Abstract
Several mosquitoes transmit human pathogens by blood feeding, with the gut being the main entrance for the pathogens. Thus, the gut epithelium defends the pathogens by eliciting potent immune responses. However, it was unclear how the mosquito gut discriminates pathogens among various microflora in the lumen. This study proposed a hypothesis that a damage signal might be specifically induced by pathogens in the gut. The Asian tiger mosquito, *Aedes albopictus*, encodes dorsal switch protein 1 (*Aa-DSP1*) as a putative damage-associated molecular pattern (DAMP). *Aa-DSP1* was localized in the nucleus of the midgut epithelium in naïve larvae. Upon infection by a pathogenic bacterium, *Serratia marcescens*, *Aa-DSP1* was released to hemocoel and activated phospholipase A2 (PLA2). The activated PLA2 increased the level of prostaglandin E2 (PGE2) in the gut and subsequently increased Ca2+ signal to produce reactive oxygen species (ROS) via dual oxidase (Duox). Inhibition of *Aa-DSP1* via RNA interference or specific inhibitor treatment failed to increase PGE2/Ca2+ signal upon the bacterial infection. Thus, the inhibitors specifically targeting eicosanoid biosynthesis significantly prevented the upregulation of ROS production in the gut and enhanced mosquito mortality after the bacterial infection. However, such inhibitory effects were rescued by adding PGE2. These suggest that *Aa-DSP1* plays an important role in immune response of the mosquito gut as a DAMP during pathogen infection by triggering a signaling pathway, DSP1/PLA2/Ca2+/Duox.

Introduction
Mosquitoes can transmit a wide range of pathogens that can cause diseases such as malaria, dengue, yellow fever, and Zika known to have a devastating impact on human health [1]. Although the use of insecticides for mosquitoes is a major tool for disease control, intensive use of insecticides leads to unexpected adverse outcomes such as environmental pollutants threatening human/wildlife health and development of insecticide resistance [2, 3]. Thus, alternative tools for mosquito control are urgently needed [4].
The Asian tiger mosquito, *Aedes albopictus*, was indigenous to tropical and subtropical areas of Asia. However, it is now regarded as one of the most rapidly spreading species in the world [1, 5]. As a capable vector, it can transmit at least 22 arboviruses including Chikungunya, Dengue, and Zika viruses, resulting in severe outbreaks worldwide [6].

Various microbes threaten mosquito survival in wild-life. Especially, the digestive tract of a mosquito is exposed to these microbes when ingesting diets. Although beneficial microbes are required for mosquito development, pathogens should be removed by gut immunity. To regulate gut immunity in a broad sense, two isoforms of a transcriptional factor, Nubbin, which is a homeodomain protein, antagonistically regulate the expression of immune-associated genes in the *Drosophila* midgut by inducing the gene expression and suppressing excessive expressions [7]. Among these immune-associated molecules, antimicrobial peptides (AMPs) and reactive oxygen species (ROS) act as two main immune effectors in the insect gut [8, 9]. AMPs play a crucial role in maintaining homeostatic microbial flora in the gut by regulating the growth of commensal microbes [10]. Upon pathogenic microbial infection, the midgut produces ROS to remove pathogens [11]. However, ROS and AMPs might have harmful effects on host insects and/or symbiotic microbes, and so their production should be tightly regulated [12].

ROS in the midgut is produced by dual oxidase (Duox) [13]. Duox contains a Ca\(^{2+}\)-binding EF domain. Its enzyme activity is induced by Ca\(^{2+}\) to produce ROS [14]. Thus, regulating ROS production can be achieved by regulating Duox activity. One regulator of Duox has been identified to be uracil released from bacterial pathogens in *Drosophila*, in which uracil acts as a pathogen-specific molecule that enables the host to distinguish uracil-releasing microorganisms as pathogens from no uracil-releasing commensals or symbionts in the gut mucosal epithelium [15]. The binding of uracil to an unidentified receptor on the midgut epithelium can trigger the PLC\(\beta\)/PKC/Ca\(^{2+}\) signal pathway to activate Duox [9]. In contrast, Duox is activated by eicosanoids in other insects such as two lepidopterans, *Spodoptera exigua* [16] and *Plutella xylostella* [17], in which inhibition of eicosanoid biosynthesis prevents ROS production in the midgut upon pathogen infection. These suggest that eicosanoids play a crucial role in gut immunity by activating Duox in insects, including *A. albopictus*.

Eicosanoids are a group of oxygenated C20 polyunsaturated fatty acids that can mediate various physiological processes including immune responses in metazoan animals [18]. Eicosanoid production is initiated by the release of free C20 polyunsaturated fatty acids from phospholipids due to the catalytic activity of phospholipase A\(_2\) (PLA\(_2\)) [19]. Among eicosanoids, prostaglandins (PGs) play a crucial role in mediating various immune responses in mosquito midgut [20]. Several PGs along with prostaglandin E\(_2\) (PGE\(_2\)) receptor and its downstream signal components found in *S. exigua* can mediate the induction of intracellular Ca\(^{2+}\) levels [21]. Thus, eicosanoids may mediate ROS production in *A. albopictus* by elevating Ca\(^{2+}\) levels.

A question is raised on how mosquito gut can discriminate pathogenic microbes from various gut microflora. We propose in this study that a novel insect damage-associated molecular pattern (DAMP) in mosquitoes might trigger gut immunity upon pathogen infection. Dorsal switch protein 1 (DSP1) is known as an insect DAMP molecule [22]. DSP1 is an orthologue of vertebrate high mobility group B1 (HMGB1) [23]. HMGB1 is a ubiquitously expressed and highly conserved nuclear protein that plays an important role in chromatin organization and transcriptional regulation in plants and animals [24]. Under stress conditions including pathogen infection, HMGB1 is released to act as a DAMP to activate the innate immune system by interacting with pattern recognition receptors (PRRs) [25]. Insect DSP1 can act as a transcription factor and a chromatin remodeling factor [23, 26]. In *S. exigua* infected by entomopathogenic bacteria, DSP1 is released from the fat body. It then activates PLA\(_2\) to elevate eicosanoids known to mediate cellular and humoral immune responses [22]. DSP1 of *Tenebrio molitor*, a coleopteran insect, can also mediate immune responses to bacterial infection [27]. Thus, we hypothesized that DSP1 in the mosquito midgut might act as a DAMP against pathogen infection. We also hypothesized that DSP1 could activate eicosanoid biosynthesis to mediate various immune responses along with Duox activation to produce ROS in the mosquito gut lumen.

### Materials and Methods

**Mosquito Rearing, Bacterial Infection, and Tissue Preparation**

*Aedes albopictus* mosquitoes were reared at 28°C with a relative humidity of 70 ± 5% under a 12 h/12 h day-night cycle. During adult stages, mosquitoes were maintained with 10% (wt/vol) sucrose solution. Two-day-old mosquitoes were fed on blood for oviposition. Larvae were reared on groundfish food supplements under the same rearing conditions. Under these conditions, *A. albopictus* underwent four larval instars (L1–L4) before pupation.
For bacterial infection, *Serratia marcescens*, a gram-negative bacterium, was cultured with a nutrient broth medium for 20 h at 28°C in a shaking (200 rpm) incubator. After centrifuging the culture at 8,000 g for 20 min, cells were washed with sterile water and used for immune challenge through a feeding assay. For oral infection of larval stages, *S. marcescens* (Sm) was added to the water at a final concentration of 10^4 CFU/mL.

For tissue preparation, whole alimentary canals were isolated from larval instars (L3–L4), pupae (2–3 days old), and adults (2–3 days old). In the case of L1–L2 larvae, the whole body was used. To prepare tissue samples, 50 L1–L2, 30 L3–L4 larvae, pupae, and adults were used.

**Chemicals and Their Application**

Arachidonic acid (AA: 5,8,11,14-eicosatetraenoic acid), deoxymethasone [DEX (11β, 16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3], naproxen [NAP (S)-(+)-2-(6-methoxy-2-naphthyl)propionic acid], esculetin (ESC: 6-hydroxy-2-methoxycoumarin), 3-ethoxy-4-methoxyphenol (EMP) as a (+)-2-(6-methylpregna-1,4-diene-3], naproxen [NAP (S)-(+)-2-(6-methoxy-2-naphthyl)propionic acid], esculetin (ESC: 6-hydroxy-7-methoxycoumarin), 3-ethoxy-4-methoxyphenol (EMP) as a specific inhibitor to DSP1 [22], and PGE2 (5Z,11α,13E,15S)-11,15-dihydroxy-9-oxoprost-5,13-dienoic acid) were purchased from Sigma-Aldrich Korea (Seoul, Korea) and leukotriene B4 (LTB4; 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). These chemicals were supplied in acetone, ethanol, or powder form. Chemicals in powder form were directly dissolved in dimethyl sulfoxide (DMSO) and stock concentrations (100 μg/µL or 10^5 ppm) were prepared. In case of chemicals in acetone or ethanol, they were dried with nitrogen gas under a fume hood and then prepared in stock concentrations (100 μg/µL or 10^5 ppm) with DMSO. Finally, a working solution (1,000 ppm) was prepared by a serial dilution using DMSO. Fura-8AM was purchased from AAT Bioquest (Sunnyvale, CA, USA) and dissolved in DMSO. Phosphate-buffered saline (PBS) was prepared with 100 mM phosphoric acid and adjusted to pH 7.4.

**Bioinformatics**

In the present study, *A. albopictus Duox* (Aa-Duox; GenBank accession number: XM_020077533.2) and DSP1 (Aa-DSP1; GenBank accession number: XP_019544674.2) were used. Protein domain structure was predicted using HMMER (https://www.ebi.ac.uk) and Pfam (http://pfam.xfam.org). Phylogenetic analyses and phylogenetic tree construction with the Neighbor-joining method were performed using MEGA6 and ClustalW programs, respectively. Bootstrapping values were obtained with 1,000 replications to support branching and clustering.

**RNA Extraction and RT-qPCR**

Total RNAs were extracted from all developmental stages of *A. albopictus* using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. To extract RNAs from whole bodies of *A. albopictus* at different developmental stages, >200 eggs, 50 L1–L2, 10 L3–L4, 5 pupa, and five adults were used. Different tissue samples (fat body, midgut, and epidermis) were isolated from 50 L4 larvae. Extracted RNAs were dissolved in 50 μL diethyl pyrocarbonate (DEPC)-treated deionized and distilled water. First-strand cDNA was synthesized from 1 μg of RNA using Maxime RT PreMix (Intron Biotechnology, Seoul, Korea) containing oligo dT primers according to the manufacturer’s instruction. The synthesized cDNA was used as a template for PCR amplification or for constructing dsRNA. Real-time quantitative polymerase chain reaction (qPCR) was carried out in a reaction volume of 20 μL using 2× SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan), 5 mM of gene-specific forward and reverse primers, and 80 ng cDNA as template. PCR amplification was performed at 95°C for 10 min for initial heat treatment, followed by 40 cycles of 98°C for 30 s, 52°C (Aa-Duox) or 55°C (Aa-DSP1) for 30 s, and 72°C for 30 s. It was then finished with a final extension step at 72°C for 7 min. Melting curves of products were obtained to confirm amplification specificity. For quantitative analysis and estimating mRNA expression levels of target genes with β-actin as a reference gene, the comparative Ct (2-ΔΔCT) method [28] was used. All experiments were independently replicated three times. All primer sequences used in this study for RT-qPCR and RT-qPCR are presented in online supplementary Table S1 (see www.karger.com doi:10.1159/000524561 for all online suppl. material).

**dsRNA Preparation and RNAi**

Double-stranded RNA (dsRNA) specific to *Aa-Duox* or *Aa-DSP1* was prepared as described in our previous study [16]. Briefly, a partial sequence was produced by PCR using gene-specific primers containing a T7 promoter sequence at the 5′ end (online suppl. Table S1). The PCR product was used as a template to generate dsRNA using a MEGAScript RNA interference (RNAi) kit (Ambion, Austin, TX, USA) according to the manufacturer’s instruction. Sense and antisense-RNA strands were synthesized using T7 RNA polymerase at 37°C for 4 h. As control dsRNA (dsCON), a 500 bp fragment of the green fluorescent protein gene was synthesized [29]. The resulting dsRNA was purified and mixed with transfection reagent Metafectene PRO (Biontex, Planegg, Germany) at a ratio of 1:1 (vol/vol) and then incubated at 25°C for 30 min to form liposomes. Five microgram of dsRNA was added to 1 mL of water medium containing L4 larvae. At different time points post dsRNA treatment, RNAi efficacy was determined by RT-qPCR as described above. Each treatment was replicated three times.

**ROS Measurement**

An OxiSelect intracellular ROS assay kit (Cell Biolabs, San Diego, CA, USA) was used to quantify ROS levels in L4 larvae of *A. albopictus*. For measuring ROS levels in the gut lumen, gut content was collected by squeezing out the midgut and centrifuged at 1,000 g for 2 min. The supernatant was mixed with 0.1× DCFH-DA (dichlorofluorescein diacetate) at the same volume. After incubation at 37°C for 30 min, the reaction product (150 μL) was then transferred to a 96-well plate. Fluorescence was read at an emission wavelength of 530 nm after excitation at 480 nm. A calibration curve was drawn using serial dilutions of dichlorofluorescein standard in TC-100 cell culture medium. ROS levels were normalized to protein amounts [30]. At 12 h after bacterial and/or chemical treatment, ROS production was assessed. Each treatment was replicated three times with independent sample preparations using five larvae per replication.

**Pathogenicity Test**

Ten L2 larvae were added to each well of a 48-well plate. Chemicals including DEX, AA, NAP, ESC, PGE2, EMP, and LTB4 were dissolved in DMSO and added to larval water at a final concentration of 1,000 ppm. Mortality in the presence or absence of Sm (10^4 CFU/mL) was checked for 4 days. All experiments were performed using three independent biological replicates.
**Secretory PLA₂ Activity**

Secretory PLA₂ (sPLA₂) activity in the carcass (excluding gut) of L4 larva was fluorometrically determined using a commercial assay kit (sPLA₂ assay kit; Cayman Chemical, Ann Arbor, MI, USA) with diheptanoyl thio-phosphatidyl choline as an enzyme-substrate following the method described by Vatanparast et al. [29]. A spectrofluorometer (VICTOR multi-label Plate reader; PerkinElmer, Waltham, MA, USA) was used to measure enzyme activity. Changes in absorbance at 405 nm of the reaction product were measured and plotted to obtain the slope of a linear portion.
of the curve. Each treatment was replicated three times with independent sample preparations using five larvae per replication. Specific enzyme activity (μmol/min/μg) was calculated by dividing the absorbance change by the protein amount used as the enzyme source for the reaction.

**Western Blotting**

For a bacterial treatment with Sm, L4 larvae were fed with Sm (10⁴ CFU/mL)-treated water for 12 h. For the inhibitory assay, EMP (1,000 ppm) was added along with Sm (10⁴ CFU/mL). At 12 h posttreatment, for each sample preparation, the carcass (excluding gut) of 10 L4 larvae was collected carefully into PBS containing a protease inhibitor cocktail (Sigma-Aldrich, Korea) and 1 mM phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, Korea). As we observed the elevated SPLA₂ activity at 12 postinfection, we selected this time point for DSP1 expression analysis. After centrifuging at 500 g for 5 min, the supernatant was collected and mixed with 4× denatured sample buffer (200 mM Tris-HCL, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, and 40% glycerol). After 5 min of denaturation at 95°C, proteins (20 μg per sample) were separated on 10% SDS-PAGE at 95 V. Separated proteins from the gel were transferred onto nitrocellulose membranes (BioRad, Hercules, CA, USA) for 50 min at 95 V in chilled transfer buffer (25 mM Tris, 190 mM glycin, 20% methanol, pH 8.5). Membranes were briefly rinsed with Tris-buffered saline containing Tween-20 (TBST) (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) and then blocked with 3% bovine serum albumin (BSA) in TBST at room temperature (RT) for 1 h. Membranes were then incubated with DSP1 antibody raised against Se-DSP1 in rabbit (Abclon, Seoul, Korea) diluted 5,000 times with TBST containing 3% BSA at 4°C overnight. After washing three times with TBST (10 min for each), the membrane was incubated with an anti-rabbit IgG-alkaline phosphatase secondary antibody (Sigma-Aldrich, Korea) at a dilution of 1:20,000 in TBST containing 3% BSA for 1 h at RT. Blots were rinsed three times with TBST. As a reference protein, α-tubulin was used. It was detected with a polyclonal antibody (GTX112141; GeneTex, Irvine, CA, USA) after dilution with BCIP (5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt)/NBT (nitro-blue tetrazolium chloride) (Sigma-Aldrich, Korea) as a substrate of alkaline phosphatase.

**Immunofluorescence Assay**

L4 larvae were fed with Sm (10⁴)-treated water. After 12 h of feeding, the midgut was collected onto glass slides. After removing gut contents, TC100 insect tissue culture medium was added immediately, followed by incubation at RT in a wet chamber for 5 min. After removing the medium, the gut sample was then fixed with 4% formaldehyde for 20 min at RT. After the fixative was replaced with PBS, the midgut was incubated at RT for 10 min. After washing with PBS three more times, the midgut was permeabilized with 0.2% Triton X-100 in PBS for 20 min at RT. After washing with PBS three times, blocking was done with 5% skimmed milk powder in PBS at RT for 20 min. A DSP1 antibody [22] raised against Se-DSP1 in rabbit was added after it was diluted with 3% BSA in PBS (1:100) followed by incubation at RT for 1 h 20 min. After washing with PBS three times, the midgut was incubated with an anti-rabbit secondary antibody (Sigma-Aldrich, Korea) diluted with 3% BSA in PBS (1:5,000) for 1 h. After washing with PBS three times, midguts were incubated with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) (Thermo Scientific, Korea) in PBS at RT for 5 min for nucleus staining. To detect PGE₂ level in the gut epithelium, 1% of PGE₂ antibody (ab2318; Abcam, Cambridge, UK) in PBS was used after blocking with 5% skimmed milk at RT for 2 h. After washing with PBS three times, gut samples were incubated with 1% anti-rabbit-FITC conjugated antibody (Thermo Fisher Scientific, Korea) in PBS at RT for 1 h. After washing with PBS three times, the nucleus was stained with DAPI (1 μg/mL) as described above. After washing with PBS three times and adding 50% glycerol, tissue samples were covered by cover glass and observed with a fluorescence microscope (DM2500; Leica, Wetzlar, Germany) at ×200 magnification. Fluorescence changes were analyzed using ImageJ software (https://imagej.nih.gov/ij). Each treatment was replicated three times, and each replication contained three larvae.

**In vitro Ca²⁺ Signaling in Response to Sm Treatment**

L4 larvae were challenged with Sm at 10⁴ CFU/mL for 12 h. Midgut tissues of challenged larvae were dissected carefully into TC100 insect medium and incubated at RT in a wet chamber for 10 min. After removing TC100, midgut tissues were then incubated with 3 μL Fura-8AM (1 mM) at RT for another 10 min. After washing with PBS three times, midgut tissues were permeabilized with 0.2% Triton X-100 in PBS for 20 min at RT. Following washing with PBS three times, the midgut was incubated with DAPI (1 μg/mL) in PBS at RT for 5 min to stain the nucleus. Finally, the midgut was washed with PBS three times and fixed with 50% glycerol. Fura signals in the midgut were observed under a fluorescence microscope (DM500; Leica, Wetzlar, Germany) at ×200 magnification. Fluorescence changes were analyzed using ImageJ software (https://imagej.nih.gov/ij). Each treatment was replicated three times, and each replication contained three larvae.

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**Fig. 1.** Molecular characterization of Aa-Duox. **a** Functional domain and phylogenetic analyses of Aa-Duox. Domains were predicted using HMMER (https://www.ebi.ac.uk) and Pfam (http://pfam.xfam.org). Predicted domains included "Pox" for peroxidase, "EF" for calcium-binding EF-hand, "TM" for transmembrane, "Ferric-reduct" for ferric chelate reductase, "FAD" for FAD-binding domain, and "NAD" for NAD-binding domain. The tree was generated by the Neighbor-joining method using MEGA 6.0. Bootstrapping values were obtained with 1,000 repetitions to support branch and clustering. Amino acid sequences were retrieved from GenBank. Accession numbers of genes are shown in online supplementary Table S2. **b** Expression profile of Aa-Duox. Expression patterns in different developmental stages: egg, first to fourth-instar larva ("L1–L4"), pupa, and adult. **c** Expression patterns and induction of Aa-Duox expression in response to bacterial challenge in indicated tissues of L4 larvae, including midgut ("Gut"), epidermis, and fat body. L4 larvae were treated with Sm (10⁴ CFU/mL) through the feeding assay for 12 h and dissected for studying expression patterns. **d** Correlation between Aa-Duox expression and ROS production in the midgut of L4 larvae. β-Actin was used as a reference. Each treatment was replicated three times with independent tissue preparations. Different letters and asterisk (*) above standard deviation bars indicate significant differences among means at type I error = 0.05 (LSD test). LSD, least squared difference.
**Gut Microbiome**

For gut bacterial density analysis, mosquito larvae at L4 instar were dissected under aseptic conditions consisting of cold anaestheticization, surface sanitization by submersion in 70% ethanol, and rinsing in sterile PBS (1 mM, pH 7.4). Entire guts were collected using 70% ethanol-cleaned forceps. DNA samples were isolated using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Extracted DNA was used as a template in qPCRs with 16S rRNA bacterial primers (online suppl. Table S1). Bacterial 16S rRNA DNA was amplified by RT-qPCR as described above with different annealing temperature 54°C for 30 s. Each measurement used 10 gut samples and was independently replicated three times. Bacterial density was expressed relatively amounts per gut in each control and treatment.

For library construction, DNA was extracted from the gut tissues of the respective dsDuox and dsCON treatments at 24 h postinfection and sent to Macrogen (Seoul, Korea) for sequencing. After performing quality control, qualified samples were proceeded to library construction using the Herculase II fusion DNA polymerase Nextera XT index kit (Illumina, San Diego, CA, USA). For cluster generation, the library was loaded into a flow cell and each fragment was then amplified into distinct, clonal clusters through bridge amplification. Then, the templates were sequenced in Illumina SBS technology. Sequencing data were converted into raw data for analysis. Raw reads were processed with Mothur version 1.43.0 (https://www.mothur.org/wiki/). Low-quality sequences such as the presence of ambiguous nucleotides, more than eight homopolymers, sequence length lower than the 2.5th percentile, and sequence length higher than the 97.5th percentile were removed. The remaining sequences were pre-clustered to reduce sequencing error (allowing one difference for every 100 bp). Chimeras were removed with VSEARCH (https://github.com/torognes/vsearch). Non-bacterial sequences were removed based on a preliminary classification using the SILVA v132 database (https://www.arb-silva.de/documentation/release-132/). Singletons were removed to avoid operational taxonomic unit (OTU: the operational definition of a species or group of species) overestimation and because of the high sequencing depth of the sampling. Bacterial diversity was estimated with the Chao1, Shannon, and Inverse Simpson indices based on OTU analysis.

**Data Analysis**

All bioassays were conducted using three independent biological replicates and plotted by mean ± standard deviation using SigmaPlot (Systat Software Inc., San Jose, CA, USA). Means and variances of treatments were compared by the least squared difference test of one-way ANOVA using the PROC GLM of SAS program [31] and discriminated at type I error of 0.05.

**Results**

Aa-Duox and ROS Production in the Midgut of A. albopictus

Interrogation to the transcriptome of A. albopictus with the Duox sequence of D. melanogaster detected a highly homologous (E value = 0.0) orthologue (Fig. 1a). This Aa-Duox ORF encoded a 1,508 amino acid sequence with conserved domains found in other Duox genes such as the Ca<sup>2+</sup>-binding domain and peroxidase domain. This identification was also supported by a phylogenetic analysis indicating that it was clustered with other dipteran Duox genes. Aa-Duox was expressed in all developmental stages with the highest expression at the pupal stage (Fig. 1b). In the larval stage, it was expressed in all three tissues (gut, epidermis, and fat body) tested in this study (Fig. 1c). Oral administration of Sm, an entomopathogen, significantly (p < 0.05) increased the expression level of Aa-Duox in the midgut but not in the epidermis or the fat body. The induction level of Aa-Duox expression was highly correlated (r = 0.85; p < 0.01) with the increase of ROS levels in the gut lumen (Fig. 1d).

**Upregulation of Aa-Duox Expression Is Controlled by DSP1 in the Midgut**

To explain the upregulation of Aa-Duox upon immune challenge, a DAMP molecule in the gut epithelium was investigated. A DSP1 ortholog of A. albopictus was predicted from its transcriptome (Fig. 2a). It encoded 448 amino acid residues and possessed two HMG boxes along with both terminal extensions. Phylogenetic analysis in-
dicated that Aa-DSP1 was clustered with other dipteran DSP1 genes. It was expressed in all developmental stages (Fig. 2b). High expression of Aa-DSP1 was found in late larval stages. Its expression was inducible upon immune challenge, especially in the gut and fat body (Fig. 2c). Aa-DSP1 was localized in the nucleus of the midgut epithelium in the naïve larvae because it overlapped with DAPI (Fig. 2d). Although it was not detected in the plasma of naïve larvae, Aa-DSP1 was released into the plasma in response to bacterial challenge (Fig. 2e). However, EMP, a specific inhibitor for DSP1 [32], prevented the release of Aa-DSP1 from the midgut nucleus (the last lane of Fig. 2e). The release of Aa-DSP1 from the nucleus was supported by the reduction in the protein amount of AaDSP1 in the gut epithelium (Fig. 2f) based on cell image (“Sm” treatment in Fig. 2d).

Aa-DSP1 Activates PLA2 to Upregulate ROS Levels in the Gut Lumen

Bacterial challenge increased PLA2 enzyme activity, induced Aa-Duox expression, and subsequently increased ROS levels in the gut lumen (Fig. 3a). However, these increases were significantly (p < 0.05) suppressed after treatment with EMP, known to inhibit Aa-DSP1 release from the gut epithelium. Such immunosuppression induced by EMP increased bacterial virulence to A. albopictus larvae in a dose-dependent manner (Fig. 3b). However, EMP alone did not give any toxicity even at 1,000 ppm to the mosquito larvae (online suppl. Fig. S1).

The influence of Aa-DSP1 on PLA2 activation was assessed by RNAi treatment. Results are shown in Figure 3c. Feeding dsRNA of Aa-DSP1 to mosquito larvae resulted in a significant reduction of Aa-DSP1 expression.
DSP1/PLA2 Induces Ca$^{2+}$ Signal in the Midgut Epithelium of A. albopictus

Bacterial infection by Sm significantly ($p < 0.05$) increased Ca$^{2+}$ levels in the midgut of A. albopictus (Fig. 5a). However, EMP treatment inhibited the induction of the Ca$^{2+}$ signal, suggesting a role of Aa-DSP1. PGE$_2$ treatment without challenge by Sm induced Ca$^{2+}$ signals in the midgut epithelium, suggesting a PGE$_2$/Ca$^{2+}$ immune signaling between Aa-DSP1 and PLA$_2$ (Fig. 5b). Indeed, RNAi specific for Aa-DSP1 suppressed PGE$_2$ induction in the gut epithelium (Fig. 5c).

Cyclooxygenase or Lipoxygenase Inhibitors Inhibit Mosquito Gut Immunity and Enhance Virulence of Sm

The influence of gut immunity modulated by eicosanoids was investigated by assessing the virulence of Sm, an entomopathogen, against A. albopictus. Mosquito larvae infected with Sm died with a red-colored appearance (Fig. 6a). The insecticidal activity of Sm increased in a dose-dependent manner (Fig. 6b). NAP or ESC treatments significantly ($p < 0.05$) increased larval mortality. However, the addition of PGE$_3$ or LT$_B_4$ significantly ($p < 0.05$) rescued such lethal effects of NAP and ESC (Fig. 6c).

**Eicosanoids Mediate Mosquito Gut Immunity**

PLA$_2$ activity induced by Aa-DSP1 release was further analyzed by investigating specific catalytic products of the enzyme. Results are shown in Figure 4. DEX, an inhibitor of PLA$_2$, prevented the induction of Aa-Duox expression and subsequent ROS increase in the gut lumen (Fig. 4a). However, the addition of AA (a catalytic product of PLA$_2$) to the group treated with DEX significantly rescued its inhibitory activities. NAP (a cyclooxygenase inhibitor) and ESC (a lipoxygenase inhibitor) showed similar inhibitory activities (Fig. 4b). However, PGE$_2$ or LT$_B_4$ (eicosanoids) significantly ($p < 0.05$) rescued inhibitory activities of these specific inhibitors. DEX, Nap, or Esc alone did not show any significant influence on the expression of Aa-Duox (online suppl. Fig. S2). These results suggest that eicosanoids PG and LT can mediate mosquito gut immunity.

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RNAi of Aa-Duox Expression Alters the Microbial Flora in the Mosquito Gut

To support the role of ROS in the gut immunity against the bacterial infection, the expression level of Aa-Duox was suppressed by the gene-specific RNAi to decrease ROS level in the gut (Fig. 7). The RNAi treatment suppressed Aa-Duox expression levels, in which more than 80% expression level was reduced at 24 h after the treatment (Fig. 7a). When the bacterial density of the gut lumen was assessed by 16S rRNA amount, the RNAi treatment specific to Aa-Duox significantly ($p < 0.05$) elevated the bacterial population (Fig. 7b). However, the bacterial diversity was reduced by the RNAi treatment because the number of bacterial species (= OTUs) decreased along with diversity indices, in which Chao1 is the richness estimate for an OTU definition, the Shannon index takes into account the number and evenness of species, and Inverse Simpson represents the probability that two randomly selected individuals in the habitat will belong to the same species (Fig. 7c). For example, Pseudomonas...
guenzennei, Flectobacillus roseus, and Rhodoferax intermedius were increased, while Pseudocidovarex intermedius, Ehrlichia ewingii, and Comamonas granuli were decreased in their densities.

**Discussion**

Pathogens are recognized in the hemocoel where PRRs discriminate against specific pathogens and trigger immune responses via immune mediators [33]. In mosquitoes transmitting Plasmodium parasites, ookinetes penetrating gut epithelium may encounter both free and membrane-bound PRRs in the hemocoel, which would promote downstream immune signaling or directly induce immune responses [34]. PRRs can activate immune deficiency pathway [35] and other major immune signaling pathways such as Toll, Jun-N-terminal kinase, and Janus kinase/signal transducers and activators of transcription [36, 37]. With respect to gut immunity, the gut epithelium in Drosophila is immunocompetent and upon enteric infection initiates innate immune responses via the immune deficiency signal pathway, mediating AMP production as well as the pathway related to Duox activation to produce ROS [38–40]. Alternatively, gut immunity is suppressed by Caudal [9] and Nubbin [7] to preserve commensal and symbiotic microbes. However, a clear pathogen recognition in the gut lumen where numerous microbes reside remained elusive. Thus, this study tested the role of HMGB1-like DSP1 as another direct damage signal to the gut epithelium in response to a bacterial pathogen.
Oral infection by Sm caused significant mortality of *A. albopictus* larvae in the present study. Nehme et al. [39] have shown that Sm can resist the systemic immune response elicited by AMPs in *Drosophila* due to the presence of lipopolysaccharide-O-antigen. However, when ingested, flies died at a rate dependent on the bacterial load in the food. ROS and AMPs produced from the insect gut are effective in defending against microbial infections [41]. Our current study showed that ROS production induced by eicosanoids was effective in defending against the bacteria in *A. albopictus*. This suggests that ROS can act as a potent immune effector against bacterial infection in the mosquito, using a microbiome analysis. This was supported by a change in bacterial flora using a microbiome analysis after RNAi specific to Aa-Duox expression. This suggests the defense role of ROS in the gut against the pathogen infection of Sm.

ROS production in *A. albopictus* was positively correlated with the expression level of Aa-Duox. Duox catalyzes NADPH-dependent oxidation to produce hydrogen peroxide and subsequent ROS. Aa-Duox expression was observed in all developmental stages, with a high expression level at the pupal stage of naïve mosquitoes. The physiological significance of such high expression of Aa-Duox at the pupal stage is unknown. Interestingly, another mosquito species, *Anopheles stephensi*, also exhibits a high expression of its Duox gene during the pupal stage [42]. In that study, RNAi of this Duox gene in *An. stephensi* suppressed its parasite *Plasmodium* development, suggesting a defensive role of Duox activity in the mosquito gut against pathogen infection. Indeed, Aa-Duox expression was inducible by Sm infection. In addition to the immune defense role of Duox in mosquitoes, Duox activity has a function of maintaining microbial homeostasis in mosquitoes. Infection by *Beauveria bassiana*, an entomopathogenic fungus, can cause dysbiosis of *An. stephensi* gut microbiota, with a significant increase in gut bacterial load and a significant decrease in bacterial diversity via downregulation of Duox expression [43].

The induction of Aa-Duox expression was caused by the release of Aa-DSP1. RNAi of Aa-DSP1 expression prevented the induction of Aa-Duox expression, which failed to upregulate ROS level in the gut lumen in response to bacterial infection by Sm. In addition, treatment with EMP, a specific inhibitor, prevented Aa-DSP1 release into plasma. EMP can bind to DSP1 of *Spodoptera exigua* and prevents its translocation out of cells [32]. Bacterial infection by Sm was accompanied by ROS generation in the present study, suggesting that a damage
signal might have occurred in the gut epithelium. In vertebrates, HMGB1 acts as a damage signal after it is released from the nucleus into the plasma where it interacts with particular receptors such as Toll-like receptors to activate immune responses [25, 44]. DSP1, an ortholog of vertebrate HMGB1, has been found in S. exigua [22]. It is known to be secreted from hemocytes or fat bodies in response to immune challenges [22]. This secreted DSP1 can interact with specific Toll receptors and activate PLA2 to induce cellular and humoral immune responses [32]. In the current study, DSP1 signal was mostly found in the nucleus of the gut epithelium of naïve A. albopictus larvae. After infection by Sm through oral feeding, DSP1 was observed in the plasma of infected larvae. However, it was not detected in the plasma of naïve larvae. This delineated that DSP1 in the nucleus of the gut epithelium was released into the hemolymph after Sm infection-mediated gut injury.

The release of Aa-DSP1 increased PLA2 activity in A. albopictus presumably to synthesize eicosanoids. Indeed, both eicosanoids PGE2 and LTB4 were effective in inducing Aa-Duox expression, subsequently elevating ROS levels in the gut lumen. In Anopheles gambiae (another mosquito), specific leukotriene called lipoxin A4 plays a role in immune priming after the mosquito midgut was infected by Plasmodium by elevating PGE2 levels in the midgut epithelium to attract hemocytes to the infection foci. In the current study, PGE2 was observed in the gut epithelium of A. albopictus. Its signal intensity was increased after bacterial infection by Sm. However, any interruption of Aa-DSP1 release failed to increase PGE2 levels in the midgut, suggesting that PLA2 was activated by Aa-DSP1 to produce PGE2. This was supported by the finding that treatment with eicosanoid biosynthesis inhibitors is known to prevent the induction of Aa-Duox expression and subsequent ROS generation, whereas the addition of PGE2 or LTB4 considerably reversed such inhibition.

The enzymatic activity of Duox, which is controlled by Ca2+ signals in the Drosophila model, is thought to upregulate ROS generation after pathogen detection [14]. Although bacterial-derived uracil has been recognized as a pathogen signal, pathogen identification by the gut epithelium remains unknown [15]. To explain this, a general damage signal molecule, HMGB1-like DSP1, was identified as a pathogen-associated molecule in the present study. It could elicit gut immunity in A. albopictus against bacterial infection. On the other hand, nonpathogenic commensal microbes occasionally induce dysregulation of gut homeostasis by stimulating ROS. In this case, another DAMP molecule triggers the immune response. This is well demonstrated in a null mutant Drosophila in a PRR gene, PGRP-SG, which facilitates overgrowth of a commensal bacterium, Lactobacillus plantarum, and the resulting excess lactic acid acting as a DAMP to produce ROS from the gut epithelium [45].

After bacterial infection, Ca2+ signal in the gut epithelium was significantly increased. PGE2 alone mediated the increase of Ca2+ signal in the gut epithelium, leading to elevated ROS levels in the gut lumen of A. albopictus. A PGE2 receptor has been identified in S. exigua [46]. Upon activation, this receptor stimulated two secondary messengers, in which cAMP was induced earlier. It was required for the subsequent induction of the Ca2+ signal [21]. Sajjadian and Kim [17] have added a role of cAMP in inducing Se-Duox expression via cAMP-responsive element-binding protein in response to PGE2 in the gut epithelium. These results suggest a working model (Fig. 8) of a gut immunity signal pathway from bacterial infection to ROS generation using the DSP1/PLA2/Ca2+/Duox signaling pathway. The role of eicosanoid in gut immunity was further supported by a pathogenicity bioassay, in which the virulence of Sm against mosquito larvae was enhanced by the addition of eicosanoid biosynthesis inhibitors.

Statement of Ethics

No approval of studies involving animals was required.

Conflict of Interest Statement

The authors have no conflicts of interest relevant to this study to disclose.

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Author Contributions

Yonggyun Kim and Shabbir Ahmed designed the experiments for this work. Shabbir Ahmed and Seyedeh Minoo Sajjadian collected the samples to perform the experiments. Shabbir Ahmed
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and Seyyed Minoo Sajjadian analyzed the data. Yonggyun Kim and Shabbir Ahmed wrote and edited the manuscript. Yonggyun Kim managed the funding. All the authors read and approved the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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