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Emi Makino, Tomoki Fukuyama, Yuko Watanabe, Risako Tajiki-Nishino, Hitoshi Tajima, Aya Ohnuma-Koyama, Naofumi Takahashi, Ryoichi Otsuka, Yoshimasa Okazaki

PII: S0955-2863(18)30550-3
DOI: https://doi.org/10.1016/j.jnutbio.2019.01.009
Reference: JNB 8123

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 4 June 2018
Accepted date: 18 January 2019

Please cite this article as: E. Makino, T. Fukuyama, Y. Watanabe, et al., Subacute oral administration of folic acid elicits anti-inflammatory response in a mouse model of allergic dermatitis, The Journal of Nutritional Biochemistry, https://doi.org/10.1016/j.jnutbio.2019.01.009

Original Research Article (The Journal of Nutritional Biochemistry)

Subacute oral administration of folic acid elicits anti-inflammatory response in a mouse model of allergic dermatitis

Authors:

Emi Makino [a]: makino@iet.or.jp
Tomoki Fukuyama [b*]: fukuyama@iet.or.jp
Yuko Watanabe [b]: yuko.19790529@gmail.com
Risako Tajiki-Nishino [b]: tajiki@iet.or.jp
Hitoshi Tajima [b]: tajima@iet.or.jp
Aya Ohnuma-Koyama [a]: koyama@iet.or.jp
Naofumi Takahashi [a]: n.takahashi@iet.or.jp
Ryoichi Otsuka [a]: ohtsuka@iet.or.jp
Yoshimasa Okazaki [a]: okazaki@iet.or.jp

Affiliations:

[a] Pathology, Toxicology Division, The Institute of Environmental Toxicology, 4321, Uchimoriya-machi, Joso-shi, Ibaraki, Japan.

[b] Laboratory of Immunotoxicology and Acute Toxicology, Toxicology Division, The Institute of Environmental Toxicology, 4321, Uchimoriya-machi, Joso-shi, Ibaraki, Japan.

*** Corresponding author:** Laboratory of Immunotoxicology and Acute Toxicology, Toxicology Division, The Institute of Environmental Toxicology, 4321, Uchimoriya-machi, Joso-shi, Ibaraki 303-0043, Japan. Tel.: +81 297 27 4628., Fax.: +81 297 27 4518.

E-mail address: fukuyama@iet.or.jp (T. Fukuyama)

Running title: Anti-inflammatory effect of folic acid

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Keywords: Folic acid, inflammation, allergic dermatitis, T cells

Abstract

Folic acid (FA) deficiency is associated with several health problems, including megaloblastic anemia and fetal neural tube defects. Therefore, supplementation with FA is strongly recommended by governments worldwide. Recent published reports indicate that FA functions in immune system maintenance. The main objective of this study is to examine possible anti-inflammatory and anti-pruritic effects of FA using a mouse model of allergic dermatitis. The mouse model was developed by repetitive sensitization to the Th2-type hapten toluene-2,4-diisocyanate (TDI). During the development of allergic dermatitis, FA was orally administered to the mice at doses of 8, 160, 1000, or 10000 μg/day for 5 weeks. The ear swelling response and scratching behavior were monitored after the TDI challenge. Serum, ear tissue, and auricular lymph node samples were isolated for further analysis 24 h after the TDI challenge. The ear swelling response was reduced in a dose-dependent manner by FA administration, and a significant change was observed at a concentration of 10000 μg/day group. Comparable results were obtained through histological evaluation and cytokine level measurement in the ear tissue samples. Oral administration of FA exhibited the inhibitory effect on T cell infiltration and T cell related cytokine production in auricular lymph nodes. Scratching behavior was not altered by FA administration. The *in vivo* evidence was corroborated by *in vitro* results, which showed that FA treatment significantly interfered with T cell proliferation in a dose-dependent manner. Our findings imply that subacute oral administration of FA elicits an anti-inflammatory response, mainly through inhibition of T cell proliferation.

1. Introduction

Folic acid (FA) is an essential nutrient that cannot be generated in the human body and therefore must be obtained from food sources. FA deficiency can lead to health problems including megaloblastic anemia and fetal neural tube defects [1-3]. Therefore, women are advised to optimize their FA intake before pregnancy to reduce the risk of neural tube defects in the fetus [4]. Since FA supplementation was recommended and food fortification laws were implemented by the US Food and Drug Administration (FDA) in 1998, the rate of infants born with neural tube defects has decreased [5, 6]. The current FDA daily requirement of FA has been set at 400 μg [5]. The Health and Medicine Division of the National Academies, American College of Obstetricians and Gynecologists (ACDC), American Academy of Family Physicians, US Public Health Service, Centers for Disease Control and Prevention (CDC), American Academy of Pediatrics, American Academy of Neurology, and American College of Medical Genetics and Genomics also recommend that women who are capable of becoming pregnant should take at least 400 μg of FA daily [7-10]. Moreover, ACDC, CDC, and several other organizations recommend that women with a history of neural tube defects or other high-risk factors take 400 μg of FA daily [11-13]. However, it is quite difficult for most women to consume even the 400 μg/day of FA from food alone. Therefore, supplementation of FA by tablet or capsule is strongly recommended to compensate for the lack of FA [14].

The effects of FA supplementation on conditions other than fetal neural tube defects have been investigated as the market of FA supplementation has expanded. Research has shown that supplementation with FA reduces side effects in patients receiving methotrexate for rheumatoid arthritis [15]. FA has also been found to reduce the mutagenicity and genotoxicity induced by benzo[a]pyrene [16]. FA has a protective role against nicotine induced cardiac injury by

reduction of COX-2 expression, decreasing TNF production and lipid peroxidation mediated cell injury [17]. Maternal FA supplementation during pregnancy protects against lipopolysaccharide-induced preterm delivery through its anti-inflammatory effects [18]. Recent investigation also implied that FA plays an important role to maintain the immune cells including T cells [15]. However, the mechanism by which FA ameliorates immune disorders has not been fully elucidated.

The main objective of this study is to examine the anti-inflammatory and anti-pruritic responses to FA using a mouse model of allergic dermatitis. In addition, several *in vitro* methods including cytokine assay using human epidermal keratinocytes and T cell proliferation were used to identify the mechanism of action.

2. Materials and Methods

2.1 Reagents

An FA deficient diet (AIN-93G with 1% succinylsulfathiazole) was purchased from EPS Ekishin Co., Ltd. (Tokyo, Japan). Folic Acid (FA), mycophenolic acid, and RPMI-1640 medium were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (LPS), Pefabloc® SC, polyinosinic–polycytidylic acid sodium salt [Poly (I:C)], and toluene-2,4-diisocyanate (TDI) were from Sigma-Aldrich (Tokyo, Japan). ^{3}H-methyl thymidine was from PerkinElmer Japan Co. Ltd. (Kanagawa, Japan). The DC Protein Assay Kit was from Bio-Rad Laboratories, Inc. (Tokyo, Japan). A Dynabeads mouse T-Activator CD3/CD28 and total IgE ELISA set were ordered from Thermo Fisher Scientific Inc. (Kanagawa, Japan). Epivita basal medium w/hydrocortisone was obtained from Cell Applications Inc. (San Diego, CA, USA). Recombinant Mouse GM-CSF was obtained from Pepro Tech, Inc. (Rocky Hill, NJ, USA).

ELISA kits for mouse and/or human IL-4, -5, -8, -9, -12, -13, -17, -33, TNFα, and TSLP were from R&D Systems (Minneapolis, MN, USA). Mouse BD Fc Block, PE-conjugated anti-mouse CD3, PE-Cy7-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD40, and the mouse T lymphocyte enrichment set-DM were purchased from BD Biosciences (Tokyo, Japan). APC-conjugated anti-mouse CD11c was from Miltenyi Biotec K.K. (Tokyo, Japan).

2.2 Mice

Six-week-old female BALB/c mice and female C57BL/6 mice were purchased from Charles River Japan Laboratories (Kanagawa, Japan). Animals were kept in a controlled environment under the following conditions: temperature of 22 ± 2°C, humidity of 50 ± 20%, and a 12 hour per day light cycle. BALB/c mice and C57BL/6 mice received a certified FA-deficient pellet diet and MF pellet diet, respectively, and water *ad libitum*. The study protocol was approved by the Institute of Environmental Toxicology Animal Care and Use Committee, fully accredited by AAALAC international (IACUC Protocol No. AC17056).

2.3 Oral administration of FA

For either general toxicity study or a mouse model of allergic dermatitis described in 2.4 *Investigation for general toxicity* or *2.5 A mouse model of allergic dermatitis*, FA was suspended in phosphate buffered saline (PBS) at doses of 8, 160, 1000, or 10000 μg/mouse/day. Oral administration of FA was performed once daily for 5 weeks. Eight μg/mouse/day was used as a control concentration equivalent to the FA concentration in a normal rodent diet [16], and group given 8 μg /mouse/day was defined as a control group for general toxicity study and allergic dermatitis model. One-hundred sixty μg/day has been used in previous mouse studies as an

excessive amount of FA [17]. As 1000 μg/day was suggested as an allowable limit for humans in a prior study [18], we used 10000 μg/day as the highest concentration.

2.4 Investigation for general toxicity

The general toxicity of FA was investigated by 5 weeks of oral administration in female BALB/c mice (n = 6/each group). The clinical symptoms, mortality, body weight,and food consumption were carefully monitored during the observation period, after which animals were euthanized under isoflurane inhalation anesthesia. All animals were subjected to hematology, necropsy, organ weight measurement, and the immune organs (thymus and spleen) were isolated for lymphocytes differentiation studies.

2.5 A mouse model of allergic dermatitis

The possible effects of FA on the immune system were evaluated using a BALB/c mouse model of allergic dermatitis, generated by repetitive topical sensitization with TDI using the methods described in a previous study [19]. Oral administration of FA was started 1 week prior to the initiation of TDI sensitization and ended on the day of TDI challenge (n = 5 or 6/each group). Immediately after the final TDI challenge to the ear auricle and rostral neck skin, scratching behavior was video monitored for 60 min. The ear swelling response was determined by subtracting the pre-TDI ear thickness from the 22-24 hours post-TDI ear thickness. Animals were euthanized immediately after measuring post-TDI ear thickness. Serum was isolated for measuring the total IgE level. The ear auricles were isolated for cytokine level determination. Single-cell suspensions were prepared from the auricular lymph nodes (LN) and were used to analyze the helper T cells, B cells and cytokine production.

2.6 Histopathology

Samples from pinna of BALB/c mice model of allergic dermatitis were fixed in 10% formalin solution, embedded in paraffin wax, sectioned at a thickness of 5 μm, and stained with haematoxylin and eosin. A semi-quantitative histopathological evaluation for dermal edema, cellular infiltration, folliculitis, hyperkeratosis and epidermal hyperplasia was performed in a blinded fashion with the following grading system: 0, within normal limits; 1, mild; 2, moderate; and 3, severe. Summed scores of the above 5 lesions were used for further statistical evaluation.

2.7 Measurements of total IgE in serum

The total IgE level in serum samples was determined by ELISA according to the manufacturer's protocol with ELISA (SPECTRA MAX 190, Molecular Devices, LLC., Tokyo, Japan.).

2.8 Cytokine determination in ear skin tissue

A portion of the pinna was frozen in dry ice. Determination of cytokine levels from the ear tissue was performed according to Stover, Fukuyama [20] with slight modification. In short, samples were homogenized in 200 μl RPMI 1640 medium containing 1 mmol/l Pefabloc using an electric homogenizer. After centrifugation, the supernatants were collected and the protein content was determined with a DC protein assay kit. IL-4, -5, -9, -13, -17, -33, TNFα, and TSLP were measured by ELISA.

2.9 Analysis of lymph nodes (LNs)

Single-cell suspensions were prepared from the LNs isolated from each mouse, and the total cell number was counted by Coulter Counter Z2 (Beckman Coulter, Inc. Brea, CA, USA). Single-cell suspensions were then used to analyze cytokine production and for FACS analysis. To stimulate the T cell receptors, we cultured single-cell suspensions with CD3 and CD28 antibodies for 96 hours. The levels of cytokines (IL-4, -5, -9, -13, and -17) in cell culture supernatants were measured by ELISA. For FACS analysis, 5×10^5 cells were incubated with 1 μg Mouse BD Fc Block followed by incubation with the monoclonal antibodies for 30 min. Cells (10,000 events) were analyzed on a FACSVerse cytometer by FACSuite software (BD Biosciences).

2.10 Cytokine release assay of human epidermal keratinocyte cell line

The human epidermal keratinocyte cell line was obtained from Cell Applications Inc. and cultured following the standard protocol using Epivita basal medium. Cells (1×10^4 cells/200 μl) were seeded onto a 96-well culture plate and pre-exposed to FA for 24 h at 0, 1, 10, 100, or 1000 μmol/l, followed by stimulation with toll-like receptor 3 ligand, Poly (I:C) (2 μg/ml) for another 24 h. The high concentration of FA did not induce cell toxicity (data not shown). The levels of IL-8 and TNFα in supernatants were measured using ELISA. Three independent experiments were performed in different passages.

2.11 Mixed leukocyte reaction assay

A mixed leukocyte reaction assay was performed as per the protocol in Beyer, Bartz [21] with slight modification. In short, highly purified bone marrow derived dendritic cells (BMDCs) generated from BALB/c mice according to the previous study [19] were pre-treated with FA at 0,

1, 10, 100, or 1000 μmol/l for 24 h. A 10 μmol/l mycophenolic acid solution was also treated as a positive control. For initiation of the mixed leukocyte reaction, T cells were isolated and enriched from the spleen of female C57BL/6 mice using a Mouse T Lymphocyte Enrichment Set-DM (this leads to a purity of more than 90% T cells). Enriched T cells (1×10^6 cells/tube) and test substance treated BMDCs (1×10^5 cells/tube) were seeded in a U-bottom 2 ml tube and incubated for five additional days. ^{3}H-thymidine was added for the final 16 h (5 μCi/well). After 5 days, mixed cells were washed twice with PBS, re-suspended with 5% trichloroacetic acid and stored at 4^{o}C for 18 h. Cell pellets were re-suspended in scintillation fluid and transferred to scintillation vials for determination of T cell proliferation (DPM, disintegrations per minute) using a liquid scintillation counter.

2.12 Statistical analyses

Data are expressed as the mean ± 1 SEM. Analysis of variance (ANOVA) was used to evaluate the results. For significant results, differences between the control and treatment groups were then assessed using Dunnett's multiple comparison test. The statistical significance was estimated at the 5% and 1% levels of probability.

3. Results

3.1 Subacute oral administration of FA did not induce any general toxicity in female BALB/c mice

The general toxicity of FA was examined using subacute oral administration for 5 weeks in normal female BALB/c mice. There were no treatment-related effects in clinical observation, mortality, final body weight, average food consumption, hematology, necropsy, or organ weight

measurement (Table 1). In addition, general immune responses such as numbers of double positive T cells in thymus, as well as T cells and B cells in the spleen, were not influenced by FA administration (Table 1).

3.2 Subacute oral administration of FA elicited an anti-inflammatory effect in a mouse model of allergic dermatitis

The contribution of FA administration to a cutaneous allergic response was evaluated using a TDI-induced allergic dermatitis model. The ear swelling response to the TDI challenge was inhibited in a dose-dependent manner by the oral administration of FA, and a significant reduction was particularly seen with the highest FA concentration (10000 μg/day) (Figure 1A and B). Downward trends in the histological scores of inflammatory response, especially dermal edema, cellular infiltration in the dermis and subcutis, and folliculitis were also found in a dose-dependent manner (Table 2 and Figure 1C). Interestingly, a dose-dependent reduction in total IgE levels in serum was observed in the excess FA treatment groups (Figure 1D). However, there was no difference in itch behavior among the groups (Figure 1E). The anti-inflammatory response in ear tissue was corroborated by the observed reduction in pro-inflammatory cytokines, including IL-4, -5, -9, -13, -17, -33, TNFα, and TSLP (Table 3).

To further examine the anti-inflammatory effect of FA in the ear auricle and serum, auricular LN activation was evaluated (Table 4). Number of Helper T cells and B cells were reduced by FA administration. When the secretion of helper T cell-related cytokines including IL-4, -5, -9, -13, and -17 was quantified by ELISA, an FA dose-dependent decrease and significant differences were observed in IL-5, -9, and -17 secretions in groups with elevated FA dosages, as compared with the control group (8 μg/day).

3.3 Exposure to FA did not alter keratinocyte activation

To corroborate the *in vivo* data, several *in vitro* experiments were carried out. Experiments with human epidermal keratinocytes were conducted to investigate whether epidermal cells are involved in anti-inflammatory effect of FA. However, IL-8 and TNFα secretions from activated keratinocytes were not influenced by pre-treatment with FA (Figure 2A and 2B).

3.4 T cell proliferation was significantly suppressed by FA exposure in vitro

The contribution of FA to T cell proliferation was examined *in vitro* using a mixed leukocyte reaction assay. Whereas FA exposure did not alter the activation of keratinocytes, significant inhibition of T cell proliferation was observed in the 100 and 1000 μmol/l treatment groups. Greater than 30% inhibition was observed in the 1000 μmol/l treatment group (Figure 3).

4. Discussion

In this study, we investigated the possible effects of FA on skin allergies using a mouse model of allergic dermatitis and several *in vitro* methods. We made several novel observations: (1) in a mouse model of allergic dermatitis, 5-week oral administration of FA significantly ameliorated the inflammatory response, but not the itch behavior; (2) pre-exposure to FA did not counteract the cytokine secretions by activated keratinocytes *in vitro*; and (3) T cell proliferation experiments yielded results consistent with those from our *in vivo* investigation, in that pre-treatment with FA interfered with T cell proliferation in a dose-dependent manner.

Although the importance of adequate FA consumption has been recognized, a comprehensive safety assessment of FA had not yet been performed. Therefore, an initial 5-week study of the oral administration of FA to female BALB/c mice was conducted to confirm the safety of FA, particularly in the context of immune system function. Our findings indicated that neither FA related deaths, clinical symptoms, body weight reductions, nor necropsy findings were observed in the FA 160, 1000 and 10000 μg/day treatment groups compared with the control group (mice given 8 μg/day FA) (Table 1). Hematology and general organ weights including the liver, kidney, brain, thymus, and spleen were also not affected by FA administration (Table 1). No significant changes in T cell and B cell numbers in the thymus or spleen were observed among the FA treatment groups. Based on our results, mice appear to adequately tolerate a 5-week oral exposure to until 10000 μg/day of FA.

We further elucidated whether 5-week oral administration of FA elicits anti-inflammatory and anti-pruritic responses in a mouse model of allergic dermatitis. Surprisingly, systemic administration of FA inhibited the ear swelling response in a dose-dependent manner. In particular, a statistically significant difference was seen in the 10000 μg/day treatment group, for which greater than 50% inhibition was noted compared with the control group (Figure 1A). This finding was corroborated by the reduced inflammatory response observed from histological evaluation (Table 2). When cytokine levels in TDI-challenged ear tissues were measured by ELISA, FA administration dose-dependently reduced levels of Th2, Th9, Th17, DC, and keratinocyte related cytokines, although the reduction was not statistically significant except for IL-33 (Table 3). On the other hand, a significant reduction in T cell infiltration was noted in auricular LNs, and cytokine production from activated T cells was also significantly reduced in a dose-dependent manner by FA administration (Table 4). Although TDI is a Th2 dominant hapten,

our previous study demonstrated that other types of Th cells, including Th9 and Th17, were activated in a TDI-induced allergic inflammation model [22]. Significant reductions in IL-9 and IL-17 production by activated T cells were observed in FA-treated mice (Table 4). Recent clinical evidence indicates that increasing levels of IL-9 and IL-17 are highly correlated with the severity of skin allergies [23, 24]; therefore, our findings imply that FA administration may improve the inflammatory response, partly through inhibiting IL-9 and IL-17 secretion. Most surprisingly, a significant reduction in the serum IgE level was noted in excess FA treatment groups following the same trend as the ear swelling response, suggesting that FA maintains not only the local immune response but also the systemic immune response, as IgE is the key player in type I allergies (Figure 1D). FA administration also led to a reduction of B cell infiltration in auricular LNs (Table 4), which correlated with downregulation of IgE levels in serum. We also evaluated whether FA administration affected the itch response. However, FA administration did not affect the itch behavior in our mouse model (Figure 1E).

Based on our findings, we hypothesized that T cells are the main contributors to the anti-inflammatory response following subacute FA administration. We attempted to confirm the *in vivo* evidence using several *in vitro* methods including keratinocytes and T cells, which are deeply involved in developing cutaneous allergies. Keratinocytes independently maintain the homeostasis of the skin by secreting cytokines such as IL-33 and TSLP [25]. Baumer and Kietzmann [26] demonstrated that the inhibitory effects of cyclosporine A and cilomilast were correlated with cytokine release by an activated human or murine epidermal keratinocyte cell line. In this study, we used the toll-like receptor agonist Poly (I:C) to activate human epidermal keratinocytes and evaluate the effect of FA on cytokine secretion by keratinocytes. However, no alterations following FA pre-exposure were detected in cytokine production by keratinocytes

(Figure 2). Subsequently, the effect of FA exposure on T cell proliferation was analyzed using an mix leucocyte reaction assay. T cell proliferation was dose-dependently reduced by FA pre-exposure compared with untreated controls (Figure 3). It has been found that the folate receptor, which has a high affinity for folic acid, is highly expressed by helper T cells, and the folate pathway may control the helper T cell responses in immunomodulatory disease [32]. However, data on this topic is limited and further study is needed.

Taken together, our findings demonstrate that subacute oral administration of FA is well tolerated and that FA impressive anti-inflammatory properties. Reduced inflammation was confirmed histologically, as we observed a reduction in cytokines relevant for the maintenance of a cutaneous allergy. Our results also demonstrate the ability of FA to significantly inhibit T cell proliferation while having minimal effect on keratinocyte function. We can conclude that inhibition of T cell function and the secretion of cytokines such as IL-4, -9, -13, and -17, which have the potential to activate B cells and promote IgE levels in serum, plays an important role in the amelioration of cutaneous allergies by FA.

Future studies using models of human chronic atopic dermatitis, such as NC/Nga mice, would be beneficial to further characterize the efficacy of FA in cutaneous allergy. NC/Nga mice have been widely used for elucidating mechanisms and new therapeutic options of AD, as lesions can be induced with a relevant allergen (house dust mite antigen) and the phenotype shares several similarities with the human counterpart (lichenification, Th2 cytokines, enhanced IgE levels, constant itch) [27].

Acknowledgements

We are grateful to Dr. Toshinori Yoshida, Dr. Hiroaki Aoyama, and Dr. Takanori Harada for their valuable suggestions.

Conflict of Interest

The authors state no conflict of interest.

Figure Captions

Fig. 1. Subacute oral administration of FA significantly reduced the ear swelling response (A). Representative pictures of ear auricles of the control group and the 10000 μg/day FA group (B). Representative histological images of ear tissues of the control group and 10000 μg/day group (C). Total IgE levels in serum were reduced following FA administration (D). No effect was observed on scratching behavior by FA administration (E). Each result (A,D, or E) is presented as the mean ± 1 SEM. n = 5 to 6 per group. *$p < 0.05$, **$p < 0.01$ (Dunnett's multiple comparison test) vs. the FA 8 μg/day group (control).

Fig. 2. Pre-exposure to FA does not counteract toll like receptor agonist-stimulated IL-8 (A) and TNFα (B) secretion by keratinocytes. Each result is the mean (pg/ml) ± 1 SEM. n = 7 per group. Dunnett's multiple comparison test vs. the vehicle-only control group (FA 0 μmol/l).

Fig. 3. FA significantly reduces T cell proliferation in a mixed leukocyte reaction assay. Each result is the mean (DPM) ± 1 SEM. n = 6 per group.
*$p < 0.05$, **$p < 0.01$ (Dunnett's multiple comparison test) vs. untreated group.

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TABLE 1

General toxicity of FA in female BALB/c mice

Results are expressed as the mean ± 1 SEM. (n = 5 or 6 per group).

Group	FA 8 μg/day	FA 160 μg/day	FA 1000 μg/day	FA 10000 μg/day
Final body weight (g)	21.2 ± 0.9	21.7 ± 1.1	21.6 ± 0.9	20.7 ± 0.6
Average of food consumption (g)	2.20 ± 0.21	2.19 ± 0.14	2.30 ± 0.25	2.06 ± 0.24
Hematology				
Ht (%);	42.1 ± 0.4	41.5 ± 0.4	42.6 ± 1.2	41.9 ± 0.8
Hb (g/dL);	14.4 ± 0.2	14.2 ± 0.3	14.6 ± 0.4	14.2 ± 0.3
RBC (10^6/μL);	9.20 ± 0.30	9.23 ± 0.37	9.34 ± 0.33	9.13 ± 0.26
Retics (10^9/L);	575.7 ± 100.3	597.4 ± 65.7	559.1 ± 39.9	544.2 ± 60.7
PLT (10^3/μL);	1025 ± 45	1037 ± 92	1126 ± 95	1036 ± 61
WBC (10^3/μL);	5.55 ± 0.76	5.38 ± 1.91	6.33 ± 2.82	4.51 ± 0.84
Organ weight				
Liver (g)	0.99 ± 0.09	0.98 ± 0.09	0.96 ± 0.06	0.90 ± 0.05
Kidney (mg)	239.5 ± 15.1	246.0 ± 45.0	244.3 ± 18.2	226.7 ± 9.8
Spleen (mg)	93.7 ± 11.0	112.3 ± 32.6	99.8 ± 12.4	85.4 ± 8.4
Thymus (mg)	43.3 ± 5.3	46.8 ± 3.3	48.1 ± 4.4	48.1 ± 4.0
$CD3^+CD4^+CD8^+$ T cells in Thymus ($\times 10^5$ cells)	4.13 ± 0.62	4.14 ± 0.74	3.57 ± 0.51	3.60 ± 0.82
$CD3^+$ T cells in Spleen ($\times 10^6$ cells)	4.23 ± 0.73	5.08 ± 0.97	4.76 ± 1.39	4.05 ± 1.14
$B220^+$ B cells in Spleen ($\times 10^6$ cels)	6.49 ± 1.81	7.30 ± 1.27	6.46 ± 1.72	5.80 ± 1.81

TABLE 2

Histological evaluation of the ear auricle to subacute oral administration of FA in a mouse model of allergic dermatitis

Histological score (0, within normal limits; 1, mild; 2, moderate; and 3, severe) was given for each finding.

Results are expressed as the mean ± SEM. n = 5 or 6 per group. Statistical analysis was not performed as n was small.

Group	TDI + FA 8 μg/day	TDI + FA 160 μg/day	TDI + FA 1000 μg/day	TDI + FA 10000 μg/day
Dermal edema	1.33 ± 0.47	1.17 ± 0.37	0.80 ± 0.40	0.80 ± 0.40
Cellular infiltration	1.83 ± 0.37	1.67 ± 0.47	1.40 ± 0.49	1.20 ± 0.40
Folliculitis	1.00 ± 0.82	1.17 ± 0.37	1.00 ± 0.89	0.40 ± 0.49
Total score	4.17 ± 1.34	4.00 ± 1.00	3.20 ± 1.17	2.40 ± 0.49

TABLE 3

Responses in the ear auricle to subacute oral administration of FA in a mouse model of allergic dermatitis

Ear sample was collected 24 hours after TDI challenge. Results are expressed as the mean ± SEM. n = 5 or 6 per group.

*$p < 0.05$ and **$p < 0.01$ (Dunnett's multiple comparison test) vs. TDI + FA 8 μg/day group (control).

Group	TDI + FA 8 μg/day	TDI + FA 160 μg/day	TDI + FA 1000 μg/day	TDI + FA 10000 μg/day
IL-4 (pg/mg protein)	177 ± 84	153 ± 51	122 ± 34	119 ± 46
IL-5 (pg/mg protein)	242 ± 85	187 ± 85	139 ± 69	142 ± 49
IL-9 (pg/mg protein)	132 ± 53	101 ± 39	73 ± 24	59 ± 19
IL-13 (pg/mg protein)	994 ± 435	920 ± 383	579 ± 151	564 ± 193
IL-17 (pg/mg protein)	564 ± 246	479 ± 268	217 ± 82	198 ± 80
IL-33 (pg/mg protein)	607 ± 247	534 ± 209	375 ± 124*	400 ± 147*
TNFα (pg/mg protein)	204 ± 79	185 ± 90	127 ± 47	127 ± 49
TSLP (pg/mg protein)	74 ± 31	68 ± 35	47 ± 14	46 ± 19

TABLE 4

Responses in the auricular lymph nodes to subacute oral administration of FA in a mouse model of allergic dermatitis

Cytokine production was measured in response to anti CD3 and CD28 stimulation. Results are expressed as the mean ± 1 SEM. (n = 5 or 6 per group).

*$p < 0.05$ and **$p < 0.01$ (Dunnett's multiple comparison test) vs. TDI + FA 8 μg/day group (control).

Group	TDI + FA 8 μg/day	TDI + FA 160 μg/day	TDI + FA 1000 μg/day	TDI + FA 10000 μg/day
$CD3^+CD4^+$ cells ($\times 10^6$ cells)	3.37 ± 0.93	2.38 ± 0.35*	2.69 ± 0.66	2.02 ± 0.21**
$B220^+$ cells ($\times 10^6$ cells)	2.97 ± 0.52	2.54 ± 0.39	3.38 ± 1.03	2.02 ± 0.41
IL-4 (pg/ml)	67.5 ± 23.1	78.6 ± 53.9	69.5 ± 36.5	37.1 ± 4.6
IL-5 (pg/ml)	32.1 ± 26.1	6.2 ± 8.2**	6.6 ± 3.1*	1.9 ± 4.3**
IL-9 (pg/ml)	141 ± 25	113 ± 53	51 ± 31**	31 ± 10**
IL-13 (pg/ml)	775 ± 376	732 ± 645	826 ± 331	129 ± 48
IL-17 (pg/ml)	146 ± 66	86 ± 26	64 ± 40*	37 ± 13**

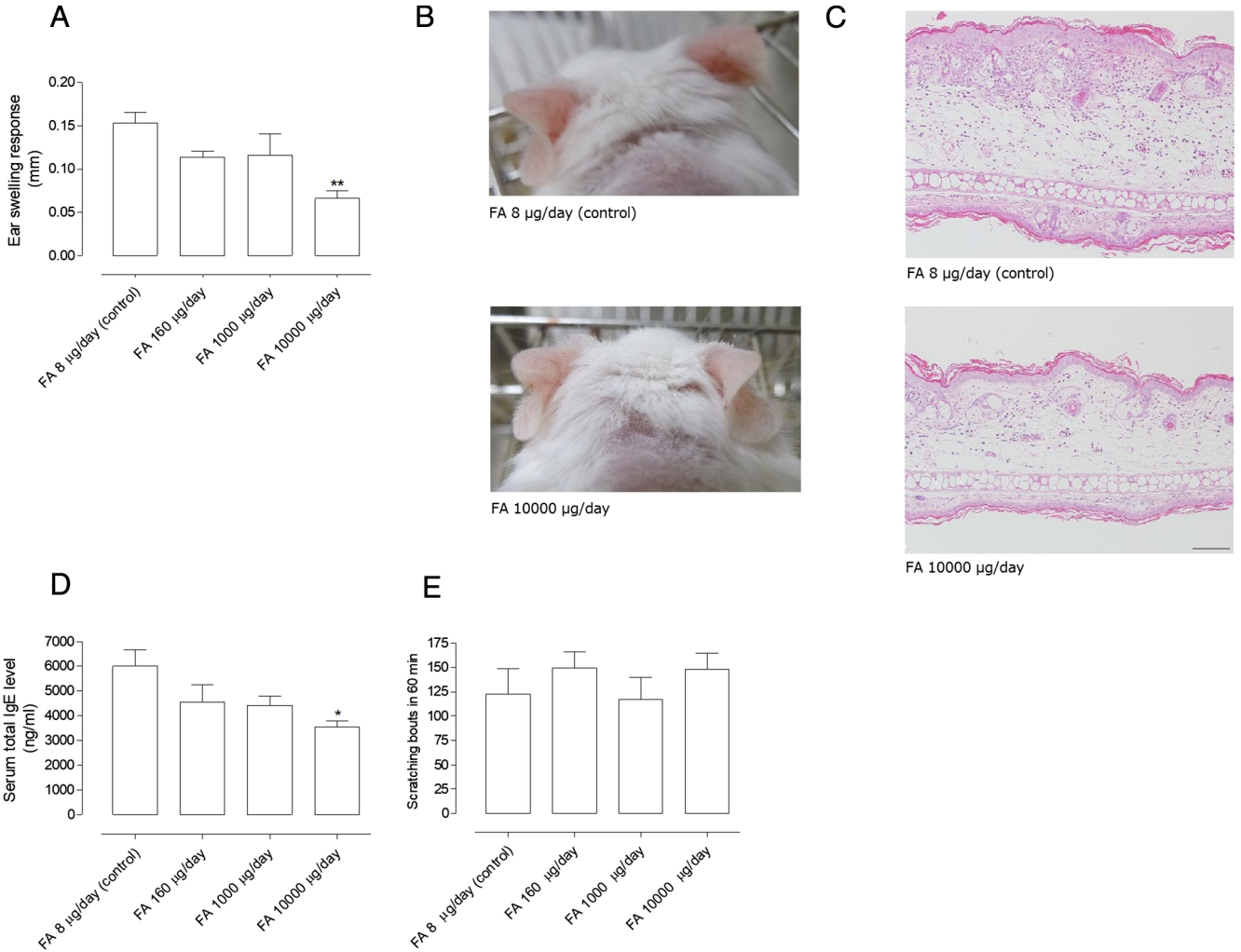
A
Ear swelling response (mm)
0.20
0.15
0.10
0.05
0.00
**
FA 8 µg/day (control)
FA 160 µg/day
FA 1000 µg/day
FA 10000 µg/day
B
FA 8 µg/day (control)
FA 10000 µg/day
C
FA 8 µg/day (control)
FA 10000 µg/day
D
Serum total IgE level (ng/ml)
7000
6000
5000
4000
3000
2000
1000
0
*
FA 8 µg/day (control)
FA 160 µg/day
FA 1000 µg/day
FA 10000 µg/day
E
Scratching bouts in 60 min
175
150
125
100
75
50
25
0
FA 8 µg/day (control)
FA 160 µg/day
FA 1000 µg/day
FA 10000 µg/day

Figure 1

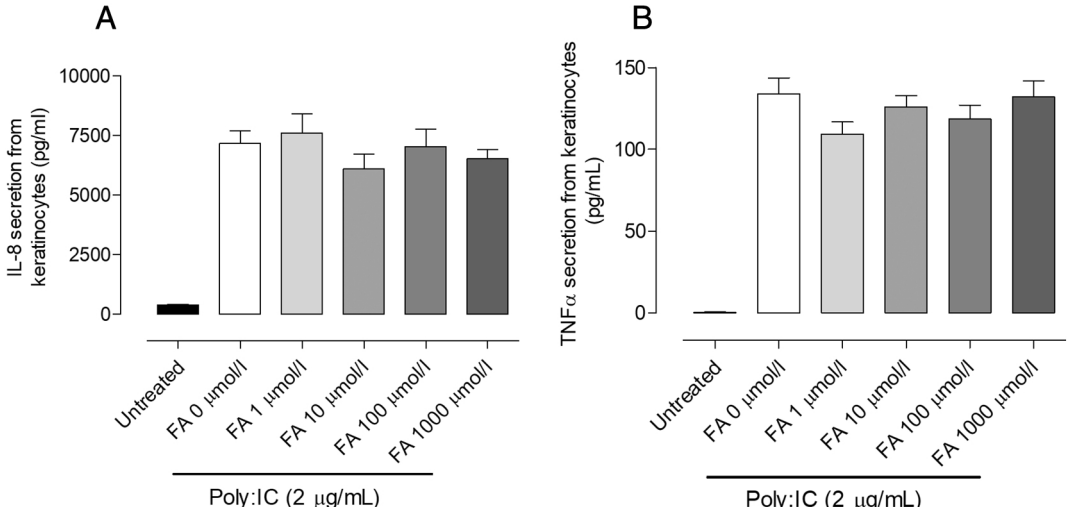
A
IL-8 secretion from keratinocytes (pg/ml)
10000
7500
5000
2500
0
Untreated
FA 0 µmol/l
FA 1 µmol/l
FA 10 µmol/l
FA 100 µmol/l
FA 1000 µmol/l
Poly:IC (2 µg/mL)
B
TNFα secretion from keratinocytes (pg/mL)
150
100
50
0
Untreated
FA 0 µmol/l
FA 1 µmol/l
FA 10 µmol/l
FA 100 µmol/l
FA 1000 µmol/l
Poly:IC (2 µg/mL)

Figure 2

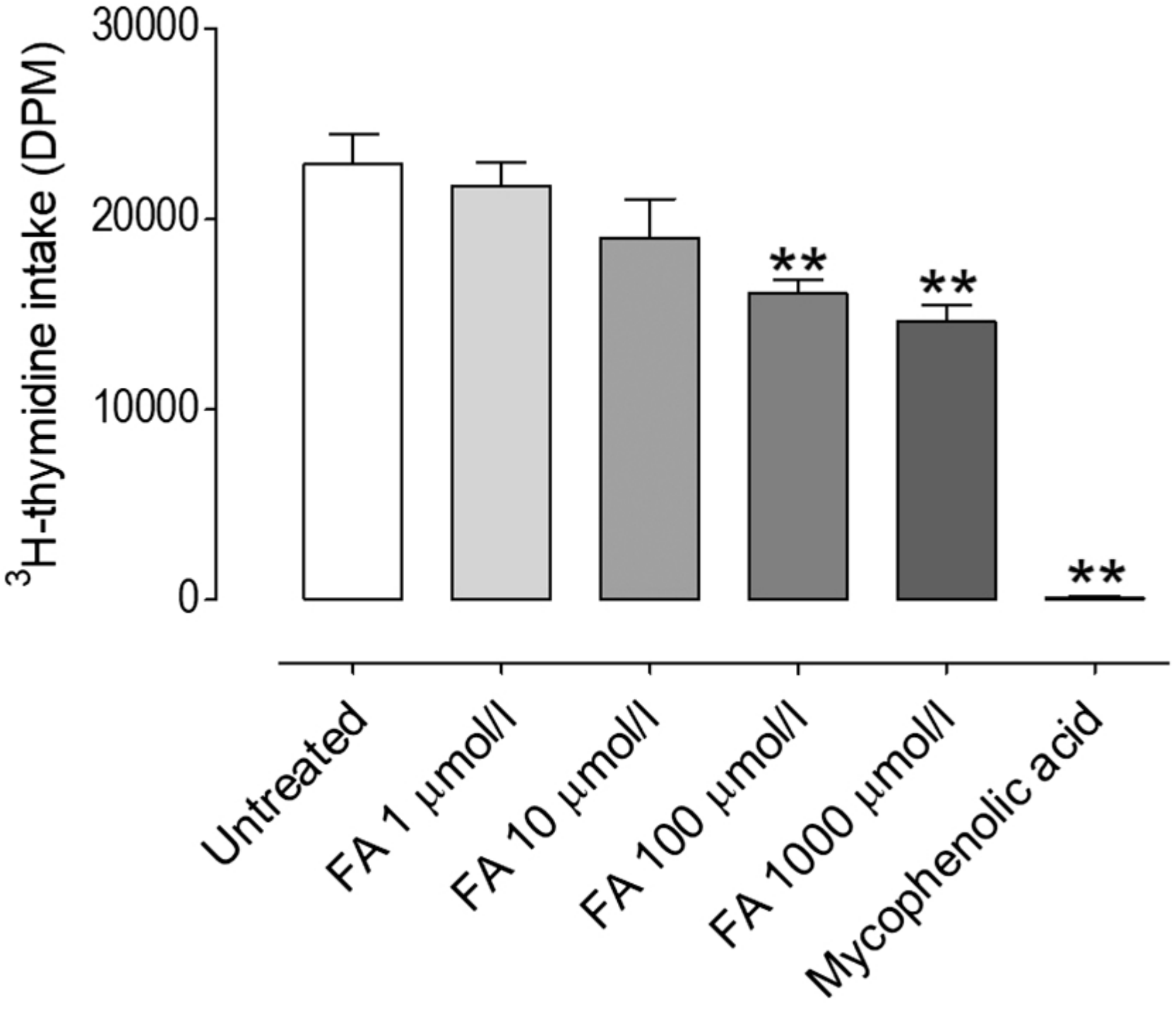
30000
20000
10000
0
^{3}H-thymidine intake (DPM)
**
**
**
Untreated
FA 1 μmol/l
FA 10 μmol/l
FA 100 μmol/l
FA 1000 μmol/l
Mycophenolic acid

Figure 3