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# Additional Analysis of Effects of Young Barley Leaf Powder on Immune Function

## —A Randomized, Double-Blind, Placebo-Controlled, Parallel-Group Study

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## Abstract

**Background:** The health benefits of young barley leaves, known for their high dietary fiber content, have been explored over several decades. In a previous study on healthy adults, we found that intake of young barley leaf powder for 8 weeks notably reduced the number of cumulative days in which participants experienced cold-like symptoms compared to a placebo. This improvement was accompanied by increased expression of HLA-DR on a specific subset of conventional dendritic cells (cDCs), namely cDC1 cells characterized by CD11c, CD4, and CD141 expression. However, it remains unclear whether these findings extend to total cDCs and whether they are associated with T cell activation. Here, we conducted an additional analysis using samples from previous research to assess the impact of young barley leaf powder on the activation of total cDCs and T cells in healthy participants. **Methods:** We performed a randomized, double-blind, placebo-controlled, parallel-group study with healthy participants. Fifty-six individuals were enrolled and randomly assigned to groups receiving either young barley leaf powder or a placebo for 8 weeks. We assessed their immune functions by measuring the expression levels of HLA-DR, CD86, and CD40 on $CD11c^+$ cDCs, and CD69 on T cells at baseline and after 8 weeks of intake. **Results:** After 8 weeks, the expression levels of HLA-DR, CD86, and CD40 on $CD11c^+$ cDCs, as well as CD69 on T cells, were significantly higher in the group consuming young barley leaf powder compared to the placebo group ($P < 0.05$). **Conclusions:** Intake of young barley leaf powder enhanced the activity of cDCs and T cells, and improved the physical condition of healthy adults.

**Keywords**

Young Barley Leaf Powder, Conventional Dendritic Cells, T Cells, Immunity, Physical Condition

## 1. Introduction

During daily life, our immune system protects us from infections caused by pathogens like viruses and bacteria [1]. Maintaining a healthy immune function is crucial for defending against infections, reducing the onset of cold-like symptoms, relieving these symptoms, and promoting overall health. The immune system is divided into two main components: innate immunity and adaptive immunity. Innate immunity provides an immediate, non-specific response to invading pathogens, primarily through the actions of macrophages and dendritic cells (DCs). In contrast, adaptive immunity targets specific antigens after receiving information from antigen-presenting cells, with T cells and B cells playing key roles in this process [2]. Dendritic cells are particularly important as they bridge innate and adaptive immunity, acting as the command center of immune response [3]. Among these cells, conventional dendritic cells ($CD11c^+$, conventional DCs: cDCs) are notable for their exceptional ability to activate T cells. They are the only antigen-presenting cells capable of inducing naïve T cells to become helper T cells and killer T cells [3].

cDCs are further divided into two distinct subpopulations: cDC1 ($CD141^+CD1c^-$) and cDC2 ($CD1c^+CD141^-$) [3] [4]. cDC1, which comprises 5% - 10% of all cDCs in humans, primarily activates type 1 helper T cells and killer T cells, which are crucial for defense against infection [3] [4]. Conversely, cDC2 mainly activates type 2 and type 17 helper T cells [3] [4], but also partially contributes to the direct activation of type 1 helper T cells and killer T cells [5] [6]. Furthermore, cytokines from type 1 helper T cells partially activated by cDC2 can enhance the killer T cell-inducing ability of cDC1 [7]-[9]. Thus, cDCs complement each other in activating various T cell types, thereby bolstering infection defense. Evaluating the overall activation capacity of cDCs is essential for understanding their role in T cell activation and immune defense against pathogens.

Young barley leaf powder is rich in dietary fiber, particularly insoluble fiber, and recent studies have highlighted its immune-boosting effects [10]-[12]. A previous placebo-controlled, randomized, double-blind, parallel-group trial in humans showed that intake of young barley leaf powder led to increased cDC1 activation, NK cell activation, and reduced cold-like symptoms [13]. However, the mechanism by which young barley leaf powder reduces cold-like symptoms, especially its impact on T cell activation via triggering cDCs, remains unclear. An earlier study focused on the cDC1 subpopulation only, neglecting the overall activity of cDCs [13], which is necessary to understand the full range of immunoregulatory effects. Since the clinical impact of cDC1 is more limited compared to

the entire cDC population, evaluating cDC1 alone may not fully capture the immune regulatory effects. In this study, we conducted additional analyses using data and samples from a previous study [13] to assess the activation of total cDCs, reflecting the broader immunomodulatory effects of consuming young barley leaf powder, as well as the consequent activation of T cells that occurs in association with enhanced cDC activity.

## 2. Materials and Methods

This study is an additional analysis using data and specimens from a previously reported study [13], focusing on newly assessed cDC and T cell activation. Both the original and current study protocols are registered with the University Hospital Medical Information Network Research Center's clinical trial system. The registration ID for the earlier study [13] is UMIN000046561, titled "A Study on the Effect of Plant-Derived Products on Immune Function—A Randomized, Double-blind, Placebo-controlled, Parallel-group Study". The current study is registered under ID UMIN000054553, titled "A Study on the Effect of Plant-Derived Products on Immune-related indicators". The primary outcomes of the current study were registered as immune-related indicators in the registry, and were not changed after the start of the study. The Ethical Committee of Kobuna Orthopedics Clinic, chaired by Toshio Kawada, approved this trial on December 23, 2021. It was conducted under physician supervision, adhering to the "Declaration of Helsinki, October 2013, WMA Fortaleza General Assembly (Brazil), as amended" and the "Ethical Guidelines for Life Science and Medical Research Involving Human Subjects" (established on March 23, 2021). The trial took place at the Nihonbashi Cardiology Clinic from December 2021 to April 2022. Additional measurements in this trial were part of the original protocol from the previous study [13], and no procedural changes occurred after the trial commenced.

### 2.1. Study Participants [13]

The target number of participants for this trial was determined using data from a previous study on the effects of young barley leaf powder on the secretion of secretory immunoglobulin A (sIgA) in saliva [12]. The calculations were based on an effect size of 0.89, a significance level of 0.05, and a power of 0.80. To account for potential dropouts, the target was set at 28 participants per group, resulting in 56 participants. Paid volunteers (19 males and 37 females) who met the inclusion criteria and did not meet any exclusion criteria were enrolled by the principal investigator. Before the study commenced, participants received a detailed explanation of the study procedure and sample measurements, and provided written informed consent.

The inclusion criteria were as follows: 1) Healthy males and females aged 20 to 64 years old; 2) Participants who can carry out self-judgment and give written informed consent voluntarily. The exclusion criteria included the following: 1) Participants who contracted or were undergoing treatment for or had a history of

serious diseases (e.g., diabetes, liver disease, kidney disease, heart disease, thyroid gland disease, adrenal gland disease, and/or metabolic disease); 2) Those who had a chronic disease and regularly used medications; 3) Those who had a history and/or a surgical history of digestive disease affecting digestion and absorption; 4) Those who were judged as unsuitable for the current study through blood tests during the screening test; 5) Those who had no subjective symptoms of upper respiratory tract infection in the last two winters; 6) Those who contracted pollinosis or allergic rhinitis and used drugs; 7) Those who could not stop using supplements and/or functional foods (including Food for Specified Health Uses or Foods with Function Claims) affecting immune functions; 8) Those who had excessive alcohol intake more than approximately 60 g/day of pure alcohol or equivalent; 9) Those who were smokers; 10) Those who had a habit of strenuous exercise such as running or soccer; 11) Those who could not stop drinking a day before each measurement; 12) Those who declared an allergic reaction to ingredients of test foods; 13) Those who were under treatment for or had a history of drug addiction and/or alcoholism; 14) Those who were shift workers and/or midnight shift workers; 15) Those who were planning to go abroad, such as an overseas trip; 16) Those who were pregnant or breastfeeding or planning to become pregnant; 17) Those who used drugs (including antiallergic drugs and antibiotics) known to affect immune function within two weeks prior to the screening test; 18) Those who donated over 200 mL of blood and/or blood components within the last four weeks prior to the screening test or over 400 mL of blood and/or blood components within the last three months prior to the screening test; 19) Those who were planning to participate and/or had participated in other clinical studies within the last month prior to the current study; 20) Those who were judged as unsuitable for the current study by the investigator for other reasons.

## 2.2. Study Methods [13]

This study was a 9-week, placebo-controlled, randomized, double-blind, parallel-group trial with a 1:1 allocation ratio, consisting of a 1-week pre-observation phase followed by an 8-week intervention phase. Participants were enrolled by the principal investigator according to predefined inclusion and exclusion criteria. A statistical analyst used computer-generated random numbers to allocate the participants according to a block randomization method (block size 4) with sex, age, and cDC activity markers as the adjustment factors. An independent controller, not otherwise directly involved in the trial, assigned participants to the two randomized groups, either the active group or placebo group, created a key code documenting the allocation, and sealed it. The sealed code was stored securely and was not opened until after the population analysis had been finalized, thereby maintaining blinding to persons other than the controller.

During the study, participants consumed two packets per day (a total of 5.4 g/day) of their assigned food, dissolved in an appropriate amount of water or hot water. They were instructed to maintain their usual lifestyle; avoid the use of sup-

plements or health foods; not change their frequency of intake if regular consumers of foods containing lactobacilli or bifidobacteria; avoid excessive alcohol consumption; and refrain from participating in other clinical studies. Before the test visit, participants were instructed to abstain from alcohol the day before, obtain sufficient sleep the night before, consume only water after 9:00 PM the night before, and avoid strenuous exercise from the day before until test completion. The use of medications was permitted only with approval from the principal investigator or a sub-investigator, except in emergencies.

All participants were given food diaries and participant diaries. The following survey items were entered daily during the intake period, starting on 1 week before intake: 1) Test food intake status; 2) Changes in living conditions; 3) Presence/absence of hospital visits; 4) Presence/absence of menstruation; 5) Presence/absence of bowel movements; 6) Presence/absence of pharmaceutical use; 7) Dietary contents.

### 2.3. Test Food [13]

The powdered active beverage was made by combining maltose with young barley leaf powder (Toyo Shinyaku Co., Ltd.). The placebo beverage, also a powdered maltose formulation without young barley leaf powder, was adjusted with coloring and flavoring to be indistinguishable in appearance from the active beverage. Both the active and placebo beverages were administered at a dose of 5.4 g per day, provided as two 2.7 g portions packaged in plain aluminum bags to ensure the blinding of the research participants and intervention providers. The active beverage provided 0.7 g of young barley leaf-derived dietary fiber per daily intake. The caloric and nutritional values of the test food were the same as those previously reported in [13].

### 2.4. Additional Analysis Test Items

The primary outcomes of the additional analysis were the activation of total cDCs and T cells.

A. Evaluation of Human Peripheral Blood T Cell Activity

Additional analysis was performed using FlowJo software (version 10.10.0; Tree Star) on data obtained from a previous flow cytometric study of activated NK cells [13] using the same participants. Peripheral blood mononuclear cells (PBMCs) collected from participants before and after the 8-week intake of either young barley leaf powder (providing 0.7 g/day of young barley leaf-derived dietary fiber) or a placebo were analyzed. Within the PBMC population, $CD45^+$ $CD56^-$ $CD3^+$ cells were identified as T cells. The mean fluorescence intensity (MFI) of CD69 in these T cells was calculated.

B. Evaluation of Human Peripheral Blood cDC Activity

cDC activity was assessed by measuring the expression levels of HLA-DR, CD86, and CD40, which are markers of cDC activity, in PBMCs collected before and after the 8-week period of consuming either young barley leaf powder or a

placebo. Following isolation of PBMCs from blood samples, the cells were stained with dead cells and antibodies for lineage markers (CD3, CD14, CD16, CD19, CD20, CD56), as well as CD11c, CD86, CD40, and HLA-DR. The stained cells were then analyzed using a flow cytometer (Attune NxT Flow Cytometer; Thermo Fisher Scientific). In the flow cytometric analysis performed with FlowJo software, $CD11c^{+}$ cells were identified as cDCs among the populations that were negative for dead cell staining and lineage markers. The mean fluorescence intensity (MFI) of HLA-DR, CD86, and CD40 in these cDCs was subsequently calculated.

### 2.5. Statistical Analysis

The analysis population was defined as the per-protocol set (PPS). Data on human peripheral blood cDC and T cell activity were compared between groups using an independent t-test for each sample's measured values and changes from baseline. In addition, a Smirnov-Grubbs rejection test was performed on changes from baseline to exclude outliers.

Statistical analyses were performed using statistical analysis software (IBM: SPSS Statistics 28), with the significance level at 5%. Data are presented as the mean ± standard error. No additional analyses such as subgroup or sensitivity analyses were performed.

## 3. Results

### 3.1. Analysis Participants [13]

A total of 56 participants (19 males and 37 females) were enrolled in this study, with no dropouts after randomization. However, 4 participants were excluded during the trial based on discontinuation criteria set by the principal investigator, leaving 52 participants who completed the study. The reasons for discontinuation were as follows: one participant in the active group experienced unrelated adverse health effects, and two more missed their scheduled examinations. One participant in the placebo group exhibited behavior compromising their reliability. A total of 8 participants were excluded from the analysis because of violations of precautions during the examination, resulting in 44 (15 males, 29 females) participants for the final analysis. The principal investigator decided to exclude these 8 participants from analysis before key opening. The exclusions were due to the following reasons: seven participants violated the trial instructions, specifically changes in their usual lifestyle (one participant in the active group and one in the placebo group had changes in their sleep habits, and one participant in the active group and four in the placebo group had changes in lifestyle rhythm (due to their work environment). Additionally, one participant in the active group developed a new illness (pollinosis) during the trial (exclusion criterion 6). The background of the analysis participants was previously reported in [13] and a flowchart of the process from inclusion to analysis is shown in **Table 1**.

The period from recruitment to the end of the follow-up for the study participants was December 2021 to April 2022, and the study was terminated when all

participants had completed the follow-up. During the period of the study, no adverse events occurred. All confirmed events were ruled out as causally related to the study foods by the investigators.

**Table 1.** Participants characteristics.

| | Placebo | Active |
|---|---|---|
| Number | 22 | 22 |
| Sex (Male/Female) | 7/15 | 8/14 |
| Age (years) | 40.0 ± 7.9 | 41.3 ± 9.2 |
| Height (cm) | 161.5 ± 6.8 | 163.5 ± 8.1 |
| Weight (kg) | 58.5 ± 8.5 | 62.2 ± 11.6 |
| BMI (kg/m$^2$) | 22.4 ± 2.4 | 23.1 ± 2.7 |
| MFI of T cell CD69 | 237 ± 20 | 236 ± 24 |
| MFI of cDC HLA-DR | 27,994 ± 6150 | 26,329 ± 5432 |
| MFI of cDC CD86 | 2963 ± 404 | 2707 ± 447 |
| MFI of cDC CD40 | 183 ± 44 | 178 ± 41 |

Values are expressed as means ± SDs. No significant difference was observed.

### 3.2. Activation of Human Peripheral Blood T Cells due to Young Barley Leaf Powder Intake

The results of analyzing CD69 expression, an indicator of T cell activation, are shown in Table 2. At this point, one clear outlier was identified in each group, so a Smirnov-Grubbs test was performed, and the two participants were identified and excluded as outliers. Afterwards, the active group showed a significant increase in the change from baseline T cell CD69 expression levels compared to the placebo group.

**Table 2.** Changes in MFI of T cell activation markers due to young barley leaf powder intake.

| | Group | *n* | Baseline | | 8 Weeks | | Changes from Baseline | |
|---|---|---|---|---|---|---|---|---|
| | | | mean ± S.E. | *P*-value (Between groups) | mean ± S.E. | *P*-value (Between groups) | mean ± S.E. | *P*-value (Between groups) |
| CD69 | Placebo | 21 | 237 ± 4 | 0.813 | 229 ± 5 | 0.232 | **−8 ± 4** | **0.038*** |
| | Active | 21 | 236 ± 5 | | 239 ± 6 | | **3 ± 4** | |

Values are expressed as means ± SEs. *Significantly different from the placebo group ($P < 0.05$).

### 3.3. Activation of Human Peripheral Blood cDCs due to Young Barley Leaf Powder Intake

The results of analyzing the expression levels of HLA-DR, CD86, and CD40, as indicators of cDC activation, are shown in Table 3. As a result, in the group comparison of the measured values after intake, the expression level of CD40 in $CD11c^+$ cDCs showed a significantly higher value in the active group compared to the placebo group. Additionally, in the group comparison of changes from baseline, the expression levels of HLA-DR, CD86, and CD40 in cDCs showed significantly higher values in the active group compared to the placebo group.

**Table 3.** Changes in MFI of cDC activation markers due to young barley leaf powder intake.

| | Group | *n* | Baseline | | 8 Weeks | | Changes from Baseline | |
|---|---|---|---|---|---|---|---|---|
| | | | mean ± S.E. | *P*-value (Between groups) | mean ± S.E. | *P*-value (Between groups) | mean ± S.E. | *P*-value (Between groups) |
| HLA-DR | Placebo | 22 | 27,994 ± 1311 | 0.347 | 23,881 ± 1375 | 0.564 | **−4112 ± 935** | **0.034*** |
| | Active | 22 | 26,329 ± 1158 | | 24,971 ± 1275 | | **−1357 ± 835** | |
| CD86 | Placebo | 22 | 2963 ± 86 | 0.053 | 2671 ± 120 | 0.390 | **−292 ± 102** | **0.032*** |
| | Active | 22 | 2707 ± 95 | | 2840 ± 154 | | **133 ± 162** | |
| CD40 | Placebo | 22 | 183 ± 9 | 0.709 | **139 ± 7** | **0.003*** | **−44 ± 9** | **0.002*** |
| | Active | 22 | 178 ± 9 | | **194 ± 16** | | **15 ± 16** | |

Values are expressed as means ± SEs. *Significantly different from the placebo group ($P < 0.05$).

## 4. Discussion

In this study, we aimed to clarify the mechanism by which young barley leaf powder suppresses the onset of cold-like symptoms, specifically the involvement of cDC activation and T cell activation. We conducted a placebo-controlled, randomized, double-blind, parallel-group trial to expand on a previous study [13]. The results showed that in participants who consumed young barley leaf powder, the expression levels of HLA-DR and co-stimulatory molecules CD86 and CD40 in cDCs, defined as $CD11c^+$, were significantly increased after 8 weeks of intake. Additionally, the expression of the activation marker CD69 in T cells was significantly increased in participants who consumed young barley leaf powder.

During T cell activation by cDCs, cDCs directly recognize and are activated by specific antigen components through the numerous pattern recognition receptors. Activated cDCs present cDC-degraded antigens on their cell surface via a major histocompatibility complex (MHC) class II (MHC-II, such as HLA-DR in humans), thereby inducing the differentiation of naïve T cells into helper T cells [3]. Additionally, activated cDCs upregulate the expression of co-stimulatory mole-

cules such as CD80, CD86, and CD40. CD80/86 activates naïve T cells, while CD40 promotes further proliferation and activation of activated helper T cells [14]. Furthermore, cDC activation and, consequently, T cell activation stimulate various immune cells through the production of diverse cytokines and chemokines, thereby contributing to the body's defense against infection [15] [16]. This activation axis of immune response aligns with results obtained from the same participant population, in which intake of young barley leaf powder suppressed the onset of cold-like symptoms [13]. Therefore, it is considered that cDCs activated by young barley leaf powder contribute to the alleviation of cold-like symptoms by inducing T cell activation through enhanced expression of MHC-II (HLA-DR) and co-stimulatory molecules (CD40, CD86).

In a previous report [13], no significant difference was observed in CD86 expression in $CD4^+CD141^+CD11c^+$ cells following young barley leaf powder intake. By contrast, in this study, an increase in CD86 expression was observed in cDCs defined as $CD11c^+$ cells following young barley leaf powder intake. Moreover, the direction of change in MFI from pre- to post-intake differed between the studies. This discrepancy is attributed to differences in how cDCs were defined as the analytical target in the two studies. The previous report [13] defined cDCs as $CD4^+CD141^+CD11c^+$ cells; however, gating based on $CD141^+$ is specific to cDC1 [3] and does not necessarily reflect the entire cDC population, which includes both cDC1 and cDC2. Accordingly, the analysis in the previous report [13] was limited to evaluating a fraction that included cDC1. By contrast, in this study, cDCs are defined as $CD11c^+$ cells, allowing us to assess the activation caused by consuming young barley leaf powder in the entire cDC population, *i.e.*, both cDC1 and cDC2. Therefore, the results of this study are considered more accurate in their evaluation of the overall activating effect of young barley leaf powder on cDCs.

Furthermore, the mechanism by which young barley leaf powder activates cDCs is thought to involve the activation of cDC2 primarily through Dectin-1 found in young barley leaf-derived dietary fiber, thereby contributing to overall cDC activation. In our previous study using mouse bone marrow-derived cDCs, young barley leaf powder and young barley leaf-derived dietary fiber exhibited equivalent potency in enhancing the expression of MHC-II and CD80/86 in cDCs [17]. Furthermore, unlike cellulose, young barley leaf-derived dietary fiber enhanced MHC-II and CD80/86 expression in cDCs and increased IL-12 production [17]. Thus, it is suggested that young barley leaf-derived dietary fiber is the main component involved in cDC activation. Dectin-1 is a pattern recognition receptor expressed in many cDCs that recognizes only specific sugar chain structures of certain dietary fibers [18] [19], and it is suggested that cDCs directly recognize the unique sugar chain structures of young barley leaf-derived dietary fiber via Dectin-1 [17]. However, cDC1 is not characterized by high Dectin-1 expression and constitutes only 5% - 10% of cDCs. By contrast, cDC2 comprises the majority of cDCs and highly expresses diverse pattern recognition receptors, including Dectin-1 [4]. Therefore, young barley leaf-derived dietary fiber is theorized to act

more strongly on cDC2 than on cDC1. Notably, cDC2 preferentially activates type 2 helper T cells and type 17 helper T cells [3] [4]; however, it has also been reported to partially contribute to the direct activation of killer T cells and type 1 helper T cells [5] [6]. Additionally, cDC2 has been reported to further enhance the killer T cell-inducing ability of cDC1 based on the activation of type 1 helper T cells [7]-[9]. Thus, young barley leaf-derived dietary fiber has a high impact on cDC2, contributing to the overall activation of cDCs and T cells.

Young barley leaf powder may indirectly affect immune function through the gut microbiota. Studies in humans have shown that a low-fiber diet alters the composition of the gut microbiota, leading to reduced helper T cell function [20]. Dietary fiber is metabolized by gut bacteria to produce short-chain fatty acids, which inhibit histone deacetylases and activate the mTOR pathway in T cells, promoting their differentiation [21]. Therefore, young barley leaf-derived dietary fiber may affect systemic immune function through the gut microbiota. In fact, studies have reported that the administration of young barley leaf powder increases the abundance of butyrate-producing bacteria and elevates fecal butyrate concentrations in mice [17].

Based on these findings, it is considered that consuming young barley leaf-derived dietary fiber contributes to the alleviation of cold-like symptoms in healthy adults by activating cDCs, which then promote T cell activation.

## 5. Conclusion

The intake of 0.7 g/day of young barley leaf-derived dietary fiber in this study was shown to activate cDCs and T cells in healthy adult men and women. This resulted in lower levels of systemic and specific localized cold-like symptoms.

## Authors' Contributions

Conceptualization, T.K. and K.T.; methodology, T.M. and N.M.; validation, T.M.; formal analysis, K.A. and N.M.; investigation, Y.F.; writing—original draft preparation, K.A., and writing—review and editing, T.M. and Y.F.; visualization, K.A. and Y.F.; supervision, K.T. and Y.I.; project administration, S.T. and T.K. All authors have read and agreed to the published version of the manuscript.

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## Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of Kobuna Orthopedics Clinic (approval date: 2 December 2021; approval number: MK-2112-05).

## Informed Consent Statement

Informed consent was obtained from all participants involved in this study.

## Data Availability Statement

The data used in this manuscript are not publicly available due to commercial restrictions but will be made available on reasonable request.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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