

RESEARCH LETTER

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Sublingual immunotherapy with Japanese cedar pollen extract induces apoptosis of memory CD4⁺ T cells

To the editor,

Seasonal allergic rhinitis (AR) caused by Japanese cedar (JC) pollen is the most common disease in Japan; moreover, the prevalence of Japanese cedar pollinosis (JCP) has been increasing over the last 2 decades and has reached 38.8% in 2019.¹ The long-term survival of memory CD4⁺ T cells plays a crucial role in the pathophysiology of allergic rhinitis. Type 2T helper (Th2) cells and type 2 follicular T helper (Tfh2) cells are important subsets of CD4⁺ T cells in type 2 inflammation.^{2,3} The imbalance of effector T cells and regulatory cells contributes to the pathophysiology of allergic disease. The prevalence of Th2 cells and Tfh2 cells is increased, while regulatory T (Treg) cells and regulatory B (Breg) cell prevalence is decreased in peripheral blood of AR patients.⁴ Treg and Breg cells suppress allergic immune responses by producing anti-inflammatory cytokines, including IL-10, IL-35 and TGF- β .⁵ B regulatory 1 (BR1) cells, human Breg cells, are characterized by a CD19⁺CD25⁺CD71⁺CD73⁻ phenotype and are essential for allergen tolerance by suppressing antigen-specific CD4⁺ T cell proliferation and inducing IgG4 production.⁶ Allergen specific immunotherapy (AIT) is the only treatment that may alter the natural course of AR. Sublingual immunotherapy (SLIT) is effective in reducing the clinical symptoms associated with AR. However, the effects of SLIT on memory CD4⁺ T cells are not well understood. Regulatory B cells express Fas ligand (FasL) and induce apoptosis in Fas-expressing T cells,⁷ and peripheral memory CD4⁺ T cells also express the Fas receptor. The purpose of this study is to demonstrate that memory CD4⁺ T cell apoptosis contributes to the clinical efficacy of SLIT.

Additional information about study methods and findings are available in the following repository (online supplement; see <https://doi.org/10.5281/zenodo.6524033>). In this study, we defined patients with total nasal symptom and medication scores (TNSMS: range 0–18) less than 5 and visual analogue scales (VAS: 0–100) less than 30 as responders to evaluate the clinical effects of SLIT. Non-responders were defined as TNSMS greater than 5 and VAS scale greater than 30. In total, of the 30 JCP patients with SLIT, 26 patients were responders and 4 were non-responders.

We examined the effects of SLIT with JC pollen extract using peripheral blood mononuclear cells (PBMCs) from patients with JCP. Analysis of microarray gene expression data was performed on memory CD4⁺ T cells, and the mRNA expression of apoptosis-related genes, as well as FasL-induced apoptosis were examined. The effects of SLIT on the prevalence of memory Th2 cells, memory Tfh2 cells and BR1 cells were examined by flow cytometry. Details of the study methods are described in the online supplement.

Microarray analysis study participants were four JCP patients, four SLIT responders and four non-responders. Memory CD4⁺ T cells were isolated from the PBMCs of JCP, responder and non-responder groups. A total of 60,901 mRNAs were detected, and differentially expressed mRNAs were screened using the following criteria: Fold Change >1 and *p* value <.05. Subsequently, the data were plotted as log2 (Fold Change) on the abscissa (x-axis) and log10 (*p* value) on the ordinate (y-axis) to show the distribution of mRNA expression in a Volcano plot (Figure 1A). When comparing the SLIT responder group to the JCP or non-responder groups, we found 42 differentially expressed mRNAs (Fold Change >1 and *p* value <.05), with 39 up-regulated (Figure 1B) and three down-regulated (Figure 1C). The memory CD4⁺ T cells from the responder group showed decreased expression of the DPF2 (D4, zinc and double plant homeodomain fingers family 2), ADAM12 (a disintegrin and metalloproteinase 12) and LOC645166 genes compared with the JCP or non-responder groups (Figure 1C).

Recent studies provide compelling evidence that NF- κ B suppresses cell death in response to a variety of apoptotic stimuli. DPF2 belongs to the d4 family of proteins, and plays important roles in NF- κ B transcriptional activation at the most downstream events of the NF- κ B noncanonical pathway.⁸ Decreased DPF2 gene expression may induce apoptosis in memory CD4⁺ T cells. We compared the mRNA expression of apoptosis-related genes in memory CD4⁺ T cells from individual SLIT responders before and after SLIT with JC pollen extract. We examined the caspase-3 and Fas genes that promote apoptosis, and the NF- κ B p100 and DPF2 genes that suppress apoptosis. The mRNA expression of caspase3 and Fas were not changed, whereas expression of NF- κ B p100 and DPF2 were significantly decreased after SLIT (Figure 1D), which is consistent with increased apoptosis of memory CD4⁺ T cells after SLIT.

Th2 cells and Tfh2 cells are important subsets of CD4⁺ T cells in type 2 inflammation and are important to evaluate the immunological effects of SLIT.^{2,3} We examined the prevalence of memory Th2 cells and memory Tfh2 cells in PBMCs from individual SLIT responders before and after SLIT using flow cytometry. The prevalence of memory Th2 cells in the total CD4⁺ T cell population of pre-SLIT patients was significantly higher than that of the healthy controls (HC) group. SLIT markedly reduced the prevalence of memory Th2 cells in the SLIT responders (Figure 2A). The prevalence of memory Tfh2 cells in the total CD4⁺ T cell population of pre-SLIT patients was significantly higher than that of the HC groups, and SLIT significantly reduced the prevalence of memory Tfh2 cells in the SLIT responders (Figure 2B).

The prevalence of a human Breg subset, IL-10 producing CD73⁺CD71⁺ BR1 cells, was reported to be increased after AIT in patients with bee venom allergy.⁷ We next examined the prevalence of BR1 cells in PBMCs of the SLIT responders. The prevalence of BR1 cells among CD19⁺ B cells from pre-SLIT patients was significantly lower than in the HC group, indicating that SLIT significantly increased the prevalence of BR1 cells in the SLIT responders (Figure 2C). The prevalence of FasL⁺ BR1 cells among the CD19⁺ B cells and that of IL-10⁺ BR1 cells were also significantly increased following SLIT (Figure 2D,E).

Breg cell subsets suppress immune responses in allergic inflammation by IL-10 production and by FasL-Fas induced T cell apoptosis.⁷ Initially, we examined the distribution of FasL positive B cells in SLIT responders and found that the ratio of FasL-positive cells was significantly increased in BR1 cells compared with CD19⁺ B cells. We next examined the effects of FasL on the apoptosis of memory CD4⁺ T cells from JCP patients using flow cytometry. FasL induced apoptosis of memory CD4⁺ T cells, and anti-FasL antibody and Z-VAD-FMK, a pan-caspase inhibitor, suppressed FasL-induced apoptosis of memory CD4⁺ T cells (Figure 2F,G).

In the present study, Microarray analysis of memory CD4⁺ T cells revealed the decreased DPF2 gene expression in SLIT responders. It has been reported that mRNA expression of DPF2 was significantly correlated with its copy number variation, suggesting that genetic variation of DPF2 gene and its expression in basophils might be associated with the efficacy of sublingual immunotherapy.⁹ DPF2 is known as an anti-apoptotic gene in the non-canonical NF- κ B signaling pathway. Our data show that mRNA expression of DPF2 and NF- κ B p100 in memory CD4⁺ T cells was significantly decreased after SLIT in the individual SLIT responders. These results indicate that SLIT induces apoptosis of memory CD4⁺ T cells by suppressing NF- κ B-mediated cell survival.

The human Breg cell subset BR1 cells were induced after SLIT and the prevalence of FasL- or IL-10-expressing BR1 cells was significantly increased. FasL stimulated the apoptosis of memory CD4⁺ T cells, which was significantly inhibited by an anti-FasL antibody and caspase inhibitor. Although the mRNA expression of Fas receptor and caspase 3 was not changed in memory CD4⁺ T cells, these results suggest that increased FasL-expressing BR1 cells induce the apoptosis of memory CD4⁺ T cells through the extrinsic FasL-Fas pathway following SLIT. Because of the limited number of collected

Key Messages

- Sublingual immunotherapy (SLIT) reduced memory Th2 cells and Tfh2 cells and increased FasL⁺ BR1 cells.
- Fas ligand (FasL) induces the apoptosis of memory CD4⁺ T cells.
- FasL-expressing BR1 cells may be involved in FasL-Fas induced apoptosis of memory CD4⁺ T cells.

BR1 and memory CD4⁺ T cells, it was not possible to examine the effects of co-cultured cells. Obtaining direct evidence of BR1 cell-induced apoptosis of memory CD4⁺ T cells will be the subject of future studies.

Memory Th2 cells and memory Tfh2 cells, the important subsets of memory CD4⁺ T cells, are rapidly activated by subsequent allergen exposure in type 2 immune responses. In the present study, the prevalence of these cells was significantly decreased after SLIT, which may result in the development of long-term clinical tolerance after SLIT.

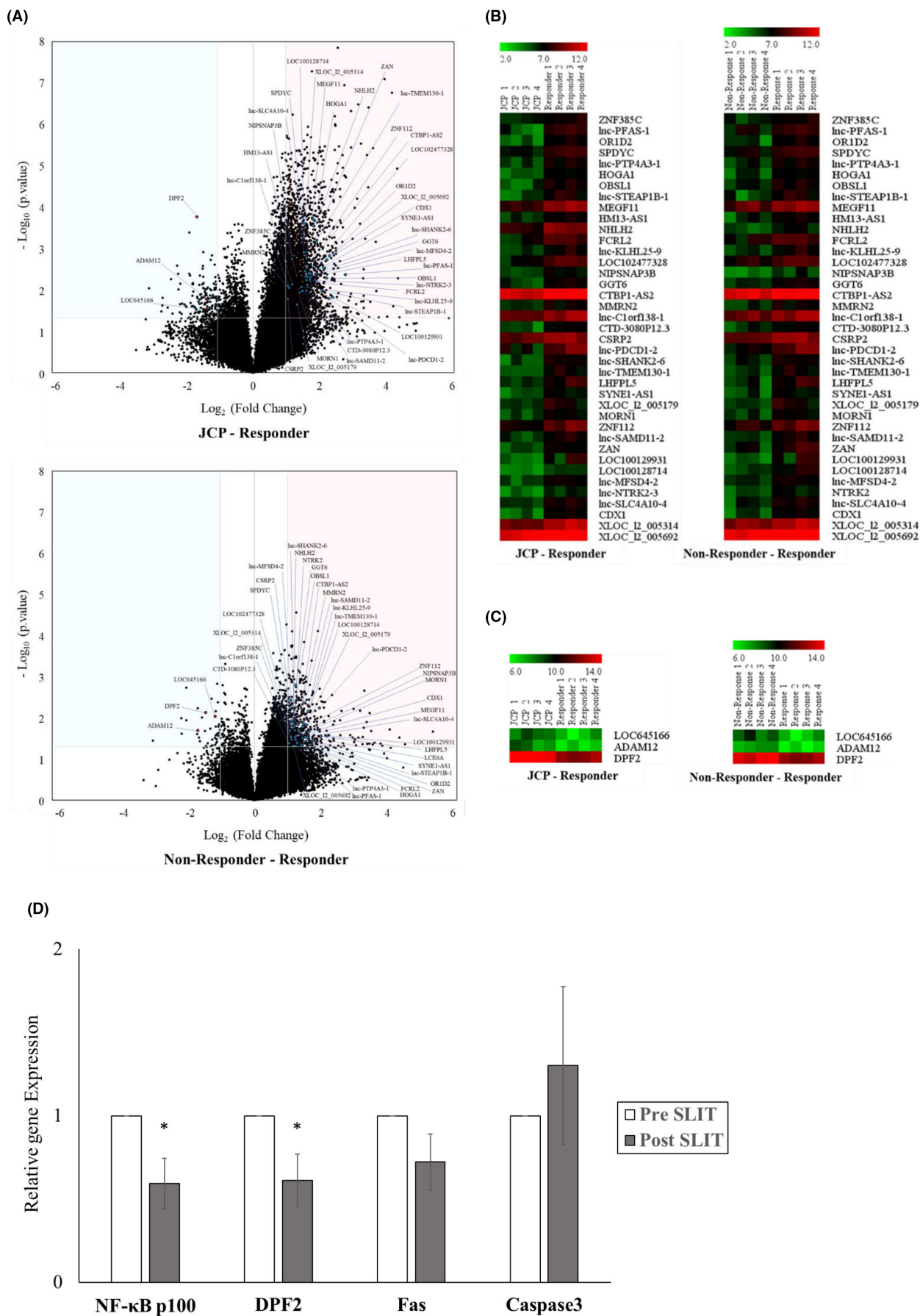
Since sublingual immunotherapy with cedar pollen extract is effective in more than 85% of patients in Japan, the number of non-responder cases was limited. Obtaining sufficient non-responder samples is an issue for future studies.

In conclusion, the present study demonstrated that SLIT with JC pollen extract induces the apoptosis of memory CD4⁺ T cells in SLIT responders. Increased FasL-expressing BR1 cells may be involved in FasL-Fas induced apoptosis of memory CD4⁺ T cells. The prevalence of memory Th2 and memory Tfh2 cells were decreased after SLIT. These findings will be useful in the development of diagnostic methods to predict SLIT efficacy and to elucidate the pathophysiology of other allergic diseases.

AUTHOR CONTRIBUTIONS

Hiroyuki Arai contributed to manuscript writing and research, Hideaki Kouzaki contributed to study design and manuscript writing, Takuya Murao, Keigo Nakamura, Kento Kawakita, Koji Matsumoto and Hirotaka Kikuoka contributed to study design, Atsushi Yuta and Takeshi Shimizu contributed to study design and writing of the manuscript. All authors read and approved the final manuscript.

FIGURE 1 (A–C) Microarray analysis of mRNA expression in memory CD4⁺ T cells from JCP, SLIT responder, and SLIT non-responder groups. (A) Volcano plot showing Fold Change and *p* value for comparisons of the JCP group versus the responder and the non-responder versus the responder group. Differentially expressed genes (Fold Change >1, *p* value <.05) are depicted; the red area indicates up-regulated genes, and the blue area indicates down-regulated genes. (B) Heatmaps illustrate the expression profiles of 39 up-regulated genes in the JCP group versus the responder group (left panel) and the non-responder group versus the responder group (right panel). (C) Heatmaps illustrate the expression profiles of three down-regulated genes in the JCP group versus the responder group (left panel) and the non-responder group versus the responder group (right panel). (D) Real-time PCR analysis of relative mRNA expression levels of apoptosis-related genes in memory CD4⁺ T cells from individual SLIT responders before and after SLIT with JC pollen extract. We targeted the caspase-3 and Fas genes that promote apoptosis, and the NF- κ B p100 and DPF2 genes that suppress apoptosis. The GAPDH gene is used as an endogenous reference. Results are shown as mean $\pm$ SEM (*n* = 9 in each group). **p* < .05



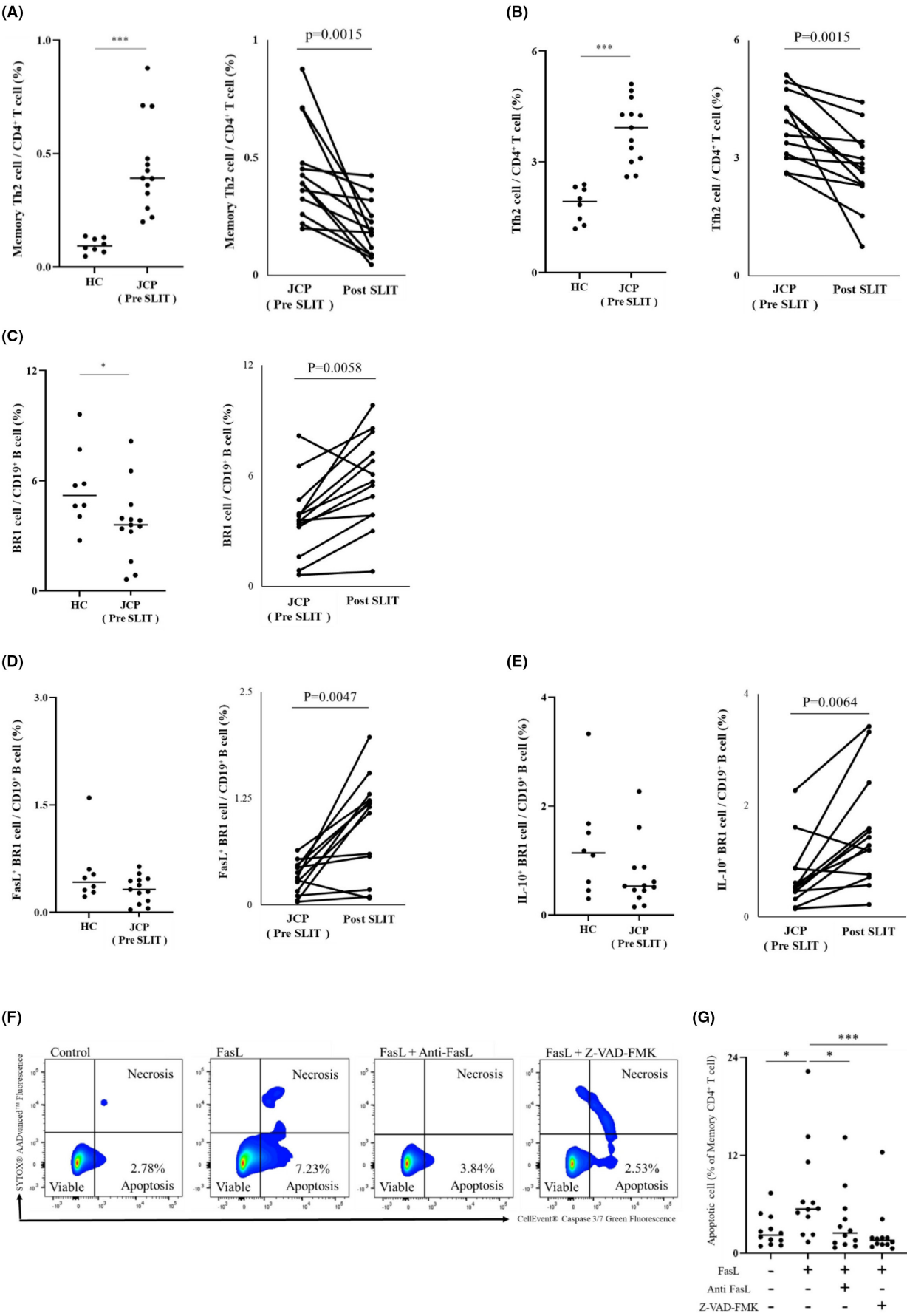


FIGURE 2 (A–B) The prevalence of memory Th2 and Tfh2 cells in PBMCs from individual SLIT responders before and after SLIT with JC pollen extract. (A) The prevalence of memory Th2 cells in CD4⁺ T cells in the HC and JCP (pre-SLIT) groups and the pre- and post-SLIT groups. (B) The prevalence of memory Tfh2 cells in the CD4⁺ T cell populations of the HC and JCP (pre-SLIT) groups and the pre- and post-SLIT groups. (C–E) The prevalence of BR1 cells, FasL⁺ BR1 cells and IL-10⁺ BR1 cells in the CD19⁺ B cell population from individual SLIT responders before and after SLIT. (C) The prevalence of BR1 cells in the CD19⁺ B cell populations of the HC and JCP (pre-SLIT) groups and the pre- and post-SLIT groups. (D) The prevalence of FasL⁺ BR1 cells in the CD19⁺ B cell population of the HC and JCP (pre-SLIT) groups and the pre- and post-SLIT groups. (E) The prevalence of IL-10⁺ BR1 cells in the CD19⁺ B cell populations of the HC and JCP (pre-SLIT) groups and the pre- and post-SLIT groups. (F–G) Effects of FasL on the apoptosis of memory CD4⁺ T cells from JCP patients. (F) Gated dot plots showing apoptotic cells in the memory CD4⁺ T cell population. Numbers indicate percentage of apoptotic cells in the gate. (G) Scatter plots summarize the observed apoptotic cell proportions in the absence or presence of anti-FasL or Z-VAD-FMK. Each dot present data obtained from one subject. * $p < .05$ and *** $p < .005$

KEYWORDS

apoptosis, BR1 cells, DPF2, memory CD4⁺ T cells, sublingual immunotherapy

ACKNOWLEDGEMENT

The authors gratefully acknowledge the work of past and present members of our laboratory. This study was supported by research grants from the Japan Society for the Promotion of Science (Grants-in-Aid for Scientific Research 19H03802).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. Additional information about study methods and findings are available in the following repository: <https://doi.org/10.5281/zenodo.6524033>.

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REFERENCES

1. Matsubara A, Sakashita M, Gotoh M, et al. Epidemiological survey of allergic rhinitis in Japan 2019. *Nippon Jibiinkoka Gakkai Kaiho*. 2020;123:485-490.
2. Endo Y, Iwamura C, Kuwahara M, et al. Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3. *Immunity*. 2011;35:733-745.
3. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+) CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011;34:108-121.
4. Kamekura R, Shigehara K, Miyajima S, et al. Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma. *Clin Immunol*. 2015;158:204-211.
5. Kouzaki H, Arikata M, Koji M, et al. Dynamic change of anti-inflammatory cytokine IL-35 in allergen immune therapy for Japanese cedar pollinosis. *Allergy*. 2020;75:981-983.
6. van de Veen W, Stanic B, Yaman G, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol*. 2013;131:1204-1212.
7. Braza F, Chesne J, Castagnet S, Magnan A, Brouard S. Regulatory functions of B cells in allergic diseases. *Allergy*. 2014;69:1454-1463.
8. Tando T, Ishizaka A, Watanabe H, et al. Requin protein links RelB/p52 and the Brm-type SWI/SNF complex in a noncanonical NF-kappaB pathway. *J Biol Chem*. 2010;285:21951-21960.
9. Gotoh M, Kaminuma O, Nakaya A, et al. Association of basophil-expressing genes with effectiveness of sublingual immunotherapy. *J Nippon Med Sch*. 2020;87:49-52.