

Curcumin Effects on Age-Related Changes in Oral Immunity: An *In Vivo* Study

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Abstract

The current study aimed to investigate the effects of ageing on oral immunity using β -defensin (DEFB) 1/2 as a marker and evaluate the effects of curcumin (CUR) on these processes. The study sample included 30 male C57BL/6J mice divided into three groups based on the treatment method used. The young control (YC) and old control (OC) groups received 0.5% methylcellulose-400 (curcumin vehicle) orally for five days, whereas the CUR group of older mice received a curcumin solution suspended in 0.5% methylcellulose-400 (dose: 3.0 mg/kg body). DEFB1/2 and immune indicator levels were measured in the saliva and salivary glands posttreatment. The saliva volume and protein content were significantly reduced in the OC group compared to the YC group. CUR administration restored these parameters, decreased DEFB1 expression in the salivary gland and increased DEFB1/2 secretion and DEFB2 expression. These findings were supported by epigenetic gene regulation and partial cytokine activation from changes in WD40 repeat protein 5, Tumour Necrosis Factor alpha and interleukin-1beta. Curcumin can partially restore age-related changes in oral immune responses and promote oral health, thereby preventing frailty in the older population through a nutritional therapeutic pathway.

Keywords: β -defensin; saliva; salivary glands; polyphenol; oral frailty

Introduction

Frailty has recently been found to directly impact the healthy lifespan of an older individual and act as a precursor to the need for care^(1,2). Frailty can lead to a decline in various physical and physiological declines that can easily lead to health disadvantages, such as weight loss, fatigue, reduced walking speed, decreased grip strength and reduced physical activity^(1,2). Similarly, oral frailty is a multifaceted condition with widespread implications for overall health and well-being in the elderly because it implies deterioration of oral tissues, immune response and microbial balance, leading to an increased susceptibility to oral diseases and conditions⁽¹⁻⁴⁾. Addressing oral frailty through preventive measures, early detection and appropriate interventions is essential to promote healthy ageing and improve the quality of life of the elderly⁽¹⁻⁴⁾. Proactive interventions for oral frailty, characterised by a decline in oral function (e.g. poor oral hygiene, dry mouth, reduced tongue-lip mobility, decreased bite force, low tongue pressure, impaired chewing function and weakened swallowing function), should aim to prevent transition to specialised treatment^(3,4). In particular, poor oral hygiene and dry mouth are not only associated with poor oral mucosal immunity but have also been reported to be related to nutritional status^(5,6), and this could likely be attributed to dysregulation of inflammatory and antimicrobial responses associated with down-regulation of regulatory receptors on the mucosal surfaces. Furthermore, evidence also suggests that age-related fluctuations in biological low-molecular-weight compounds can lead to the development of periodontitis and other oral diseases through various underlying molecular mechanisms^(7,8). Oral frailty can be addressed through preventive measures, early detection and appropriate interventions as they mainly focus on oral immunological defence mechanisms and their role in maintaining oral health and its resilience^(9,10). Therefore, oral immunity, traditionally evaluated using established markers such as IgA and, more recently, oral peptides such as β -defensins, plays an important role in disease status^(9,10). Although numerous studies have demonstrated the effects of drugs, certain food items and fluctuations in antigen (e.g. bacteria and viruses) levels on mucosal immunity, very few have examined the effects of ageing to date^(10,11).

Oral immunity factors such as β -defensins (DEFB) are typically produced by the gingival tissues and play an important role in innate immunity^(12,13). They include subtype 1 (DEFB1), which are constitutively expressed, and subtype 2 (DEFB2) which are induced under certain conditions such as infections or inflammation only⁽¹²⁻¹⁴⁾. However, no studies to date have examined variations in DEFB expression and secretion levels in the oral cavity in

response to ageing. Defensins are typically induced through pathways that are similar to inflammatory cascades (including toll-like receptors, myeloid differentiation factor 88 and tumour necrosis factor receptor-associated factor 6), with mechanisms regulating transcription levels through DNA and histone modifications also playing a role. However, there is limited evidence on the effects of ageing on these processes⁽¹⁴⁻¹⁶⁾.

Curcumin (CUR) is a polyphenolic compound that has been shown to have anti-inflammatory effects in mice with inflammatory bowel disease, and also immune-regulatory effects in response to drug-induced inflammation in humans⁽¹⁷⁻²²⁾. It increases the expression of antimicrobial peptides stimulated by blood cell components, thus activating innate immunity⁽²¹⁾; induces epigenetic gene regulation by inhibiting DNA methyltransferases⁽²²⁾; and exhibits antiaging effects by acting as an antioxidant or Sirtuin modulator^(9,21,23). However, despite stimulating various immune responses, polyphenolic compounds such as CUR exhibit poor water-solubility and very low absorption rates in the gastrointestinal tract⁽²⁴⁾. Therefore, their efficacy as nutritional supplements is often limited by their low biological availability, as demonstrated by numerous *in vitro* and *in vivo* studies^(19,25,26). However, oral administration of CUR has been shown to affect the gastrointestinal mucosal surfaces through toll-like receptors and similar pathways, regardless of the absorption levels⁽²⁷⁾. Therefore, we hypothesised that CUR absorption would result in immunomodulatory effects in the gastrointestinal tract.

Although a direct effect of CUR on oral ulcers has not yet been demonstrated⁽²⁸⁾, an improvement in the oral environment through the regulation of oral flora and promotion of the body's defence mechanisms is expected upon its administration. Therefore, the current study examined changes in DEFB and related cascades in response to ageing and evaluated the effects of CUR on the oral immunity of older mice, with the aim of establishing convenient salivary markers that can inform predisease strategies targeted at extending healthy lifespan through dietary habits.

Experimental methods

Chemicals

Curcumin (CUR) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were used at the highest grade available and without further purification.

Animals

The study sample included 30 male mice (C57BL/6J; weight: 19–40 g; age: 7 or 70 weeks) that were obtained from Jackson Laboratory, Hokkaido, Japan. For the young age group, time of sexual maturity was selected as the criterion. This age range represents a stage in the mice's life cycle where they have reached sexual maturity but are still considered young adults⁽²⁹⁾. In contrast, the criterion for the old age group was based on a specific age in weeks. This age corresponds to a point in the mice's lifespan where changes associated with ageing can be reliably detected across various biomarkers in almost all animals⁽²⁹⁾. For experiments involving older animals, the sample size was set according to older mice, referring to The Mouse Phenome Database (<https://phenome.jax.org/>), to consider the environment, feed and health conditions at the time of rearing. The mice were housed in plastic cages under standard laboratory conditions, including a constant temperature of 23°C ± 2°C and a 12:12-hour light–dark cycle, and ad libitum access to water and food (standard rodent chow diet) was provided. The cage measured 235 × 353 × 160 mm (width × depth × height), the flooring was Shepherd's® Specialty Blend™ (Shepherd Specialty Papers, Inc.), and there were five animals/cage. The experimental animal protocol was approved by the Committee of the Laboratory Animal Center (No. 2022-011), and all procedures were carried out in accordance with the Guiding Principles for the Care and Use of Experimental Animals at Hokkaido University of Science. CUR (dose: 3.0 mg/kg body) was dissolved in 0.5% methylcellulose-400 and 1 mL/kg was administered orally using a micropipette. To ensure uniformity in body weight, the animals were randomly divided into the young control (YC; age: 7 weeks; n = 10); old control (OC; age: 70 weeks; n = 9); and curcumin (CUR; age: 70 weeks; n = 10) groups (Figure S1). An acclimation period of 1 week was considered in all animal experiments. Herein, 7-week-old mice were acclimatised for 1 week and 70-week-old mice for 70 weeks after purchase at 60 weeks. One 70-week-old mouse died during the acclimatisation period; therefore, the sample size at the start of treatment was n = 9 in OC. The dose of CUR was set in a way that it did not exceed the acceptable daily intake (ADI) of

CUR established by the World Health Organization⁽³⁰⁾. The animals were anesthetized after administration of food reagents for 5 days. A mixture of pharmaceutical-grade medetomidine hydrochloride (Domitor®; NIPPON Zenyaku Kogyo Co., Ltd., Fukushima, Japan); midazolam hydrochloride (Dormicum®; Astellas Pharma Inc., Tokyo, Japan); and butorphanol tartrate (Vetorphale®; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) was created using concentrations of 0.75 mg/kg, 4.0 mg/kg and 5.0 mg/kg, respectively. These drugs were diluted in sterile saline and administered using an intraperitoneal injection. After euthanasia, the submandibular and parotid glands were sampled, stored at -80°C , and appropriately processed prior to analysis.

Saliva collection and measurement

Following intraperitoneal administration of 1 mg/kg of pilocarpine-HCl (Sampiro 1%; Santen Pharmaceutical Co., Ltd., Osaka, Japan) after anaesthesia⁽³¹⁾, the saliva samples were collected strictly from the oral cavity using a micropipette continuously for 10 min. Thereafter, the samples were immediately placed on ice, centrifuged at $10,000 \times g$ for 2 min at 4°C and subsequently stored at -80°C until analyses were performed. The β -defensin1/2 (DEFB1/2) levels were then detected using enzyme-linked immunosorbent assay (ELISA), whereas bicinchoninic acid assay was used to determine the protein concentration.

Enzyme-linked immuno sorbent assay (ELISA)

DEFB secretion was determined using β -defensin 1 and β -defensin 2 ELISA kits (MyBioSource, USA) as per the manufacturer's instructions. No changes were made to the procedure; however, the saliva samples were diluted 50-fold in phosphate-buffered saline and assayed thereafter.

Western Blotting

The control and treated tissue lysates (10 μg protein/lane) were separated using 4%–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad Laboratories Inc., Hercules, CA, USA). The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc.) and analysed using Western Blotting with anti-DEFB1, anti-DEFB2 and anti-cystic fibrosis transmembrane conductance regulator (CFTR) (1:500) antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). The membrane was blocked for 1 h using 5% EzBlock Chemi (ATTO, Tokyo, Japan), and then incubated for

15–18 hour using the appropriate primary antibodies at 4°C. The blots were washed with Tween-20 (Bio-Rad Laboratories Inc.) containing phosphate-buffered saline and incubated with appropriate secondary antibodies for 1 h at 22°C–25°C. The protein bands were visualised using enhanced chemiluminescence Western Blot detection reagents (Bio-Rad Laboratories Inc.)⁽³²⁾.

Real-time quantitative PCR

Total RNA was extracted from the tissues using the ISOGEN II reagent (NIPPON Gene Co. Ltd., Tokyo, Japan). Thereafter, single-stranded cDNA was prepared from 1 µg total RNA using ReverTra Ace (Toyobo, Osaka, Japan), and reverse transcription-quantitative polymerase chain reactions (RT-qPCR) were performed using the QuantStudio™5 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with KAPA SYBR™ FAST (NIPPON Genetics Co. Ltd., Tokyo, Japan) as per the manufacturer's protocol. Supplementary Table 1 shows the sequences of the specific primers used.

Data analysis

All statistical analyses were performed using the statistical software package Excel (Bell Curve for Excel v.3.20). Data were presented as mean ± standard deviation and were analysed for statistical significance using one-way analysis of variance followed by Tukey's test to compare means of two or more groups, and Welch's t test for between two groups. P-values <0.05 or <0.01 were considered statistically significant.

Results

The effects of CUR on age-related changes in saliva volume and protein content.

The OC group exhibited a significant decrease in salivary volume per minute and salivary protein content compared to the YC group (P<0.01; Figures 1A, B). However, administration of CUR in the OC group significantly increased these parameters, suggesting that it exerted a restorative effect on the salivary secretory capacity (P<0.01; Figures 1A, B).

The CFTR, responsible for the regulation of pH and salt concentration in the oral mucosa, exhibited significantly decreased expression in the submandibular glands of the OC group compared to the YC group. However, restoration of this decreased expression was observed in the CUR group (Figure 1C).

Effects of CUR on DEFB1/2 secretion and expression in old mice.

Examination of the effects of CUR on DEFB1/2 in the OC group showed a significant increase in DEFB1 secretion levels in the saliva (p-value < 0.05; Figure 2A), and a significant decrease in the protein and gene expression levels of DEFB1 in submandibular gland tissues (P<0.05; Figures 2B, C). Furthermore, CUR also increased DEFB2 secretion in the saliva (P=0.065; Figure 2D) and protein (P<0.05; Figure 2E) and gene (P<0.01; Figure 2F) expression levels in the submandibular and parotid glands. These findings confirm that CUR induces transcription and translation of DEFB2 in the salivary glands.

The effects of CUR on age-related changes in immunomodulatory capacity.

Changes in intracellular signalling, epigenetically related genes and cytokines were examined to enable elucidation of the mechanisms underlying the differential effects on constitutively and indelibly expressed DEFB1 and DEFB2. Intracellular signalling variations were assessed by measuring the myeloid differentiation factor 88 (*Myd88*) and tumour necrosis receptor-associated factor 6 (*Traf6*), while epigenetic gene regulation was confirmed by measuring WD40 repeat protein 5 (*Wdr5*). The results showed a significant reduction in gene levels (P<0.01; Figure 3A), indicating age-related effects. Furthermore, CUR significantly decreased *Myd88*, *Traf6* and *Wdr5* levels in the submandibular glands of the OC group (P<0.01; Figures 3B–D).

Furthermore, examination of variations in cytokines including tumour necrosis factor- α (*Tnfa*) and interleukin 1 β (*IL-1 β*), which exhibit similar cascades or DEFB2-inducing abilities in the submandibular gland, showed a significant decrease in expression levels as a result of ageing. CUR administration further decreased *IL-1 β* gene expression (P<0.05; Figures 3E, F) and restored *Tnfa* gene expression. However, the final *Tnfa* levels observed did not exceed that of the control groups, indicating an immune stimulus that was not inflammatory in nature.

Discussion

The current study identified age-related changes in oral immunity and examined the effects of CUR administration and its underlying mechanisms. In oral immunity, we focused on DEFB1/2, which is related to innate immunity, from tissue expression to secretion, as an indicator. Reports stating the effects of curcumin on immune responses were used to determine the CUR dosage⁽³³⁻³⁵⁾. In mice, 100 mg/kg body weight of turmeric extract can

suppress ovalbumin-induced anaphylaxis, and even 3 mg/kg body weight of curcumin, converted from the fact that curcumin contains approximately 3% in the turmeric extract, affected immunity⁽³³⁾. This suggests that the biological activity is sufficient even at ADI doses. In particular, the effect of curcumin on Th2 cells was discussed in the corresponding report; however, the effect on innate immunity, such as DEFB1/2, was not fully investigated⁽³³⁾; therefore, this study was conducted with curcumin dose of 3 mg/kg body weight administered orally.

In this study, CUR restored the reductions in saliva volume/min and salivary protein content observed in the OC group. Decreased saliva volume may lead to decreased oral function, exacerbating frailty, further exacerbating oral health problems, and increasing susceptibility to dental caries, gingivitis, oral mucosal lesions and taste changes^(3,4). This may result in weight loss and malnourishment due to decreased food intake⁽⁴⁾. Saliva volume is reduced in patients with hyponutrition, suggesting that saliva volume may play a role in the vicious cycle^(5,6,36). Therefore, from an oral hygiene perspective, supporting the frailty of the elderly by adequate saliva secretion may not only enhance the mechanical function of digestion and absorption but also help mitigate potential adverse effects^(4-6,36). Saliva secretion can be divided into two categories: that controlled by the autonomic nervous system and that secreted during processes, such as mastication⁽³⁷⁾. The former is present in a certain amount in the oral cavity and is involved in the control of sensory stimuli such as taste, and the saliva measured in this study falls into this category⁽³⁷⁾. Therefore, amount of saliva can determine age-related abnormalities, leading to a decrease in food intake and progression of frailty, mainly weight loss, due to the loss of enjoyment of taste. However, the restoration of autonomic nervous system-controlled saliva by pilocarpine stimulation by CUR in this study indicates that it can improve taste abnormalities, suggesting the possibility of nutritional intervention for frailty. Moreover, proteins in saliva, which control taste perception, are altered by tasty foods⁽³⁸⁾, and this study confirms that the amount of immune substances present was also altered by CUR, making food-based oral hygiene interventions for oral frailty a realistic possibility. Because this study was conducted in animals, results commonly observed in humans (such as oral lesions and weight loss) were not obtained. This is a limitation while assessing the association between oral frailty and frailty in terms of food intake, dietary goals, and number of taste receptors, which may be hampered by differences in biological species differences between (laboratory animals and humans)^(3,4,36). Therefore, as discussed in detail below, further nutritional interventions may be warranted to investigate

the effects of ageing and CUR on the regulators and biomarkers of salivary components. Previous studies suggested that immune senescence associated with ageing predominantly affected adaptive immunity rather than innate immunity⁽³⁹⁾. Furthermore, examination of the gene expression ratios in various organs through analysis of data from the Human Protein Atlas Project (v.21.1; <https://www.proteinatlas.org/>) showed higher expression levels in the salivary glands, suggesting that DEFB played a significant role in oral immunity (Figure S2)⁽⁴⁰⁾. Therefore, the investigation of age-related alterations in innate immunity, which serves as the initial defence mechanism against antigens, is imperative, to demonstrate the utility of DEFB as a short-term oral immune indicator. The current study examined changes in oral immune function due to ageing by focusing on the quality and quantity of saliva produced.

Notable changes in salivary salt concentration and pH due to ageing have been reported previously⁽⁴¹⁾, with studies examining the pathophysiology of cystic fibrosis demonstrating that increased salt concentration on the mucosal surface and decreased pH led to a reduction in antimicrobial peptide activity⁽⁴²⁾. Furthermore, age-related changes in salivary glands, including a decrease in acinar cells, fibrosis and lymphocyte infiltration, have been shown to decrease salivary secretion⁽⁴³⁾. In the current study, CUR treatment was seen to restore the age-related decrease in CFTR protein expression in the submandibular glands, and this was accompanied by recovery of the salivary flow rate and protein concentration. Although both the submandibular and sublingual glands were examined in the current study, CFTR protein expression recovery was not observed in the former and this discrepancy could likely be attributed to the fact that the sublingual glands became the predominant source of proteins during saliva collection using pilocarpine⁽⁴⁴⁾. Considering the pivotal role of CFTR protein expression in salivary composition, particularly with regard to maintenance of the antimicrobial activity of DEFB proteins, it can be inferred that qualitative changes in salivary components contributed to this process.

Although CUR restored the DEFB1 secretion levels to a certain extent, the overall levels were still relatively low in the salivary gland tissue, potentially due to morphological changes associated with ageing that prevented complete recovery⁽⁴⁵⁾. Therefore, evaluation of age-related changes in natural immunity requires examination of variations in DEFB2 which is known to be indelibly expressed and is reflective of immune reactivity. The sublingual gland is highly responsive to immune stimulation, suggesting that the polyphenols may act as ligands and activate the immune system^(11,46). Additionally, polyphenols exhibit poor

translocation into the cells when existing as aglycones⁽²⁴⁾. In the current study, the CUR was suspended as an aglycone before administration, suggesting that the observed effects originated from extracellular immune stimulation.

Polyphenols have been shown to modulate immune responses via host defence peptides in various organs and tissues⁽¹¹⁾, and the mechanisms involved include activation of the MAPK pathway, induction of sphingosine-1-phosphate, inhibition of KEAP1 leading to induction of NRF2, epigenetic gene regulation and metabolic regulation of gut microbiota⁽¹¹⁾. The current study found that CUR activated the main immunity and inflammatory factors such as MyD88 without inducing inflammation, indicating partial immune stimulation. In addition, they decreased IL-1 β , which induced multiple cytokine networks and selectively increased TNF α , which was secondarily induced. These findings were consistent with previous studies that reported DEFB2 induction by TNF α ⁽⁴⁷⁾, demonstrating restoration of the expression of both genes within the salivary gland. Furthermore, while CUR showed different results compared to its anti-inflammatory effects assessed by TNF α as an indicator in mice, interestingly, the inflammatory state in older individuals, who are at risk for chronic inflammation, appears to operate through a mechanism distinct from that of pathological or drug-induced inflammation^(17,18,48). CUR has also been reported to act on G-protein coupled receptors, suggesting a cascade that is distinct from immune stimulation by immunological ligands^(49,50). Evaluation of histone methylation showed inhibition of *Wdr5* gene expression and, consequently, acetylation which controlled the observed transcriptional levels⁽⁵¹⁾.

Although previous studies have used DNA methyltransferase and histone deacetylase enzymes as indicators of epigenetic gene control with polyphenols⁽²¹⁾, the effects of *Wdr5* were previously unknown. The variations in the effects of CUR could likely be attributed to DNA and histone modifications that regulate the selective recovery of cytokines and result in differential effects of DEFB1 and DEFB2 on the same chromosome. However, the current study was limited by its inability to directly observe methylation/acetylation of histones or methylation at the DNA level. Future studies should aim to examine methylation status in the promoter regions of genes that exhibited altered expression in the current study (e.g. DEFB1 and DEFB2) or examine the symmetrically varied results of IL-1 β and TNF α to gain a deeper understanding of epigenetic gene control in the older.

The findings of this study can inform clinical practice by potentially serving as a marker for immune-related diseases. For example, oral flares and fluctuations in the oral environment due to infections require monitoring, similar to autoimmune diseases. The use of

saliva samples gained popularity during the novel corona virus infectious disease, emerged in 2019 (COVID-19) pandemic and this was accompanied by rapid advancements in measurement techniques for viral antigens. However, there are a limited number of markers available for the elucidation of mechanisms underlying the development of autoimmune oral disorders associated with COVID-19⁽⁵²⁾, and future studies should aim to identify inflammatory markers and examine the possible outcomes of epigenetic gene regulation to facilitate establishment of diagnostic reference values for salivary markers in humans. At the very least, tracking oral health status using salivary molecular markers such as DEFB2 has the potential to facilitate early detection of oral frailty in the general population, even in the absence of medical expertise. This, in turn, could promote self-care practices, including the incorporation of specific food ingredients, to sustain overall health^(9,53,54).

In conclusion, the findings of this study showed that salivary secretion declined with age, and the changes in the quality and quantity of saliva may be directly linked to infection control. Moreover, CUR administration in older mice showed suppression of inflammatory signals, epigenetic regulation through suppression of histone methylation and partial induction of inflammatory cytokines in the salivary gland tissues. Increased secretion and expression of DEFB2 helped strengthen the protective response to foreign antigens in older individuals who are susceptible to infection, suggesting that it may be used as a biomarker to monitor the oral environment. These findings confirm the hypothesis that CUR exerts significant effects on the gastrointestinal tract, regardless of absorption. To link the significance of DEFB variation to health effects, attention must be paid to variation in oral bacteria; however, this was not possible in this study. Alternatively, while it has been reported that orthodontic acrylic additives containing CUR have antimicrobial activity against *S. mutans* and *C. albicans* biofilms⁽⁵⁵⁾, it is also consistent with the antimicrobial spectrum of DEFB⁽⁵⁶⁾, which indicates that this action is mediated by DEFB. Therefore, it is expected that this is DEFB-mediated action. The significance can also be considered from the potential host effects of antimicrobial peptides. We have previously demonstrated the direct host effects of an antimicrobial peptide, α -defensin5, on CFTR in the intestinal tract and the utility of polyphenols in cancer therapy⁽⁵⁷⁻⁵⁹⁾. Further studies to better elucidate the potential host effects of DEFB are required in future. Medical interventions are typically associated with higher risks in older individuals who have lower tolerance to treatment measures. Therefore, the findings of the current study, which showed that innate immunity can be regulated through food components, can prevent progression of disease and provide insight into

appropriate dietary habits during the treatment period. CUR is expected to help prevent frailty in the older population by improving the oral environment and partially restoring the age-related decrease in innate immune responses.

Author contributions

Ippe Uemura: Conceptualisation, formal analysis, investigation, writing–original draft, writing–review & editing, visualisation; **Natsuko Takahashi-Suzuki:** conceptualisation, investigation, supervision, writing–original draft, writing–review & editing, funding acquisition; **Akari Sano:** investigation; **Shogo Yamada:** investigation; **Akifumi Nakata:** investigation, writing–review & editing; **Takashi Satoh:** supervision, writing–review & editing.

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Supplementary data

This article contains supplementary data.

Declaration of interests

The authors declare no conflicts of interest.

Abbreviations

ADI, acceptable daily intake; CFTR, cystic fibrosis transmembrane conductance regulator; CUR, curcumin; DEFB, β -defensin; ELISA, enzyme-linked immuno sorbent assay; IL-1 β , interleukin-1beta; Myd88, myeloid differentiation factor 88; OC, old control; Pr, parotid glands; RT-qPCR, reverse transcription-quantitative polymerase chain reactions, Sm, submandibular; ; Tnfa, Tumour Necrosis Factor alpha; Traf6, tumour necrosis factor receptor-associated factor 6; Wdr5- WD40 repeat protein 5; YC, young control.

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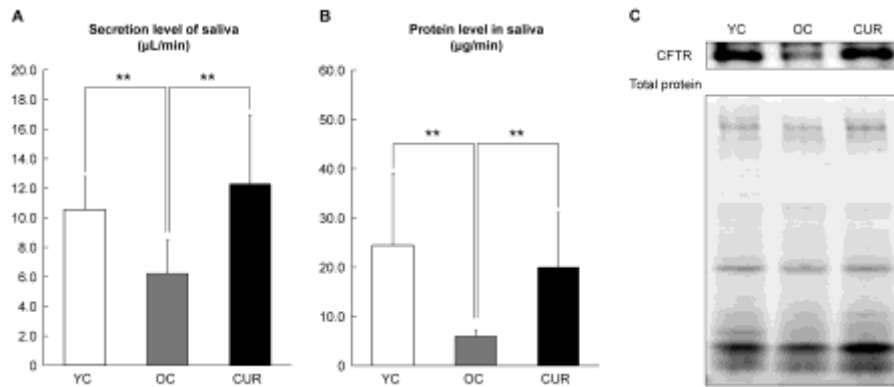


Figure 1: The effects of CUR on age-related changes in salivary volume and protein content. The bar graphs show the mean values and standard deviations observed after >2 independent experiments (n = 7–10). * and ** indicate statistical significance at a level of $p < 0.05$ and $p < 0.01$, respectively. The results are presented as follows:

- (A) Saliva secretion level (µL/min)
- (B) Protein levels in saliva (µg/min)
- (C) Expression of CFTR protein by Western Blotting

Abbreviations: CUR, curcumin; YC, Young control; OC, Old control; DEFB, beta-defensin; CFTR, cystic fibrosis transmembrane conductance regulator

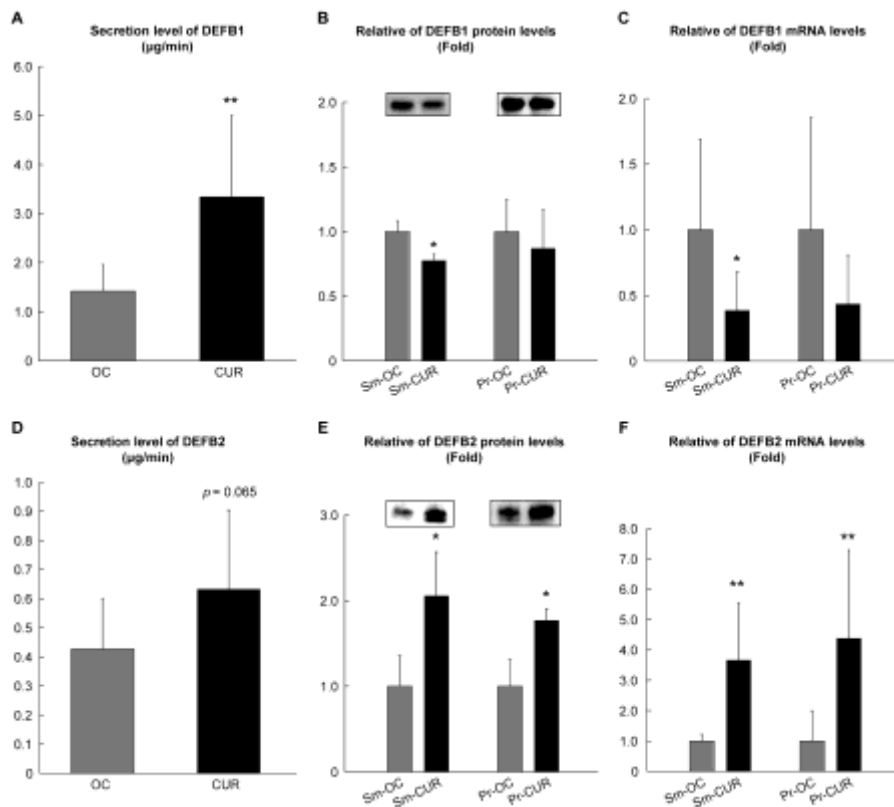


Figure 2: Effects of CUR on DEFB1/2 secretion and expression in old mice.

The bar graphs show the mean values and standard deviations observed after >2 independent experiments (n = 7–10). * and ** indicate statistical significance at a level of $p < 0.05$ and $p < 0.01$, respectively. The results are presented as follows:

- (A) DEFB1 secretion level (µg/min)
- (B) Relative to DEFB1 protein levels (Fold)
- (C) Relative to *Defb1* mRNA levels (Fold)
- (D) DEFB2 secretion level (µg/min)
- (E) Relative to DEFB2 protein levels (Fold)
- (F) Relative to *Defb2* mRNA levels (Fold)

Abbreviations: CUR, curcumin; OC, Old control; Sm, submandibular; Pr, parotid glands; DEFB, beta-defensin.

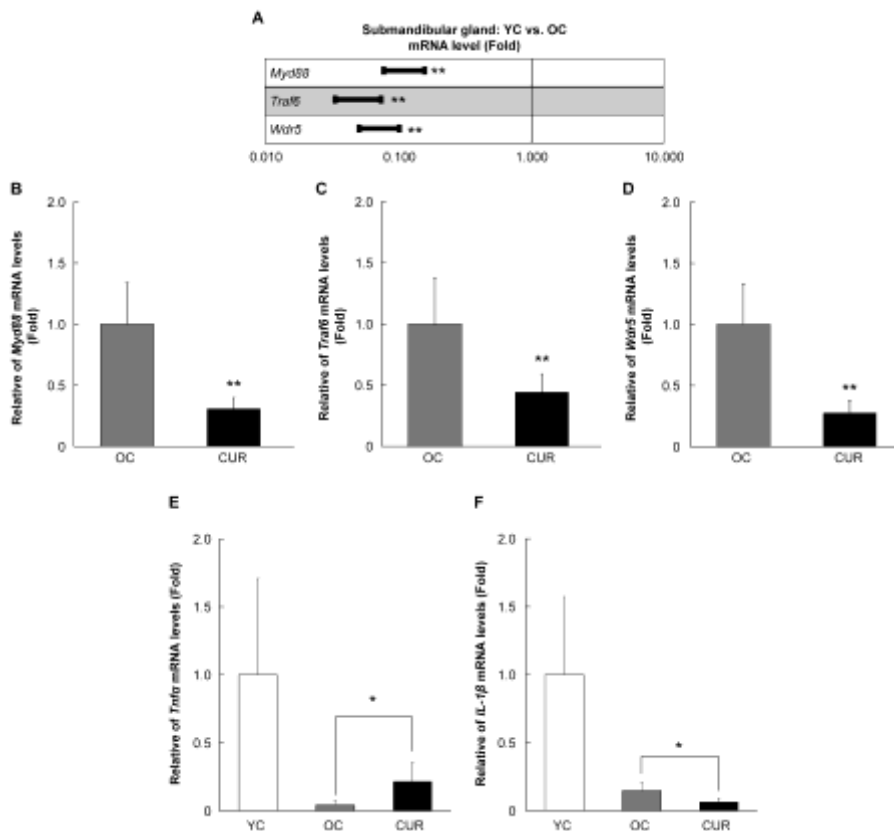


Figure 3: The effects of CUR on age-related changes in immunomodulatory capacity.

The bar graphs show the mean values and standard deviations observed after >2 independent experiments (n = 7–10). * and ** indicate statistical significance at a level of $p < 0.05$ and $p < 0.01$, respectively. The results are presented as follows:

- (A) mRNA variation in the old control group relative to the young control group (Fold)
- (B) Relative to *Myd88* mRNA levels (Fold)
- (C) Relative to *Traf6* mRNA levels (Fold)
- (D) Relative to *Wdr5* mRNA levels (Fold)
- (E) Relative to *Tnfa* mRNA levels (Fold)
- (F) Relative to *IL-1β* mRNA levels (Fold)

Abbreviations: CUR, curcumin; YC, Young control; OC, Old control; Myd88, myeloid differentiation factor 88; Wdr5- WD40 repeat protein 5; Traf6, tumour necrosis factor receptor-associated factor 6; Tnfa, Tumour Necrosis Factor alpha; IL-1β, interleukin-1beta.