Discovery of aspirin-triggered eicosanoid-like mediators in a *Drosophila* metainflammation blood tumor model

Silvio Panettieri¹,²,§, Indira Paddibhatla³,⁴,*,§, Jennifer Chou⁴, Roma Rajwani⁴, Rebecca S. Moore⁴,†, Tamara Goncharuk⁴, George John¹,²,¶ and Shubha Govind⁴,⁵,¶

**ABSTRACT**

Epidemiologic studies have linked the use of aspirin to a decline in chronic inflammation that underlies many human diseases, including some cancers. Aspirin reduces the levels of cyclooxygenase-mediated pro-inflammatory prostaglandins, promotes the production of pro-resolution molecules, and triggers the production of anti-inflammatory electrophilic mono-oxygenated (EFOX) lipid mediators. We investigated the effects of aspirin in fruit fly models of chronic inflammation. Ectopic Toll/NF-κB and JAK/STAT signaling in mutant *D. melanogaster* results in overproliferation of hematopoietic blood progenitors resulting in the formation of granuloma-like tumors. Ectopic JAK/STAT signaling also leads to metabolic inflammation. We report that aspirin-treated mutant flies experience reduction in metabolic inflammation, mitosis, ectopic immune signaling, and macrophage infiltration. Moreover, these flies synthesize 13-HODE, and aspirin triggers 13-oxoODE (13-EFOX-L₂) production. Providing the precursor of 13-HODE, linoleic acid, or performing targeted knockdown of the transcription factor STAT in inflammatory blood cells, boosts 13-EFOX-L₂ levels while decreasing metabolic inflammation. Thus, hematopoietic cells regulate metabolic inflammation in flies, and their effects can be reversed by pharmaceutical or dietary intervention, suggesting deep phylogenetic conservation in the ability of animals to resolve inflammation and repair tissue damage. These findings can help identify novel treatment targets in humans.

**KEY WORDS:** Eicosanoid, EFOX, 13-HODE, 13-oxoODE, NF-κB, Fatty acid, Aspirin, Systemic inflammation, Resolution, Macrophages, *Drosophila*

**INTRODUCTION**

As part of the innate immune system of animals, acute inflammation targets parasites and microbes and repairs injured tissues. Circulating macrophages and neutrophils migrate to sites of infection or injury where they initiate acute inflammation either to phagocytose and clear invading organisms, or to sequester parasite or cellular debris. Mammals and *Drosophila melanogaster* rely on the archetypal pro-inflammatory NF-κB signaling pathway to activate the inflammation response (Lawrence, 2009). Canonical NF-κB signaling employs Toll and Toll-like receptors (TLRs), which, when bound by pathogen-derived ligand in mammals, mediate the release (from their I-κB inhibitors) and nuclear import of nuclear factor (NF)-κB. NF-κB activates pro-inflammatory pattern of gene expression (Baker et al., 2011; Lawrence, 2009). Pathogen receptors in flies also activate the Toll-NF-κB signaling pathway for host defense (Cherry and Silverman, 2006; Govind, 2008; Govind and Nehm, 2004; Lemaître and Hoffmann, 2007). A key target of pro-inflammatory NF-κB activation in mammals is the cyclooxygenase-2 (COX-2; also known as prostaglandin-endoperoxide synthase 2, PTGS2) promoter. COX-2 catalyzes the rate-limiting step in the conversion of the polyunsaturated C20:4 ω-6 fatty acid (FA) arachidonic acid (AA) to pro- and anti-inflammatory prostanooids (Groeger et al., 2010). AA, an important component of the phospholipids in the plasma membrane, is derived from the dietary omega (ω)-6 FA linoleic acid (LA). NF-κB-mediated induction of COX-2 integrates eicosanoid-mediated signaling with NF-κB signaling. Toll and NF-κB signaling also responds to free fatty acid levels in the blood. For example, saturated (but not unsaturated) fatty acids activate TLR4 and its downstream mediators, and NF-κB p65 (also known as RELA) activation triggers COX-2 expression (Lee et al., 2001, 2003). Since Toll receptors sense both pathogens and nutrients in mammals, diet and energy balance are closely integrated with innate immunity and inflammation (Baker et al., 2011; Hotamisligil, 2006; Shapiro et al., 2011). Aspirin (acetylsalicylic acid, ASA) inhibits the pro-inflammatory effects of prostanooids by acetylating COX-1 and -2 activities (Alfonso et al., 2014).

The second biochemical effect of aspirin is to promote the resolution of acute inflammation. A new class of specialized pro-resolving mediators (SPMs) are formed in the presence of acetylated COX-2. Derived from C20 or C22 ω-3 and ω-6 FAs, SPMs include lipoxins, resolvins, protectins and maresins. SPMs boost efferocytosis, promote phagocytosis of microbes, and limit neutrophil-mediated tissue damage; reviewed in Serhan et al. (2015). In human cells, acetylated COX-2 also triggers the production of a family of electrophilic mono-oxygenated (EFOX) lipid mediators, including 17- and 13-EFOX, derived from ω-3 C22:6 docosahexaenoic acid (DHA) (Delmastro-Greenwood et al., 2014). These dual anti-inflammatory and pro-resolution roles of aspirin explain how dietary ω-3 FAs or aspirin stalls inflammation and supports well-being in humans (Delmastro-Greenwood et al., 2014; Groeger et al., 2010). They also expose the diversity of eicosanoid and eicosanoid-like lipid mediators and the complexity of their cell-specific effects in maintaining normal organism-level physiology. How are the pro- and anti-inflammatory effects of...
eicosanoid family members precisely integrated with the metabolic and immune pathways in vivo? How are their effects unified from cellular to tissue and organism levels (e.g. from inflammatory macrophages to adipose tissue)? Because of their inherent diversity and cellular complexity, vertebrate models do not lend themselves to straightforward resolution of such questions.

To systematically address these issues, we used *D. melanogaster* models with proven hallmarks of chronic inflammation (Ci). In one such Ci mutant (*Ubc9*, which encodes the E2 SUMO-conjugating enzyme), Toll-NF-κB dependent regulatory mechanisms underlying acute inflammation is dysregulated. Ci in *Ubc9* is characterized by (1) high levels of the pro-mitotic and pro-inflammatory cytokine and Toll ligand Spätzle (Spz) and the Spätzle Processing Enzyme (SPE); (2) constitutive expression of the Toll-NF-κB target gene *Drosomycin* (*Drs*) in the liver-like larval fat body; (3) niche-independent hematopoietic overgrowth of the lymph gland progenitors; and (4) loss of basement membrane integrity of the fat body and blood cell infiltration into the diseased tissue (Kalamaz et al., 2012; Paddibhatla et al., 2010). Similar defects have been described in *hopscotch*/*tum* mutants with hyperactive JAK-STAT signaling (Chiu et al., 2005; Harrison et al., 1995; Luo et al., 1995).

Flies also react to metabolic stress in ways similar to humans. In response to a high-sugar diet (but not to excess dietary fat or protein), *Drosophila* develop signs of metabolic inflammation (or metainflammation) where cellular and molecular phenotypes resemble the pathophysiology of type-2 diabetes mellitus (Baker et al., 2011; Musselman et al., 2011). High-sugar-fed larvae exhibit an altered metabolism and the cells of their fat body organ display larger-sized lipid droplets (LDs, which store and mobilize fat in animal cells; Musselman et al., 2011). Therefore, mechanistically, sugar-induced metabolic dysregulation in flies resembles insulin resistance in humans (Kim and Choe, 2014; Musselman et al., 2011; Shapiro et al., 2011).

Given that flies exhibit hallmarks of parasite-induced inflammatory and diet-induced metainflammatory responses, we hypothesized that these phenotypes are sensitive to aspirin, and aspirin might activate and coordinate the intrinsic healing mechanisms of the flies as it does in mammals. While flies lack FAs longer than C18 (AA, DHA, etc.) (Shen et al., 2010) and eicosanoids have not been reported from *Drosophila*, prostaglandin administration rescues the loss-of-function phenotype of a human COX-1-like fly gene peroxisitonein (pxt) (Tootle and Spradling, 2008), suggesting that flies possess primordial eicosanoid-based mechanisms through which aspirin might act. In addition, C20 polyunsaturated fatty acid (PUFA)-fed flies generate hydroxyeicosatetraenoic acid (13-hETE) (Tan et al., 2016), an AA metabolite, suggesting that flies express enzymes for eicosanoid synthesis. Our computational studies show that many functional enzymes for eicosanoid biosynthesis are encoded in the fly genome, including two other COX-like enzymes in addition to Pxt (Scarpati et al., 2019). We therefore analyzed inflammation readouts in flies after aspirin treatment and searched for anti-inflammatory C18-derived PUFAs.

Here, we show that aspirin has dose-dependent and molecular-, cell-, and organ-specific effects with striking parallels to its effects in mammals. We also show, for the first time, that flies produce the LA-derived PUFA, 13-HODE, and aspirin increases 13-HODE levels, while also triggering the production of its oxidized analog 13-oxoODE (herein called 13-EFOX-L$_2$). The efficacy of aspirin in resolving inflammation and repairing tissue damage in fruit flies suggests genetic and biochemical conservation underlying these processes across the animal kingdom. Furthermore, like flies, mammals may produce LA-derived electrophilic FAs, with beneficial anti-inflammatory roles in organs with high LA levels. Unbiased genetic screens in *Drosophila* that report the links between inflammation, diet and drug action, therefore, might have translational significance in identifying new therapies for inflammation-based human diseases.

**RESULTS**

Wild-type Canton S flies tolerate a wide range of orally administered aspirin. We found that at 5 mM aspirin, 65% of the animals eclosed to adulthood while 42% of pupae became adults at 55 mM. In the experiments described below, 0.5 mM to 1 mM aspirin was added to fly food (see Materials and Methods for additional details). Aspirin uptake was confirmed by covalently conjugating it to Rhodamine B. Dissected hematopoietic and fat body cells did not retain the unconjugated dye *ex vivo*, but Rhodamine B–aspirin (RB-ASA) localized to the perinuclear regions in both cell types (Fig. S1).

**Aspirin inhibits hematopoietic overgrowth and restores regulation of Toll-NF-κB signaling in Ubc9 mutants**

We introduced 0.5 nM or 5.5 μM aspirin to the diet of developmentally synchronized cultures of heterozygote and inflammatory tumor-growing homozygous *Ubc9* mutant larvae, and examined its effects on tumor abundance (penetration) and sizes (tumor projection area or expressivity), and gene expression. Aspirin (0.5 nM and 5.5 μM) treatment resulted in a remarkable increase in tumor-free animals (0–2 tumors/animal compared to three or more tumors in untreated animals; Fig. 1A,B,D). At both aspirin concentrations, tumor projection area shrank by more than 60% (Fig. 1C,E). Tumor penetrance remained unaffected with salicylic acid (SA) treatment (see Materials and Methods).

We have previously shown that *Ubc9* tumors are derived from overproliferating lymph gland lobes (Kalamarz et al., 2012). Reduction in tumor penetrance by aspirin treatment (Fig. 1A–E) was also reflected in the shrinkage of lymph gland lobes and in the restoration of lymph gland morphology (Fig. 1F). Relative to heterozygous lymph glands, *76B>*GFP-marked mutant *Ubc9* lymph glands have an expanded GFP-positive progenitor population with many inflammatory lamellocytes (Fig. 1F) (Kalamarz et al., 2012). Treatment with 0.5 mM aspirin reduced the progenitor and mature blood cell populations (Fig. 1F). The size of the lymph gland lobes was reduced; while the projection areas of anterior lobes did not differ significantly (*P* = 0.34), the first set of posterior lobes shrank in size (*P* = 0.005; 20.907.04±1838.24 μm$^2$ in untreated versus 9221.45±3238.40 μm$^2$ in 0.5 mM aspirin treated mutant larvae, *mean±s.d.*; three biological replicates, four to six lobes per replicate). Thus, administration of aspirin restricted lymph gland overgrowth and tumor development.

Constitutive signaling in *Ubc9* fat bodies results in the activation of *Drs-GFP* as well as infiltration of macrophages and lamellocytes on the diseased fat body (Chiu et al., 2005; Paddibhatla et al., 2010). Both these phenotypes are rescued by aspirin. At day 6, there was a ~40% decrease in the percentage of *Drs-GFP*-positive larvae (0.5 nM ASA; Fig. 1G,H). This decrease in *Drs-GFP* expression correlated with reduced infiltration of blood cells into the fat body adipocytes (Fig. 1I). Thus, it appears that like in mammalian cells (Kopp and Ghosh, 1994), aspirin inhibits chronic inflammation in flies by modulating the Toll-NF-κB axis.

**Aspirin counteracts constitutive immune signaling in hopscotch*/*tum* blood cells; it is anti-mitotic, and improves organismal viability**

To corroborate the effects of aspirin in another genetic background and understand how it promotes healing and tissue repair, we turned...
to the hopscotch<sup>Tum-l</sup> (hop<sup>Tum-l</sup> or simply Tum-l) mutant, where JAK-STAT signaling is hyperactive due to a dominant gain-of-function point mutation (Luo et al., 1995). In these mutants, hyperactive STAT activity expands the hematopoietic progenitor population (Anderson et al., 2017; Harrison et al., 1995; Luo et al., 1995) and lymph gland dysplasia leads to tumor development and partial lethality (Irving et al., 2005) (Fig. 2A–C). Microarray analysis revealed high expression of key Toll pathway genes (<i>SPE</i>, <i>spz</i>, <i>Toll</i> and <i>Dif</i>) in hop<sup>Tum-l</sup> mutants (Fig. 2D) (Irving et al., 2005).

To determine whether the expanded hematopoietic population is fundamental to chronic inflammation in hop<sup>Tum-l</sup> mutants and whether systemic effects compromise viability, we depleted STAT and Toll pathway gene expression (<i>SPE</i>, <i>spz</i>, <i>Toll</i> and <i>dl</i>) in this expanding hematopoietic population by means of RNAi knockdown (KD; hop<sup>Tum-l</sup> <i>msn</i>&gt;<i>mcD8-GFP</i>, RNAi; Fig. 2A). We compared the viability of KD animals with outcrossed hop<sup>Tum-l</sup> mutants without KD. Viability of progeny from the <i>y</i> <i>w</i> outcross was no different if white, ebony or GFP RNAi was expressed (Fig. S2A). In contrast, compared to the control <i>y</i> <i>w</i> outcross, hop<sup>Tum-l</sup> mutants were more viable when pro-inflammatory STAT or NF-κB signaling was knocked down. The most significant gains were seen with <i>STAT</i>, <i>SPE</i> and <i>spz</i> knockdown in blood cells (Fig. 2E). These results suggest a potential downstream or cooperative role of the Spz-Toll-Dorsal/Dif axis with STAT in hop<sup>Tum-l</sup>-blood cells. Aspirin treatment (1 μM) of hop<sup>Tum-l</sup> animals reduced the mitotic index in circulating blood cells (Fig. 2F). These results confirm that, as such, the effects of aspirin are not limited to a specific genetic background, but instead its biological effects must be mediated by molecular pathways common to both mutants.

Immune responses in mammals can modulate insulin signaling and nutrient storage (Cressler et al., 2014), and conversely, immune...
Fig. 2. Aspirin is anti-inflammatory in hopTum-l larvae and improves viability. (A) Schematic showing immune organs present in a hopTum-l msn>mCD8-GFP larva. The expanded hematopoietic cells are found in the hemolymph and around the fat body. An msn-GAL4 transgene allows targeted manipulation of gene expression in progenitors of inflammatory lamellocytes. The effects of such manipulation on tumorigenesis, viability and metainflammation can thus be examined. (B) GFP-positive hematopoietic tumors (arrows) in 6-day-old hopTum-l msn>mCD8-GFP whole larvae. Scale bar: 1 mm. (C) An aggregate of mutant blood cells from a drop of hemolymph gently bled from a 6-day-old third-instar larva showing GFP-positive lamellocytes and GFP-negative macrophages (white arrows). Only these two cell types are present in this preparation. Scale bar: 50 μm. (D) Microarray data summarized from reference (Irving et al., 2005) performed on hopTum-l revealing that Toll pathway components are transcriptionally active. A significant increase (>1.5-fold) was observed for SPE, spz, Toll and Dif genes. (E) Relative viability of adult hopTum-l msn>mCD8-GFP males at 27°C with RNAi compared to viability of control hopTum-l males from an outcross of hopTum-l/FM7 females and y w males without RNAi. Bars represent means±s.e.m.; P<0.05 (Student’s t-test) for all pairwise comparisons across three biological replicates. The number of males scored for a given replicate ranged from 76 to 279. (F) Mitotic index in blood cell smears of hopTum-l msn>mCD8-GFP animals after 1 μM aspirin (ASA) treatment (means±s.d.; three biological replicates, 20 larvae per replicate, and more than 800 macrophages per treatment). **P<0.01 (Student’s t-test). (G) Representative images of Nile Red-stained larval fat body adipocytes from untreated y w, and untreated and 1 μM aspirin-treated hopTum-l msn>mCD8-GFP animals. Scale bars: 30 μm. (H) Average number of large, medium or small LDs per cell from fat bodies of untreated y w animals, and untreated and 1 μM aspirin-treated hopTum-l msn>mCD8-GFP animals. At least 30 fat body cells per animal were scored and means±s.d. was computed using six animals. Three biological replicates were performed. **P<0.001 (Student’s t-test.). (I,J) Confocal images of Rhodamine–phalloidin and Hoechst 33258-stained fat body from untreated (I) or 1 μM aspirin-treated (J) hopTum-l msn>mCD8-GFP animals. Fewer aggregates and tumors (arrows) were present on the organ from treated animals and the organ morphology is closer to normal. (K) Larval-to-adult viability of hopTum-l msn>mCD8-GFP animals upon systemic aspirin treatment (means±s.d. is shown across four biological replicates; n=38 larvae per replicate). **P<0.05 (Student’s t-test).
pathways are activated in response to nutrient stress (Gregor and Hotamisligil, 2011). We wondered whether LD sizes are affected in hop\textsuperscript{Tum-1} mutants and if aspirin might reverse these effects. In flies, the diabetes-like metabolic state is reflected in larger LDs in larval fat bodies of animals consuming high dietary glucose (Musselman et al., 2011). Adipocytes of the hop\textsuperscript{Tum-1} fat body form many large and distorted LDs without added dietary glucose (Fig. 2G,H). Additionally, the mutant fat body loses integrity and intercellular adhesion; several portions of the organ detach from the main organ (Fig. 2I). However, administration of 1 µM aspirin restored a significant portion of the small LD population and there were fewer large LDs (Fig. 2G,H); aspirin also restored the overall morphology of the organ (Fig. 2I,J). These results suggest that as in vertebrate models and in human trials, where anti-inflammatory therapies restore metabolic function (Bozza et al., 1996; Gregor and Hotamisligil, 2011), aspirin treatment in flies also reduces metabolic stress.

Encouraged by the strong and consistent anti-tumor and anti-inflammatory effects of aspirin at the molecular, cellular and organ levels in two inflammation models, we asked whether these effects might translate to benefits at the organism level and whether aspirin improves the viability of hop\textsuperscript{Tum-1} animals. 1 nM and 1 µM aspirin improved the viability of hop\textsuperscript{Tum-1} animals to adulthood (i.e. the proportion of flies that eclosed compared to the no treatment controls) (Fig. 2K) and these gains paralleled viability increments in the targeted knockdown experiments (Fig. 2E). This correlation suggests that the salutary effects of aspirin are, in part, realized by blocking the systemic pro-inflammatory effects of blood cells. We hypothesized that, in addition to reducing mitosis and shrinking the pro-inflammatory blood cell population, aspirin might trigger oxylipid production whose anti-inflammatory effects may be realized in other parts of the body and possibly the entire organism. We tested this idea in biochemical experiments.

**Discovery of 13-HODE and 13-EFOX-L\textsubscript{2} in fly larvae**

Because D. melanogaster lack FAs longer than C18 (Shen et al., 2010), we searched for non-classical anti-inflammatory lipid mediators derived from unsaturated C18 precursors linoleic acid (LA) and α-linolenic acid (ALA), present in the normal fly diet (Shen et al., 2010). Oxidized derivatives of LA and ALA [13-hydroxyoctadecadienoic acid (13-HODE) and 13-hydroxyoctadecatrienoic acid (13-HOTrE), respectively; see structures in Fig. 3A and Fig. S3A] are bioactive and exert potent anti-inflammatory effects in mammals (Bozza et al., 1996; Delmastro-Greenwood et al., 2014; Dennis and Norris, 2015; Groeger et al., 2010; Serhan et al., 2015). In liquid chromatography tandem mass spectrometry (LC-MS/MS)-based targeted lipidomics of larval extracts, we did not detect 13-HOTrE in wild-type or mutant larvae, but discovered that untreated third-instar wild-type, control, and hop\textsuperscript{Tum-1} larvae produce 13-HODE at levels comparable to mammalian cells (∼40 nM; Fig. 3B; Figs S2D and S3B,D; Yuan et al., 2010).
Application of an enantiomer-specific antibody in ELISA experiments (Enzo Life Sciences) confirmed the presence of 13(S)-HODE in both isolated larval fat bodies and the remaining organs of untreated larvae, suggesting its wide occurrence in the body. Chiral LC-MS/MS analysis resolved the R and S enantiomers of 13-HODE, and showed that 13(S)-HODE was twice as abundant as 13(R)-HODE regardless of genetic background or aspirin treatment (Fig. 3A). We detected 13-EFOX-L2 in only hopTum-l mutants at high aspirin concentrations (1 μM and 1 mM) but not in control animals (Fig. 3C; Fig. S2E). The appearance of 13-EFOX-L2 correlated with the rise in 13-HODE in response to aspirin treatment of mutants (Fig. 3B), supporting the idea that 13-HODE is a substrate for 13-EFOX-L2 synthesis in vivo (Fig. 3A).

The discovery of 13-HODE and 13-EFOX-L2, two LA-derived oxylipids, in larval extracts prompted us to examine whether hopTum-l mutants would benefit from dietary addition of LA (13-HODE and 13-EFOX-L2 are chemically not stable enough for such experiments). Addition of 5 mM LA to the diet increased the larval-to-adult viability of hopTum-l mutants from 35% to up to 90% while 5 mM myristic acid addition had almost no effect (Fig. S3E). Unlike 1 μM and 1 mM aspirin, 5 mM LA treatment did not yield a significant increase in 13-HODE (Fig. 3B), but 13-EFOX-L2 was clearly detected in LA-treated control animals and it was 7-fold higher in LA-treated hopTum-l mutants than in their aspirin-treated counterparts (Fig. 3C). These correlations suggest that (1) 13-HODE and 13-EFOX-L2 levels can be modulated by dietary modification in the absence of aspirin, and (2) the in vivo effects of aspirin and LA are realized differently. Overall, however, the levels of 13-HODE and 13-EFOX-L2 correlated with reduced inflammation and improved the survival of hopTum-l mutants. We did not detect other members of the EFOX-D or -E families identified in mammalian cells (Groeger et al., 2010) in larval extracts, perhaps because EFOX-D and -E derive from C20 precursors, not found in flies (Shen et al., 2010).

A blood cell-derived signal affects metainflammation and EFOX levels in drug-free hopTum-l animals

STAT92E, the sole STAT family member present in flies, acts downstream of Hopscotch, the only Janus kinase found in flies. Previous reports of cell-autonomous effects of hopTum-l (Anderson et al., 2017; Hanratty and Ryerse, 1981; Harrison et al., 1995) and the tumor-suppressive property of STAT loss-of-function mutations (Hou et al., 1996; Yan et al., 1996) suggested that STAT KD (msn>STATRNAi) in the hopTum-l background should relieve tumor burden. We found that tumor penetrance in hopTum-l males is significantly reduced with msn>STATRNAi (Fig. 4A), and this reduction may be due, in part, to reduced mitosis in msn>STATRNAi macrophages (Fig. 4B). The msn promoter is active in many lymph gland progenitors as early as 56 h (second larval instar; Tokusumi et al., 2009b) and given that the macrophage/lemelocyte lineages have a shared origin (Anderson et al., 2017; Stofanek et al., 2010), we hypothesized that mutant blood cells influence the systemic health of animals. Targeted STAT or even spz KD (msn>STATRNAi or msn>spzRNAi) in the hyperplastic hopTum-l blood cell population rescued metainflammatory inflammation in larval adipocytes (Fig. 4C,D), while control RNAi did not (Fig. S2B,C).

This rescue of metabolic inflammation in hopTum-l mutants by STAT KD suggested a correlation to anti-inflammatory oxylipins. We observed an increase in 13-HODE (from 43 nM to 130 nM) and in 13-EFOX-L2 (from undetectable to 14.0 nM) in an LC-MS/MS quantification of hopTum-l msn>STATRNAi animals (Fig. 4E,F). The levels of these oxylipids remained unchanged in control RNAi animals (Fig. S2D,E). These in vivo 13-HODE and 13-EFOX-L2 levels are consistent with corresponding levels in mammalian cells (Groeger et al., 2010; Yuan et al., 2010). Together, our observations suggest that a STAT-dependent pro-inflammatory signal from hematopoietic cells affects immune and metabolic health of flies by regulating 13-HODE and 13-EFOX-L2 levels.

DISCUSSION

Basic science and clinical discoveries have revealed the pervasive role of inflammation in human disease. Many of these studies expose the intimate links between diet, drugs and disease development (Baker et al., 2011; Hotamisligil, 2006; Serhan et al., 2015; Shapiro et al., 2011). Our studies of the effects of aspirin in fruit flies show that aspirin treatment reduces pro-inflammatory cytokine-producing cells and metabolic inflammation. We further show that these changes correlate with an increase in anti-inflammatory oxylipids.

The anti-mitotic effects of aspirin appear to be important to its salutary effects in flies. In both Ubc9 and hopTum-l flies, spz expression is high (Irving et al., 2005; Paddibhatla et al., 2010) and hence the Toll-NF-κB signaling is activated. Thus, selective spz KD in hopTum-l blood cells improves organismal viability, much as aspirin treatment does. We envision two possible mechanisms of action: (1) aspirin may block the activities of COX-like enzymes (Scarpati et al., 2019; Tootle and Spradling, 2008), which may, in turn, act through the NF-κB/STAT axis in hopTum-l blood cells to reduce levels of pro-mitotic/pro-inflammatory proteins, or (2) it may directly block the observed cell cycle progression, for example, by interacting with cell cycle proteins. The anti-mitotic effects of aspirin and reduction in tumorgenesis in flies are consistent with reported salutary effects in epidemiological studies of cancer patients (Elwood et al., 2009; Qiao et al., 2018; Schreinemachers and Everson, 1994). Identifying the direct biochemical targets of aspirin in fly cells (including putative COX enzymes) and understanding its action through these targets will provide new insights into the biochemical effects of aspirin in human cells.

Our biochemical and genetic experiments with the hopTum-l larvae revealed the existence of 13-HODE and 13-EFOX-L2 in flies. Furthermore, these experiments showed that untreated mutants and aspirin-treated mutants differ from the wild-type animals in distinct ways: (1) 13-HODE is detected in untreated w controls and hopTum-l mutants; its levels are higher in aspirin-treated controls and hopTum-l mutants, as well as in msn>STAT92E KD animals; (2) 13-EFOX-L2 is not detected in untreated control or mutant animals and is found only in aspirin-treated animals or in msn>STATRNAi animals. Because LD morphologies in these mutant animals with high 13-HODE and 13-EFOX-L2 are restored, we propose that pro-inflammatory signals arising from blood cells adversely affect the metainflammatory state...
of the animal. Reducing such signals results in high levels of 13-HODE and EFOX-L2, reducing metainflammation and improving viability. This interpretation suggests that aspirin treatment of affected mutant flies reverses inflammatory phenotypes by tapping into molecular-genetic pathways that are otherwise inactive in wild-type animals. This suggestion provides new avenues for treating inflammatory diseases, but needs to be substantiated further in flies and other organisms.

How might 13-HODE and 13-EFOX-L2 work in flies? The chemistry of 13-HODE in mammals may suggest some mechanisms to be explored. Data from three biological replicates with 28–52 males in each replicate are represented. The total number of tumors observed ranged from 72 to 189. The P-values of unweighted average tumor number comparisons (irrespective of size) were also significant (P=0.02 and 0.03 for the TRiP and VDRC lines, respectively). (A) Mean±s.e.m. number of tumors (weighted for size, see Materials and Methods) per control (hop<sup>Tum^i</sup> msn>mCD8-GFP) male or per hop<sup>Tum^i</sup> msn>mCD8-GFP male STAT<sup>RNAi</sup> line (27°C). Pairwise comparisons of each experimental class with controls indicate significant differences. **P<0.05 (Student's t-test). Data from three biological replicates are shown. The number of tumors observed ranged from 72 to 189. The P-values of unweighted average tumor number comparisons (irrespective of size) were also significant (P=0.02 and 0.03 for the TRiP and VDRC lines, respectively). (B) Mean±s.e.m. percentage of PH3-positive macrophages in the hemolymph of male larvae is shown. Data were obtained from three biological replicates; hemolymph was examined from 6–10 animals in each replicate. Males were from the hop<sup>Tum^i</sup> msn>mCD8-GFP from y w outcross or from the cross with TRiP 31318 females. **P<0.05 (Student's t-test). (C,D) Nile Red-stained fat bodies from hop<sup>Tum^i</sup> msn>mCD8-GFP without or with STAT<sup>RNAi</sup> or spz<sup>RNAi</sup> expressed in overgrowing blood cells. Average number of large, medium or small LDs per cell from these fat bodies are quantified in D. Bars represent mean±s.d. from three biological replicates, with more than 100 cells per replicate were scored. Three animals per replicate were examined. **P<0.001 (Student's t-test). (E,F) 13-HODE and 13-EFOX-L2 levels in larval extracts of control (y w) hop<sup>Tum^i</sup> msn>mCD8-GFP without or with STAT<sup>RNAi</sup> (mean±s.d. across four replicates; n>200 larvae per replicate). **P<0.05 (one-way ANOVA).
2009). Aspirin’s anti-mitotic and anti-inflammatory effects observed in flies are comparable to its effects in mammals and suggest broad mechanistic similarities in vertebrates and invertebrates. Our work suggests that LA-derived electrophilic fatty acids with anti-inflammatory properties must be also produced in mammalian organs rich in LA. Such a discovery will reinforce the value of the Drosophila model system for not only understanding disease progression mechanisms but also for understanding how disease progression (tumor development, dietary stress, and inflammation) can be abated by the animals’ intrinsic genetic and biochemical mechanisms.

**MATERIALS AND METHODS**

**Fly lines and crosses**

Ubc9 strains (1) y w; Ubc9<sup>4-2</sup>FRT40Ac/CyO y<sup>-</sup>, (2) y w; Ubc9<sup>9</sup>FRT40Ac/CyO y<sup>-</sup>, (3) y w; Drs-GFP, Iver<sup>-1</sup>/CyO y<sup>-</sup>, and (4) y w; Drs-GFP Iver<sup>-1</sup>/CyO y<sup>-</sup> are described in Chiu et al. (2005). The 76B>GFP inserts in the Ubc9 background are described in Kalamarz et al. (2012). The X-linked temperature-sensitive hop<sup>Tum-1</sup> mutation (Luo et al., 1995) was recombined with X-linked msnf9-GAL4 insert (Tokusumi et al., 2009a). The mCD8-GFP reporter transgene [Bloomington Drosophila Stock Center (BDSC) no. 5137] was introduced in this background to distinguish macrophages (GFP-negative) from GFP-positive lamellocytes. The final genotype of this stock is: y w hop<sup>Tum-1</sup> msn-GAL4/FM7; UAS-mCD8-GFP and is referred to as hop<sup>Tum-1</sup> msn-mCD8-GFP herein for short. UAS‘‘knockdown’’ lines are as follows. Control UAS-RNAi lines used were: white [Drosophila Transgenic RNAi Project (TRIP) lines 25785 or 28980], ebony (TRIP line 28812) and GFP (TRIP line 41554). white knockdown was validated with the eyeless-GAL4 driver and ebony knockdown was confirmed with universal daughterless-GAL4 (da-GAL4) driver. Loss of the red pigmentation in eyes of ey>UAS RNAi flies and darker adult body color in da>UAS RNAi flies was observed (not shown). The EGFP.shRNA construct was validated in Neumüller et al. (Neumüller et al., 2012).

UAS-STAT<sup>RNAi</sup> lines used for Signal transducer and activator of transcription (STAT2E) were 31318 (TRIP1), 31317 (TRIP2), and Vienna Drosophila Resource Center (VDRC) GD 43867. The TRIP 31318 strain was used for biochemistry. The line for Spätzle processing enzyme (SPE) knockdown was w; UAS-SPE<sup>RNAi</sup>/CyO (VDRC v30972). For spätzle (spz) knockdown y w; UAS-spz<sup>RNAi</sup> VDRC v105017 and TRIP 28538 lines were used and for Toll (Tt) knockdown UAS-TTR<sup>RNAi</sup>/MKRS TRIP 31044 (TRIP1) and 31477 (TRIP2) were used. UAS-d<sup>RNAi</sup> TRIP (27650) was used for dorsal (dl) knockdown.

**Gene knockdown in hop<sup>Tum-1</sup> flies**

Because of the semidominant X-linked mutation, fewer hop<sup>Tum-1</sup>/Y males survive relative to their F<sup>M7</sup>/Y siblings. This genetic background is therefore suitable for biochemical and genetic interaction experiments. y w hop<sup>Tum-1</sup> msn-GAL4/FM7; UAS-mCD8-GFP females were crossed either with UAS-RNAi males or with y w/Y control males. Effects of RNA interference (STAT, SPE, spz, Toll or dl) on viability in the y w hop<sup>Tum-1</sup> msn-GAL4/FM7; UAS-mCD8-GFP animals were normalized relative to the y w outcross. The proportion of hop<sup>Tum-1</sup>/Y (mutant) sons relative to F<sup>M7</sup>/Y (control) sons is defined as relative rescue. In additional crosses with control UAS-RNAi for white (two lines), ebony or GFP, we found that in all four pairwise comparisons, the ratio of surviving hop<sup>Tum-1</sup>/Y RNAi sons relative to F<sup>M7</sup>/Y balancer-carrying sons was statistically indistinguishable from this ratio derived from the y w/Y outcross (Fig. S2A).

**Chemical treatments**

**Salicylic acid treatment**

SA is the principal metabolite of aspirin and lacks the functional acetyl group characteristic of ASA. SA was dissolved in DMSO to make a 10 M solution. The final DMSO content in the fly food was negligible, being four orders of magnitude lower than the reported cytotoxic DMSO concentration in Drosophila larvae (~0.3% w/w; Nazir et al., 2003).

SA treatment of Ubc9 mutants did not ameliorate tumor penetrance of Ubc9 mutants. At 1 mM and 50 mM concentrations, all animals (n=10 and n=13, respectively) developed tumors and produced aggregates, much like the untreated controls. None of the mutants was tumor free. This result was also reflected in infiltration indices (number of macrophages adhering to 100 fat body cells) where no significant difference between SA-treated (1.58±0.10 macrophages for 1 mM and 2.46±0.27 macrophages for 50 mM SA, mean±s.e.m.) and control treatment (2.64±0.34 macrophages, no DMSO, or 2.18±0.06 macrophages, with DMSO) was observed (P>0.05). In each case, 100 fat body cells were examined for infiltration by macrophages.

**DMSO treatment**

No significant difference was observed in tumor penetration between ‘no treatment’ or ‘DMSO-only’ treatment classes of Ubc<sup>9</sup> animals. Under both conditions, all animals developed tumors and/or aggregates. Infiltration index was also statistically indistinguishable (see numbers in the previous section).

**Aspirin treatment**

The sources of aspirin for Ubc<sup>9</sup> and hop<sup>Tum-1</sup> treatments were different. For Ubc<sup>9</sup> larvae, one 325 mg Bayer aspirin tablet was ground finely and 1 mg/ml of this powder was dissolved in an appropriate amount of water and the suspension was stirred vigorously for 15 min and filtered. An appropriate volume of aspirin solution was added to fly food to yield aspirin concentrations of 0.5 mM or 5.5 μM.

Drug purity in these tablets was determined as follows: A 325 mg tablet was ground to a fine powder and suspended in ice-cold methanol with vigorous stirring for 10 min. It was vacuum-filtered with a Buchner funnel. The solvent was dried under a gentle stream of nitrogen; the white solid powder was further dried under high vacuum, overnight. High purity of the compound (>99.8%) was verified via LC-MS and nuclear magnetic resonance (NMR) analyses. Results matched data reported in the literature (Byrd and Donnell, 2003).

For hop<sup>Tum-1</sup> larvae, a 10 M aspirin (≥99.9%, Sigma-Aldrich) solution in DMSO was diluted in water to achieve a final aspirin concentration of 1 mM, 1 μM or 1 mM in fly food. The final DMSO content in the fly food was significantly lower than levels known to be toxic in Drosophila larvae (~0.3% w/w; Nazir et al., 2003). An appropriate volume of the resulting aqueous solution was added directly to fly food and thoroughly homogenized with an electric mixer for 15 min. Larvae were reared at 27°C and collected for lipid extraction, viability assays, or dissections, 5 days after egg laying, unless stated otherwise.

Viability of 0.5 M and 5.5 M α-salicylic acid-treated y w animals remained as high as untreated y w animals (98–100%) and no toxic effects were observed, whereas addition of 1 mM aspirin in fly food resulted in partial lethality at pupal stages. Aspirin administration did not rescue recessive lethality of Ubc9 mutants. To examine the effects of aspirin on larval-to-adult viability, the number of hop<sup>Tum-1</sup> msn>mCD8-GFP adults emerging from a known number of second- and third-instar larvae (untreated or treated with 1 mM, 1 μM, or 1 mM of aspirin) was scored.

**Linoleic acid and myristic acid treatment**

An appropriate volume of pure LA (≥99.9%, Sigma-Aldrich) was added directly to fly food for a final concentration of 5 mM. The mixture was homogenized into fly food for 15 min. y w and hop<sup>Tum-1</sup> msn>mCD8-GFP larvae were reared at 27°C for 5 days and collected for lipid extraction or viability assays. Myristic acid (MA,≥99.9%, Sigma-Aldrich) was similarly administered, also at 5 mM final concentration.

**Synthesis of Rhodamine B-conjugated aspirin and visualization**

In a 150 ml two-neck round bottom flask, 2 g (4 mmol) of Rhodamine B was dissolved in 16 ml (10 mmol, 2.5 equivalent) of phosphoryl chloride (POCl<sub>3</sub>) and the mixture was vigorously stirred at 110°C for 2 days. Subsequently, POCl<sub>3</sub> was thoroughly distilled off under vacuum after which 60 ml of molecular sieve-dried acetonitrile was added to the flask along with 2.1 g (11.3 mmol, 2.8 equivalent) of mono-Boc-protected piperezine and 0.7 ml (5 mmol, 1.2 equivalent) of triethylamine. The mixture was stirred at room temperature for 24 h, and then for 12 h under reflux. Once thin layer chromatography showed complete conversion of the starting material,
triethylamine was distilled off and the crude product was dissolved in 20 ml of trifluoroacetic acid and stirred for 2 h at room temperature. The solvent was removed and the crude product was purified via flash chromatography (the initial eluting mixture 5.5:1 CHCl₃:CH₂OH changed gradually to a 2:1 ratio). After collecting the correct fractions, the solvent was removed using a rotary evaporator and the final product was precipitated by dissolving it in 2 ml of methanol and then adding it to 50 ml of diethyl ether, drop-wise.

After drying it under vacuum, 150 mg of this intermediate (1 equivalent) was dissolved in 10 ml of DMSO to which 54 mg of aspirin (0.3 mmol, 1 equivalent), 153 μl of triethylamine and 410 mg of HBTU (1 mmol, 3.3 equivalent) were added in this order. The mixture was stirred for 24 h at room temperature under nitrogen. At the end of this period, 150 ml of 0.5 M sodium bicarbonate (NaHCO₃) were added, followed by 100 ml of ethyl acetate. The biphasic mixture was transferred into a separatory funnel. Upon vigorous stirring, the organic phase was recovered, whereas the aqueous phase was extracted three times with ethyl acetate. The combined organic fractions were dried over Na₂SO₄. After filtration on a cotton plug, the solvent was removed by rotary evaporator almost to dryness. The crude product was dissolved in 2 ml of ethyl acetate, which was added to 150 ml of diethyl ether drop-wise at room temperature. After keeping the solution at 4°C for 1 h, the product was recovered via filtration and dried in vacuum to yield 160 mg of Rhodamine B-conjugated aspirin {Rh-ASA, 80% yield over two steps; 1H-NMR: 400 MHz, δ (ppm): 1.13 (12H, t), 2.08 (3H, s), 3.39 (12H, q), 3.50 (4H, t), 3.60 (4H, t), 5.34 (1H, d), 6.13 (2H, s), 6.19 (2H, d), 6.84 (2H, dd), 7.24 (1H, s), 7.25 (3H), 7.39 (1H), 7.48 (1H, dd), 7.83 (1H, d), 7.92 (1H, d); MS: [M-Cl]⁻ 444.24 m/z}. A working solution (800 μM Rh-ASA in phosphate-buffered saline (PBS, pH 7.2)) was applied to dissected larval tissues for 30 min at room temperature in a humidified chamber. Negative control samples were exposed to a mixture of 800 μM Rhodamine B plus 800 μM aspirin solution in PBS.

Analysis of aspirin-treated animals

Tumor penetrance and expressivity

The effects of aspirin on the presence and abundance of tumors (penetrance) and tumor sizes (expressivity) were scored in 8-day-old Ubc9 animals. Age-matched third-instar heterozygous or mutant Ubc9 larvae were chosen without bias to sex from a timed (6–24 h) egg lay. Developmentally delayed larvae (smaller in size than majority of mutants) were not selected for analysis. In three independent experiments, all available mutant untreated or aspirin-treated animals were first scored for the absence/presence of visible tumors and then scored for tumor sizes in the majority of mutants. The number of single blood cells adhering above the glass surface. (5) Using clean tweezers, three to four pieces of the stained fat body pieces were excised from the tape and peeled off using clean tweezers. The exposed rectangular area becomes the well for the sample. (4) A drop of Vectashield was placed in the well and evenly spread on the glass surface. (5) Using clean tweezers, three to four pieces of the stained fat

mitotic index (see below).

Mitosis

For scoring the mitotic index in hop¹⁰⁸⁸⁻¹ msn>mcD8-GFP blood cells (without or with aspirin), rabbit anti-PH3 (1:200; Cat #06-570, Lot #2465253, EMD Millipore) antibody was visualized with anti-rabbit alkaline phosphatase-linked secondary antibody (1:5000; Product #31340, Lot #Q5226802, Thermo Fisher Scientific). Alkaline phosphatase staining was performed with 125 μg/ml BCIP and 250 μg/ml NBT from Promega. Mitotic indices of control hop¹⁰⁸⁸⁻¹ msn>mcD8-GFP larvae (from y w crosses) and hop¹⁰⁸⁸⁻¹ msn>mcD8-GFP animals expressing STATnin (TRiP 31318) were determined using the same methods, except that blood cells from only male larvae were examined in each case.

Reporter gene expression

Changes in Drs-GFP expression in third-instar larval fat bodies were assessed at 6 days and 8 days after egg laying. The entire fat body was carefully dissected and mounted on a slide. The status of GFP expression in the organ was scored under a Zeiss Axioplan 2. AxioVision LE 4.5 software was used to process images.

Infiltration index

Third-instar larval fat bodies were dissected from untreated control and aspirin-treated larvae. Dissected tissues were fixed and stained with Hoechst 3258 and TRITC-labeled phalloidin. Samples were mounted in 50% glycerol in PBS (pH 7.4). The number of single blood cells adhering above or below the fat body were scored as described in Paddibhatla et al. (2010) under a Zeiss Axioscope fluorescence microscope.

Slide preparation for fat body lipid droplet analysis

Fat body LDs were detected after fixation and staining with Nile Red (N3013, Sigma-Aldrich) as described by Greenspan et al. (1985). Samples were kept in PBS (pH 7.2) throughout the procedure. Samples were counterstained with the nuclear dye Hoechst 33258 and were mounted in Vectashield (both from Invitrogen Molecular Probes). To preserve the endogenous LD sizes, slides carrying fat body samples were prepared as follows. Fixed, Nile Red- and Hoechst 33258-stained fat body samples were prepared as follows. Fixed, Nile Red and Hoechst 3258-stained fat body pieces were mounted on slides with a spacer to prevent distortion of LDs and maintain normal tissue morphology. A 4.5 cm strip of single-sided Scotch tape was placed in the center of the slide. (2) A thin, even layer of Vaseline was applied onto the tape with a paintbrush. (3) Using the corner of a razor blade, a 1 cm×3 cm rectangle was excised from the tape and peeled off using tweezers. (The exposed rectangular area becomes the well for the sample.) (4) A drop of Vectashield was placed in the well and evenly spread on the glass surface. (5) Using clean tweezers, three to four pieces of the stained fat
bodies (in PBS, pH 7.4) were transferred into the well, avoiding overlap. (6) A glass cover slip was then gently placed over the samples and uniformly pressed; the Vaseline adheres the tape to the coverslip. The sample in the Vectashield should now occupy the well. (7) Clear nail polish was gently applied to seal the coverslip. While the prepared slides can be stored at 4°C, optimal results were obtained when samples were imaged immediately. Samples were imaged on a Zeiss LSM 510 or 710 confocal microscope. LD sizes were classified as follows: small (radius (r)<3.1 μm); medium (3.1<r<5 μm); large (r>5 μm).

Biochemistry
For oxidized lipids in *Drosophila* larvae, we used solid-phase extraction methods (Massey and Nicolaou, 2013). At least 200 age-matched animals were transferred from fly food onto a fine-sieve mesh, washed with water, then 70% ethanol, and then again with water, and gently dried with a Kimwipe. Samples were weighed and stored at ~80°C until further use.

Materials for lipidomics
Materials used were as follows. Solid-phase extraction STRATA SPE cartridges: C18-E (500 mg, 6 ml; Cat. No. 8B-5001-HCH); non-chiral RP-HPLC column: Phenomenex Luna 3 μm C18(2) 100A 150×2.0 mm; Chiral HPLC column: Chiralcel OD-RH 5 μm 150×2.1 mm; MS system: 4000 QTRAP (Applied Biosystems), HPLC system: Shimadzu Prominance HPLC (Shimadzu USA); NMR instrument: Varian Mercury-300. MS-grade water and 10 ml of hexane. The lipids were recovered with 12 ml of cold methyl formate, which was evaporated on ice and in the dark under a gentle stream of nitrogen. The purified lipids were dissolved in 1 ml of cold methanol.

Sample preparation
The amounts of solvents and internal standards before solid-phase extraction used for 200 mg of larvae are presented below; these amounts were adjusted where larval weight exceeded 200 mg. For lipid extraction, larvae were transferred into a 1 ml pre-chilled glass Dounce grinder and homogenized thoroughly at 0°C with a glass pestle along with 500 μl of cold methanol. The pestle was rinsed twice with 100 μl of cold methanol. 4 ml of ice-cold was added to obtain a 15% methanol solution. Subsequently, 50 ng of deuterated internal standards were added to the homogenate, which was left on ice in the dark. After 15 min the homogenate was centrifuged at 4°C and 3,700 g for 10 min. The supernatant was recovered and kept on ice. The pH was then adjusted to 3 by adding 10–20 drops of 0.1 M HCl and 2.0 ml of the acidic extract were immediately loaded onto the SPE column, which was previously conditioned with 20 ml of methanol and 20 ml of water. The cartridge was washed with 20 ml of ice-cold 15% methanol in water, 20 ml of water and 10 ml of hexane. The lipids were recovered with 12 ml of cold methyl formate, which was evaporated on ice and in the dark under a gentle stream of nitrogen. The purified lipids were dissolved in 1 ml of cold degassed ethanol, and stored at ~80°C until the LC-MS analysis.

Lipid derivatives were analyzed on a reverse phase-high pressure liquid chromatography coupled to a mass spectrometer (RP-HPLC MS/MS). The non-chiral LC-MS runs were performed after diluting the extract 10-fold in ethanol. All samples were analyzed with a gradient solvent system consisting of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The flow rate was 250 μl/min and the gradient used was the following: hold at 35% B for 3 min, then 35–90% B in 23 min, then 90–100% B in 0.1 min, hold for 5.9 min and 100–35% B in 0.1 min, and finally hold for 7.9 min. The oven temperature was 40°C. Chiral analyses were performed in isocratic elution with 35% A and 65% B for 25 min. The flow rate was 250 μl/min and the oven temperature was 40°C.

LC-MS analyses were performed in Multiple Reaction Monitoring (MRM) mode whose optimal parameters and most-abundant fragments were obtained experimentally for 13-HODE, 13-HOTrE and 13-EFOX-L2 by utilizing commercially available synthetic standards. The settings used are shown in Table S1. Standard curves were prepared using 13-HODE and 13-EFOX-L2 as standards and 13-HODE-d4 and 13-EFOX-L2-d3 as internal standards. Analyte:internal standard peak area ratios were used for quantification. Comparison of retention times and the use of at least two MS transitions per compound rendered the detection reliable and sensitive (Fig. S3B,C and Fig. S4). Data were analyzed by one-way ANOVA.

Statistical analysis
Experiments were replicated three or more times. Where results were quantified, at least three biological repeats were performed. The numbers of animals tested are indicated in the text or in the Figure legend. For pairwise comparison, either one-way ANOVA or the t-test was applied. P<0.05 was considered to be significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary information
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