Rose flavor compound β-damascone regulates dendritic cell-mediated immunoresponses by modulating the NRF2 pathway and ameliorates contact hypersensitivity

Running title: β-damascone suppresses DC activation

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Abstract

Numerous pharmacologically beneficial compounds have been isolated from natural products derived from plants, which are often characterized as phytochemicals and are used in flavors, spices, and fragrances. Here we show that β-damascone, a major ingredient of rose fragrance, was selected from an aroma library as a candidate compound that suppresses antigen-dependent T cell activation. β-Damascone inhibited dendritic cell (DC)-related responses, including DC-induced Th1 development, TLR ligand-induced production of inflammatory cytokines in DCs. β-Damascone treatment increased the levels of NRF2 protein and *Hmox1* mRNA in DCs. *Nrf2*$^{-/-}$ DCs possessed Th1-induction activity and higher IL-12p40 production activity even in the presence of β-damascone in comparison with *Nrf2*$^{+/-}$ DCs. Finally, we found that β-damascone intake suppressed ear swelling of contact hypersensitivity model mice. Taken together, we conclude that the rose aroma compound β-damascone, which suppresses DC-mediated immunoresponses by activating the NRF2 pathway, could be useful to ameliorate immunorelated diseases.

Keywords: β-damascone, dendritic cells, NRF2

Abbreviations: Ab; antibody, Ag; antigen, APCs; antigen presenting cells, BMDCs; bone marrow-derived dendritic cells, CHS; contact hypersensitivity, DCs; dendritic cells, DNCB; 2,4-dinitrochlorobenzene, DNFB; 2,3-dinitrofluorobenzene, NRF2; NF-E2-related factor 2, OVA; ovalbumin, p.o.; per os

Introduction

Since ancient times, humans have obtained pharmacological compounds from natural products derived from plants and bacteria. Phytochemicals, which are produced in plants to resist various stresses, including UV and insect pests, exhibit beneficial effects, such as antioxidant, anticancer, and anti-inflammatory effects, on humans and mammals. Because some phytochemicals are scenting and are used as flavors and flagrances, we expected that unidentified immunomodulators could be found in aroma compounds.

Dendritic cells (DCs), the most typical antigen presenting cells (APCs), contribute to both innate and adaptive immunity. Activation of DCs by pathogens leads to the release of cytokines and chemokines from DCs, and DC-mediated expansion of antigen-specific T cell clones. Therefore, DC activation is essential for host defense against infection, but hyper and/or lasting activation of DC-mediated immunoresponses causes inflammation and autoimmune diseases.

A number of studies regarding the immunomodulatory effects of phytochemicals have been reported (Mainardi, Kapoor and Bielory 2009). Resveratrol is the most well-known polyphenolic stilbenoid (Baur et al. 2006, Jang et al. 1997) and exhibits effects on the prevention of inflammation-related diseases by suppressing TLR signaling-induced expression of proinflammatory genes (Malaguarnera 2019). Regarding scent phytochemicals, several food ingredients, including curcumin (Kunnumakkara et al. 2017) and menthol (Oz et al. 2017), are expected to possess therapeutic potential.

In the current study, we first performed screening to find novel immunomodulators by analyzing the effect on APC-dependent T cell activation and selected β-damascone, a major ingredient of rose fragrance, from approximately 150 kinds of aroma compounds.

Next, we investigated the molecular mechanisms by which β-damascone regulates antigen (Ag)-dependent T cell proliferation and found that β-damascone suppressed DC-mediated immunoresponses and that activation of the transcription factor NF-E2-related factor 2 (NRF2)-pathway in DCs by β-damascone is one of the causes. Finally, we confirmed the effect of β-damascone on immunoresponses *in vivo* using a contact hypersensitivity (CHS) mouse model. Taken together, these results indicate that β-damascone, which suppresses immunoresponses mainly targeting DCs, exhibits beneficial effects on the prevention and/or treatment of immunorelated diseases.

Results

β-damascone was identified as an immunomodulator suppressing DC-mediated T cell activation

To find novel immunomodulators, we performed 2-step screening using an aroma-compound library (**Supplementary Table SII**). By the 1st screening, in which APC-dependent T cell proliferation was assayed, 22 candidates were selected from 150 compounds as immunosuppressors, and 3 compounds were passed through the 2nd screening evaluating the suppressive effect on IL-2 release from OVA-pulsed OT-II splenocytes (**Supplementary Figure S1**). Considering several factors, such as IC_{50}, we finally selected #177 β-damascone (**Figure 1A**), a major component of rose aromas. In accordance with preliminary observations in the abovementioned screening, β-damascone suppressed OVA-induced IL-2 production from OT-II splenocytes (**Figure 1B**) and APC-dependent proliferation of $CD4^+$ T cells (**Figure 1D**) in a dose-dependent manner at concentrations much lower than those showing cytotoxicity (**Figure 1C**). Although APC-independent T cell proliferation caused by stimulation with plate-coated anti-CD3 and anti-CD28 Abs was moderately suppressed by β-damascone (**Figure 1E**), considering that the development of Th1 cells induced by OVA-pulsed DCs (**Figure 1F**) but not by CD3/CD28 stimulation (**Figure 1G**) was significantly suppressed by β-damascone, we focused on the effect of β-damascone on the function of DCs.

β-damascone suppressed LPS-induced activation of DCs

We evaluated the effects of β-damascone on LPS-induced activation of DCs by analyzing the cytokine expression levels and cell surface expression levels of APC-related molecules in BMDCs. As shown in **Figure 2A**, β-damascone apparently

reduced the mRNA levels of IL-6, IL-12p40, and TNF-α in DCs, which was increased following LPS stimulation, in a dose-dependent manner. The concentrations of these inflammatory cytokines in the culture media of LPS-stimulated DCs were also decreased in the presence of β-damascone (**Figure 2B**), suggesting that suppression of the LPS-induced transactivation of cytokine genes by β-damascone was reflected in protein release. In addition, flow cytometric analysis revealed that LPS-induced upregulation of MHC class II and CD86 was also suppressed in β-damascone-treated DCs (**Figure 2C**). We also confirmed that the increased phosphorylation of IκB in LPS-stimulated DCs was reduced by the pretreatment with β-damascone (**Figure 2D**). These results indicate that β-damascone exhibits a suppressive effect on LPS-induced inflammatory responses and antigen presentation activity in DCs.

The effects of β-damascone on the activation of DCs via other TLRs

The effects of β-damascone on the activation of DCs caused by stimulants other than LPS were examined by using polyI:C, R-848, and CpG. Quantification of mRNA levels (**Figure 3A-3C**) and determination of protein concentrations (**Figure 3D-3F**) revealed that the transactivation and subsequent protein production of IL-6, IL-12p40, TNF-α, and IL-23p19 in BMDCs, which were induced by stimulation via TLR3, TLR7/8, or TLR9, were significantly suppressed by β-damascone. These results suggest that β-damascone exhibited suppressive effects against various TLR ligand-induced activation of DCs.

Involvement of the NRF2 pathway in the suppressive effects of β-damascone on DCs

Some substances derived from plants, especially phytochemicals (e.g., sulforaphane), often activate the NRF2 pathway by directly acting on Keap1, resulting in the induction of antioxidant and anti-inflammatory responses (Kensler et al. 2013). To clarify whether β-damascone activates the NRF2 pathway in DCs, we determined the NRF2 protein and *Hmox1* mRNA levels in DB-treated DCs. As shown in **Figure 4A**, a Western blot analysis revealed that the amount of NRF2 protein was increased in DCs in the presence of β-damascone and peaked at 1 h after the addition of DB to the culture medium. The mRNA levels of *Hmox1*, which is a target gene of NRF2, were markedly upregulated by β-damascone much higher than those induced by LPS (**Figure 4B**). These results showing the increase in NRF2 protein and *Hmox1* mRNA levels in β-damascone-treated DCs suggest the possibility that β-damascone activates the NRF2 pathway in DCs. Then, we investigated the roles of NRF2 in DB-mediated modification of DC function by using *Nrf2*$^{-/-}$ DCs. First, it was confirmed that the β-damascone-induced increase in *Hmox1* mRNA levels in DCs was almost abolished by NRF2 deficiency (**Figure 4C**). When OVA-pulsed *Nrf2*$^{-/-}$ BMDCs were cocultured with OT-II CD4^{+} T cells under a Th1-polalizing conditions, the suppression of Th1 development by β-damascone was not observed, whereas control (*Nrf2*$^{+/-}$) DC-dependent Th1 development was significantly suppressed in the presence of β-damascone (**Figure 4D**), as was the case for WT BMDCs (**Figure 1E**). Furthermore, IL-12p40 release from LPS-stimulated DCs tended to increase by *Nrf2* deficiency in DCs, and the suppressive effect of β-damascone on IL-12p40 production was markedly reduced in *Nrf2*$^{-/-}$ BMDCs compared with that in *Nrf2*$^{+/-}$ BMDCs (**Figure 4E**). These results demonstrate that DC functions, including Th1 induction and IL-12 production, were suppressed by β-damascone depending on activation of the NRF2 pathway in DCs.

Oral administration of β-damascone ameliorates contact hypersensitivity in mice

Finally, we investigated whether β-damascone, which inhibits DC-mediated inflammation in *in vitro* experiments, exhibits a biologically significant effect on immunorelated diseases by using CHS model mice (**Figure 5A**). First, we examined the effects of various doses of β-damascone on ear swelling symptoms in DNFB-treated mice and found that oral administration of β-damascone at 10 or 50 mg/kg per day from 2 days before sensitization suppressed ear swelling (data not shown). Under optimized doses, the suppressive effect of β-damascone on ear swelling was observed not only after challenge but also after rechallenge (**Figure 5B**), accompanied by a tendency of reduced mRNA levels of *Il6* in the lesional skin (data not shown). These results indicate that orally administered β-damascone modulates DC-induced immunoresponses *in vivo*.

Discussion

In the current study, we identified β-damascone from approximately 150 compounds as a novel immunomodulator through 2-step screening monitoring of DC-mediated immunoresponses. By conducting *in vivo* experiments using a mouse model, we demonstrated that oral intake of β-damascone, which activates the NRF2 pathway, ameliorated CHS inflammation. NRF2 plays a role as a master regulator of antioxidant and anti-electrophilic responses by transactivating the genes encoding antioxidant enzymes and drug metabolizing enzymes. In a previous study using a 2,4-dinitrochlorobenzene (DNCB)-induced CHS model, NRF2-deficient mice exhibited more severe inflammation accompanied by increased chemokine production and neutrophil recruitment in the skin than control mice (Helou et al. 2019). This observation that NRF2 deficiency deteriorates CHS may be coincident with our results, indicating the important roles of NRF2 in DC-mediated immune responses. In addition to CHS, various immunorelated inflammatory diseases and autoimmune diseases, including colitis and psoriasis, are reported to be exacerbated in NRF2 knockout mice (Khor et al. 2006, Ogawa et al. 2020, Yoh et al. 2001, Johnson et al. 2010). Although the effects of NRF2 deficiency are not restricted to DC function, because NRF2 is ubiquitously expressed, these studies of NRF2-deficient mice support the possibility that β-damascone could be useful for the prevention and/or treatment of inflammatory diseases and autoimmune diseases. We found that β-damascone significantly suppressed TLR7-mediated gene expression and protein production of IL-6, IL-12p40, and TNF-α in BMDCs (**Figure 3C** and **3F**). Because a topical application of imiquimod (Schön and Schön 2007, Gilliet et al. 2004), a ligand of TLR7 and TLR8, causes psoriasis-like pathology in mouse skin through activation of dermal DCs and Langerhans cells (van

der Fits et al. 2009), we expect that β-damascone ameliorates psoriasis, even from the point of view of modification of DC function. We will evaluate the effects of β-damascone on various immunorelated diseases, such as psoriasis, inflammatory bowel disease, and multiple sclerosis using a mouse model in the near future.

Our results using *Nrf2*$^{-/-}$ DCs indicated that the suppressive effects of β-damascone on IL-12 production in DCs and Th1 development activity of DCs are dependent on NRF2. In contrast, the suppressive effects of β-damascone on the production of TNF-α and IL-6 were still observed in *Nrf2*$^{-/-}$ DCs, suggesting that β-damascone modulates DC function through additional target(s) other than NRF2. It has been reported that the induction of the proinflammatory cytokine genes, *Il1a*, *Il1b*, and *Il6* is inhibited by NRF2, which binds to these genes and subsequently inhibits the recruitment of RNA polymerase II to target genes in macrophages (Kobayashi et al. 2016). However, under our experimental conditions, we could not find a significant contribution of NRF2 in the suppression of the production of IL-6 and TNF-α in DCs. Further detailed analysis to clarify other target(s) of β-damascone could lead to the development of novel anti-inflammatory immunomodulators.

In the current study, we identified β-damascone as the most effective compound from an aroma library and confirmed that oral administration of β-damascone ameliorated CHS. Although we obtained β-damascone as a natural compound candidate of immunomodulator in the current study, there is room for improvement; briefly, β-damascone could be developed to a drag carrying stronger activity and lesser toxicity by chemical structural modification. Further research using an organic chemical approach considering β-damascone as a lead compound should be conducted to develop a novel immunomodulator.

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Materials and Methods

Mice and cells

C57BL/6 and Balb/c mice were purchased from Japan SLC (Hamamatsu, Japan), and OT-II mice were obtained from The Jackson Laboratory (USA). *Nrf2*$^{-/-}$ mice were previously generated (Itoh et al. 1997). Mice were maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with the guidelines of the Institutional Review Board of Tokyo University of Science. The current study was specifically approved by the Animal Care and Use Committees of Tokyo University of Science: K21004, K20005, K19006, K18006, K17009, and K17012. Bone marrow-derived DCs (BMDCs) were generated from whole BM cells by cultivation in RPMI-1640-based media supplemented with 20 ng/mL mGM-CSF (BioLegend, San Diego, CA, USA) as previously described (Kanada et al. 2011). Naïve CD4^{+} T cells were isolated from the spleen by using the MojoSort Mouse Naïve CD4^{+} T Cell Isolation Kit (#480040, BioLegend). CD4^{+} T cells isolated from the OT-II spleen were cocultured with BMDCs, which were generated from C57BL/6 mice and were preincubated with OVA peptide 323-339 (POV-3636-PI, Peptide Institute, Inc., Osaka, Japan). To stimulate CD4^{+} T cells with plate-coated antibodies (Abs), anti-CD3ε Ab (clone 145-2111C, BioLegend) and anti-CD28 Ab (clone 37.51, TONBO Bioscience) were used. For Th1 polarization, 10 ng/mL mIL-12 (PeproTech Inc., Rocky Hill, NJ, USA) and 10 μg/mL anti-IL-4 Ab (clone 11B11, BioLegend) were added to the culture media. Th2 polarization was induced with 20 ng/mL IL-4 (PeproTech) and 10 μg/mL anti-IL-12 Ab (clone C17.8, BioLegend).

Damasone-β (#34059, Vigon International, East Stroudsburg, PA, USA) was diluted with DMSO. LPS (#L3024, Wako), poly-I:C (#P0913, Sigma), R848

(AG-CR1-3582-M005, AdipoGen, Liestal, Switzerland), and CpG (ODN1826, InvivoGen, San Diego, CA, USA) were used to stimulate BMDCs. DAPI (#11034-56, Nacalai Tesque Inc., Kyoto, Japan) was used to determine cell viability.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of mouse IL-2, IL-6, TNF-α, and IL-12p40 were determined by ELISA kits purchased from BioLegend (#431004, #431315, #430915, and #431604, respectively).

Flow cytometry

To analyze the proliferation of T cells, CFSE (eBioscience Inc., San Diego, CA, USA) was used. Cell surface MHC class II and CD86 on BMDCs were stained with anti-I-A/I-E-PerCP (clone M5/114.15.2, BioLegend) and anti-CD86-PE (clone GL-1, BioLegend), respectively. Intracellular IFN-γ and IL-4 were stained with anti-IFN-γ-PE/Cyanine7 (clone XMG1.2, BioLegend), and anti-IL-4-PE (clone 11B11, BioLegend), respectively, with anti-CD4-FITC (clone GK1.5, BioLegend) after treatment with the Fixation Buffer (#420801, BioLegend) and the Intracellular Staining Perm Wash Buffer (#421002, BioLegend). Fluorescence was detected by a MACS Quant Analyzer (Miltenyi Biotech) and analyzed with FlowJo (Tomy Digital Biology, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as previously described(Kitamura et al. 2012) with anti-phospho-IkBα (Ser32) (clone 14D4, Cell Signaling), anti-NRF2 (clone D1Z9C,

Cell Signaling), and anti-β-actin (clone AC-15, Sigma-Aldrich) Abs.

Quantitative RT-PCR

Total RNA was extracted from BMDCs using the ReliaPrep RNA Cell Miniprep System (#Z6012, Promega, Madison, USA) and from the skin using ISOGEN (#311-07361, Nippongene, Tokyo, Japan). Synthesis of cDNA, and quantitative PCR were performed as previously described (Ito et al. 2021). The nucleotide sequences of the primer sets are listed in **Supplementary Table SI**.

CHS model

Mice were sensitized on shaved abdominal skin with 25 μl 0.5% (w/v) DNFB in acetone/olive oil (4:1) and were challenged with an application of 20 μl 0.25% DNFB on the ear at 5 days after sensitization. Ear thickness was measured by a caliper.

Statistical analysis

To compare two samples, a two-tailed Student's t-test was used (Figure 1F, and 1G). To compare more than two samples, one-way ANOVA-followed by Tukey's multiple comparison test (Figure 1B, 1D, 1E, 2A, 2B, 3A-F, 4B-E, 5B, and 5C) or Dunnett's multiple comparison test (Figure 2C) was used. P values < 0.05 were considered significant.

Authorship Contribution

Contribution: N.K. performed experiments, analyzed data, and wrote the paper; H.O. performed experiments and analyzed data; K.N. analyzed data and wrote the paper; M.A. N.I. and T.Y. performed experiments; G.I. and M.Y. provided experimental tools; M.H. analyzed data and wrote the paper; C.N. designed research and wrote the paper.

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Legends

Figure 1. Effects of β-damascone on DC-mediated activation of T cells.

A. Structure of β-damascone.

B. The concentrations of IL-2 in the culture media of OT-II splenocytes. A total of 1.0 x 10^5 cells obtained from the whole spleen of OT-II were maintained in the presence or absence of 500 ng OVA peptide and the indicated concentration of β-damascone (β-D) in 200 μL culture media for 72 h.

C. Cell viabilities of DCs and T cells in the presence of β-damascone. BMDCs and naïve $CD4^+$ T cells were preincubated with the indicated concentrations of β-damascone for 24 h. Cell viability was judged with DAPI staining.

D. Division of OT-II $CD4^+$ T cells cocultured with OVA-pulsed BMDCs. CFSE-labeled OT-II $CD4^+$ T cells (5.0 x 10^4) were cocultured with or without C57BL/6 BMDCs (1.0 x 10^4), which were pretreated with 500 ng OVA peptide, in the presence or absence of the indicated concentrations of β-damascone in 200 μL of culture media for 72 h.

E. Division of $CD4^+$ T cells via stimulation with anti-CD3 and anti-CD28 Abs. $CD4^+$ T cells, which were isolated from the spleen of C57BL/6 mice, were labeled with CFSE, and then incubated with plate coated Abs as described in the Materials and Methods.

F. Effects of β-damascone on the frequencies of Th1 and Th2 cells developed under polarization conditions in a DC-dependent manner. OT-II naïve $CD4^+$ T cells were cocultured with OVA-pulsed C57BL/6 BMDCs under Th1 (top) or Th2 (bottom) polarization conditions in the presence or absence of β-damascone (30 μM) for 72 h as described in the Materials and Methods section using a gating strategy shown in **Supplementary Figure S2A**.

G. Frequencies of Th1 and Th2 cells induced under polarization conditions in an

APC-independent manner. C57BL/6 naïve T cells were stimulated with anti-CD3 and anti-CD28 Abs under Th1 (top) or Th2 (bottom) polarization conditions.

The Tukey-Kramer test was used (**B**, **D**, **E**, **F** and **G**). *; $p < 0.05$, **; $p < 0/01$, n.s.; not significant.

Figure 2. β-Damascone suppressed LPS-induced activation of DCs.

A. Messenger RNA expression levels in BMDCs.

B. Concentrations of cytokines in the culture media of BMDCs.

C. Cell surface expression levels of MHC class II and CD86 on BMDCs.

D. A Western blot profile of phosphorylated IκB.

BMDCs pretreated with 50 μM β-damascone for 24 h were stimulated with 100 ng/mL LPS. The cells were harvested at 3 h and 24 h after LPS stimulation to determine mRNA levels (**A**) and cell surface protein expression levels (**C**), respectively, and culture media were collected at 24 h after stimulation to measure the concentration of cytokines (**B**). For Western blot analysis, cells at 1 h after LPS stimulation were lysed, and aliquots containing a total of 10 μg protein were added to each lane (**D**). The data represent the mean ± SEM of three independent experiments (**A**, **B**, and **C**) performed in triplicate samples (**A** and **B**). A gating strategy of flowcytometric analysis of BMDCs is shown in **Supplementary Figure S2B**.

The Tukey-Kramer test (**A**, and **B**) or Dunnett's test (**C**) was used. *; $p < 0.05$, **; $p < 0/01$.

Figure 3. Suppressive effects of β-damascone on the stimulation of DCs by various TLR ligands.

The effects of β-damascone (50 μM) on mRNA expression levels (**A**, **B**, and **C**) and protein release of cytokines (**D**, **E**, **F**) in BMDCs stimulated with polyI:C (**A** and **D**), CpG (**B** and **E**), or R848 (**C** and **F**). The data represent the mean ± SEM of three independent experiments performed in triplicate samples.

The Tukey-Kramer test was used. *; $p < 0.05$, **; $p < 0/01$.

Figure 4. β-Damascone activated the NRF2 signaling in DCs, and NRF2 deficiency reduced the suppressive effects of β-damascone.

A. A Western blot profile showing NRF2 protein levels in BMDCs following treatment with β-damascone. BMDCs were incubated in the presence of 50 μM β-damascone for the indicated hours.

B. The mRNA levels of *Hmox1* in BMDCs. BMDCs cultured in the presence or absence of 50 μM β-damascone for 24 h were incubated with or without 100 μg/mL LPS for an additional 3 h.

C. The mRNA levels of *Hmox1* in BMDCs generated from NRF2-deficient mice ($Nrf2^{-/-}$) and control mice ($Nrf2^{+/-}$).

D. Th1 development induced by coculture with $Nrf2^{-/-}$ BMDCs or control BMDCs.

E. LPS-induced cytokine production from $Nrf2^{-/-}$ BMDCs and control BMDCs.

The data represent the mean ± SEM of three independent experiments performed in triplicate samples. The Tukey-Kramer test was used. *; $p < 0.05$, **; $p < 0/01$, n.s.; not significant.

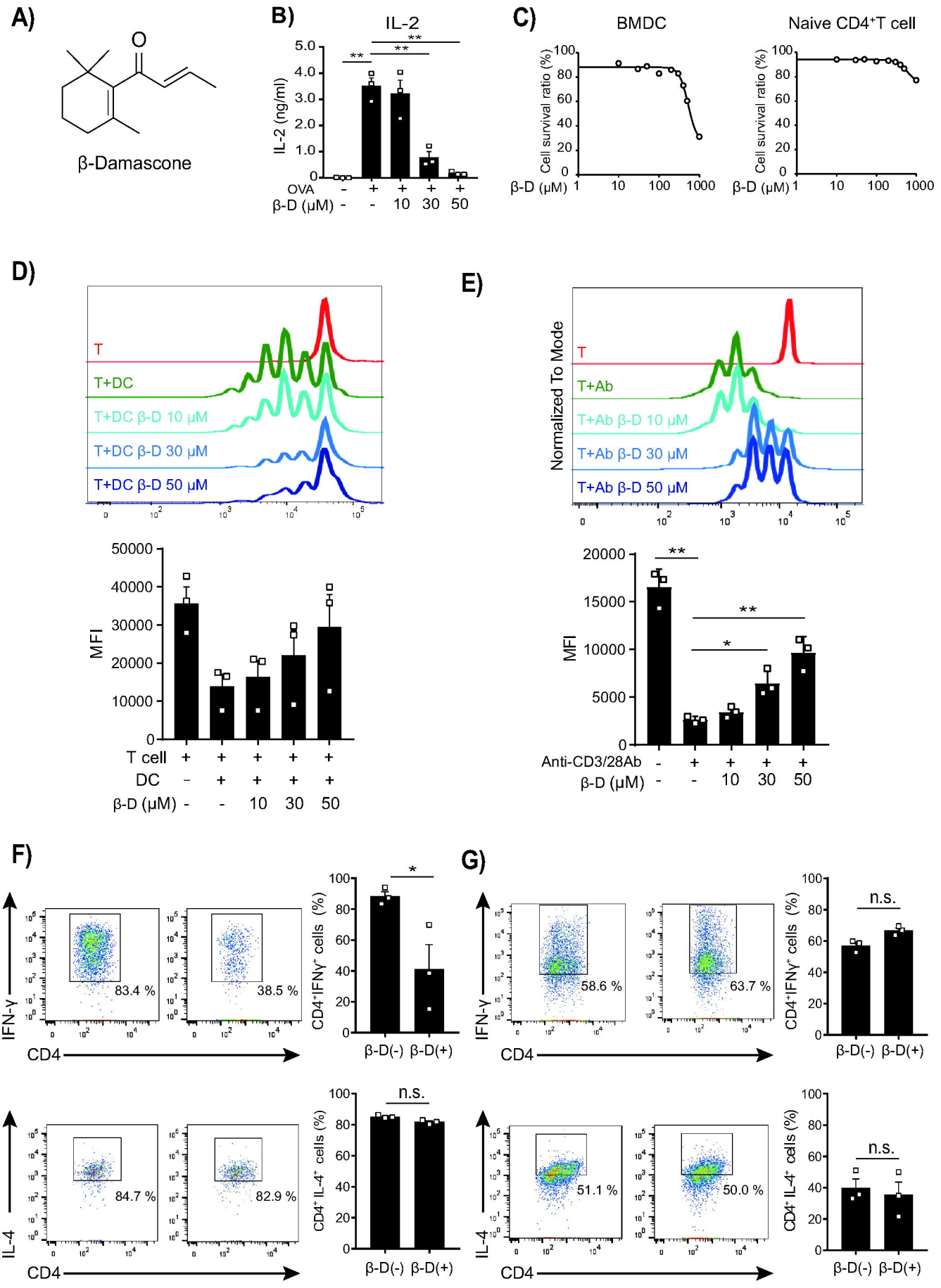
Figure 5. Oral administration of β-damascone ameliorated the pathology of CHS.

A. Schematic of the oral administration schedule of β-damascone in the DNFB-induced CHS model. The indicated amounts of β-damascone in 200 μl saline were orally administered every day. p.o.; per os.

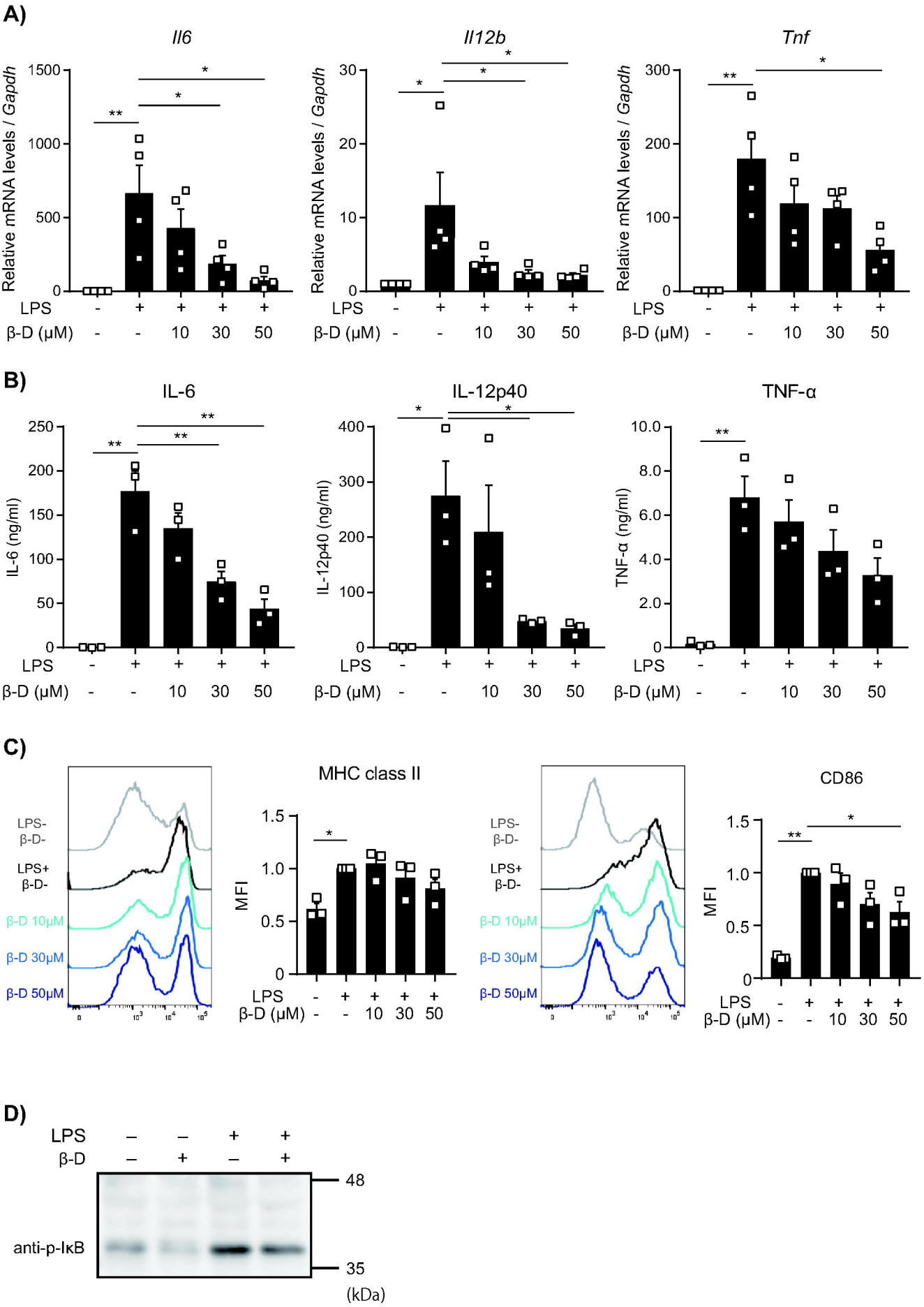
B. Ear swelling of CHS mice at the indicated time points.

(Ear swelling) = (ear thickness after challenge) – (ear thickness before the first challenge)

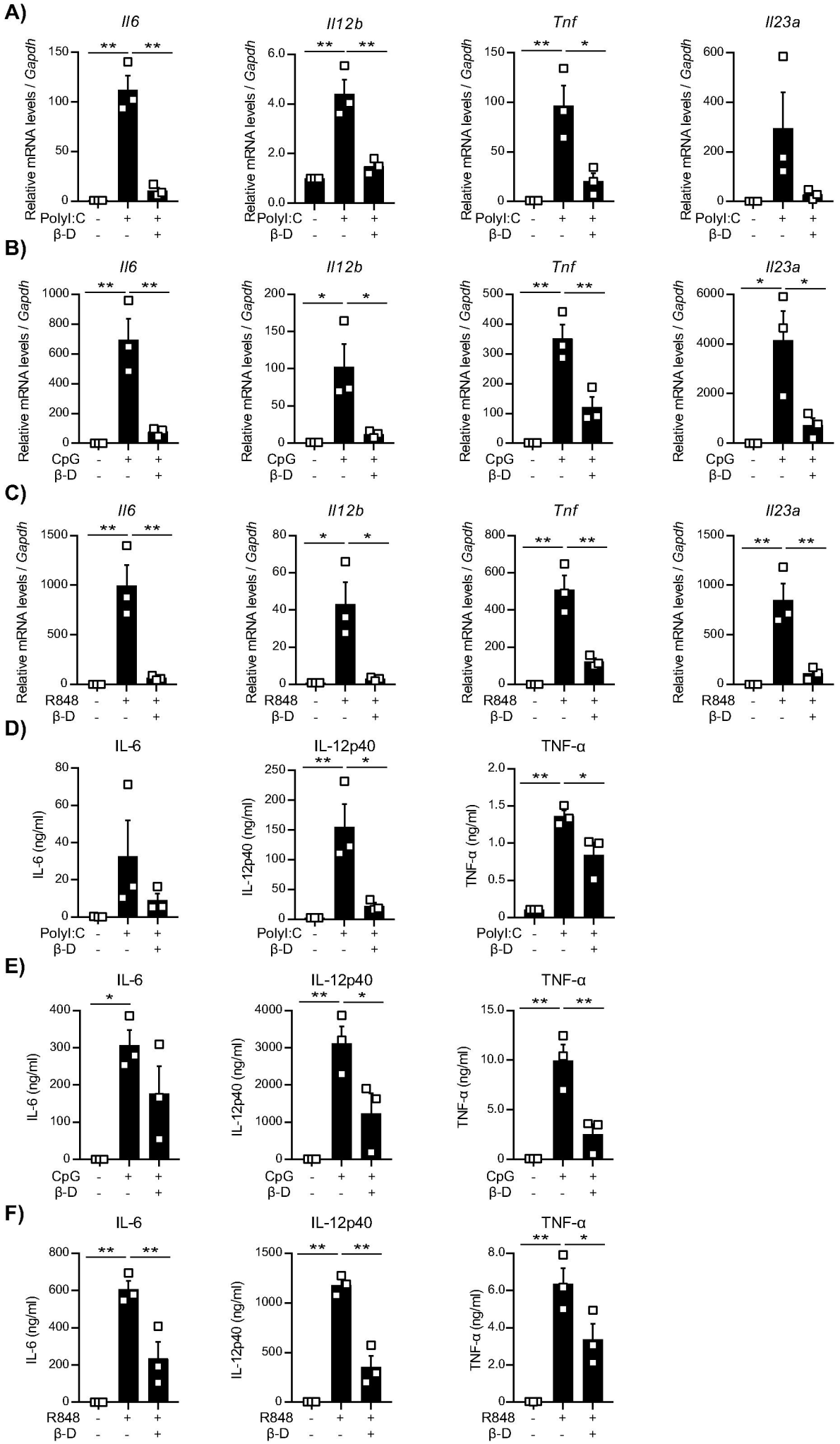
Data were pooled from 2 independent experiments. n = 9-10.



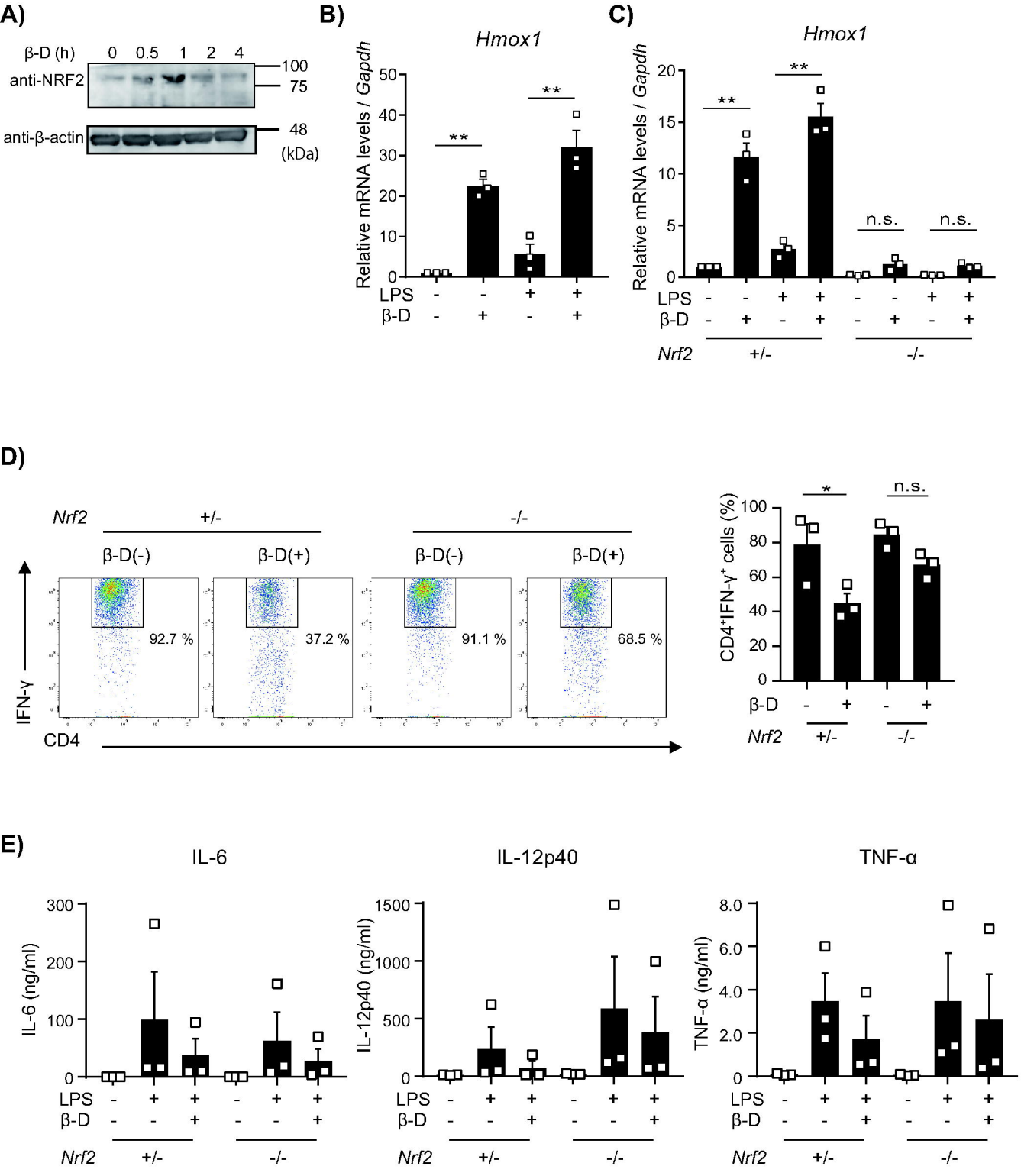
Kodama et al. Figure 1



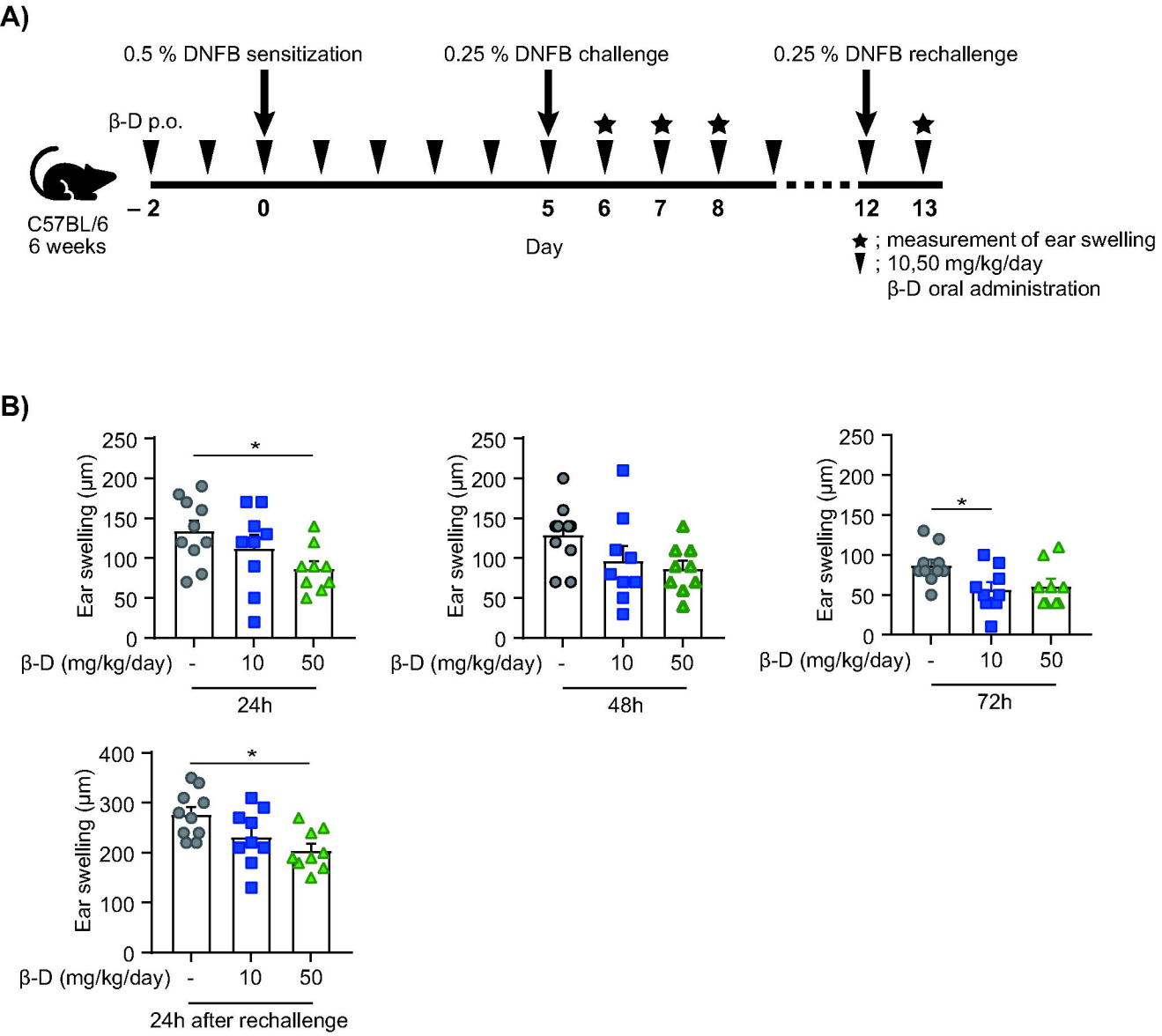
Kodama et al. Figure 2



Kodama et al. Figure 3



Kodama et al. Figure 4



Kodama et al. Figure 5