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Research Article

Title: Synergistic anti-allergy activity using a combination of *Enterococcus faecalis* IC-1 and luteolin

Short Title: Synergistic anti-allergy effects

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Abstract

Allergies are global issues, however, medical intervention for allergy treatment is limited. Recent studies have focused on allergy prevention with food components. Both lactic acid bacteria *Enterococcus faecalis* IC-1 (IC-1) and the flavonoid luteolin have been shown to have an anti-allergic effect. Following the concept of diet diversity and synergistic effects, the purpose of this study was to explore the anti-allergic activity of a combination of IC-1 and luteolin. A combination of IC-1 and luteolin had anti-allergic effects in passive cutaneous anaphylaxis reaction and strongly inhibited mast cell degranulation in a Caco-2/RBL-2H3 cells co-culture system. Oral administration with a combination of IC-1 and luteolin significantly decreased blood IgE content in ovalbumin (OVA)-induced allergic mice and alleviated symptoms with an allergy attack. These might have been achieved through correcting Th2 shifting with the allergy state since a combination of IC-1 and luteolin increased the IFN-γ content in blood. Furthermore, *in vitro* differentiation of the Th2 subset confirmed that a combination of IC-1 and luteolin inhibited naïve T cell differentiate into a Th2 subset through inhibition of Th2 transcription factor GATA-3 expression. The combination also promoted the gene expression level of IFN-γ in mice bone marrow-derived dendritic cells. These results suggested a synergistic anti-allergic effect using a combination of IC-1 and luteolin both in reducing IgE content and in inhibiting mast cell degranulation during the allergy state.

Key Words:

Anti-allergy; *Enterococcus faecalis* IC-1; food allergy; lactic acid bacteria; luteolin; polyphenol

Abbreviations: APC, antigen presenting cells; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; LAB, lactic acid bacteria; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis reaction; STAT, signal transducer and activator of transcription; TLR, toll-like receptor

1. Introduction

The global incidence of allergic diseases has increased, especially in developed countries. Allergic diseases are caused by hypersensitivity of the immune system. Hypersensitivity can be divided into 4 types, namely: immediate hypersensitivity (type I), cytotoxic reaction (type II), immune complex reaction (type III), and delayed hypersensitivity (type IV) (Janeway et al., 2001). Type I hypersensitivity, which is also known as an allergy and is represented by food allergies and allergic asthma, is the most commonly seen type of humans.

Type I hypersensitivity is initiated by the allergens, including food components, pollens and dusts. When the allergen gets access into the human body, it is recognized by antigen presenting cells (APC) and presented to naïve helper T cell (Th0), thereby activating it. Activated Th0 differentiates into T helper 2 cell (Th2) rather than T helper 1 cell (Th1). Th2 is capable of secreting Th2 cytokines including Intelukin-4 (IL-4), IL-5 and IL-13, which can promote B cells to process immunoglobulin (Ig) class switch recombination leading to accumulation of IgE. When the same allergen invades again, the binding of the allergen to IgE activates the Fcε Receptor I (FcεRI) on mast cells and cause mast cells degranulation, resulting in the release of chemical mediators like histamine (Janeway et al., 2001). The effects induced by histamine include smooth

62 muscle contraction, increased vascular permeability, and prostaglandin generation
63 (White, 1990), which in turn lead to symptoms like skin reactions (urticarial, eczema
64 and angioedema), respiratory tract reactions (rhinitis and bronchitis), gastrointestinal
65 tract (intestinal cramps and diarrhea) and the worst, anaphylactic shock (Janeway et al.,
66 2001).

67 Although certain types of medicines have been developed to treat allergy, the option
68 is still quite limited. For example, disodium cromoglycate (cromolyn), a well-known
69 mast cell stabilizer, has been widely used in the clinical field for asthma treatment.
70 However, it only has preventive effect which means the medicine has to be taken a week
71 before the individual is exposed to the allergen. It also has no effect on alleviating
72 symptoms in asthmatic attack (Patel & Theoharides, 2017).

73 Even though some food components including cow's milk and peanut are easy to
74 trigger allergy reaction (Wood, 2016), other food components have been shown to have
75 anti-allergy effect. Flavonoid, a secondary product of vegetables and plants (Castell et
76 al., 2014), has been shown to have a variety of biological effects such as anti-tumor,
77 antioxidant, anti-allergic, anti-microbial, and anti-angiogenic properties (Shimada et al.,
78 2006). Luteolin, a flavonoid contained in celery, green pepper, perilla leaf and
79 chamomile, is also well known for its anti-inflammatory and anti-allergic activities.

80 Animal researches showed that luteolin can ameliorate allergy symptom and reduce Th2
81 cytokine including IL-4 and IL-13 in mice with allergic asthma induced by ovalbumin
82 (OVA) (Jang et al., 2017). Previous research also showed luteolin can inhibit human
83 cultured mast cell degranulation through the inhibition of Ca^{2+} influx and protein kinase
84 C (PKC) activation (Kimata et al., 2000). However, the bioavailability of luteolin is
85 quite low since as a flavonoid aglycons, it is conjugated to glucuronic acid or sulfuric
86 acid by epithelial cells in intestinal absorption (Murota et al., 2002), which makes it
87 difficult for luteolin to have its effects in human body.

88 Lactic acid bacteria (LAB) are the other popular candidates for regulating immune
89 system. LAB is a group of Gram-positive, anaerobic bacteria including *Enterococcus*,
90 *Lactobacillus*, *Streptococcus*, and *Lactococcus*, which has been widely used in the
91 fermentation and storage of milk, meat and vegetables (Makarova et al., 2006; Stiles &
92 Holzapfel, 1997). Numbers of researches had demonstrated that LAB can ameliorate
93 allergic symptoms through decrease of the serum levels of antigen specific IgE in
94 animal models. LAB can influence immune system by products like metabolites, cell
95 wall component and nucleic acid (Oelschlaeger, 2010), which makes it reasonable that
96 not only the live LAB (Schiavi et al., 2011; Ai et al., 2016) known as 'probiotics', but
97 also the heat-killed LAB (Segawa et al., 2008; Peng & Hsu, 2005) can have anti-allergic

98 effect. The anti-allergic effect of LAB is strain dependent with distinguished
99 mechanisms and the most popular one is through mediating Th1/Th2 cytokine
100 expression and balance (Fujiwara et al., 2004).

101 It has been suggested that LAB can switch the Th2 dominance to Th1 response in
102 allergy state. This may be achieved by stimulating Th1 differentiation and secretion of
103 Th1 cytokines including interferon (IFN)- γ and IL-12, which have antagonisms for IgE
104 production promoted by IL-4 (Ongol et al., 2008). *Enterococcus faecalis* IC-1 (IC-1) is
105 a resident lactic acid bacterium in the human intestine and it has been reported to
106 enhance immune activity in its inactivated form (Nakase et al., 2017). Previous
107 researches demonstrated that IC-1 can reduce serum IgE content in OVA-induced
108 allergic model and inhibit mast cell degranulation through *in vitro* Caco2/RBL-2H3
109 cells co-culture system (Yamashita et al., 2016).

110 Diversity is an important character of human dietary habit and it is reasonable that
111 different combinations of food components may have various effects including
112 synergistic effect, additive effect, and antagonistic effect. This study was intended to
113 explore the anti-allergic activity using a combination of IC-1 and luteolin and provide a
114 safe and effective guide for people who have trouble with food allergies.

115

116 **2. Materials and Methods**

117

118 ***2.1. Reagents***

119 Dulbecco's modified Eagle's medium (DMEM, high glucose), Evans blue, luteolin and

120 aluminum hydroxide gel were purchased from Wako Pure Chemical Industries (Osaka,

121 Japan). RPMI 1640 medium, Minimal Essential Medium (MEM) non-essential amino

122 acids (NEAA) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine

123 serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-

124 dinitrophenyl (DNP) IgE, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and albumin from

125 chicken egg white lyophilized powder (OVA) were purchased from Sigma (St. Louis,

126 MO, USA). DNP-bovine serum albumin (DNP-BSA) was purchased from Cosmo Bio

127 (Tokyo, Japan). Anti-2,4,6-trinitrophenyl (TNP) IgE (clone: C38-2) was purchased from

128 BD Biosciences (Franklin Lakes, NJ, USA). 2,4,6-Trinitrochlorobenzene was purchased

129 from Tokyo Chemical Industry (Tokyo, Japan). *Enterococcus faecalis* IC-1was

130 purchased from Chichiyasu Pharmaceutical Co., Ltd. (Hiroshima, Japan) as a

131 commercial product which was killed by heat.

132

133 ***2.2. Mice***

134 Mice were purchased from Japan SLC (Shizuoka, Japan). BALB/c mice (6 weeks,
135 female) were used in the OVA-induced allergic model experiment and the *in vitro* Th2
136 differentiation experiment. BALB/c mice (7 weeks, female) were used in the passive
137 cutaneous anaphylaxis reaction (PCA). C57BL/6N mice (6 weeks , male) were used to
138 generate bone marrow-derived dendritic cell. All mice were maintained in Kobe
139 University Life Science Laboratory with specific pathogen-free conditions, stable
140 temperature (23 ± 2°C) and humidity (55 ± 10%). Mice had access to protein chow and
141 water freely. All animal experiments were approved by the Animal Experiment Ethnics
142 Committee of Kobe University (approval number: 28-10-02-R1).

143

144 ***2.3. Cell culture***

145 Rat basophilic leukemia cell line, RBL-2H3 cells (American Type Culture
146 Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented
147 with 10% (v/v) heat-inactivated FBS (57°C, 30 min), 2 g/L $NaHCO_3$ (Nacalai Tesque,
148 Kyoto, Japan), 100 μg/mL streptomycin (Thermo Fisher Scientific), 100 U/mL
149 penicillin (Sigma-aldrich). The human intestinal epithelial cell line, Caco-2 cells
150 (American Type Culture Collection) were cultured in DMEM (4.5g/L glucose)

151 supplemented with 10% FBS, 1% MEM-NEAA, 100 μg/mL streptomycin, 100 U/mL

152 penicillin. All cells were incubated in a humidified 5% CO_2 incubator at 37°C.

153

154 ***2.4.* Passive Cutaneous Anaphylaxis (PCA) Reaction**

155 Mice were orally administered with IC-1 (1×10^7 cfu/day), luteolin (200 μg/day) or a

156 combination of the two, each with the same concentration as above, dissolved in 100 μL

157 0.5% sodium carboxymethyl cellulose (CMC, Nacalai Tesque) solution, respectively.

158 Ten days after oral administration, mice were sensitized by subcutaneous injection with

159 0.1 μg/ear anti-DNP IgE dissolved in 10 μL PBS into both ears. After 24 h, mice were

160 challenged with 0.2 mg DNP-albumin dissolved in 100 μL PBS containing 1mg Evans

161 blue by intravenous injected to the caudal vein. Binding of DNP-BSA to anti-DNP IgE

162 stimulates mast cell to degranulate and causes increased vascular permeability, leading

163 to leakage of Evans blue. After 30 min, mice were sacrificed by cervical dislocation and

164 both ears were collected. The ears were cut into small pieces and soaked in formamide

165 for 24 h to extract Evans blue from the ears. The quantity of Evans blue was determined

166 by measuring absorbance at 610 nm (V-630, Jasco Corp., Tokyo, Japan). The severity of

167 the allergy was evaluated by the quantity of Evans Blue.

168

169 ***2.5.* β-Hexosaminidase activity assay**

170 RBL-2H3 cells were adjusted to the concentration of 2×10^6 cells /mL and dispensed
171 into 96-well plates at a concentration of 2×10^5 cells/well. Cells were incubated over-
172 night in a humidified 5% CO2 incubator at 37°C. Cells were sensitized with anti-TNP
173 IgE for 2 h. Cells were washed twice with Siraganian buffer before adding different
174 concentrations of IC-1 (1×10^7, 1×10^8 cfu/mL) or luteolin (12.5, 25, 50 μM) to each well
175 and incubated for 1 h, followed by a challenge with the antigen for 1 h. The reaction
176 was stopped by cooling in an ice bath for 10 min. The supernatant (50 μL) was
177 transferred into 96-well microplate and incubate with 50 μL of substrate solution (2 mM
178 p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.2 M citrate buffer at pH 4.5) for 1 h.
179 The reaction was stopped by adding stop solution (0.2 M glycine-NaOH at pH 13). The
180 absorbance was measured at 405 nm with a microplate reader (SH-9000, Corona
181 Electric Co., Hitachi, Japan).

182

183 ***2.6.* Caco-2/RBL-2H3 cells co-culture system**

184 Caco-2/RBL-2H3 cells co-culture system was done in accordance with a previous
185 study (Yamashita et al., 2016), with some modifications. Caco2 cells were dispensed
186 into 24-well Transwell insert plates (0.33 cm^2, 0.4 μm pore size, Corning Costar Corp.,

187 Cambridge, MA, USA). Cell culture mediums were changed every 3 days until the

188 transepithelial electrical resistance (TEER) value of Caco2 cells reaches 300 $\Omega \cdot cm^2$

189 measured by the Millicell-ERS Voltohmmeters (Merck KGaA, Darmstadt, Germany),

190 which is considered fully differentiated (Srinivasan et al., 2015). RBL-2H3 cells were

191 adjusted to the concentration of 2×10^4 cells /mL and dispensed into 24-well plate at a

192 concentration of 2×10^5 cells/well. RBL-2H3 cells were incubated over-night in a

193 humidified 5% CO2 incubator at 37°C. RBL-2H3 cells were sensitized with anti-TNP

194 IgE for 2 h. The inserts with Caco2 were placed on the top of RBL-2H3. Caco2 and

195 RBL-2H3 cells were washed twice with Siraganian buffer before adding IC-1 (0.5×10^8,

196 1×10^8 cfu/mL), luteolin (30 μM) or combination of the two, each with the same

197 concentration as above to Caco2 inserts well, co-cultured Caco2 and RBL-2H3 for 6 h.

198 Caco2 inserts were removed and RBL-2H3 were challenged with antigen for 1 h. The

199 remaining procedure was the same with β-hexosaminidase activity assay.

200

201 ***2.7.* Ovalbumin-induced allergic model**

202 Mice were orally administered with IC-1(1×10^8 cfu/day), luteolin (200 μg/day) or

203 combination of the two, each with the same concentration as above dissolved in 100 μL

204 0.5% CMC solution respectively. Seven days after oral administration, mice were

205 sensitized with 10 μg OVA + 1mg $Al(OH)_3$ adjuvant dissolved in 300 μL phosphate-
206 buffered saline (PBS) by intraperitoneal injection. The sensitizations were continued
207 once in 5 days for 2 more times. Five days after last intraperitoneal sensitization, mice
208 were challenged with OVA by intravenous injection with 3 μg OVA dissolved in 100 μL
209 PBS to trigger allergy response. Rectal temperature changes were measured every 10
210 min from 0 to 90 min after OVA challenge to estimate allergic reaction (Tsujimura et al.,
211 2008). One day before each OVA sensitization or challenge, blood samples were
212 collected from caudal vein. On the final day, blood samples were collected using cardiac
213 puncture.
214
215 ***2.8.* Enzyme-linked immunosorbent assay (ELISA)**
216 Blood samples were stored at 4°C overnight after placed at 22-25°C for 30 min.
217 Serum was collected by centrifugation of blood samples in 1.5 mL eppendorf tube at
218 11,000 x g (10,000 rpm in a AF-2724A rotor, Kubota 6200 centrifuge, Kubota Corp.,
219 Tokyo, Japan) at 4°C for 10 min. Total IgE contents were measured using a Mouse IgE
220 ELISA Set (BD Bioscience, San Jose, CA, USA). OVA-specific IgE were measured
221 using DS Mouse IgE ELISA (OVA) (DS Pharma Biomedical, Osaka, Japan). IFN-γ was
222 measured using IFN-gamma Quantikine ELISA Kit (R&D systems, Minneapolis, MN,

223 USA). All procedures were specifically following manufacturer's protocol, respectively.

224

225 *2.8.1 IgE*

226 Diluted Capture Ab was added to 96 well-plate at 100 μL/well and incubate

227 overnight at 4°C. The wells were washed with Wash Buffer for 3 times, then 200 μL

228 Assay Diluent was add to each well and incubated 1 h at room temperature (RT). The

229 wells were washed with Wash Buffer for 3 times, then 100 μL standard or sample was

230 added to each well and incubated 2 h at RT. The wells were washed with Wash Buffer

231 for 5 times, then 100 μL Working Detector was added to each well and incubated 1 h at

232 RT. The wells were washed with Wash Buffer for 7 times, then 100 μL Substrate

233 Solution was added to each well. After incubated 30 min at RT in dark, 50 μL Stop

234 Solution was add to each well. The absorbance was read at 450 nm and the

235 concentration of the IgE was calculated from the standard curve.

236

237 *2.8.2 OVA-specific IgE*

238 The Buffer was added into the Microplate at 150 μL/well before 15 μL sample or

239 Standard solution was added into each well and incubated for 10 min at RT. The aliquot

240 solution was added to each well at 110 μL/well and incubated in dark for 60 min at RT.

241 The wells were washed with Wash buffer for 3 times, then 100 μL OVA-enzyme

242 conjugate was added to each well and incubated in dark for 30 min at RT. The wells

243 were washed with Wash buffer for 3 times, then 100 μL Substrate was added to each

244 well. After incubated in dark for 30 min at RT, 100 μL Stop solution was added to each

245 well. The absorbance was read at 450 nm and the concentration of the OVA-specific IgE

246 was calculated from the standard curve.

247

248 *2.8.3 IFN-γ*

249 The Assay Diluent RD1-21 was added into the Microplate at 50 μL/well before

250 50 μL standard, control, or sample was added into each well and incubated for 2 h at

251 RT. The wells were washed with Wash buffer for 5 times, then 100 μL Mouse IFN-γ

252 Conjugate was added to each well and incubated for 2 h at RT. The wells were washed

253 with Wash buffer for 5 times, then 100 μL Substrate Solution was added to each well.

254 After incubated in dark for 30 min at RT, 100 μL Stop solution was added to each well.

255 The absorbance was read at 450 nm and the concentration of the IFN-γ was calculated

256 from the standard curve.

257

258 ***2.9.* Spleen cell isolation and in vitro differentiation of Th2 subset**

259 Spleen cell isolation and *in vitro* differentiation of Th2 subset were performed in
260 accordance with a previous study (Flaherty & Reynolds, 2015), with some
261 modifications. Balb/c mice were sacrificed by cervical dislocation and disinfected in
262 75% ethanol for 5 min. The spleens were isolated and carefully transferred into dish
263 containing RPMI 1640 medium. The needle of a 1-mL syringe was inserted into the
264 spleen and it was rinsed with the media until its surface turned to white. The cell
265 suspension in the dish was collected and centrifuged at 1,500 g for 3 min. The
266 supernatants were discarded and the cell pellets were resuspended with red blood cell
267 (RBC) lysis buffer (NH_4Cl , tris(hydroxymethyl)aminomethane) to lyse the RBC. The
268 cell suspensions were centrifuged again and the supernatants were discarded. The cell
269 pellets were resuspended with RPMI 1640 medium supplied with 55 μM 2-
270 mercaptoethanol (Gibco BRL), 5 ng/mL IL-2 and 10 ng/mL IL-4 (R&D Systems,
271 Minneapolis, MN, USA). The cells adjusted to be at the concentration of 1×10^6
272 cells/mL. Cell suspensions were seeded in 24-well plate pre-coated with 2 μg/mL anti-
273 CD3 (Bio X Cell, West Lebanon, NH, USA) and 2 μg/mL anti-CD28 (Bio X Cell) at
274 1×10^6 cells/well, and then were added IC-1 (1×10^8 cfu), luteolin (10μM) or combination
275 of IC-1 and luteolin were added into different wells and cultured in a humidified 5%
276 CO2 incubator at 37°C for 96 hours. The cells were re-stimulated with 1μg/mL of anti-

277 CD3 for 4 hours. The total cell RNA was isolated with Sepasol®-RNA I Super G

278 (Nacalai Tesque) and used for detecting gene expression.

279

280 ***2.10.* Generation of bone marrow-derived dendritic cell**

281 Generation of bone marrow-derived dendritic cell (DC) was performed in

282 accordance with a previous study (Inaba et al., 1992), with some modifications.

283 C57BL/6N mice were sacrificed by cervical dislocation. The tibias and femurs were

284 carefully removed and disinfected in 75% ethanol for 5 min, and then transferred into

285 dish containing RPMI 1640 medium. The two ends of the bone were cut off and a

286 needle of a 1 mL syringe was inserted into the bone cavity to remove the bone marrow

287 until the surface of bone turned to white. The cell suspensions in the dish were collected

288 and centrifuged at 1,500 g for 3 min, and the supernatants were discarded. The cell

289 pellets were resuspended with RBC lysis buffer to lyse the RBC. The cell suspensions

290 were centrifuged again, the supernatants were discarded, and pelleted cells were

291 resuspended with RPMI 1640 medium with 55 μM 2-mercaptoethanol, 10 ng/mL IL-4

292 and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D

293 Systems). The cells were counted and adjusted the concentration to 1×10^7 cells/mL. The

294 cell suspensions were plated in dish at 3×10^7 cells/dish. The culture medium was

295 replaced 48 hours later to remove the unattached cells and supplied with new medium.

296 On day 6th, the semi-suspended cells and loosely attached cells were considered

297 immature DC. The immature DCs were counted and the concentration was adjusted to

298 1×10^6 cells/mL and plated in 24-well plate at 1×10^6 cells/well. IC-1 (1×10^8 cfu),

299 luteolin (10 μM) or combination of IC-1 and luteolin were added into different wells

300 and cultured for 72 hours. The total cell RNA was isolated with Sepasol®-RNA I Super

301 G (Nacalai Tesque) and used for detecting gene expression.

302

303 ***2.11.* Real-time PCR**

304 Quantitative PCR was performed using a 7500 Fast Real Time PCR system (Life

305 Technologies, Carlsbad, CA, USA) and FastStart Universal Probe Master (ROX)

306 (Roche Diagnostics, Basel, Switzerland) according to the manufacture's protocol.

307 TaqMan gene expression assay were purchased from Applied Biosystems and assay

308 identifications (IDs) involve are as follows; mouse β-actin Assay ID: Mm00607939_s1,

309 mouse GATA-3 Assay ID: Mm00484683_mH, mouse TBX21 Assay ID:

310 Mm00450960_m1, mouse IL-4 Assay ID: Mm00445259_mH and mouse IFNG Assay

311 ID: Mm01168134_mH. Double delta Ct analysis was used to process gene expression

312 level. β-actin was used as endogenous control.

313

314 ***2.12.* Statistical Analysis**

315 All the data were shown as mean ± standard deviation (M ± SD). To compare with

316 any two groups, statistical significance was analyzed using the Student's t-test. To

317 compare with more than two groups, statistical significance was analyzed using the

318 Tukey-Kramer test. Statistical significance was defined as $p < 0.05$.

319

320 **3. Results**

321

322 ***3.1. Anti-allergic effect by combination of IC-1 and luteolin in passive cutaneous***

323 ***anaphylaxis reaction***

324 Passive cutaneous anaphylaxis reaction (PCA) is a common animal model for

325 evaluating type I hypersensitivity. As shown in Fig. 1, oral administration of

326 combination suppressed the leakage of Evans blue in the ears of mice while IC-1 alone

327 or luteolin alone group had no significant inhibition effect, indicating that simultaneous

328 administration of IC-1 and luteolin may alleviate allergic symptom through inhibiting

329 mast cell degranulation.

330

331 ***3.2. Inhibition of Luteolin and IC-1 on mast cell degranulation***

332 Since PCA is directly induced by mast cell degranulation, β-hexosaminidase
333 activity assay was performed to confirm the effect of IC-1 and luteolin on inhibiting
334 mast cell degranulation *in vitro*. As shown in Fig. 2A, luteolin had concentration-
335 dependent inhibition on mast cell degranulation. On the other hand, IC-1 showed no
336 inhibition effect on mast cell degranulation (Fig. 2B).

337

338 ***3.3. Inhibition on mast cell degranulation in the Caco-2/RBL-2H3 cells co-culture***
339 ***system by combination of IC-1 and luteolin***

340 Both IC-1 and luteolin have to encounter with intestine barrier when entering
341 digestive tract. Yamashita et al. (2016) used an *in vitro* co-culture model comprised of
342 Caco-2/RBL-2H3 cells to evaluate anti-allergic effects of food components through the
343 intestine. As shown in Fig. 3, the combination of IC-1 (0.5×10^8 cfu/mL) and luteolin
344 (30 μM) showed inhibition effect in Caco-2/RBL-2H3 cells co-culture system while
345 their single form failed to inhibit mast cell degranulation.

346

347 ***3.4. Effect on allergic symptom in OVA-induced allergic model by combination of IC-***
348 ***1 and luteolin***

349 Apart from PCA, OVA-induced active systemic anaphylaxis is another common
350 animal model for type I hypersensitivity. To confirm whether luteolin or IC-1 can
351 alleviate allergic symptom, rectal temperature change after OVA challenge for 90 min
352 was monitored (Tsujimura et al., 2008). As shown in Fig. 4, the rectal temperature of
353 mice in OVA group dropped dramatically and hit the low point (7.3 ± 0.5°C) at 60 min
354 after intravenous injection with OVA. The rectal temperature recovered gradually after
355 60 min but did not return to normal level at 90 min after OVA challenge. Both luteolin
356 and IC-1 groups showed lower temperature decrease and faster recovery rate compared
357 to OVA group but neither of them was able to return to normal level at 90 min after
358 OVA challenge. On the other hand, the rectal temperature in combination group hit the
359 nadir point (3.3 ± 1.0°C) at 40 min and the decrease level was significantly lower than
360 OVA group from 40 to 70 min after injection of OVA. It also showed the highest
361 recovery rate and returned to normal at 90 min after OVA challenge. These results
362 indicated that combination of IC-1 and luteolin can alleviate symptom with an allergy
363 attack.
364
365 ***3.5. Effect on IgE production in OVA-induced allergic model by combination of IC-1***
366 ***and luteolin***

367 It was ascertained that combination of IC-1 and luteolin significantly alleviated
368 allergy symptom (Fig. 4). IgE is the major immunoglobin for initiating mast cell
369 degranulation to trigger Type I hypersensitivity reaction (Janeway et al., 2001). Previous
370 studies demonstrated that both luteolin and LAB can suppress IgE production under
371 allergy state in animal experiments (Ai et al., 2016; Fujiwara et al., 2004; Kimata et al.,
372 2000; Peng & Hsu, 2005; Segawa et al., 2008). To confirm whether the combination of
373 IC-1 and luteolin can reduce IgE production in active systemic anaphylaxis (ASA), total
374 IgE content was monitored during OVA experiment. As shown in Fig. 5A, total IgE
375 content in blood of mice that administrated with OVA and $Al(OH)_3$ adjuvant (OVA
376 group) increased continually. Mice orally administered only with IC-1 or luteolin also
377 showed a tendency to suppress total IgE production compared to OVA group. However,
378 the combination group, which was orally administered simultaneously with IC-1 and
379 luteolin, showed the lowest total IgE among 5 groups and significantly different with
380 OVA group at 17 days.
381 Specificity against a particular protein is an important character of IgE, meaning
382 that a certain type of IgE only triggers allergic reaction under the stimulating of the
383 same allergen (Janeway et al., 2001). In order to confirm whether the combination of
384 IC-1 and luteolin also can reduce OVA-specific IgE production, blood content of OVA-

385 specific IgE was measured. As shown in Fig. 5B, the combination group also
386 significantly suppressed OVA-specific IgE compared to OVA group. These data
387 suggested that combination of IC-1 and luteolin has enhanced anti-allergic effect
388 compared to their single form.
389
390 ***3.6. Effect on IFN-γ content in OVA-induced allergic model by combination of IC-1***
391 ***and luteolin***
392 Th2 response is the major immune response under allergic state. Th2 cytokine IL-4
393 is known to promote B cell to process immunoglobulin (Ig) class switch recombination
394 from IgM to IgE. On the other hand, IFN-γ, a typical Th1 cytokine, is antagonistic to
395 IgE production promoted by IL-4. Since combination of IC-1 and luteolin has been
396 confirmed to suppress IgE production in allergic state (Fig. 5), IFN-γ content in blood
397 was further measured. As shown in Fig. 6, the combination group showed highest IFN-γ
398 content compared to other groups. These results may suggest that the simultaneous
399 administration of IC-1 and luteolin might improve Th1/2 balance toward Th1
400 dominance.
401
402 ***3.7. Inhibition on naïve T cell differentiate into Th-2 subset by combination of IC-1***

403 ***and luteolin***

404 To confirm whether combination of IC-1 and luteolin can improve Th2 shifting
405 toward Th1 dominance in allergy state, *in vitro* differentiation into Th2 subset was
406 investigated using spleen cells. Th1 related gene T box transcription factor (T-bet) and
407 IFN-γ, and Th2 related gene GATA-3 and IL-4 were measured by RT-PCR. As shown
408 in Fig. 7, combination and luteolin group significantly suppressed GATA-3 expression.
409 They also showed tendency of suppressing IL-4 expression. IC-1 showed no effect both
410 on GATA-3 and IL-4. Combination and IC-1 group showed significant increase in Th1
411 cytokine IFN-γ expression level. Interestingly, the expression level of T-bet, the
412 transcription factor specifically expressed in Th1 did not increase in spite of IFN-γ
413 increase (Larsen et al., 2016). These results suggested that combination of IC-1 and
414 luteolin could inhibit naïve T cell differentiation into Th-2 subset through inhibiting
415 Th2 transcription factor GATA-3 expression.

416

417 ***3.8. Effect on IFN-γ gene expression in bone marrow-derived DC by combination of***
418 ***IC-1 and luteolin***

419 Result in Fig. 7 shown combination of IC-1 and luteolin had no improvement on
420 Th1 cell differentiation since Th1 transcription factor T-bet remained unchanged,

421 suggesting other cells may contribute to the increased IFN-γ expression. Apart from T
422 cells, spleen cells also consist of DC, macrophages and plasma cells. Even though IFN-
423 γ is mainly secreted by Th1 cell, it could also be secreted by DC (Moretto et al., 2007).
424 To confirm whether combination can promote DC to secrete IFN-γ, bone marrow-
425 derived DCs were cultured with IC-1, luteolin or combination. As shown in Fig. 8,
426 combination of IC-1 and luteolin showed increased IFN-γ gene expression in DC
427 compared to other groups treated with IC-1 alone, luteolin alone or nothing.
428
429 **4. Discussion**
430
431 Even though the allergic diseases have become a global issue, there is still lack of
432 effective and safe treatment. For example, allergy immunotherapy, which has been
433 recommended by current clinical guidance is reported to be of good efficacy in allergy
434 treatment (Larsen et al., 2016). However, the allergy immunotherapy can only be
435 applied to patient with clear allergen diagnosis and its safety is still on the debate. In
436 addition, the anti-allergic effect of food components have gained increasing attention in
437 the last decades. Both luteolin and IC-1 had been shown to have anti-allergic effect
438 (Jang et al., 2017; Kimata et al., 2000; Yamashita et al., 2016) and this study has shown
439 that the combination of IC-1 and luteolin had synergistic anti-allergic activity.

440 The combination of IC-1 and luteolin alleviated symptom in PCA reaction
441 (Fig. 1). Since PCA reaction is mainly induced by mast cell degranulation, these results
442 suggested that the combination had inhibition effect on mast cell degranulation. In β-
443 hexosaminidase activity assay, which was used to evaluate the direct inhibit effect of
444 IC-1 and luteolin, only luteolin inhibited β-hexosaminidase activity (Fig. 2A). These
445 results stay consistent with previous study that luteolin can inhibit mast cell
446 degranulation through the inhibition of $Ca2^{+}$ influx and PKC activation (Kimata et al.,
447 2000). However, the inhibition effect of luteolin could not be observed in Caco2/RBL-
448 2H3 cells co-culture system (Fig. 3). This may be due to the low bioavailability of
449 luteolin which is partially metabolized when passed through the monolayer of Caco-2
450 cell, a cell model for mimicking small intestine (Murota et al., 2000; Nishitani et al.,
451 2013). On the other hand, even though IC-1 showed no inhibition on mast cell
452 degranulation when directly applied to RBL-2H3 cells (Fig. 2B), it showed inhibition
453 effect in Caco2/RBL-2H3 cells co-culture system (Fig. 3A). These results suggested
454 that some compounds derived from small intestine epithelial cell through recognizing
455 IC-1 inhibited mast cell degranulation. On the basis of results from PCA (Fig. 1), β-
456 hexosaminidase assay (Fig. 2) and Caco2/RBL-2H3 cells co-culture system (Fig. 3), it
457 can be concluded that the combination of luteolin and IC-1 had enhanced anti-allergic

458 activity by inhibition on mast cell degranulation with the individual pathway in each
459 compound, respectively.
460 In OVA-induced allergic model, the combination of IC-1 and luteolin
461 significantly alleviated allergic symptom (Fig. 4). Among all the 5 types of
462 immunoglobulins, IgE is the major antibody in Type I hypersensitivity to cause allergic
463 symptoms. In according with the result of allergic symptoms (Fig. 4), the combination
464 of IC-1 and luteolin also suppressed total IgE and OVA-specific IgE production in
465 blood (Fig. 5). The production of IgE is the result of immunoglobulin class switch
466 recombination (CSR). Initially, B cell is only programmed to express IgM and/or IgD,
467 but upon immunization, B cell starts to undergo CSR and achieves ability of expressing
468 IgM, IgG or IgE (Snapper & Mond, 1993). There are several factors that could regulate
469 B cell CSR, of which cytokine micro-environment is the most important factor. For
470 example, bacteria or virus inflammation increases Th1 cytokines, IFN-γ concentration,
471 and leading to IgG production; parasites infection increases Th2 cytokine, IL-4
472 concentration, and leading to IgE production; the mucosa is enriched with TGF-ß1,
473 which is important in IgA production (Bode et al., 2004). IL-4 and IFN-γ are working in
474 an antagonistic manner. It has been shown that IFN-γ antagonizes IL-4-induced B cell
475 switching to IgE isotype (Hasbold et al., 1999). Different types of cytokines secreted by

476 different subsets of T cell. Fig. 6 showed that the combination group had the highest
477 IFN-γ content in the blood, which suggested that the combination group may improve
478 Th2 shifting toward Th1 dominance in the allergy state.

479 Generation of different $CD4^+$ T cell subsets requires different signal
480 transduction pathway (Jenner et al., 2009). IFN-γ signaling leads to activation of the
481 signal transducer and activator of transcription (STAT) 4, which will activate T-bet, an
482 important regulator of Th1 differentiation. IL-4 signaling leads to phosphorylation of
483 the STAT6 and activate GATA-3, an important regulator of Th2 differentiation. *In vitro*
484 Th2 subset-induced experiment using the cells isolated from spleen showed that the
485 combination of IC-1 and luteolin significantly suppressed GATA-3 whereas IFN-γ
486 expression increased significantly (Fig. 7). The expression level of T-bet did not
487 increase in spite of IFN-γ increasing (Fig. 7), which implied that the combination did
488 not promote Th0 differentiation into Th1. These results suggested that other cells may
489 contribute to the increased IFN-γ expression in the combination group.

490 Even though IFN-γ is mainly secreted by Th1 cell, it could also be secreted by
491 other immune cells such as DC. DC is a specialized APC that plays an important role
492 both in innate and adaptive immune response. It has been shown that IFN-γ produced by
493 DCs is important for priming gut intraepithelial lymphocyte response (Moretto et al.,

494 2007). To confirm whether DC is the resources of increasing IFN-γ in combination
495 group, bone marrow-derived DC was cultured simultaneously with IC-1 and luteolin.
496 As shown in Fig. 8, the combination of IC-1 and luteolin increased IFN-γ gene
497 expression in DC.

498 Previous works showed that IC-1 could be recognized by Toll-like receptor
499 (TLR) 2 expressed on Caco-2 cells (Yamashita et al., 2016). TLRs are pattern
500 recognition receptors (PRRs), which have been reported to be important for LAB to
501 execute immune response. TLRs are widely expressed not only on immune cells
502 including DC, but also on other tissue cells such as intestinal epithelium (Ey et al.,
503 2009). Upon stimulation of TLRs, various adaptor proteins are recruited, and a series of
504 signaling cascades are activated to release specific cytokines such as IFN-γ depending
505 on different cells and situations (Lebeer et al., 2010). Since the increase of IFN-γ was
506 also observed in IC-1 group (Fig. 7 and 8), there is a high possibility that IC-1 is
507 recognized by TLR2 expressed on DC leading to an increase secretion of IFN-γ.
508 Through suppressing Th2 cytokine, IL-4 production, and increasing Th1 cytokine, IFN-
509 γ production (Fig. 5C, 6D and 7), the combination of IC-1 and luteolin strongly
510 inhibited IgE production in the allergy state (Fig. 5).

511 Both IC-1 and luteolin contribute to allergic diseases but with limitation. When

512 administered with IC-1 or luteolin alone, neither of them is able to significantly
513 alleviate allergic symptom (Fig. 1 and 4) or suppress IgE production (Fig. 5). Luteolin
514 only had the ability to suppress Th2 differentiation (Fig. 7) while IC-1 only had the
515 ability to increase IFN-γ (Fig. 7 and 8). However, by synergistically working with each
516 other, the combination of IC-1 and luteolin had an enhanced anti-allergic effect.
517

518 **5. Conclusion**

519 The combination of IC-1 and luteolin promoted DC to secrete IFN-γ and
520 inhibited Th0 differentiation into Th2 subset through suppressing GATA-3 expression.
521 The increase of Th1 cytokine, IFN-γ and the decrease of Th2 cytokine, IL-4 suppressed
522 IgE production to alleviate allergic symptoms. In addition, intestine epithelial cells
523 (IECs) recognized IC-1 and possibly released a type of compound(s) which can work
524 synergistically with luteolin to inhibit mast cell degranulation. In conclusion, this
525 research confirmed the synergistic anti-allergic activity using a combination of IC-1 and
526 luteolin.
527

528 **Conflict of Interest**

529 The authors have declared no conflicts of interest.

530

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532

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664 Figure legends

665

666 Figure 1. Anti-allergic effect by combination of IC-1 and luteolin in passive cutaneous

667 anaphylaxis reaction

668 Mice were oral administration with IC-1 (1×10^7 cfu/day), luteolin (200 μg/day)

669 or the combination of the two, each with the same concentration as above, respectively

670 for 10 days. On day 10, mice were sensitized by subcutaneous injection of anti-DNP

671 IgE into both ears and exposed to DNP-BSA containing 1% Evans blue to induce

672 passive cutaneous anaphylaxis reaction. Different superscript letters indicate the

673 statistical significance between groups ($p < 0.05$). Values are presented as means ± SD

674 (n=4-5).

675

676 Figure 2. Inhibition of Luteolin and IC-1 on mast cell degranulation

677 Different concentrations of luteolin (12.5, 25, 50 μM) or IC-1 (1×10^7, 1×10^8

678 cfu/mL) was applied to RBL-2H3 cell after sensitized with anti-DNP IgE and followed

679 by challenged with TNP-BSA. Different superscript letters indicate the statistical

680 significance between groups ($p < 0.05$). Values are showed as means ± SD (n=5).

681

682 Figure 3. Inhibition on mast cell degranulation in the Caco-2/RBL-2H3 cells co-culture

683 system by combination of IC-1 and luteolin

684 Caco2/RBL-2H3 cell co-culture system was used to evaluate anti-allergic

685 effects of IC-1, luteolin or the combination of IC-1 and luteolin through the intestine.

686 IC-1 (1×10^8 cfu/mL), luteolin (30 μM) or the combination of the two, each with the

687 same concentration as above, was applied to apical side of Caco2 cell and co-cultured

688 with RBL-2H3 which was sensitized with anti-DNP IgE for 6 hours, and then RBL-2H3

689 cells were challenged with TNP-BSA. Different superscript letters indicate the statistical

690 significance between groups ($p < 0.05$). Values are showed as means ± SD (n=4-6).

691

692 Figure 4. Effect on allergic symptom in OVA-induced allergic model by combination of

693 IC-1 and luteolin

694 Mice were oral administration with IC-1 (1×10^8 cfu/day), luteolin (200 μg/day)

695 or the combination of the two, each with the same concentration as above, for 24 days.

696 Mice were challenged with OVA by intravenous injection to trigger allergy response

697 after sensitization. Rectal temperature change was measured every 10 min from 0 to 90

698 min after OVA challenge to estimate allergic reaction. *($p < 0.05$), significantly different

699 from the group only administrated with OVA. Values are showed as means ± SD (n=5).

700

701 Figure 5. Effect on IgE production in OVA-induced allergic model by combination of

702 IC-1 and luteolin

703 Mice were orally administered with IC-1 (1×10^8 cfu/day), luteolin (200

704 μg/day) or the combination of the two, each with the same concentration as above, for

705 24 days. *($p < 0.05$), significantly different from the group only administrated with

706 OVA. Values are showed as means ± SD (n=5). U.D indicates under detection.

707

708 Figure 6. Effect on IFN-γ content in OVA-induced allergic model by combination of

709 IC-1

710 Mice were orally administered with IC-1 (1×10^8 cfu/day), luteolin (200

711 μg/day) or the combination ofthe two, each with the same concentration as above, for

712 24 days. Serum was collected on the final day using cardiac puncture. Different

713 superscript letters indicate the statistical significance between groups ($p < 0.05$) . Values

714 are showed as means ± SD (n=3).

715

716 Figure 7. Inhibition on naïve T cell differentiate into Th-2 subset by combination of IC-

717 1 and luteolin

718 Spleen cells were isolated from mice and cultured with IC-1 (1×10^8 cfu/mL), luteolin

719 (10 μM) or the combination of the two, each with the same concentration as above, in

720 the Th-2 subset-induced environment for 96 h. Cells were collected and then Th1 and

721 Th2 related gene T-bet, IFN-γ, GATA-3 and IL-4 were measured by real time-PCR

722 respectively. Different superscript letters indicate the statistical significance between

723 groups ($p < 0.05$) . Values are showed as means ± SD (n=4-5).

724

725 Figure 8. Effect on IFN-γ gene expression in bone marrow-derived DC by combination

726 of IC-1 and luteolin

727 Immature DC was generated from bone marrow by providing precursor cells

728 with GM-CSF and IL-4. Immature DC was cultured with IC-1 (1×10^8 cfu/mL), luteolin

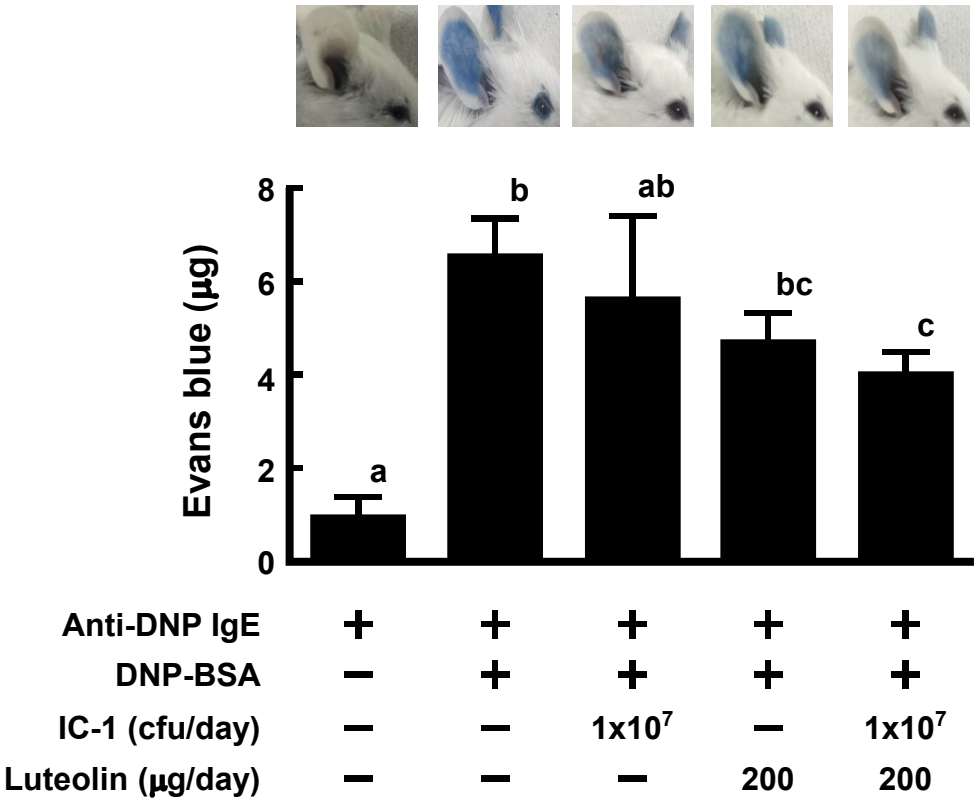
729 (10 μM) or the combination of the two, each with the same concentration as above.

730 After 72 h, cells were collected. IFN-γ was measured by RT-PCR. Different superscript

731 letters indicate the statistical significance between groups ($p < 0.05$). Values are showed

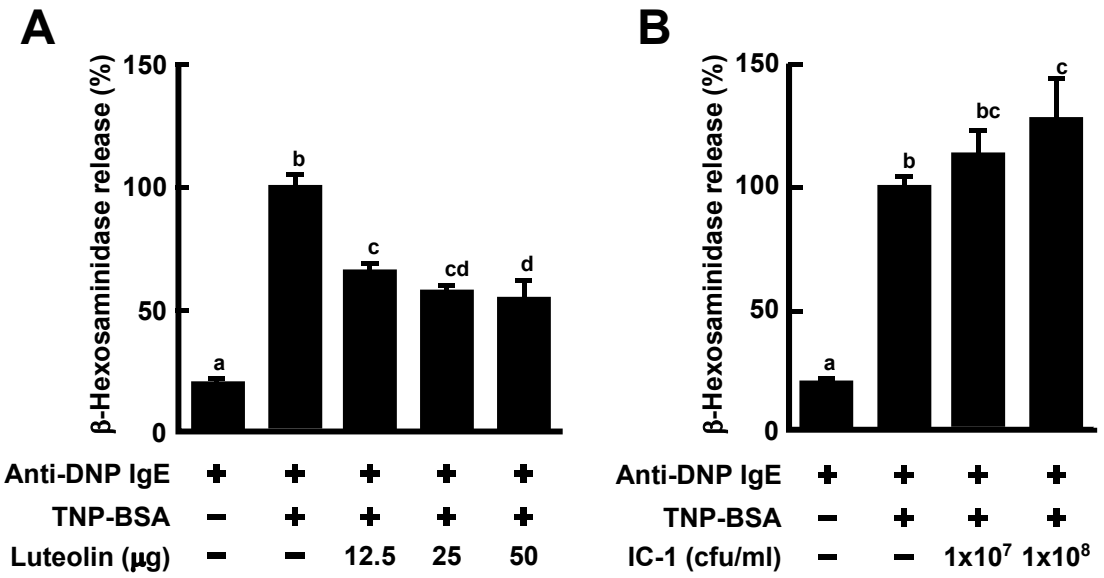
732 as means ± SD (n=3).

733



Up

Han *et al.*, Fig. 1



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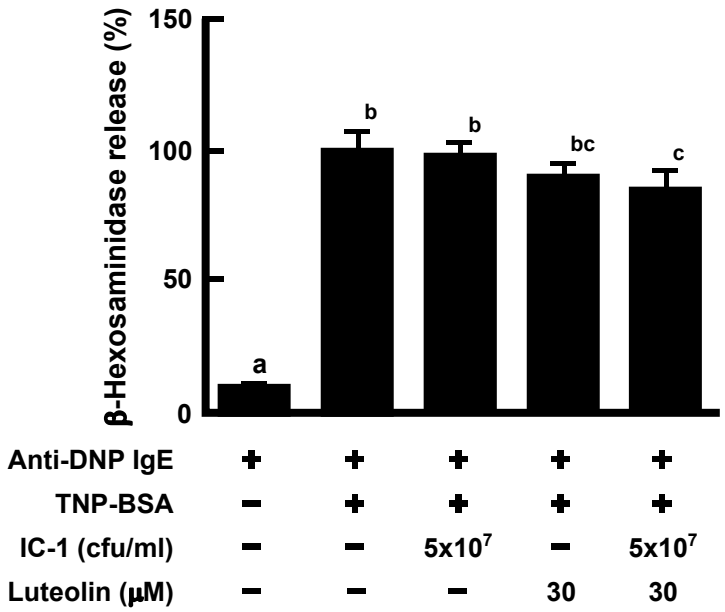

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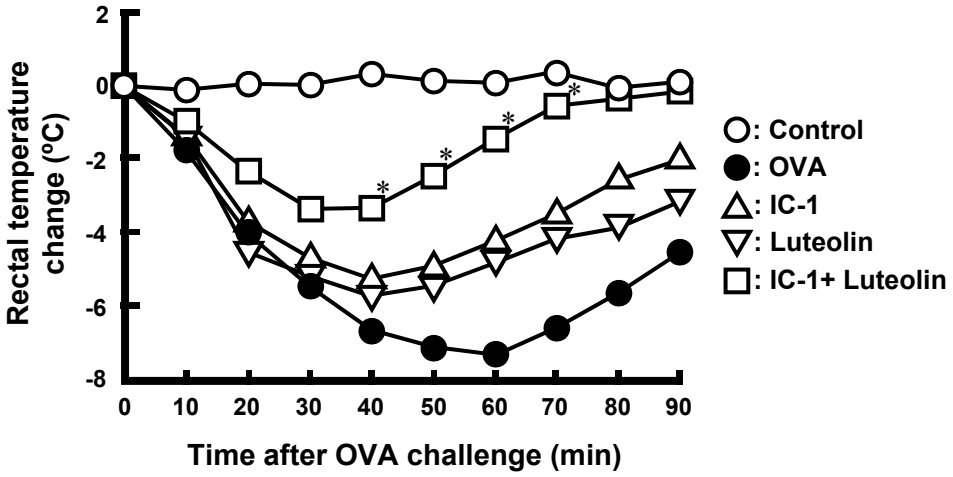

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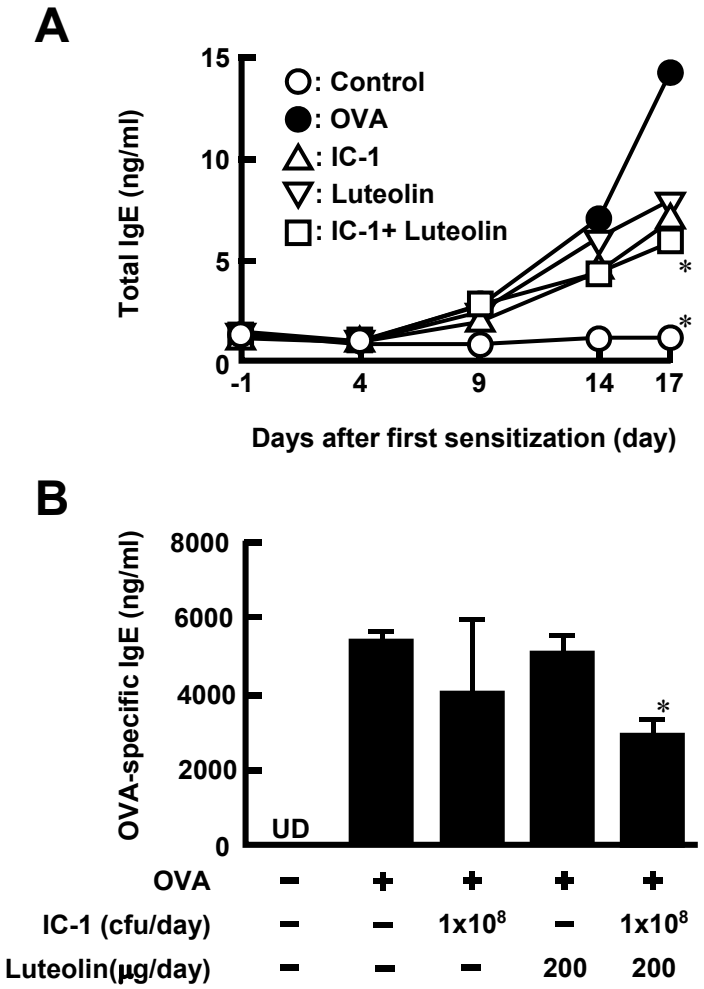
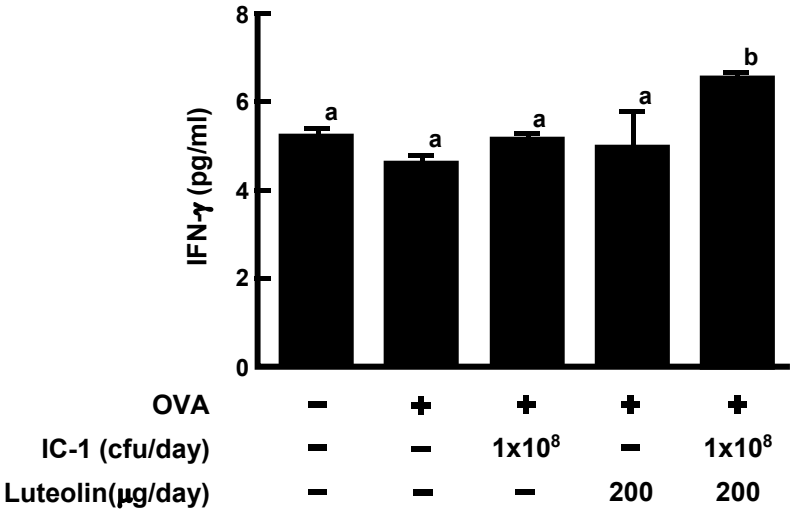
Han *et al.*, Fig. 4



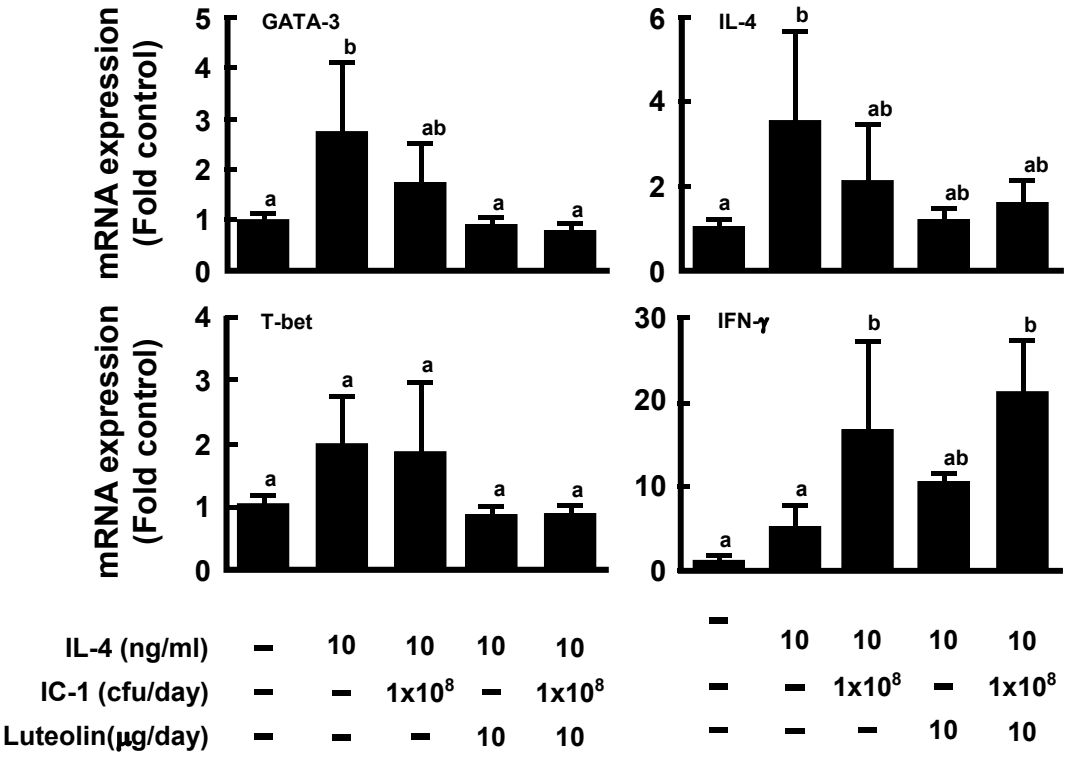
Up

Han *et al.*, Fig. 5



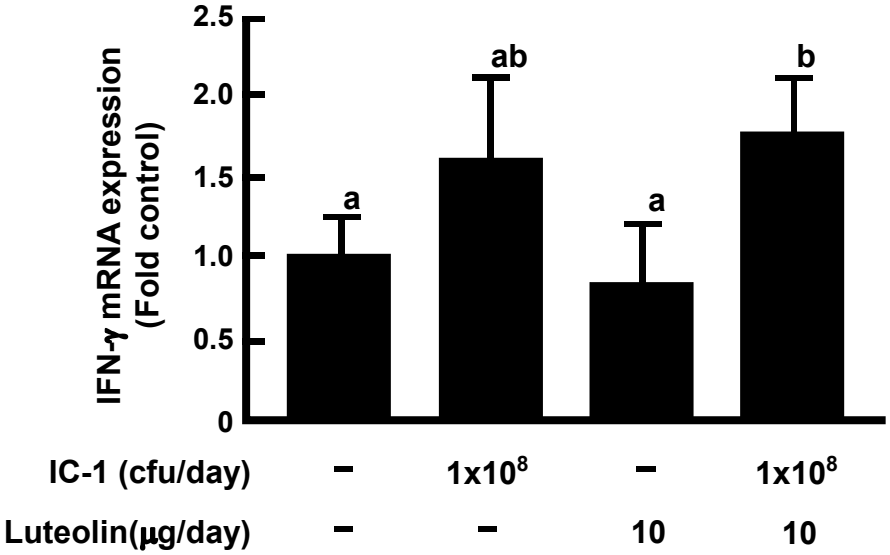
Up

Han *et al.*, Fig. 6



Up

Han *et al.*, Fig. 7



Up

Han *et al.*, Fig. 8