

Propolis induces Ca²⁺ signaling in immune cells

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Propolis possesses several immunological functions. We recently generated a conditional Ca²⁺ biosensor yellowameleon (YC3.60) transgenic mouse line and established a five-dimensional (5D) (x, y, z, time, and Ca²⁺ signaling) system for intravital imaging of lymphoid tissues, including Peyer's patches (PPs). To assess the effects of propolis on immune cells, we analyzed Ca²⁺ signaling *in vitro* and *in vivo* using CD11c-Cre/YC3.60^{lox} transgenic mice, in which CD11c⁺ dendritic cells (DCs) specifically express YC3.60. We found that propolis induced Ca²⁺ signaling in DCs in the PPs. Intravital imaging of PPs also showed that an intraperitoneal injection of propolis augmented Ca²⁺ signaling in CD11c⁺ cells, suggesting that propolis possesses immune-stimulating activity. Furthermore, CD11c⁺ cells in PPs in mice administrated propolis indicated an increase in Ca²⁺ signaling. Our results indicate that propolis induces immunogenicity under physiological conditions.

Key words: calcium signaling, gut, imaging, propolis, B cell, dendritic cell

INTRODUCTION

Propolis is produced by honeybees and is composed of resin, pollen, and other constituents from plants around beehives together with wax secreted by the bees. Therefore, the constituents of propolis collected worldwide are wildly diverse depending on the regions. Since ancient times, it has been used as an internal and external medicine worldwide. Propolis exhibits multiple biological functions, such as immunomodulatory, antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal, antiviral, and antiparasitic functions, and is consumed as a health food [1–5]. Among the various types of propolis, Brazilian green propolis is well studied because plants for its constituents have been clarified [6]. Brazilian green propolis has been reported to prevent and alleviate pollen allergy in human studies [7, 8]. Furthermore,

it functions to alleviate colds and recovery from colds [9]. It is also effective for improving immune system, especially in aged mice [10]. It has an adjuvant activity in vaccination of herpes simplex virus in cows [11] and mice [12, 13]. Moreover, propolis prevents the production of inflammatory cytokines and elevates anti-inflammatory cytokines in mice [14]. To clarify the biological properties of propolis, further physiological analyses are required.

Calcium ions (Ca²⁺) are universal second messengers that have multiple functions in most cells. In the immune system, the stimulation of immune receptors, including the B-cell antigen receptor, induces intracellular Ca²⁺ mobilization concomitant with other signaling events such as the phosphorylation of cellular substrates [15–18]. Ca²⁺ signaling regulates the mitogen-activated protein kinase, nuclear factor of activated T cells, and nuclear factor-κB pathways in B cells and plays a crucial role in B-cell development and function during humoral immune responses [15, 17].

We generated a conditional mouse model that expresses the Ca²⁺ indicator yellowameleon 3.60 (YC3.60). YC3.60 is a double-chromophore indicator that employs Förster/fluorescence resonance energy transfer between the cyan fluorescent protein (CFP) and a circularly permuted variant of the yellow fluorescent protein (YFP) Venus [19]. Ca²⁺ signaling is monitored by measuring the ratio of YFP to CFP

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(YFP/CFP). Therefore, YC3.60 is suitable for *in vivo* whole-body imaging in mice, particularly for identifying migrating immune cells.

Although propolis has health-promoting benefits, its mechanism of action is not completely understood. Therefore, to evaluate the effect of propolis on the immune system, *in vitro* and *in vivo* Ca^{2+} signaling in immune cells was evaluated using calcium biosensor transgenic mice. This transgenic mouse line conditionally expresses YC3.60 to visualize the temporal and spatial dynamics of Ca^{2+} signaling in immune cells, enabling the analysis of specific-cell functions under both normal physiological and pathological conditions. Here we analyzed the physiological effects of propolis on the gut immune cells using intravital imaging in Ca^{2+} biosensor mice.

MATERIALS AND METHODS

Propolis

A Brazilian green propolis ethanol extract, including 55% propolis extract as a solid content, was obtained from Yamada Bee Company, Inc. (Okayama, Japan). The extract was standardized to contain a minimum of 8.0% artemillin C and a minimum of 0.14% culifolin.

YC3.60 reporter mice

The floxed YC3.60 reporter (YC3.60^{fllox}) mouse line [20] was crossed with a CD11c-Cre mouse line [21], resulting in CD11c⁺ cell-specific YC3.60 expression in YC3.60^{fllox}/CD11c-Cre mice because of the loss of the loxP-flanked neomycin cassette. The YC3.60^{fllox} mouse line was also crossed with an IgG1-Cre mouse line [22], resulting in IgG1⁺ cell-specific YC3.60 expression. CD19-Cre/YC3.60^{fllox} mice were described previously [20]. All mice were maintained in our animal facility under specific pathogen-free conditions according to the guidelines of the Tokyo Medical and Dental University for animal care.

Flow cytometry

Flow cytometry analysis was performed using a MACSQuant Analyzer (Miltenyi Biotec). VioletFluor™ 450-labeled anti-B220, FTIC-labeled anti-CD4, and APC-labeled anti-CD86 antibodies were purchased from TONBO Biosciences, and phycoerythrin (PE)-labeled anti-CD69 antibodies were purchased from BioLegend. Dead cells were excluded by propidium iodide staining. Data analysis was conducted with FlowJo (FlowJo, LLC).

In vitro culture assay

Spleen cells from C57BL/6 mice (3×10^6 cells) were suspended in RPMI-1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol, 0.3 g/L L-glutamine, and 10% fetal bovine serum and seeded at 1 mL/well into 24-well culture plates. Cells were incubated in 5% CO_2 at 37°C for 48 hr.

Fluorescent microscopy

Organs and tissues were observed under an M165 FC fluorescent stereoscope with an FL600 (Leica).

Intravital and in vitro microscope

PPs and IECs from anesthetized mice were imaged. PPs were surgically exposed, immobilized on a microscope stage, and maintained at 37°C [20]. For image acquisition, a Nikon A1 laser scanning confocal microscope with a 20 × objective and NIS-Elements AR software was used, as previously described [20]. We also used dichroic mirrors (DM457/514) and two band-pass emission filters (482/35 for CFP; 540/30 for YFP). The YFP/CFP ratio was obtained by excitation at 458 nm. PE and Alexa-647 were excited at 488 nm and 633 nm, respectively, and DM405/488/561/640 and band-pass emission filters (525/50, 595/50, and 700/75) were used for image acquisition. Images of purified spleen cells in PBS were also obtained as described above. Acquired images were analyzed using the Nis-Elements software (Nikon).

In vivo stimulatory assay

We injected 50 µg of propolis in PBS into the peritoneal cavity of mice. After 2 hr, mice were subjected to intravital imaging analysis. PBS was intraperitoneally injected into the mice as a control, as previously described [20].

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test. A *p* value of <0.05 was considered statistically significant.

RESULTS

Effects of propolis on splenocytes

There are various types of propolis worldwide. Here, we used an ethanol extract of Brazilian propolis provided by Yamada Bee Company, Inc. Although the effect of propolis on splenocytes has been previously reported [23], the results of the study were controversial possibly because of the use of different constituents of propolis. Therefore, in this study, we first tested the effect of the propolis that we used on splenocytes. Splenocytes from C57BL/6 mice were cultured, and various doses of propolis were applied. The viability and expression of lymphocyte activation markers were then measured. Propolis did not have a remarkable effect on the viability of the splenocytes or on the expression of CD86 on B cells and CD69 on T cells. LPS, used as a positive control, induced CD86 and CD69 expression and increased the viability of the splenocytes (Fig. 1). These data suggest that Brazilian propolis does not possess lymphocyte proliferation activity, at least *in vitro*.

Propolis induces Ca^{2+} signaling in B cells and dendritic cells in vitro

We evaluated the effect of propolis on Ca^{2+} signaling in various immune cells. Monocyte-derived cells such as

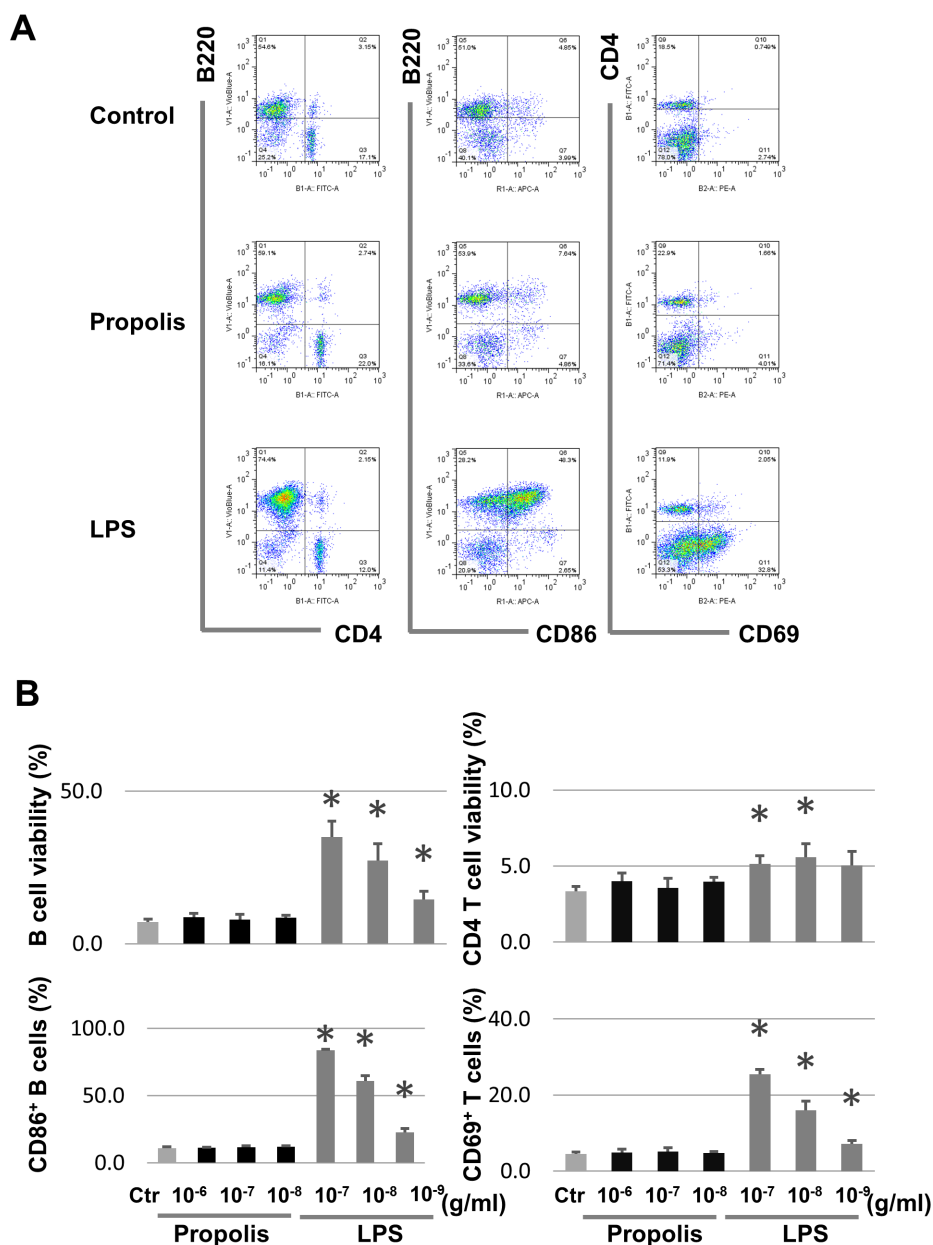


Fig. 1. Effect of propolis on survival and activation of spleen cells.

(A) Effect of propolis on activation of spleen cells. Spleen cells were cultured without or with propolis for 2 days, and CD86 and CD69 expressions were analyzed. Cells were cultured with LPS as a positive control for B cells. (B) Dose dependency of propolis on survival and activation of spleen cells. *p<0.05 (t-test).

dendritic cells (DCs) express various receptors such as Toll-like receptors, NOD family receptors, RIG family receptors, and lectin receptors to regulate pathogens [24]. CD11c is a well-known surface marker of DCs; other subpopulations of cell types such as B cells and macrophages also express CD11c on their cell surface. To examine whether propolis induces Ca²⁺ signaling in DCs, we crossed YC3.60^{flox} mice [20] with CD11c-Cre mice [21]. We then measured YC3.60

expression. In Peyer's patches (PPs), CD11c-Cre/YC3.60^{flox} mice showed extrafollicular localization of YC3.60-expressing cells, whereas CD19-Cre/YC3.60^{flox} and IgG1-Cre/YC3.60^{flox} mice showed YC3.60-expressing cells in the follicular and second follicular regions, respectively (Fig. 2A). In the spleen, YC3.60-expressing cells were sparsely located in the T-cell regions (Fig. 2B). These results indicate that DCs express YC3.60 in the CD11c-Cre/YC3.60^{flox} mice.

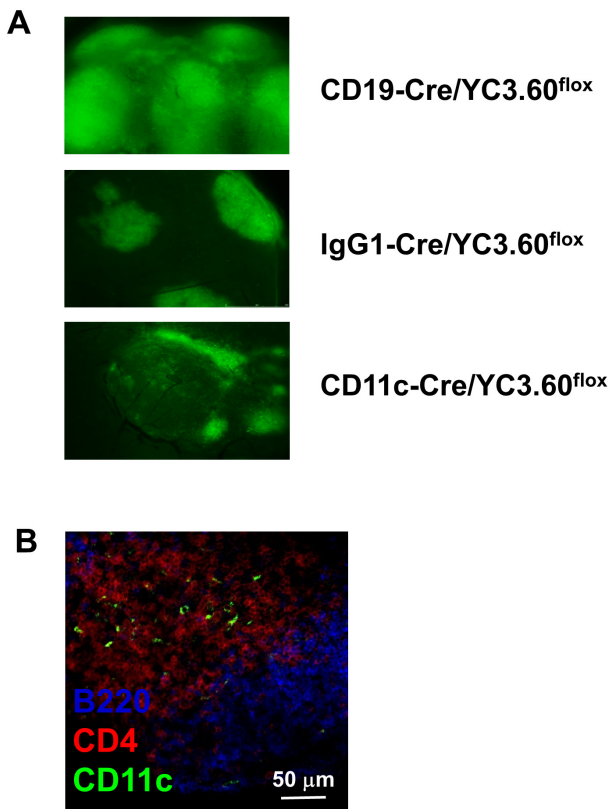


Fig. 2. Property of CD11c-Cre/YC3.60^{fl}ox mice.

(A) Representative images of Peyer's patches of cell-specific YC3.60 mice. Peyer's patches were analyzed by fluorescent microscopy ($n > 3$ mice). YC3.60-expressing cells are shown in green. (B) YC3.60-expressing cells in the spleen of CD11c-Cre/YC3.60^{fl}ox mice. The spleen was fixed with 4% PFA and then sliced and stained with anti-B220 mAb (blue) and anti-CD4 mAb (red). YC3.60-expressing cells are shown in green.

Next, we examined whether propolis induces Ca²⁺ signaling in DCs. As shown in Fig. 3A, propolis induced Ca²⁺ signaling in bone marrow-derived DCs from the CD11c-Cre/YC3.60^{fl}ox mice. Intracellular Ca²⁺ concentrations of DCs were transiently elevated just after adding propolis, and the Ca²⁺ concentrations immediately decreased (Fig. 3B). We also examined whether propolis stimulates B cells, as another type of immune cells. Propolis also induced transient Ca²⁺ signaling in B cells from CD19-Cre/YC3.60^{fl}ox mice (Fig. 4). These results strongly suggest that propolis directly stimulates B cells.

Propolis induces Ca²⁺ signaling in dendritic cells in vivo

We demonstrated that propolis induces Ca²⁺ signaling in immune cells *in vitro*. Next, we examined the immunostimulatory action of propolis *in vivo*. We injected propolis into the peritoneal cavity of CD11c-Cre/YC3.60^{fl}ox mice and then analyzed Ca²⁺ signaling in DCs using intravital imaging. DCs in PPs of propolis-injected mice exhibited

elevated Ca²⁺ concentrations compared with those of control mice that exhibited low intracellular Ca²⁺ concentrations (Fig. 5A and B). Cells exhibiting high intracellular Ca²⁺ concentrations robustly migrated (Fig. 5C and D and Supplementary Movie 1). These results suggest that propolis activates DCs.

Oral administration of propolis elevates Ca²⁺ signaling in dendritic cells

Propolis stimulates B cells and DCs. To assess the effect of orally administered propolis on DCs *in vivo*, we measured Ca²⁺ signaling in DCs using intravital imaging. After free administration of 1% propolis for 1 week, we performed an intravital imaging analysis of PPs in CD11c-Cre/YC3.60^{fl}ox mice. Compared with DCs in control mice, intracellular Ca²⁺ concentrations in DCs were increased by approximately three fold (Fig. 6). At the steady state concentration in pathogen-free mice, approximately 1% DCs exhibited high intracellular Ca²⁺ concentrations, suggesting that propolis regulates immune cells by modulating Ca²⁺ signaling.

DISCUSSION

In this study, we evaluated the effect of propolis on immune cells by measuring intracellular Ca²⁺ signaling in DCs using conditional calcium biosensor YC3.60 transgenic mice. We found that propolis directly induced Ca²⁺ signaling in B cells and DCs. Furthermore, we demonstrated that propolis exhibited stimulatory activity *in vivo*. In addition, the oral administration of propolis stimulated DCs in PPs. Altogether, our data clearly indicated that propolis possesses immune-stimulating properties.

We also demonstrated, for the first time, that Brazilian propolis induced Ca²⁺ signaling in immune cells. A main constituent of Brazilian green propolis, Artepillin C, activates transient receptor potential ankyrin 1 (TRPA1) channels, which induce Ca²⁺ flux [25]. As lymphocytes express various TRP channels [26], propolis-induced Ca²⁺ signaling in B cells may be mediated by TRP channels such as TRPA1. However, the inhibitory effects of propolis on Ca²⁺ signaling have also been reported. Constituents of propolis, such as CAPE and pinocembrin, prevent TCR-mediated and angiotensin II-induced Ca²⁺ signaling in endothelial cells, respectively [27, 28], although these compounds were rare in Brazilian green propolis. Thus, propolis appears to contain various constituents that exhibit contradictory biological functions. Further investigations are required to clarify the distinctive roles of propolis in Ca²⁺ signaling.

Although propolis induced Ca²⁺ signaling in immune cells, it did not induce the expression of activation markers on lymphocytes and their proliferation. Propolis-induced Ca²⁺ signaling may exhibit synergistic effects and modulate or augment other signaling pathways [1–5]. Thus, the effects of propolis on multiple biological functions may be because of its multiple constituents.

We previously established a 5D (x, y, z, time, and Ca²⁺

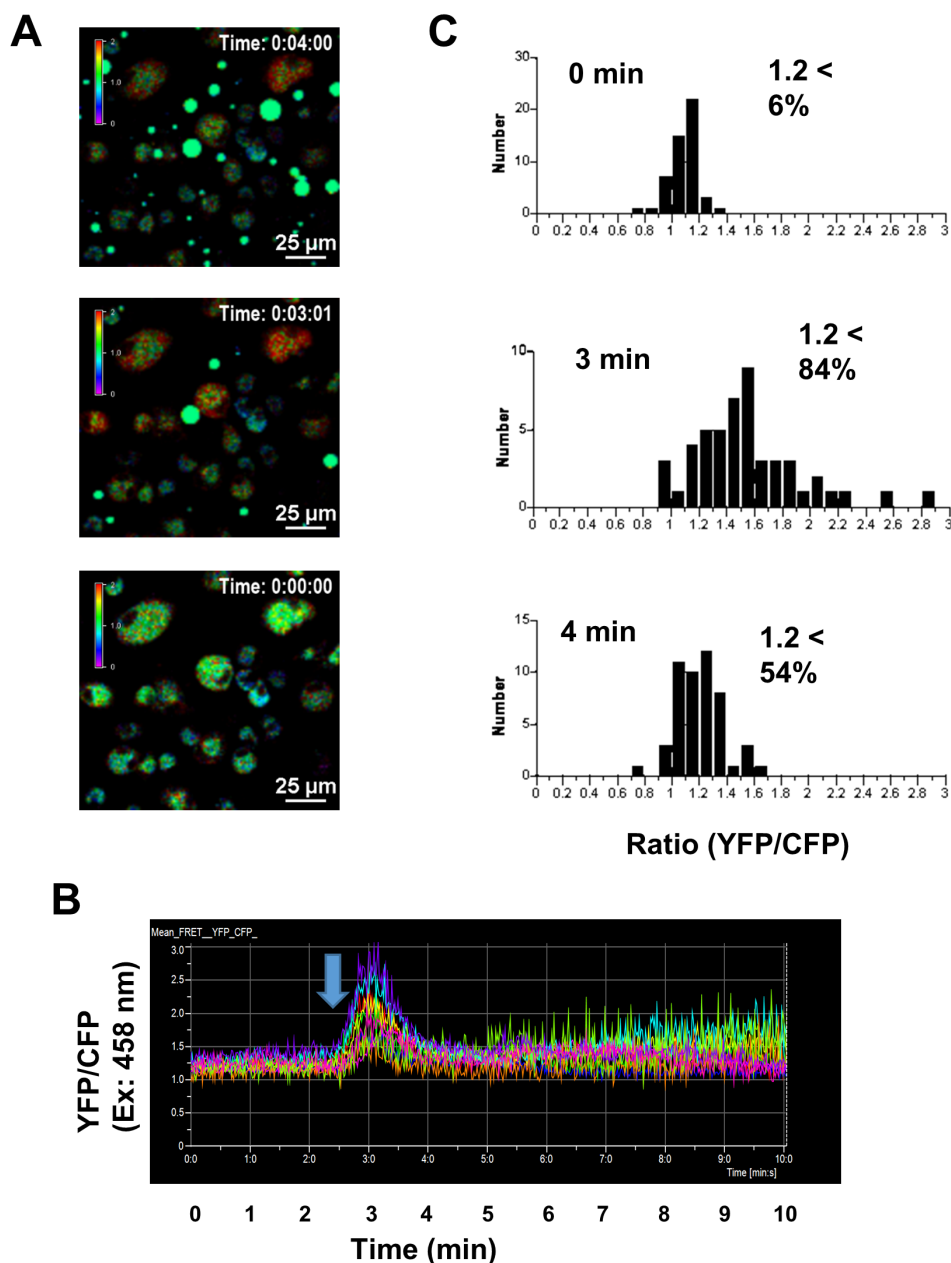


Fig. 3. Ca^{2+} signaling images mediated by propolis in DCs *in vitro*.

(A) Representative Ca^{2+} signaling images in DCs from CD11-Cre/YC3.60 mice. Radiometric images (YFP/CFP at excitation of 458 nm) are shown. Propolis in PBS (final concentration: 10 μ M) was added to the cell culture at the time point of 2 min 40 sec. A rainbow parameter indicates relative Ca^{2+} concentrations. (B) Time course for fluorescence intensities of the YFP/CFP ratio on excitation at 458 nm in the cells (n=10; frame=855, 1-sec interval). (C) Distribution of intracellular Ca^{2+} concentrations of randomly selected cells at the indicated time points (n=100). The results are representative of three experiments.

signaling) intravital imaging system in calcium biosensor mice [20]. Using this mouse model, we revealed that propolis directly induced Ca^{2+} signaling in immune cells. As IECs secrete cytokines that regulate immune cells [29], propolis may indirectly regulate immune responses through IECs. Furthermore, we showed that orally administered propolis

activated DCs, suggesting that these DCs activate T cells and/or B cells via their cognate interactions. Thus, propolis may affect immune responses both directly and indirectly. These observations may account for the previously reported multifunctional properties of propolis [23, 30].

Our line of conditional YC3.60 mice enabled the analysis

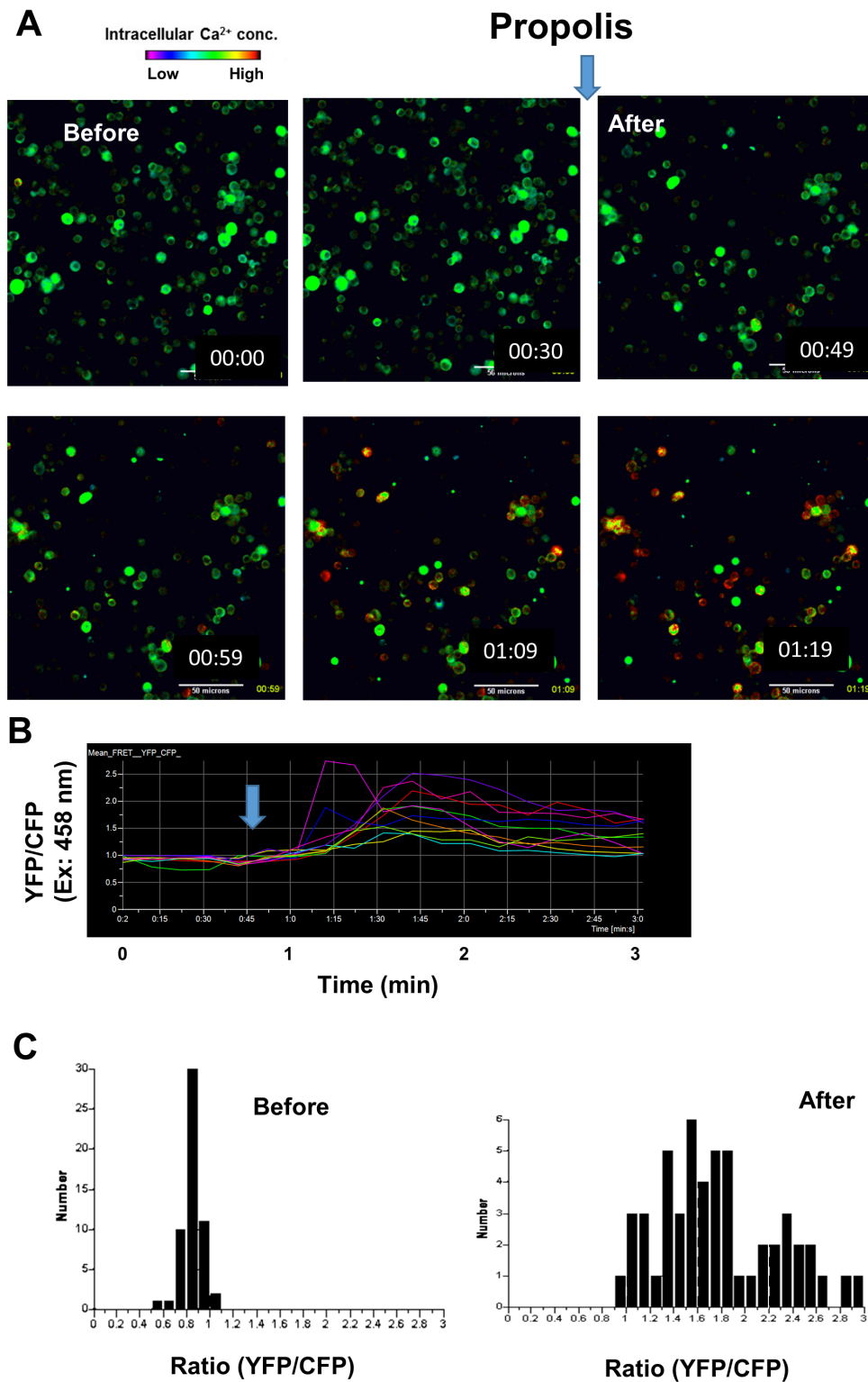


Fig. 4. Ca^{2+} signaling images mediated by propolis in B cells *in vitro*.

(A) Representative Ca^{2+} signaling images in spleen B cells from CD19-Cre/YC3.60 mice. Ratiometric images (YFP/CFP excitation at 458 nm) are shown. Propolis in PBS (final concentration: 10 μM) was added to the cell culture at the indicated time point. A rainbow parameter indicates relative Ca^{2+} concentrations. (B) Time course for fluorescence intensities of the YFP/CFP ratio on excitation at 458 nm in the cells ($n=10$). (C) Distribution of time-integrated intracellular Ca^{2+} concentrations of randomly selected cells ($n=10$; scale bar, 25 μm ; frame=19, 10-sec interval). These are representative results of three experiments.

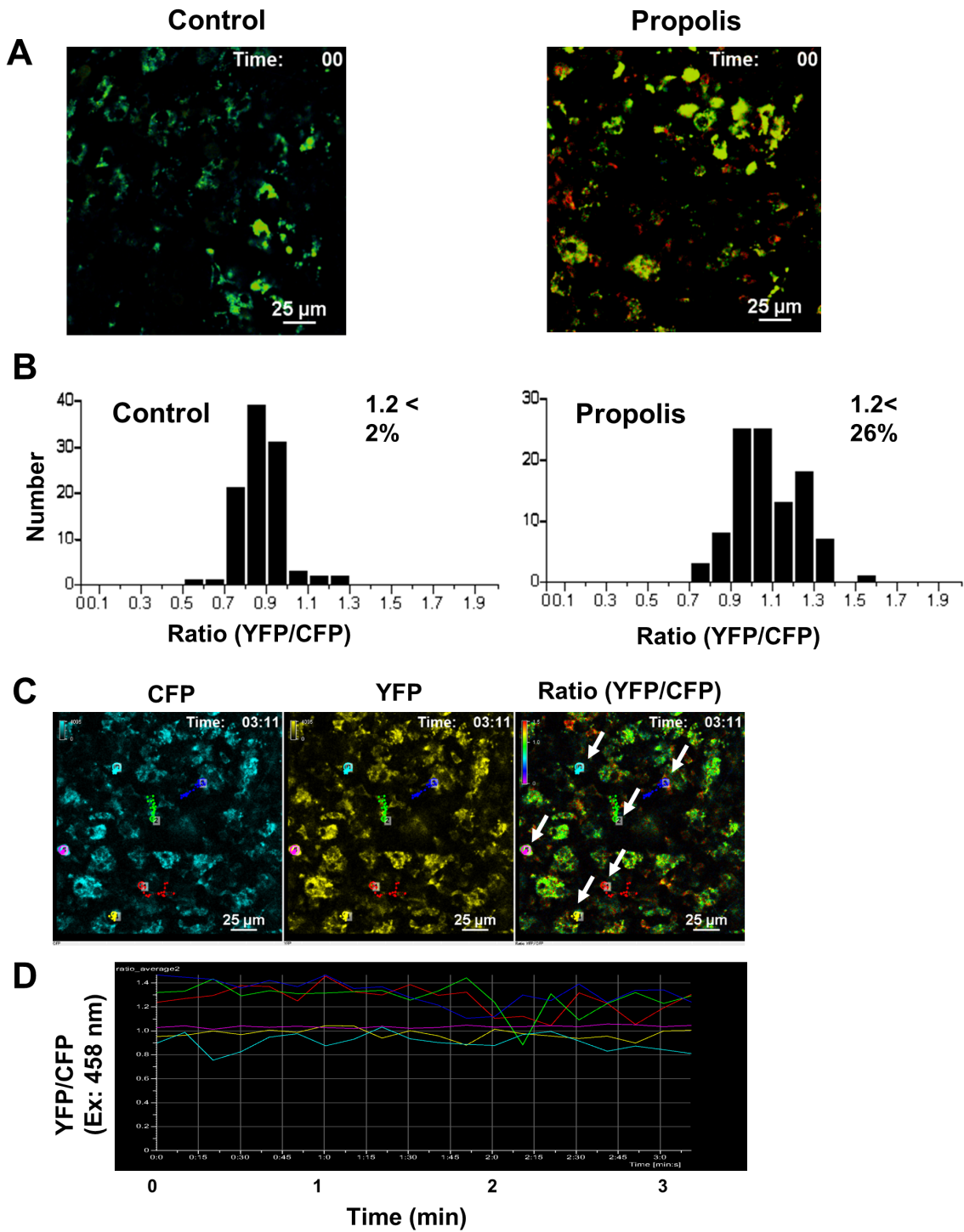


Fig. 5. Representative Ca^{2+} signaling images in Peyer's patches of the propolis-injected CD11c-Cre/YC3.60^{fllox} mice.

(A) Image of Peyer's patches of a CD11c-Cre/YC3.60^{fllox} mouse in which propolis was injected intraperitoneally. Two hours after injection, the mouse was subjected to intravital imaging. Representative Ca^{2+} signaling image in the Peyer's patches of a YC3.60^{fllox}/CD11c-Cre mouse injected with PBS as a control (left) and propolis/PBS (right). Only radiometric images (YFP/CFP excitation at 458 nm) are shown. Results are representative of at least three independent experiments (n=3 mice; scale bars, 25 μ m; [left] frame=34, 2 sec interval; [right] frame=20, 10-sec interval). (B) Distribution of intracellular Ca^{2+} concentrations in randomly selected cells (n=100). (C) Tracking images of DCs in Peyer's patches of a CD11c-Cre/YC3.60^{fllox} mouse intraperitoneally injected with propolis. Analyzed cells (right panel in A) are indicated by arrows. (D) Time course for fluorescence intensities of the YFP/CFP ratio on excitation at 458 nm in the cells. Results of the cells indicated in (C) are shown.

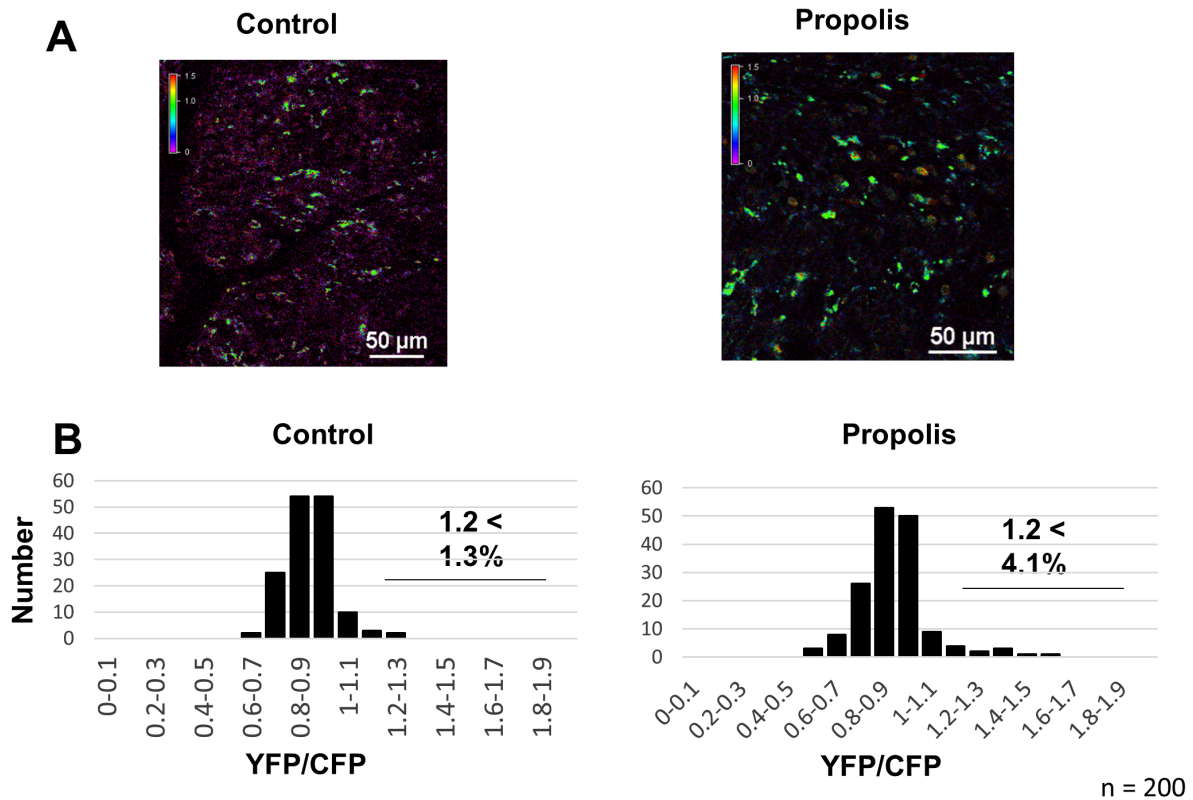


Fig. 6. Representative Ca^{2+} signaling images in Peyer's patches of CD11c-Cre/YC3.60^{lox} mice administered propolis.

(A) Image of PPs of a CD11c-Cre/YC3.60^{lox} mouse after administration of propolis for one week. Representative Ca^{2+} signaling image in the PPs of a CD11c-Cre/YC3.60^{lox} mouse (right). Only radiometric images (YFP/CFP excitation at 458 nm) are shown (scale bars, 50 μm). (B) Distribution of intracellular Ca^{2+} concentrations of randomly selected regions (n=200). This is a representative image from >3 mice.

of propolis-induced Ca^{2+} signaling in specific cells under physiological conditions. For a comprehensive understanding of the biological effect of propolis, further studies are necessary.

AUTHOR CONTRIBUTIONS

T.A., S.Y., H.T., N. M. T., T. O. and H.K. designed the research, and T.A. wrote the manuscript; T.A., S.Y., H.T., N.M.T. and T.K. performed the experiments, analyzed the data and prepared the figures.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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