

Epigallocatechin-3-gallate suppresses neutrophil migration speed in a transgenic zebrafish model accompanied by reduced inflammatory mediators

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Background: Polyphenol catechins from green tea, particularly (–)-epigallocatechin-3-gallate (EGCG), exhibits numerous beneficial health effects, although the mechanisms remain unclear.

Methods: In this study, the mechanism of EGCG-mediated healing in an experimentally injured zebrafish model was examined at the cellular and molecular level using confocal microscopy and gene expression analysis.

Results: The mechanisms of action of EGCG were shown to involve: (1) reducing neutrophil response (accumulation, travel speed, and distance) and (2) downregulating the expression of IL-1 β , TNF α , and related signaling pathways. As determined by dynamic time-lapse tracking studies, the local accumulation of neutrophils with high migration speeds after wounding (n=33 cells, v=0.020 μ m/s, d=37.8 μ m), underwent significant reduction following treatment with EGCG doses of 300 μ M (n=22 cells, v=0.013 μ m/s, d=39.5 μ m) and 600 μ M (n=18 cells, v=0.008 μ m/s, d=9.53 μ m). Reverse transcription polymerase chain reaction studies revealed that several signature genes in the IL-1 β , TNF α , and related signaling pathways were downregulated after EGCG treatment.

Conclusion: The convenience, transparency, and simplicity of the zebrafish model facilitate tracking of fluorescent neutrophils in real time, in order to monitor inflammation, and assess the impact of therapeutic agents.

Keywords: green tea, innate immunity, animal models, IL-1, TNF

Introduction

Acute inflammation is an important early immune response that protects the host against injury and pathogen invasion.¹ Inflammation is initiated when activated stromal cells and other innate immune cells secrete specific mediators and cytokines or chemokines into surrounding extracellular space, thus promoting the recruitment of leukocytes to stressed tissues.² Pro-inflammatory neutrophils are rapidly recruited after infection, and are aggressive in removing invading microorganisms. Fifty or more pro-inflammatory molecules are localized on the plasma membrane of neutrophils and within their intracellular granules, including oxygen metabolites, proteolytic enzymes, and other chemical mediators that effectively destroy any living tissues with which they come in contact.³ Neutrophils also release type I interferon and cyclin-dependent kinase to sustain inflammation.^{1,4,5} Under normal conditions, neutrophils also promote wound healing. After eliminating target organisms, activated neutrophils generate anti-inflammatory agents such as lipoxin, macrophage-derived resolvins, and protectins, and secretory leukocyte protease inhibitors to attenuate further leukocyte infiltration and accumulation.^{6–8}

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Interventions targeting neutrophils are being developed to complement treatments for autoinflammatory and autoimmune conditions.^{9,10}

Conventional studies involving neutrophils require isolating the cells under natural conditions or indirectly studying the cells by examining their excreted metabolites, although advances in genetic modulation techniques now enable both steps to be conducted in a single model organism, such as the zebrafish, *Danio rerio*. Traditionally, mice have been the most popular animal model in immunology research. However, the costs associated with facilities for maintaining mice and conducting large-scale projects are typically high, and this can negatively impact efforts to meet the high demand for testing new drugs. Zebrafish possess several attributes that make an attractive candidate in research, including their rapid growth rate, high fecundity, and versatility in image analyses. Optical transparency, which enables direct visualization of biological processes, has made zebrafish a particularly useful model for studying embryonic development and organogenesis since the 1980s.¹¹ The availability of clones of homozygous fish and ease of breeding have made zebrafish a promising tool for vertebrate genetic analysis.¹² Genetic studies conducted using gamma-irradiated fish mutants have identified genes associated with human disorders.^{13–16} Recent interest has also focused on utilizing zebrafish in high throughput screening of therapeutic compounds. For example, the weißherbst zebrafish mutant develops hypochromic anemia and is thus useful as a disease model for drug screening.^{15,16}

Indeed, zebrafish provide a versatile model for studying immune responses. As they are exposed to pathogens at an early age, most fish larvae possess a highly developed innate immune system. The innate immune system develops as soon as 2 days post-fertilization, and the adaptive immune system is functional by 4–6 weeks post-fertilization.¹⁷ Since thymic organogenesis and compartmentalization into the medulla and cortex are well conserved in zebrafish,¹⁸ the immune system is “fully armed” with cells of major hematopoietic lineages (erythrocyte, leukocytes, myeloid cells). Genome-wide mutagenesis screening studies revealed that hematopoiesis and lymphopoiesis in zebrafish are similar to the processes in the human bone marrow.^{19,20} A library of 24 toll-like receptors has been described in zebrafish, including 10 orthologs of human toll-like receptor family members.^{21,22}

Reporter fish with fluorescent-labeled immune cells have been developed to track macrophages, neutrophils,

and epithelial cells.²³ Since studying neutrophils could open up opportunities for identifying drugs with anti-inflammatory effects, many studies have investigated the use of *mpx* mutants with GFP-labeled neutrophils as a testing platform. One popular experimental design involves simple tail resection to induce inflammation and monitoring of real-time trafficking of neutrophils inside living, translucent fish.²⁴ Based on their high fecundity and translucent vasculature, zebrafish represent a straightforward, cost-effective model organism for testing chemical compounds and rapidly assessing responses at the cellular level.

There is considerable interest in using natural compounds for treating chronic and acute inflammation. Green tea is a traditional drink known for its anti-inflammatory effects. The major catechin in green tea, (–)-epigallocatechin-3-gallate (EGCG), accounts for most of the anti-inflammatory activity.²⁵ EGCG suppresses multiple pro-inflammatory pathways, including the NF- κ B pathway and allergic IgE-mediated histamine release.^{26,27} The anti-inflammatory properties of EGCG have been demonstrated by its effects on reducing trans-endothelial migration of cultured neutrophils and inhibiting the production of reactive oxygen species.^{28,29} In the present study, we demonstrate the utility of transgenic zebrafish as a suitable model for anti-inflammatory drug testing using EGCG as a modulator.

Materials and methods

Zebrafish husbandry

Tg(*mpx:gfp*) zebrafish were obtained from Zebrafish International Resource Center, Eugene, Oregon, and maintained on a 14 hr/10 hr light/dark cycle at 28.5°C in a zebrafish facility (Center for Nuclear Receptor and Cell Signaling, University of Houston, Houston, TX) equipped with Tecniplast (West Chester, PA) tank system. After natural spawning, embryos were grown in a 28°C incubator with daily renewal of fish medium (E3) and removal of spoiled embryos until desired maturation was reached for experimentation. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NAP 2011) and the animal use protocol was approved by the Institutional Animal Care and Use Committee at the University of Houston.

Tail resection, EGCG treatment

At 4 days post fertilization (dpf), Tg(*mpx:gfp*) larvae were anaesthetized in approximately 200 μ g/mL (0.02%) MS-222

(Millipore Sigma, Burlington, MA, USA)-infused E3. All media were sterile, and razor blades were cleaned with 70% ethanol before use. Tail-fin resection was performed using a sterile disposable razor blade at the distal-most point of the pigment gap. Fish recovered in 12-well plates with 10 fish per well and 2 mL of fresh medium infused with 300 μ M or 600 μ M EGCG (Millipore Sigma, Burlington, MA, USA).

Short-time-frame imaging

After 4–5 hrs, a total of 3–4 of untreated or treated, anesthetized larvae were mounted laterally on a glass slide and imaged using AxioObserver D.1 epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA). For quantification of neutrophil accumulation, manual cell counts and intensity measurements were performed. For manual counting, two researchers were randomly assigned to count the number of cells in the region extending up to 100 μ m from the transection site. For intensity measurements, the total intensity in the same spot was measured using Fiji ImageJ (NIH, Madison, WI, USA) with correction for background signal. The following formula was used for quantification: adjusted total fluorescence = integrated density – (area of selected cell \times mean background fluorescence). The data were fed into GraphPad Prism (GraphPad, San Diego, CA, USA) for analysis by unpaired Student's *t*-test and one-way ANOVA, with *p*-values of 0.001, 0.01, and 0.05 considered significant. Graphs show mean values \pm SEM for 10 fish.

Prolonged time-lapse imaging

Low-melt agarose (1%) (Bio-Rad, Hercules, CA, USA) was brought to liquid state at 40°C and infused with EGCG to a final concentration of 300 or 600 μ M. Anesthetized larvae were quickly mounted alive in infused low-melt agarose in a six-well glass-bottom plate (MatTek, Ashland, MA, USA). Time-lapse images were acquired using super-resolution Olympus FV1000 multiphoton confocal laser scanning microscope (Olympus, Center Valley, PA, USA) equipped with a 20 \times objective, at a time span of 1 min over at least 200 mins after tail-fin resection. Migration velocity and distance traveled were calculated and plotted using the manual tracking plug-in of Fiji ImageJ.

Gene expression

A total of nine untreated and EGCG-treated 4 dpf larvae were anesthetized and RNA isolated according to

SingleShot mRNA kit instructions (Bio-Rad, Hercules, CA, USA). RNA was pooled from three fish for each condition, to obtain sufficient RNA. The concentration of isolated mRNA was checked using a DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA). cDNA was synthesized using iScript (Bio-Rad, Hercules, CA, USA) following manufacturer instructions, followed by qPCR in 10 μ L total volume of SsoAdvanced Universal SYBR Green supermix, using 200 ng cDNA for each gene. Primers for 34 zebrafish-specific inflammation-related genes (Table 1) were designed and pre-spotted in 96-well PrimePCR plates (Bio-Rad, Hercules, CA, USA). qPCR was performed in CFX96 cycler under PrimePCR setting: initialized as 10 mins–95°C, followed by 40 cycles 15 s–95°C, 1 min–60°C, with a melt curve cycle. Relative expression was calculated and normalized against three housekeeping genes: actin beta 1 *actb1*, elongation factor 1 *elf1*, and tubulin alpha *tuba*.

Table 1 Genes interrogated by RTPCR, modulated by tail resection and EGCG treatment

Gene	Gene change after	Gene change after	T-test
	Tail resection ^a	EGCG-treatment ^b	
Gene	Mean \pm SE (%)	Mean \pm SE (%)	<i>p</i> -Value
<i>ATF2</i>	69.1 \pm 6.5%	37.8 \pm 2.5%	0.18
<i>CHUK</i>	128.5 \pm 12.5%	85.8 \pm 6.0%	0.31
<i>CP</i>	147.1 \pm 31.1%	154.9 \pm 25.0%	0.42
<i>F3A</i>	101.9 \pm 21.4%	281.3 \pm 45.2%	0.23
<i>HMOX1</i>	375.6 \pm 82.5%	531.1 \pm 81.0%	0.66
<i>IL-8A</i>	125.8 \pm 7.6%	112.2 \pm 9.3%	0.66
<i>IL1B</i>	233.4 \pm 15.0%	49.5 \pm 4.5%	0.03
<i>IL1RAPLIA</i>	144.9 \pm 31.5%	78.7 \pm 10.1%	0.47
<i>IRF1A</i>	87.8 \pm 20.0%	40.3 \pm 5.8%	0.43
<i>M17</i>	145.8 \pm 11.5%	73.9 \pm 3.2%	0.11
<i>NFKB1</i>	125.0 \pm 8.1%	108.3 \pm 3.7%	0.50
<i>NFKBIB</i>	94.3 \pm 4.8%	72.8 \pm 2.6%	0.22
<i>PTGS2A</i>	232.5 \pm 19.4%	141.7 \pm 22.2%	0.27
<i>SERPINE1</i>	257.9 \pm 84.5%	83.0 \pm 5.8%	0.48
<i>TNFB</i>	127.4 \pm 4.5%	81.8 \pm 4.9%	0.04
<i>TRAF6</i>	410.8 \pm 113.0%	131.2 \pm 37.6%	0.41
<i>UBB</i>	129.1 \pm 11.0%	82.4 \pm 2.0%	0.21

Notes: ^aThe percent expression level after tail resection represents mean \pm SE of *n*=3 replicates (each representing three fish), with the level in control fish (no tail resection) being normalized to 100%. ^bThe percent expression level after EGCG treatment represents mean \pm SE of *n*=3 replicates (each representing three fish), with the level in control fish (no tail resection) being normalized to 100%. The corresponding *t*-test *p*-values are listed in the last column. Significant changes are signified by bold.

Abbreviation: EGCG, epigallocatechin-3-gallate.

Statistics

Data were analyzed and plotted using GraphPad Prism 5. Kolmogorov–Smirnov test was used to assess the normality of the data. For comparisons of multiple groups, ANOVA test and subsequent post-test pairwise comparisons were used, employing the unpaired *t*-test, with *p*-values <0.05 considered significant. Preliminary treatment studies revealed ~34 neutrophils in control fish, and 20 neutrophils in treated fish, with a standard deviation of 6. Power calculations reveal that even a sample size of 3 per group would be sufficient to detect differences between groups at 0.05 level of significance, with 80% power. Hence, 3–10 fish were included in all studies.

Results

Static quantification of neutrophil recruitment demonstrates suppressive impact of EGCG

Tg(mpx:gfp) zebrafish are useful as a model of inflammation because they allow the tracking of GFP-labeled neutrophils within the transparent fish.²⁴ Using this model, the

process of inflammation and anti-inflammatory effects of EGCGs was captured in real time. In brief, 4 dpf larvae were anesthetized for tailfin-resection and mounted live for tracking neutrophil responses. Animated recordings of neutrophil migration or accumulation were obtained using an inverted fluorescence confocal microscope for up to 4 hrs after tail resection. Untreated fish exhibited an increased number of neutrophils at the injury site compared to fish treated with 300 or 600 μM EGCG (Figure 1A–C). At the 4 hrs mark, we found the highest number of neutrophils in untreated fish, with an average of 33 cells (Figure 1D), and this number was significantly lower in fish exposed to 300 μM EGCG (*n*=22 cells, *p*=0.003) or 600 μM EGCG (*n*=18 cells, *p*=0.0006). As the neutrophils were fluorescent, we confirmed these findings by quantifying the fluorescence intensity at the local injury site using automated ImageJ software. The intensity declined from 2.8×10^6 LU in untreated fish to 1.8×10^6 LU (*p*=0.17) and 1.2×10^6 LU (*p*=0.04) in fish treated with 300 or 600 μM EGCG (Figure 1E). EGCG at doses >900 μM were lethal to the larvae, even with short exposure; hence, the maximum dose used in this study was 600 μM.

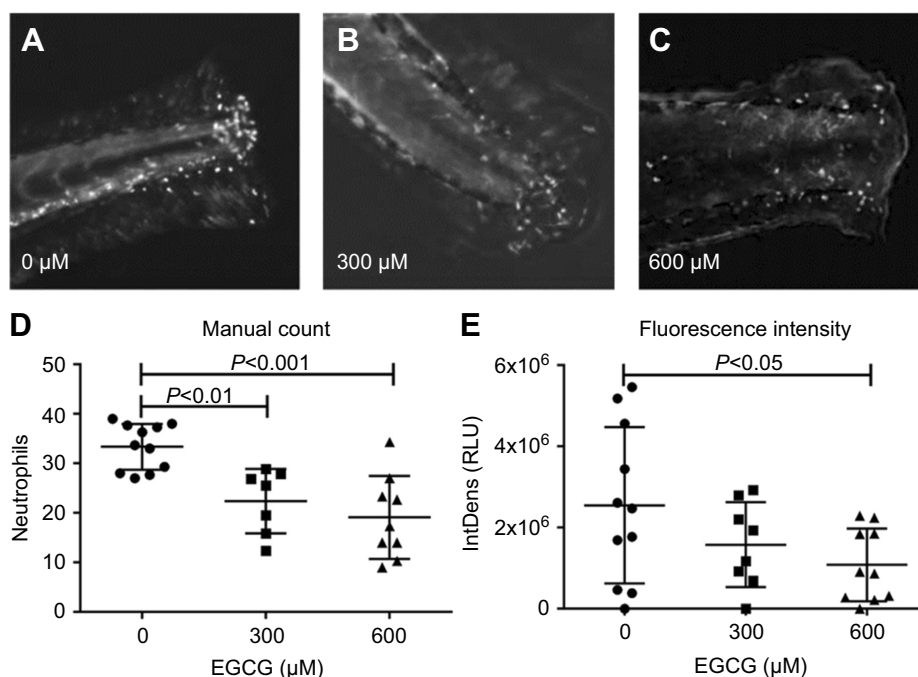


Figure 1 EGCG reduced neutrophil accumulation in tail-resected larvae. (A–C) 4-dpf zebrafish were tail-resected and incubated with EGCG for 4 hrs. Fluorescent neutrophils were imaged within the region of interest (ROI) (5 mm from resected line). Overall, more neutrophil accumulation events were observed in untreated control zebrafish compared to EGCG-treated fish (A–C). Plotted in (D) are the number of neutrophils and plotted in (E) is the fluorescence intensity observed in one ROI. The data show a consistent dose-dependent effect of EGCG. On average, 33 neutrophils were recruited to the resection site without EGCG, but this dropped to 22 and 18 cells, or from 2.8×10^6 light units (LU) to 1.8 and 1.2×10^6 LU, in fish treated with 300 or 600 μM EGCG (mean ± SD, *n*=10, *t*-test).

Abbreviation: EGCG, epigallocatechin-3-gallate.

EGCG reduces the frequency of neutrophil migration and dynamic motion following injury

For time-lapse tracking analyses, neutrophil migration to the injury site was monitored after tail resection, with or without EGCG treatment. Neutrophils were tracked over the first 2 hrs post-injury, with or without EGCG, using confocal fluorescence microscopy, and the data were analyzed using Fiji Manual Tracking. All neutrophils in the untreated group ($d=37.8 \mu\text{m}$) traveled a greater distance than cells from fish treated with 600 μM EGCG ($d=9.53 \mu\text{m}$, $p=0.0002$), but a similar distance compared with neutrophils from fish treated with 300 μM EGCG ($d=39.5 \mu\text{m}$, ns) (Figure 2). The average velocity of neutrophil migration in the untreated fish ($v=0.020 \mu\text{m/s}$) was significantly greater than that in fish treated with EGCG at 300 μM ($v=0.013 \mu\text{m/s}$, $p=0.008$) or 600 μM ($v=0.008 \mu\text{m/s}$, $p=0.001$), as shown in Figure 2. EGCG treatment reduced leukocyte infiltration in terms of recorded tracks, average speed, and mean distance traveled by these cells, as illustrated in Figure 3. The attendant movie illustrates the dynamic attenuation of the neutrophil recruitment associated with EGCG treatment, fixed frames of which are shown in Figure 3.

Identifying EGCG targets in inflammation signaling pathways, in tail-fin resected zebrafish

Based on the published data on how EGCG impacts various pathways, we examined a select set of 34 genes implicated in inflammation, including IL-1 and NF- κB

driven pathways. Following tail resection, 15 out of the 34 genes interrogated were elevated at least 10%, compared to fish with intact tails (Table 1); these are presented in Figure 4A in red bold font. Next, we asked which genes were downregulated by EGCG. Of the tail resection induced genes, 12 genes were downregulated at least 10% following EGCG treatment, as indicated by green boxes in Figure 4. Of these EGCG-induced changes, two reductions attained statistical significance, namely IL1B ($p=0.026$) and TNFB ($p=0.045$). Overall, as evident from Figure 4, several inflammatory cytokines (eg, IL-1 β , TNF β , IL-6) and inflammation related signaling molecules (eg, IL1R-APa, TRAF6, IRF1a, etc.) showed a trend toward upregulation following tail resection, but reduction following EGCG administration, though most of these changes failed to attain statistical significance.

Discussion

Neutrophils are among the earliest immune cells recruited in response to tissue injury. The zebrafish model provides researchers unique opportunities to visualize neutrophil migration in real time. In this study, we examined the protective effects of EGCG against inflammation in tail-fin-resected model of transgenic zebrafish. In our study, we observed a sizable average number of neutrophils (an average of 33 cells) being recruited to the wound site, which was indicative of a normal inflammatory response to injury. Because previous reports documented that neutrophil recruitment persists for approximately 8 hrs after wounding, with a peak at 4 hrs, we examined neutrophil recruitment at 4 hrs after resection.²⁴ Our data indicate reductions

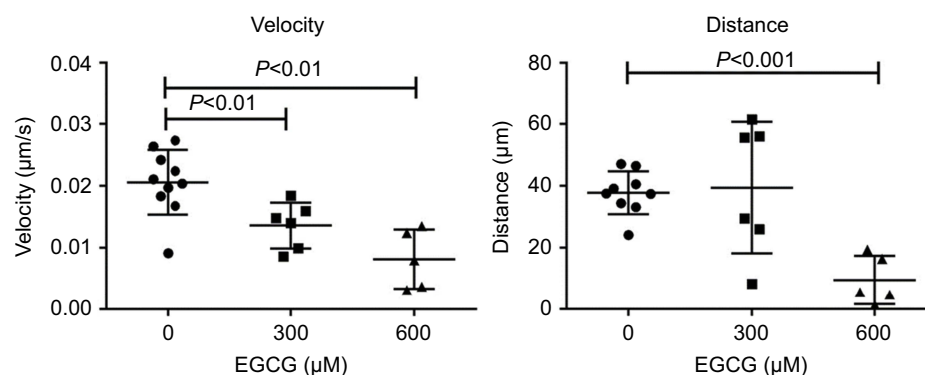


Figure 2 EGCG suppressed neutrophil migration speed and traveled distance. The larvae at 4 dpf larvae were tail-resected and mounted in EGCG infused agar for 2 hr imaging. From the time-lapse records, neutrophils were detected by fluorescence microscopy. Motion parameters including velocity and distance were extracted using Fiji Manual Tracking. More dynamic tracks were recorded in the untreated control fish within the ROI, compared to the EGCG treated fish, in terms of the number of active tracks, distance, and speed. Quantitative analysis of event speed (LEFT) and distance (RIGHT) in ROI of zebrafish treated with vehicle and EGCG (300 and 600 μM) are plotted (mean \pm SD, $n=6-10$, t -test).

Abbreviations: EGCG, epigallocatechin-3-gallate; ROI, region of interest.

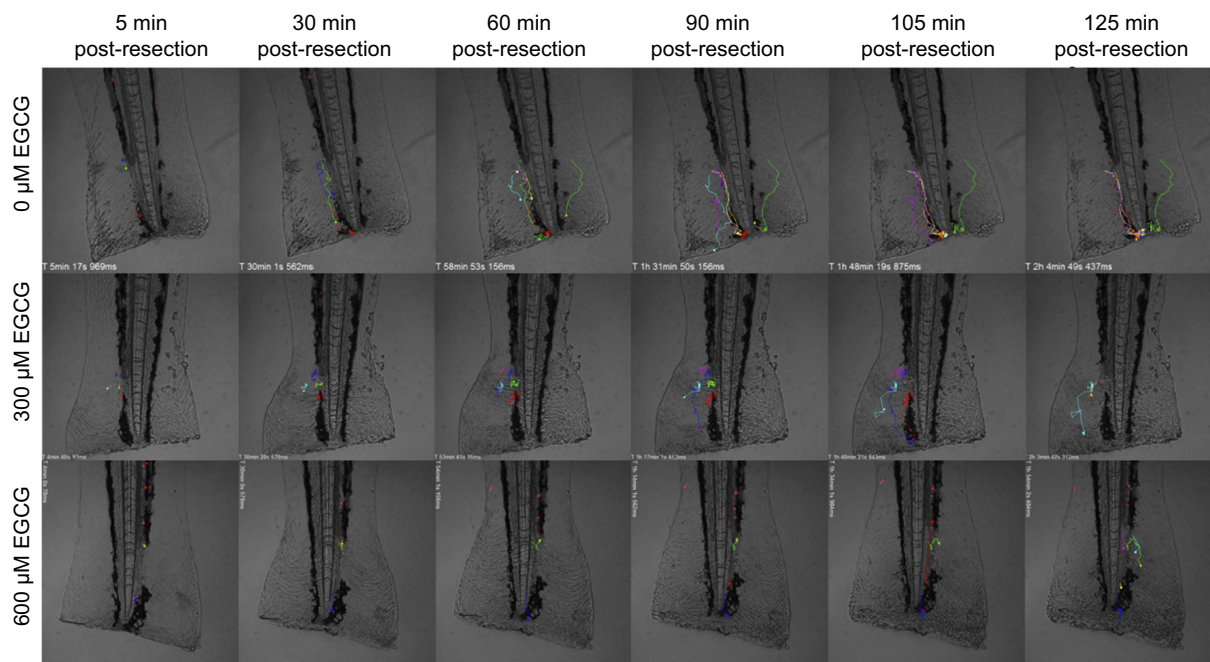


Figure 3 EGCG suppressed neutrophil movement toward the inflamed region. Extracted frames from time-lapse fluorescence tracking records showed less defined tracks in fish treated with EGCG. A dynamic view of these events can be seen in the attached movie (Supplementary animations).

Abbreviation: EGCG, epigallocatechin-3-gallate.

from an average of 33 cells in untreated fish to an average of 22 and 18 cells in fish treated with EGCG at 300 or 600 μM (~31% and 43% reduction), respectively. Live recording of neutrophil recruitment also clearly demonstrated the delay in the recruitment of neutrophils. The frequency of qualified neutrophil migration tracks in untreated fish was twice as high as that seen in EGCG-treated fish. The speed and distance traveled were also lower in EGCG-treated fish. These findings are consistent with previous reports of the inhibitory effect of EGCG on neutrophil function and mobility.^{28,29}

Neutrophils are recruited by signals released from damaged and necrotic cells. The process of neutrophil extravasation from the vasculature into the tissues is governed by chemokines and cytokines, including IL-1 β ,^{30,31,33} CXCL8,³² hydrogen peroxide,²³ and lipid mediators. It is also well known that neutrophils are capable of processing and producing active IL-1 β . EGCG dampens multiple innate signaling pathways including histamine release,²⁷ reactive oxygen species production,²⁸ and production of chemoattractants such as CINC1, IL-8,²⁹ TNF- α ^{25,34}, and IL-1 β .

Indeed, IL-1 β induced-inflammation has been reported to be reversed by EGCG treatment in other model systems.^{34,35} Among the 34 RNAs we interrogated, wounding injury induced expression of 15 molecules in the IL-1 β and related

inflammation signaling pathway, though these failed to attain statistical significance. In the EGCG-treated group, it was found that EGCG effectively reduced the expression of 12/15 wounding-induced molecules, with the suppression of IL-1B and TNFB (or TNF α 2) attaining significance. Indeed, both IL-1 and TNFB are important mediators of inflammation.³⁶ The expression of these two pro-inflammatory cytokines has synergistic effects, with many reports documenting IL1B inducing biological active TNF α production.³⁷ For more than two decades, pharmacological blockage of IL-1 (ie, Anakinra, Canakinumab, etc.) constitute major treatment modalities in inflammatory diseases.³⁸ At the same time, inhibiting TNF α has been effective in accelerating wound healing and subduing inflammation.^{39,40}

These studies underscore the utility of the transparent Tg zebrafish as an ideal model to use for tracking migration of any tagged cells, as exemplified by the neutrophil-focused analyses presented here. Second, these studies resonate with the accumulating literature that indicate that EGCG is potent at suppressing inflammation, by subduing IL-1, TNFB, and related pro-inflammatory molecules. Further studies are warranted to examine EGCG-induced molecular changes in this model more comprehensively, and to unravel the molecular mechanisms by which neutrophil migration is controlled. Finally, since Tg integration site can potentially

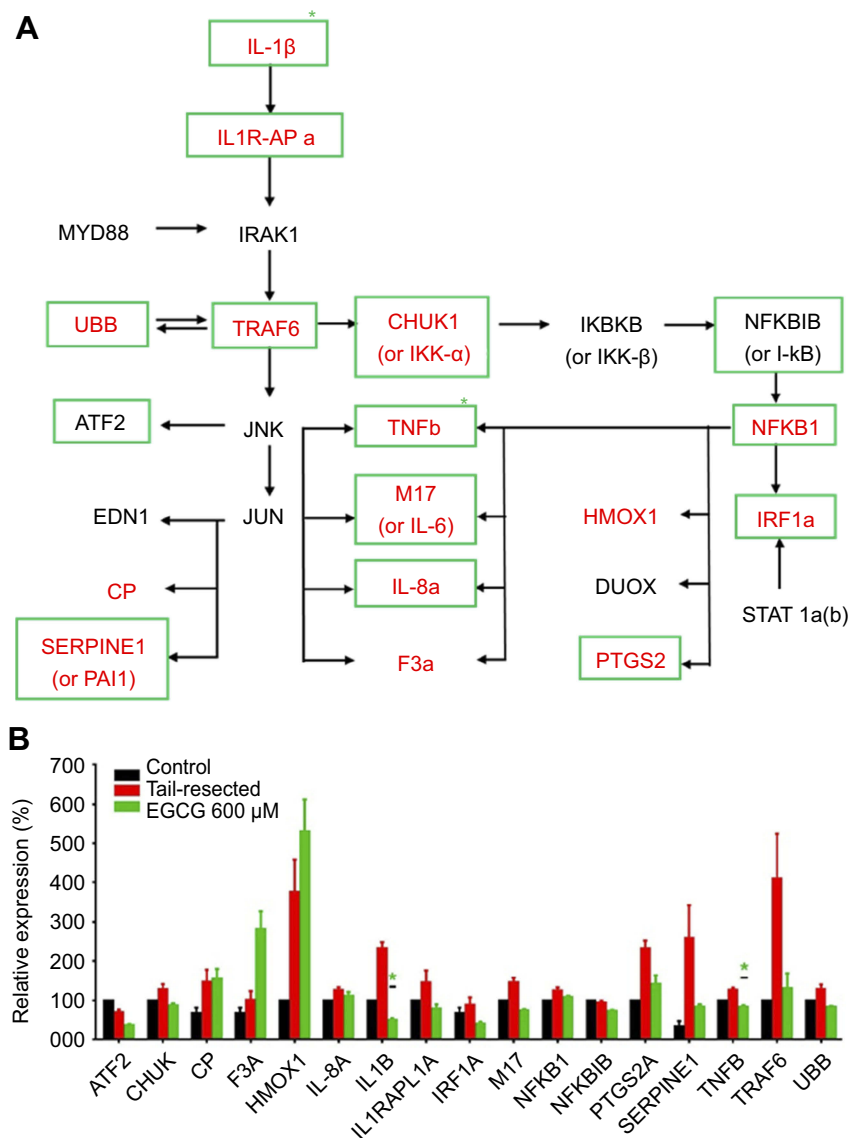


Figure 4 Impact of EGCG on wounding-induced genes in the interleukin 1 β and related signaling pathway. A group of nine tail-fin resected zebrafish were exposed to EGCG for 4 hrs. mRNA was collected and pooled (three fish RNA per pool) for RTPCR amplification of a library of 34 candidate genes in the interleukin-1 beta and related signaling pathway, as detailed in Table 1. The pathway diagram shown in (A) represents likely gene interaction relationships between the interrogated genes, constructed based on the literature review. Only genes that exhibited at least 10% change compared to the control group are plotted. The RNAs corresponding to the molecules in red-bold letters (in "a") were upregulated following tail-fin resection by at least 10%, compared to the control fish. The RNAs corresponding to the molecules within green boxes were downregulated at least 10% after EGCG treatment (in "a"). Plotted in (B) are the mean (\pm standard error) gene expression changes in three replicates (three fish per replicate), with all data normalized to the expression levels in control un-resected, untreated fish. Green asterisks mark significant fold-changes. In tail-resected zebrafish, wounding led to the upregulation of 15 inflammatory genes, of which 11 were subdued following EGCG treatment, of which IL1B and TNFB attained statistical significance.

Abbreviations: EGCG, epigallocatechin-3-gallate; RTPCR, reverse transcription polymerase chain reaction.

affect phenotypes, it is also important to confirm these findings using additional independent Tg lines or other neutrophil tagged Tg models, in future studies.

Data Availability

All data are freely available by contacting the corresponding author.

Disclosure

The authors report no conflicts of interest in this work.

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