

None declared.

Figures

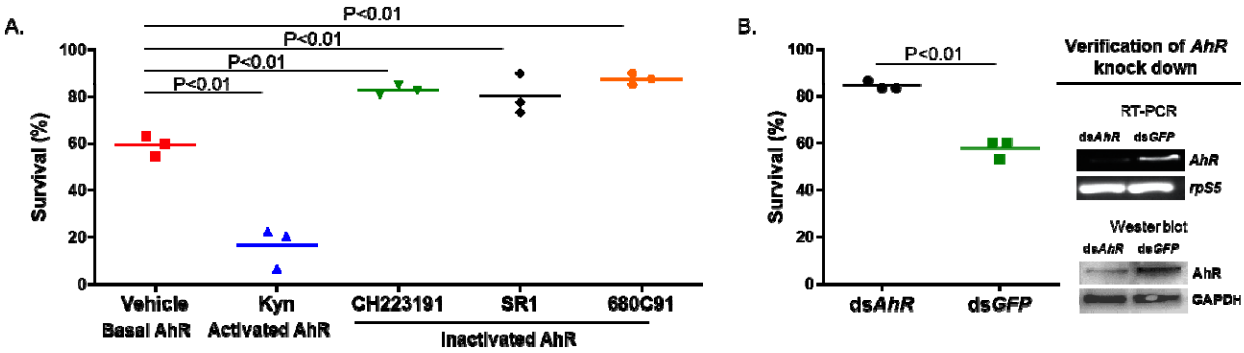

Figure 1. Mosquito survival upon bacterial challenge when AhR activity is manipulated pharmacologically or genetically. (A) DMSO vehicle control mosquitoes have with basal AhR activity; the AhR was activated by ligand Kyn-feeding. The AhR was inactivated by feeding AhR antagonists- (CH223191, SR1) or TDO inhibitor 680C91. The survival upon *Serratia* challenge was compared between the respective treatments. Data from three replicate experiments were presented. Each dot represents a cohort with 50 mosquitoes, average survival (%) was denoted by the line. The survival difference was tested by the Chi square test. **(B)** *AhR* gene was silenced by dsAhR, knockdown was verified by RT-PCR and Western blot. Mosquito survival upon *Serratia* challenge was compared between dsAhR and dsGFP control cohorts using the Chi square test. Data from three replicate experiments were presented.

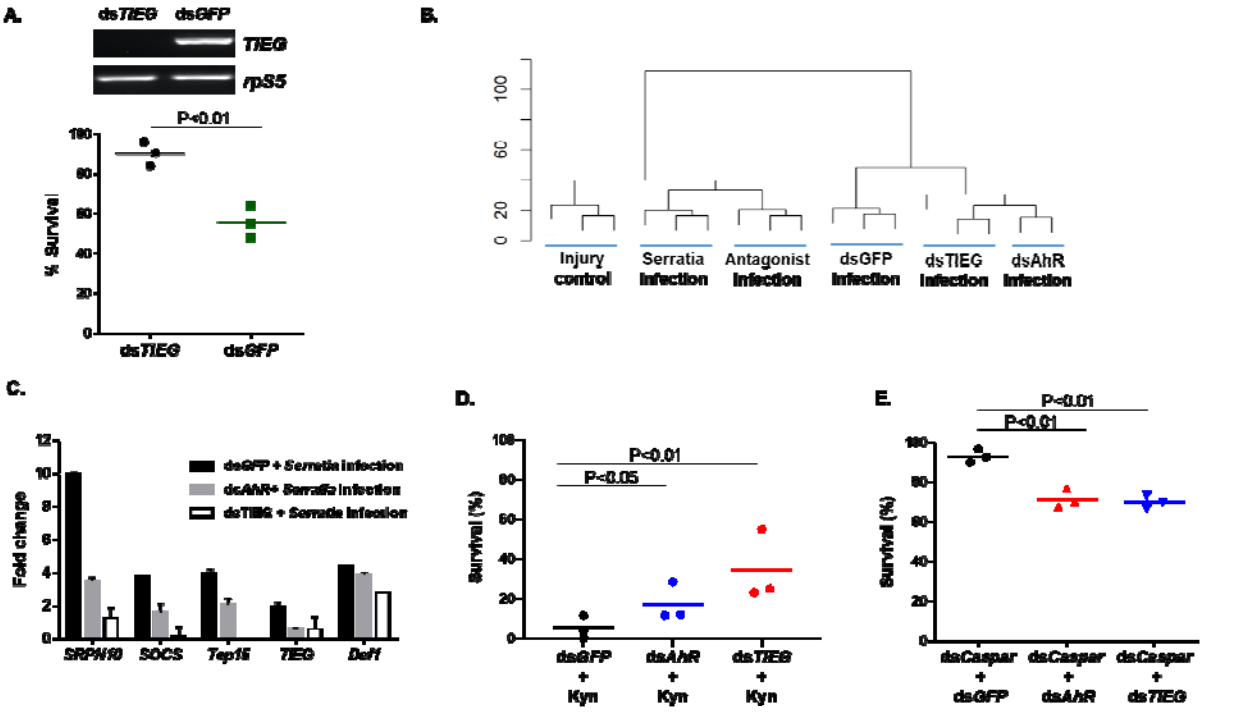

Figure 2. **TIEG is mediating immunomodulation downstream of AhR.** **(A)** The survival upon *Serratia* infection was higher in the ds*TIEG* treated cohort than that in the ds*GFP* control, data were generated from three replicates (Chi square test, P<0.01). The *TIEG* knockdown was verified by RT-PCR. **(B)** The transcriptomes of dsTIEG and dsAhR cohorts demonstrated a similar pattern in response to the *Serratia* challenge. The scale bar represents the distance between the clusters. **(C)** Validation by qPCR of selected AhR dependent and in genes in mosquitoes in which *AhR* or *TIEG* was silenced. **(D)** The lower survival associated with Kyn mediated immune suppression was reversed by either *AhR* or *TIEG* silencing. Data were generated from three replicates. Each cohort had 30 mosquitoes. Newly emerged mosquitoes were injected with respective dsRNA for gene knockdown, and mosquitoes were maintained on 10% sugar with Kyn (30µM). The treated mosquitoes were challenged with *Serratia* at day 4 post knockdown. The survival was compared by the Chi square test. **(E)** The effect of dsAhR and dsTIEG on the survival of mosquitoes in which the IMD was overactivated by dsCaspar. Survival at day 3 post gene silencing was compared by the Chi square test. The data were generated from three replicates. Each cohort had 30 mosquitoes for the treatment.

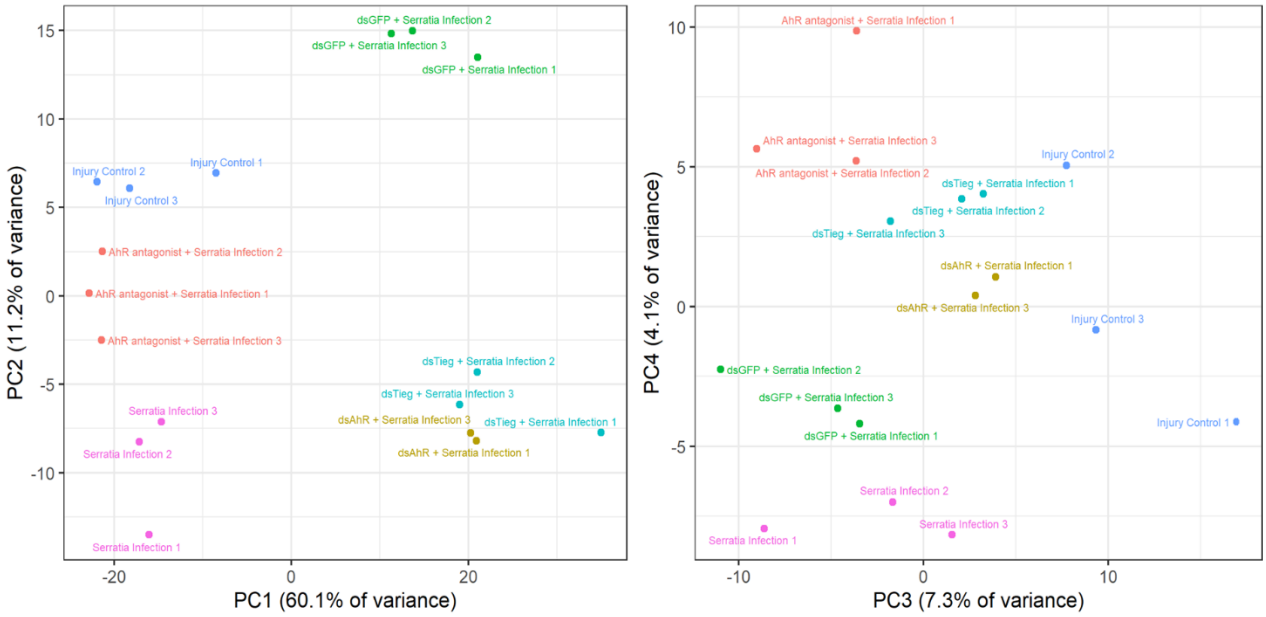

Figure 3. Principal component analysis (PCA) of transcriptomes. The PC1 and PC2 explained the major variance of transcriptomes with different treatments. The impact of AhR antagonist and dsAhR and dsTIEG on AhR activity in the infection context was better represented by PC3 and PC4. See text for more interpretation.

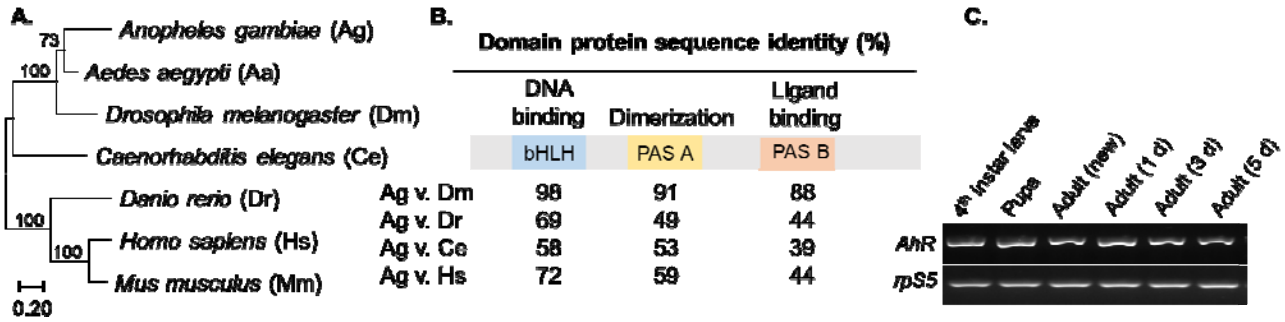

Figure S1. **(A)** The AhR phylogenetic tree inferred from amino acid sequence comparison using Neighbor-Joining method. Boostrap values were displayed on nodes. The scale bar indicates the genetic distance. **(B)** The level of conservation of domains bHLH, PAS A and PAS B, represented by identity of protein sequence. **(C)** *AhR* is constitutively expressed in larvae, pupae and adults (newly emerged, 1-, 3-, 5-day old), assayed by RT-PCR, *rpS5* was used as cDNA input control.

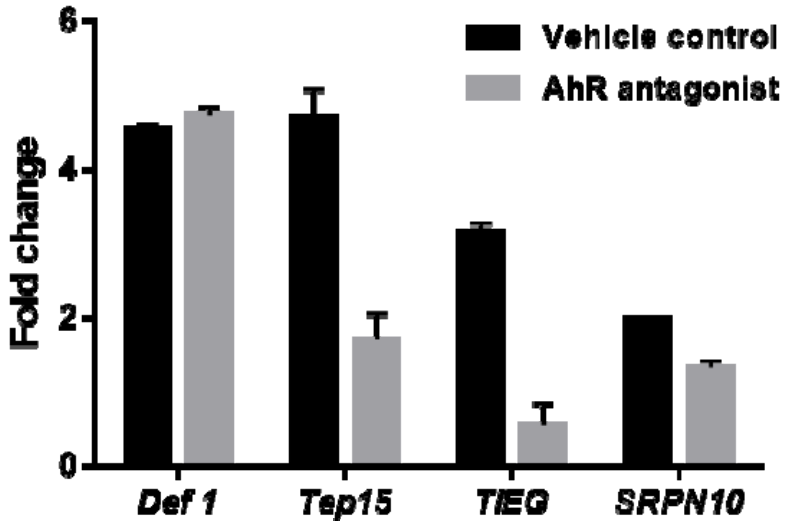

Figure S2. Transcriptional validation of selected genes by qRT-PCR. Selected genes that were identified in the transcriptome of the AhR antagonist/Serratia infection sample was chosen for the validation. Def1 represents a AhR independent gene, and *Tep15* and *TIEG* and *SRPN10* were AhR dependent genes. Fold change was calculated relative to injury control and the abundance of each transcript with respect to endogenous control rps5. Error bars represent SEM.

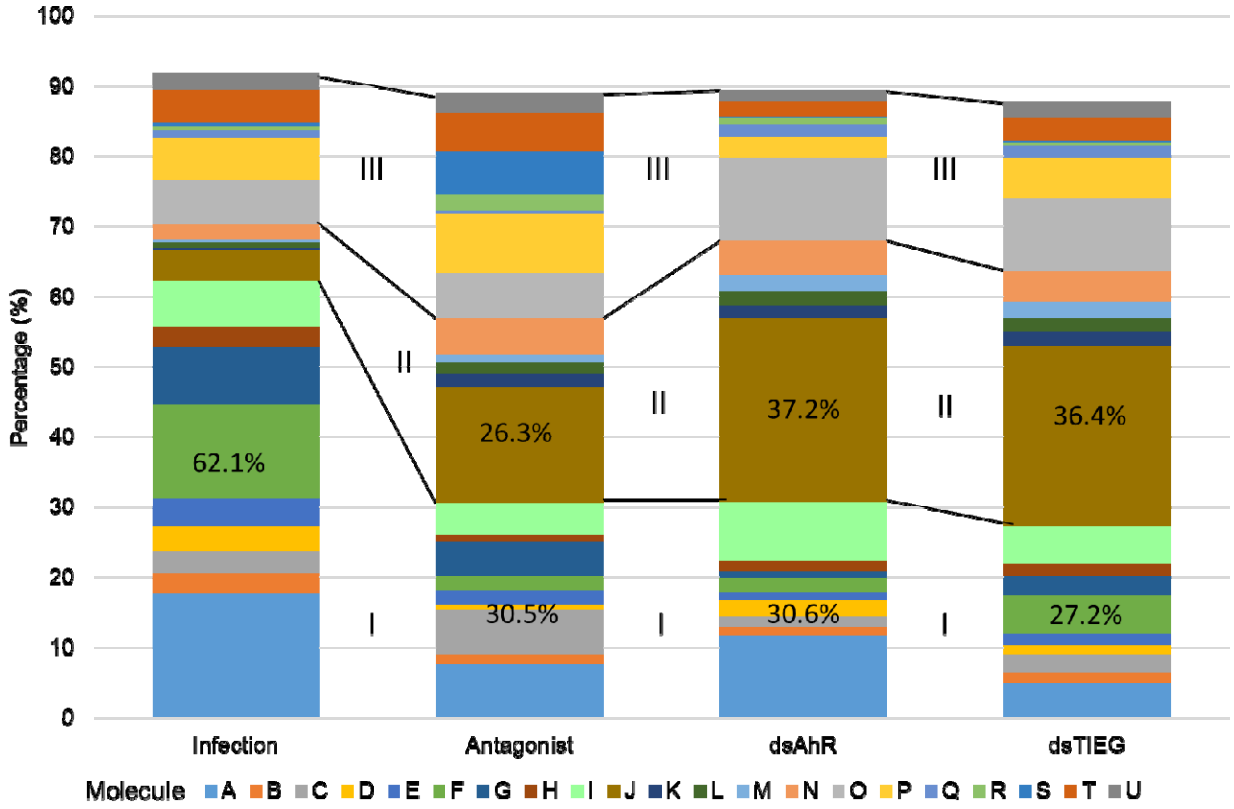

Figure S3. Co-expression modules analysis. The transcriptomic response in the cohorts of AhR antagonist, dsAhR and dsTIEG upon infection demonstrate similar impacts on co-expression modules. The number of genes in Category I modules was enriched in Infection cohort but was reduced to half in the AhR inactivated cohorts, while the number of genes in Category II modules was expanded in the AhR inactivated cohorts. The patterns represent the shared effects of AhR manipulation by both pharmacological and genetic approaches.

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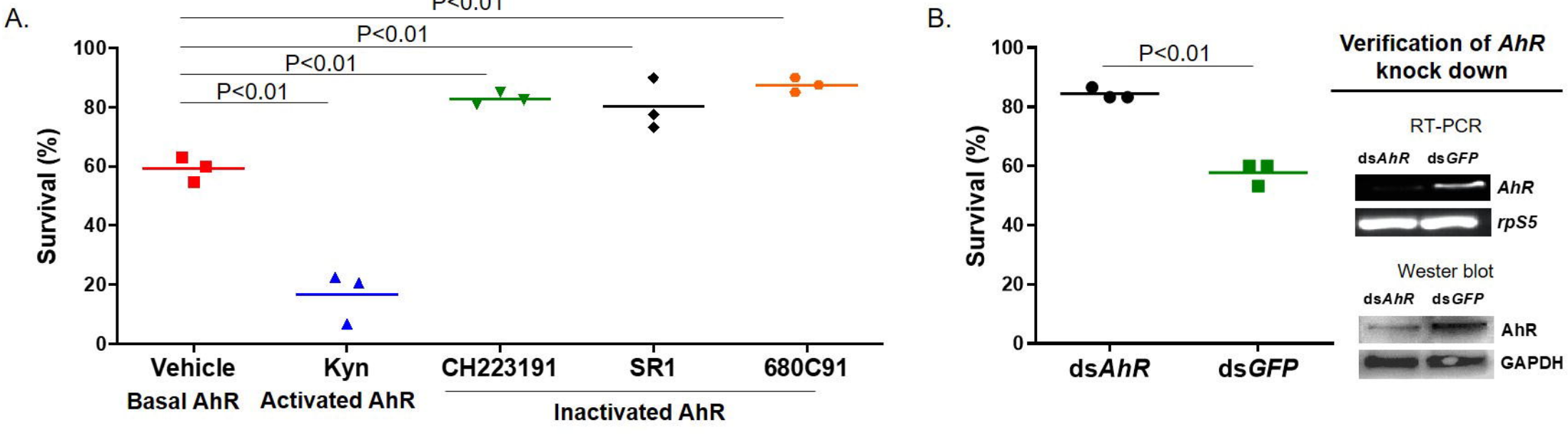
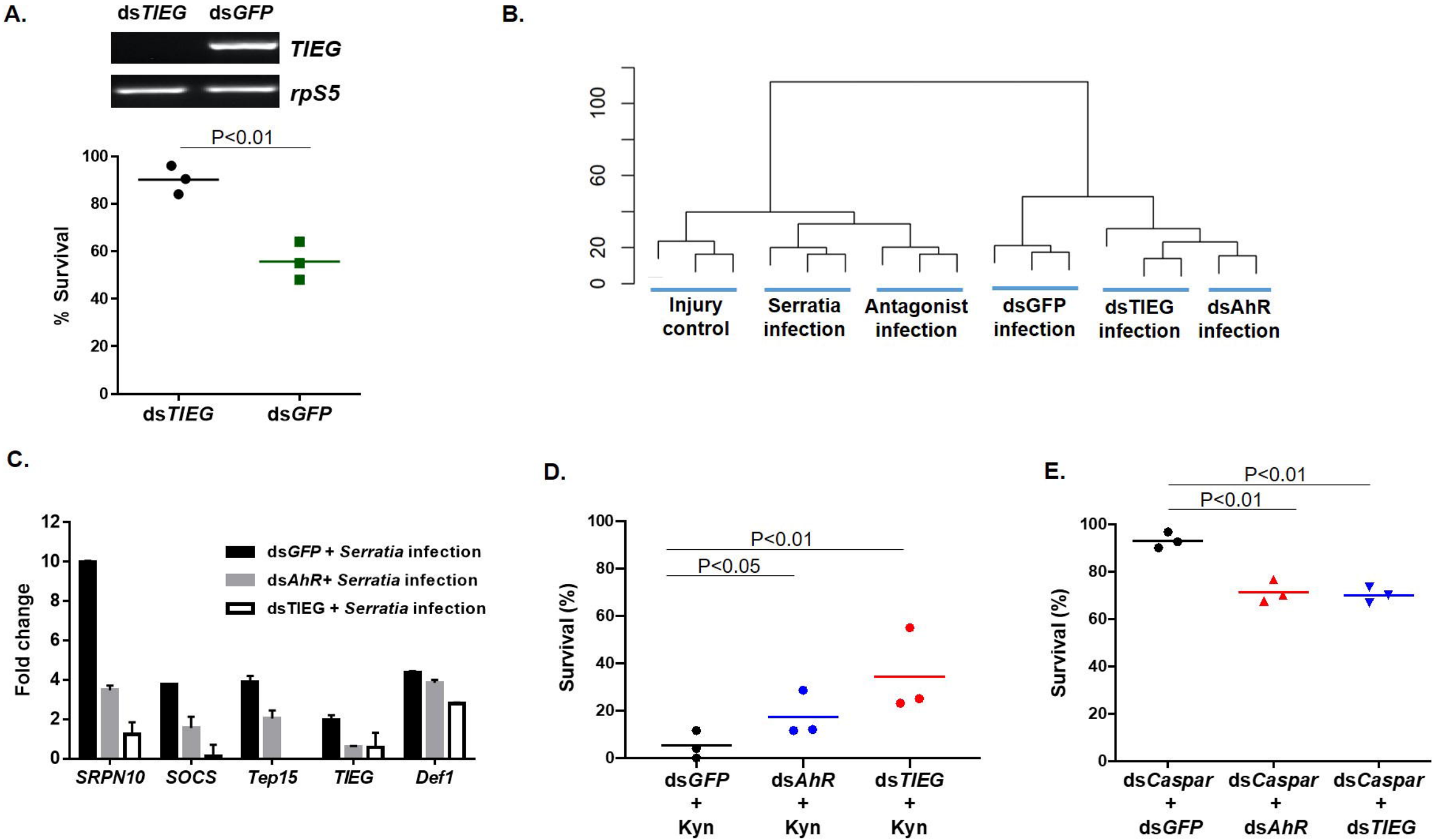
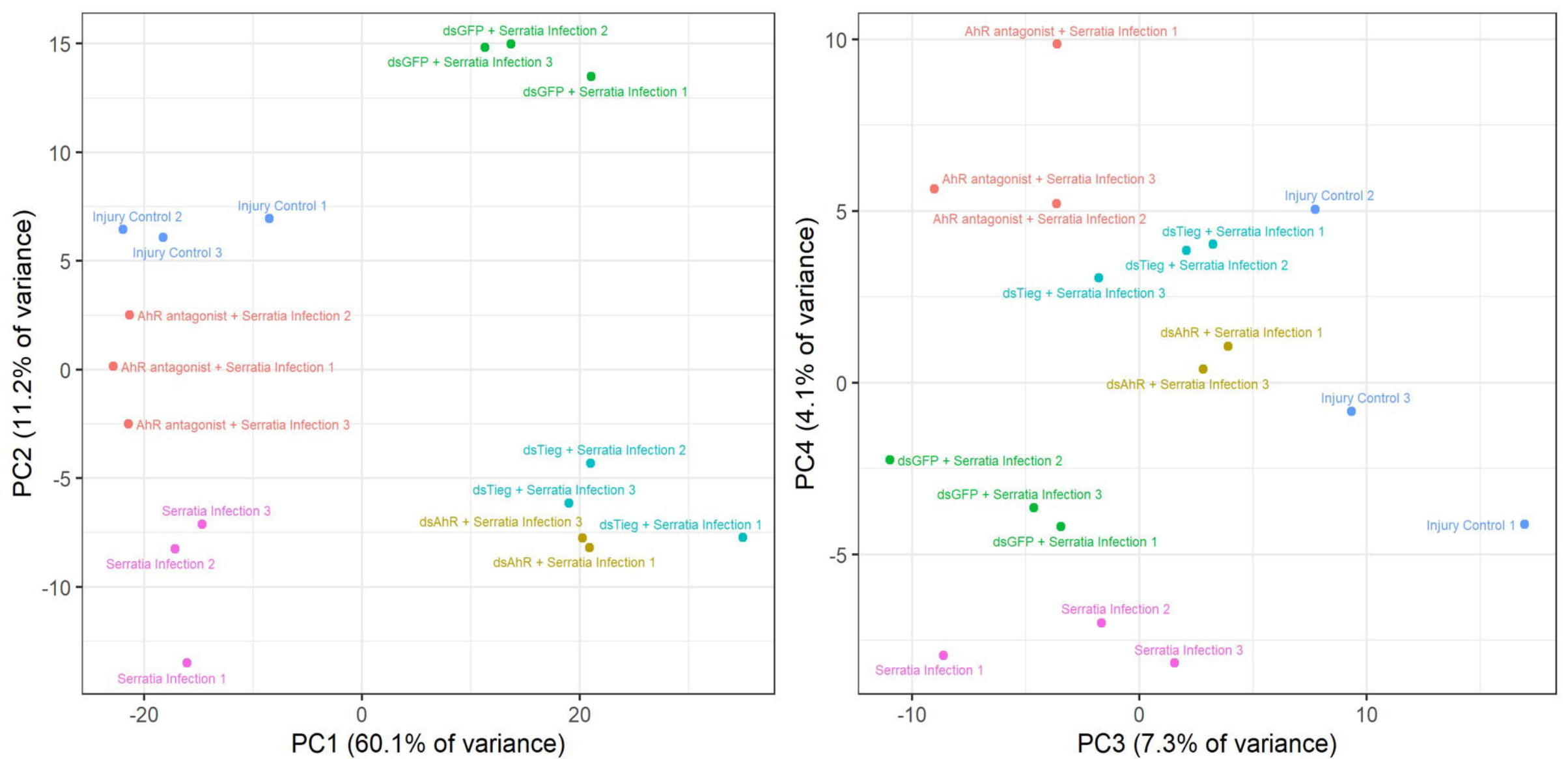
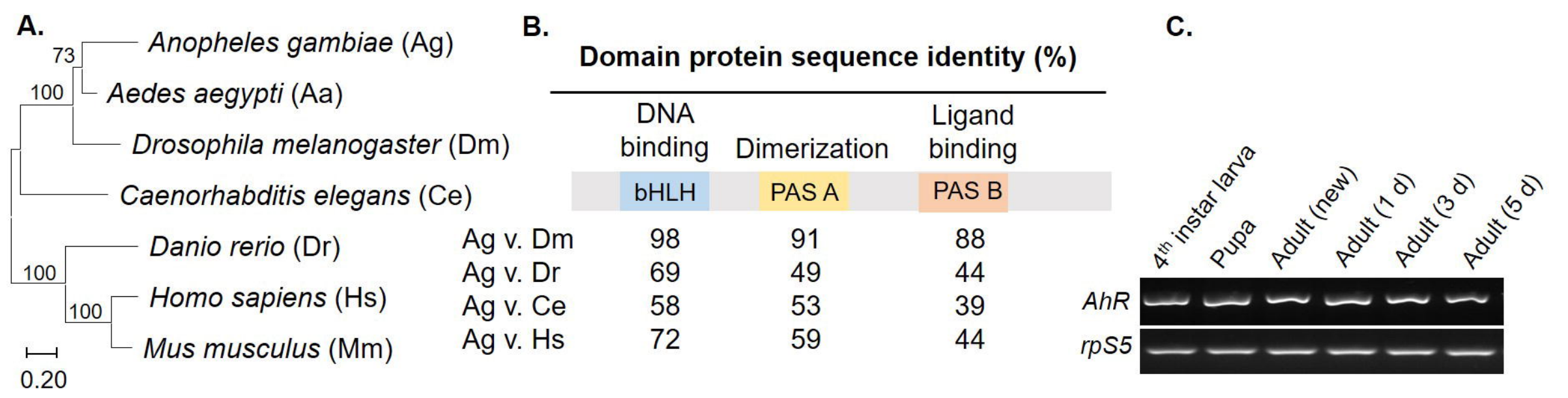
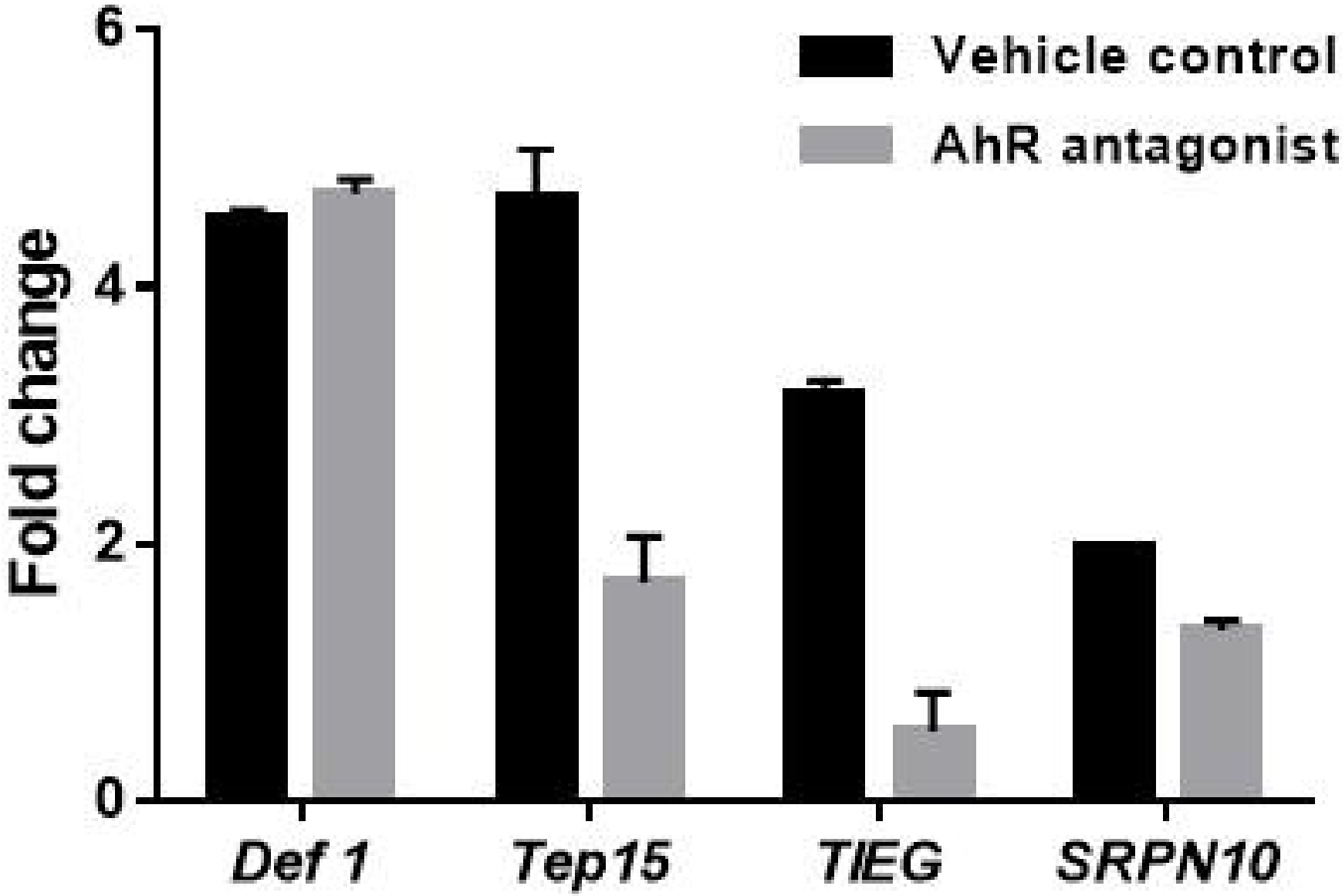
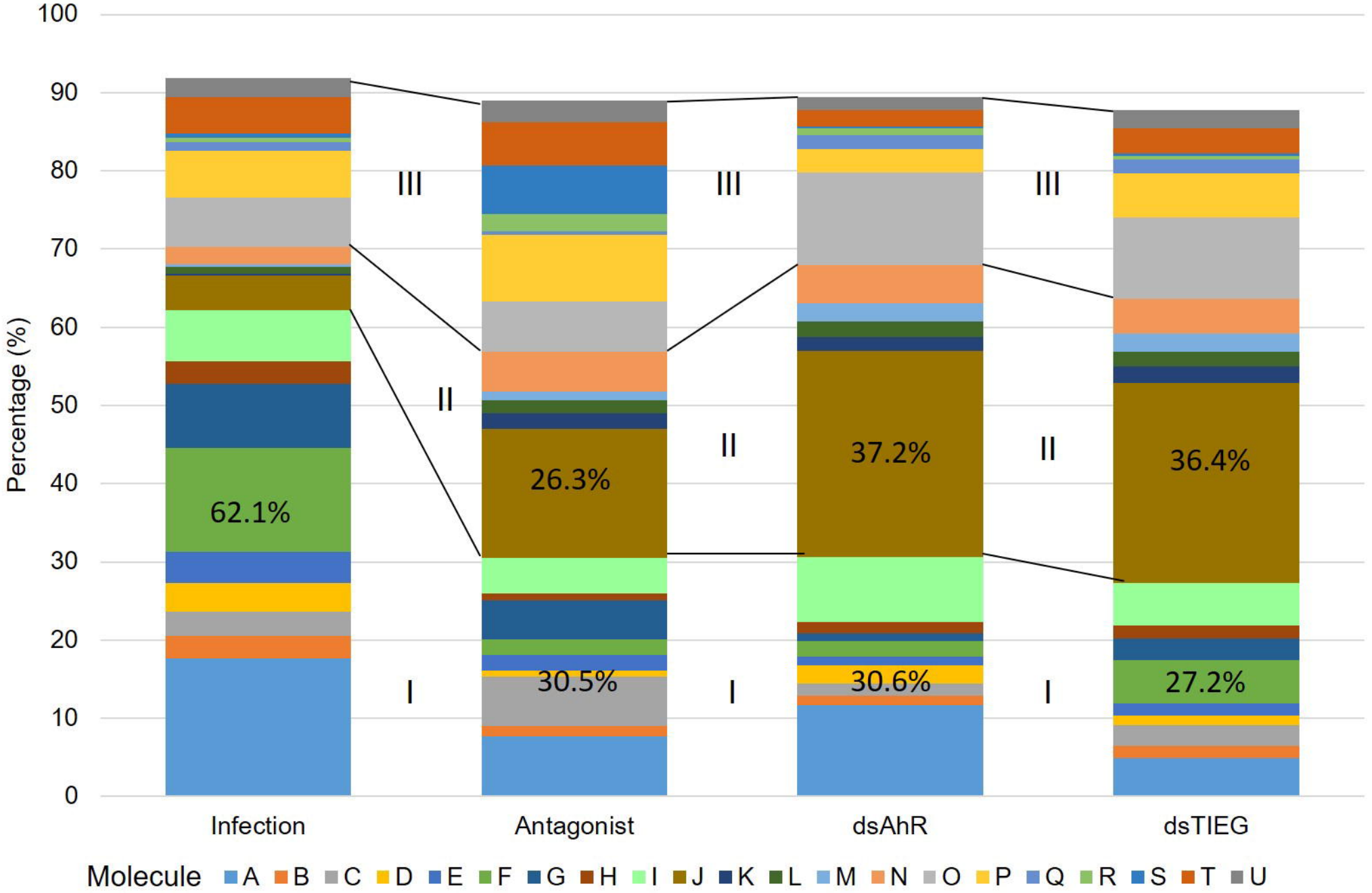

A.
P<0.01
P<0.01
P<0.01
P<0.01
Survival (%)
100
80
60
40
20
0
Vehicle
Basal AhR
Kyn
Activated AhR
CH223191
SR1
680C91
Inactivated AhR
B.
P<0.01
Survival (%)
100
80
60
40
20
0
dsAhR
dsGFP
Verification of AhR knock down
RT-PCR
dsAhR
dsGFP
AhR
rpS5
Wester blot
dsAhR
dsGFP
AhR
GAPDH


A.
dsTIEG
dsGFP
TIEG
rpS5
P<0.01
% Survival
100
80
60
40
20
0
dsTIEG
dsGFP
B.
100
60
20
0
Injury control
Serratia infection
Antagonist infection
dsGFP infection
dsTIEG infection
dsAhR infection
C.
Fold change
12
10
8
6
4
2
0
dsGFP + Serratia infection
dsAhR+ Serratia infection
dsTIEG + Serratia infection
SRPN10
SOCS
Tep15
TIEG
Def1
D.
Survival (%)
P<0.01
P<0.05
dsGFP + Kyn
dsAhR + Kyn
dsTIEG + Kyn
E.
Survival (%)
P<0.01
P<0.01
dsCaspar + dsGFP
dsCaspar + dsAhR
dsCaspar + dsTIEG


dsGFP + Serratia Infection 2
dsGFP + Serratia Infection 3
dsGFP + Serratia Infection 1
Injury Control 2
Injury Control 1
Injury Control 3
AhR antagonist + Serratia Infection 2
AhR antagonist + Serratia Infection 1
AhR antagonist + Serratia Infection 3
dsTieg + Serratia Infection 2
dsTieg + Serratia Infection 3
Serratia Infection 3
dsAhR + Serratia Infection 3
dsTieg + Serratia Infection 1
Serratia Infection 2
dsAhR + Serratia Infection 1
Serratia Infection 1
15
10
5
0
-5
-10
-20
0
20
PC2 (11.2% of variance)
PC1 (60.1% of variance)
AhR antagonist + Serratia Infection 1
AhR antagonist + Serratia Infection 3
AhR antagonist + Serratia Infection 2
Injury Control 2
dsTieg + Serratia Infection 1
dsTieg + Serratia Infection 2
dsTieg + Serratia Infection 3
dsAhR + Serratia Infection 1
dsAhR + Serratia Infection 3
Injury Control 3
dsGFP + Serratia Infection 2
dsGFP + Serratia Infection 3
dsGFP + Serratia Infection 1
Injury Control 1
Serratia Infection 2
Serratia Infection 3
Serratia Infection 1
10
5
0
-5
-10
10
PC4 (4.1% of variance)
PC3 (7.3% of variance)


A.
Anopheles gambiae (Ag)
Aedes aegypti (Aa)
Drosophila melanogaster (Dm)
Caenorhabditis elegans (Ce)
Danio rerio (Dr)
Homo sapiens (Hs)
Mus musculus (Mm)
73
100
100
100
0.20
B.
Domain protein sequence identity (%)
DNA binding
Dimerization
Ligand binding
bHLH
PAS A
PAS B
Ag v. Dm 98 91 88
Ag v. Dr 69 49 44
Ag v. Ce 58 53 39
Ag v. Hs 72 59 44
C.
4th instar larva
Pupa
Adult (new)
Adult (1 d)
Adult (3 d)
Adult (5 d)
AhR
rpS5


Vehicle control
AhR antagonist
Fold change
6
4
2
0
Def 1
Tep15
TIEG
SRPN10


100
90
80
70
60
50
40
30
20
10
0
Percentage (%)
III
II
I
62.1%
26.3%
30.5%
37.2%
30.6%
36.4%
27.2%
Infection
Antagonist
dsAhR
dsTIEG
Molecule
A
B
C
D
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F
G
H
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K
L
M
N
O
P
Q
R
S
T
U