

ORIGINAL PAPER

Human $\gamma\delta$ T cells modulate the mite allergen-specific T-helper type 2-skewed immunityS. Korematsu*, Y. Tanaka^{†‡}, T. Nagakura^{*†}, N. Minato[†] and T. Izumi*^{*}Department of Brain and Nerve Science, Division of Pediatrics and Child Neurology, Oita University Faculty of Medicine, Oita, Japan, [†]Laboratory of Immunology and Cell Biology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan and [‡]PRESTO, JST, Saitama, JapanClinical and
Experimental
Allergy

Summary

Background $\gamma\delta$ T cells have been described as one of immune regulators in patients with infection, malignancy, and allergy.**Objective** To elucidate the ability of $\gamma\delta$ T cells as an allergen immunotherapy candidate, the effectiveness of human $\gamma\delta$ T cells in allergen-specific T-helper type 2 (Th2)-type T cells was evaluated *in vitro*.**Methods** House dust mite-specific Th2-type T cell clones, Bacillus Calmette–Guerin (BCG)-specific Th1-type T cell clones, and $\gamma\delta$ T cell lines were established from the peripheral blood mononuclear cells of two patients with allergic rhinitis. The effectiveness of $\gamma\delta$ T cells and BCG-specific Th1-type T cell clones in the modulation of allergen-specific Th2 cells in terms of their cytokine productions was evaluated.**Results** In response to cognate antigens, the $\gamma\delta$ T cell lines demonstrated a proliferation and production of IFN- γ that exceeded that of BCG-specific Th1-type T cell clones (mean stimulation index: 14.5 vs. 2.8, mean IFN- γ : 130.5 vs. 10.0 pg/mL). When the $\gamma\delta$ T cell lines and mite-allergen-specific Th2 clones were co-cultured with each other, only the levels of IL-4 (mean, –87%) decreased, but not the levels of IL-5 and IL-13, with an increasing concentration of $\gamma\delta$ T cell antigen and IFN- γ production (mean, +730%).**Conclusion** These results demonstrated that $\gamma\delta$ T cells derived from allergic patients might thus have a partial ability to modulate allergen-specific Th2-skewed immunity.**Keywords** $\gamma\delta$ T cells, BCG, C8/119S, Der f 2, Th1/Th2

Submitted 18 August 2006; revised 13 August 2007; accepted 14 August 2007

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Introduction

It has been speculated that the prevalence of allergic diseases may be associated with a reduction in the number of infectious diseases such as mycobacteria [1–3]. This hygienic hypothesis and T-helper type 1 (Th1)/Th2 imbalance theory [1–3] seem to be potentially useful for the establishment of successful and effective allergen immunotherapy. Although, in an animal model, Bacillus Calmette–Guerin (BCG)-specific Th1 cells suppressed the development of allergen-specific Th2 cells [4–6], immunotherapy using these Th1 cells resulted in a poor efficacy in sensitized human allergic patients [7–12].

On the other hand, human $\gamma\delta$ T cells also act as immune regulators in mycobacterial infections [13–15], and the regulation of allergic diseases by $\gamma\delta$ T cells has been reported [16–20]. However, $\gamma\delta$ T cells have so far only

rarely been examined as an allergen immunotherapy candidate. In this report, the effectiveness of $\gamma\delta$ T cells on the modulation of allergen-specific Th2 cells was evaluated.

Materials and methods

Patients

Lymphocyte donors were 30 (A1) and 40 (A2)-year-old male patients with allergic rhinitis (AR). They both had mite-specific IgE antibodies in sera. Non-allergic healthy donors consisted of 23 (C1) and 25 (C2)-year-old males. All donors received a BCG vaccination during their childhood, and none of them had any distinct history of tuberculosis infection.

Allergens and antigens

Recombinant *Dermatophagoides farinae* 2 (Der f 2), one of the major mite allergens, and its mutant C8/119S [21, 22], were kindly provided by Asahi Breweries Ltd (Tokyo, Japan) and Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), respectively. BCG (Nippon BCG Manufacturing Co. Ltd, Tokyo, Japan) was grown in Middlebrook 7H9 medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA), supplemented with 0.5% glycerol (Nacalai Tesque Inc., Kyoto, Japan), 0.05% Tween 80 (Nacalai Tesque Inc., Kyoto, Japan), and 10% OADC (Becton Dickinson Microbiology Systems) at 37 °C for 1 week, killed in 75% ethanol for 20 min, disrupted using an ultrasonicator for 4 min on ice, and then was centrifuged in a refrigerated centrifuge at 4 °C for 20 min. The resultant supernatant was transferred to a microcentrifuge tube and stored at -80 °C until it was used.

In order to establish $\gamma\delta$ T cell lines, 2-methyl-3-butenyl-1-pyrophosphate, one of the non-peptide analogues of BCG-derived pyrophosphomonoester antigens, was synthesized as described previously [13–15]. The compound was converted to a sodium form using Dowex 50W $\times$ 8, 100–200 mesh, cation exchange column chromatography (Muromachi Technos Co. Ltd, Tokyo, Japan), and stored at -20 °C until it was used.

Cell culture and derivation of T cell clones

Peripheral blood samples derived from two patients (A1 and A2) with AR and two healthy donors (C1 and C2) after obtaining their informed consent were diluted with phosphate-buffered saline and subjected to density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences Corp., Piscataway, NJ, USA). The purified peripheral blood mononuclear cells (PBMC) were incubated at 3×10^6 cells/well in 24-well culture plates in 1.5 mL of modified Yssel's medium supplemented with 0.25% human serum albumin (Sigma-Aldrich Co., St Louis, MO, USA), 100 IU/mL of penicillin (Meiji Seika Kaisha Ltd, Tokyo, Japan), and 0.1 mg/mL of streptomycin (Meiji Seika Kaisha Ltd), in the presence of varying concentrations of antigens (Der f 2; 1 μ g/mL, BCG extract; 0.3% and 2-methyl-3-butenyl-1-pyrophosphate; 10 μ M) and 30-Gy-irradiated freshly collected autologous PBMC as antigen-presenting cells (APC).

On day 16, 2-methyl-3-butenyl-1-pyrophosphate-specific $\gamma\delta$ T cell lines were harvested, with the purity being 95% or higher judging from the cluster of differentiation (CD) 3⁺ cell population determined by flow cytometry. Der f 2-specific and BCG-specific T cell clones were established by limiting dilution.

Proliferation assay

Antigen-specific T cells (5×10^4 cells/well) and 30-Gy-irradiated APC (1.5×10^5 cells/well) were incubated at

37 °C in 96-well round-bottom plates in the presence of various concentrations of antigens for 36 h. The cells were pulsed with [³H]-thymidine (2 μ Ci/well), incubated for 12 h, and then harvested. Next, the incorporation of radioactivity into DNA was measured by a γ -counter (Perkin Elmer Life Sciences Japan, Tokyo, Japan). The stimulation index (SI) was used to determine the proliferation ratio with a maximum response/mock-stimulated response. The results were confirmed in three independent experiments.

Co-culture assay

Either $\gamma\delta$ T cell lines or BCG-specific T cell clones (5×10^5 cells/well) were co-cultured with autologous Der f 2-specific T cell clones (5×10^5 cells/well) at 37 °C in the presence of varying concentrations of relevant antigens (C8/119S, BCG and/or 2-methyl-3-butenyl-1-pyrophosphate) and 30-Gy-irradiated autologous APC (1.5×10^6 cells/well) in 96-well round-bottom plates for 2 days. The results were confirmed in three independent experiments.

Enzyme-linked immunosorbent assay

T cells were co-cultured as described above for 2 days, and then the culture supernatants were harvested and stored at -80 °C until they were used. IL-4, IL-5, IL-13, and IFN- γ productions into the culture medium were determined by ELISA (Amersham Biosciences).

Results

Differential proliferation profiles of peripheral blood mononuclear cell in response to distinct antigens

As shown in Fig. 1a, the PBMC derived from two allergic patients responded to Der f 2 (closed circle; patient A1 and closed triangle; patient A2; mean SI, 6.4 and 4.4, respectively), while no stimulatory responses were observed in two healthy donors (open circle; healthy donor C1 and open triangle; healthy donor C2; mean SI, 1.5 and 1.5, respectively). BCG extracts failed to induce a proliferation in PBMC from allergic patients (mean SI, 2.0 and 1.4, respectively), whereas the PBMC from healthy donors did exhibit such a proliferation (mean SI, 6.7 and 6.4, respectively) as shown in Fig. 1b. On the other hand, both PBMC from the patients (mean SI, 8.6 and 7.6, respectively) and the healthy donors (mean SI, 10.6 and 9.0, respectively) showed an equal proliferation in response to 2-methyl-3-butenyl-1-pyrophosphate (Fig. 1c).

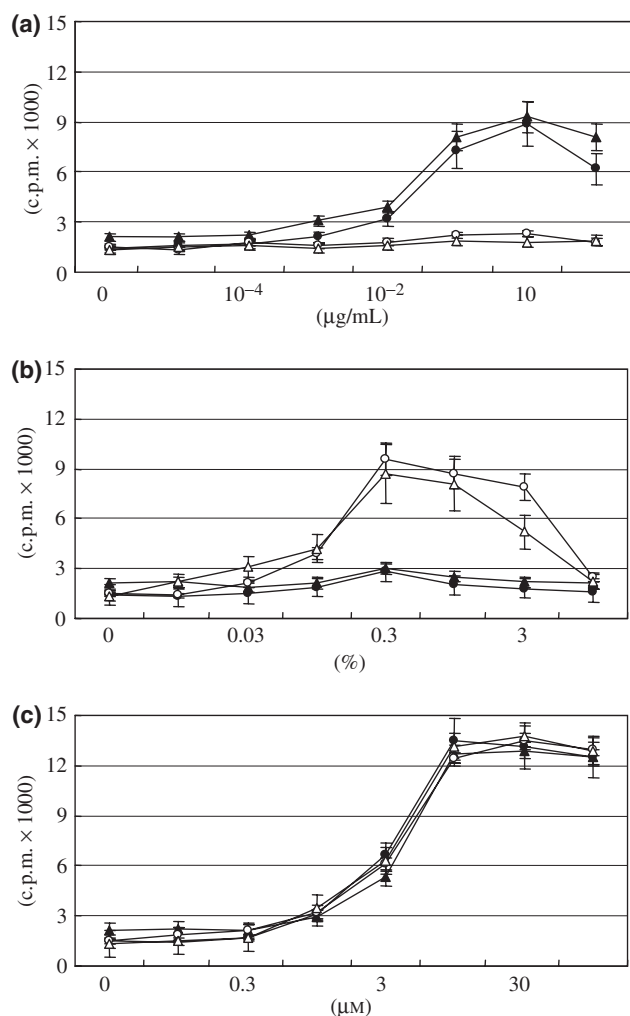


Fig. 1. Comparison of peripheral blood mononuclear cells (PBMC) responsiveness to *Dermatophagoides farinae* 2 (Der f 2), Bacillus Calmette–Guerin (BCG) proteinaceous antigens, and non-peptide pyrophosphomonoester antigens between allergic patients and healthy individuals. Proliferation of PBMC derived from patients with allergic rhinitis (AR) in response to house dust mite allergen, Der f 2 (a), BCG proteinaceous antigen (b), and 2-methyl-3-butenyl-1-pyrophosphate (c). The PBMC purified from peripheral blood samples of two patients with AR (closed circle, patient A1 and closed triangle, patient A2) and two healthy donors (open circle, healthy donor C1 and open triangle, healthy donor C2) were cultured in the presence of varying concentrations of the antigens, respectively. The bar lines show the mean and standard deviation of independent conducted experiments three times.

Derivation and characterization of antigen-specific T cell clones and lines

Two independent Der f 2-specific T cell clones from two allergic patients were established and named D-A1 (a Der f 2 clone derived from a patient A1, closed circle) and D-A2 (a Der f 2 clone derived from a patient A2, closed triangle), respectively. Der f 2-specific T cell clones were not generated from two healthy donors because of a lack of reactivity to the allergen. Both the Der f 2-specific T cell

clones were CD3^+ , CD4^+ , CD8^- , $\text{V}\gamma 2^-$, and $\text{V}\delta 2^-$ Th2 cells (data not shown). The T cell clones proliferated in response to Der f 2 in a dose-dependent manner (mean SI, 18.8 and 18.2, respectively) (Fig. 2a) and produced IL-4 (black bars) instead of IFN- γ (dotted bars), thus indicating them to be Th2 cell clones as shown in Fig. 3.

Likewise, three BCG-specific T cell clones, named B-A1 (a BCG clone derived from a patient A1; closed circle), B-C1 (a BCG clone derived from a control donor C1; open circle), and B-C2 (derived from a control donor C2; open triangle), were established from one allergic patient and two healthy donors. All the BCG-specific T cell clones were CD3^+ , CD4^+ , CD8^- , $\text{V}\gamma 2^-$, and $\text{V}\delta 2^-$ Th1 cells

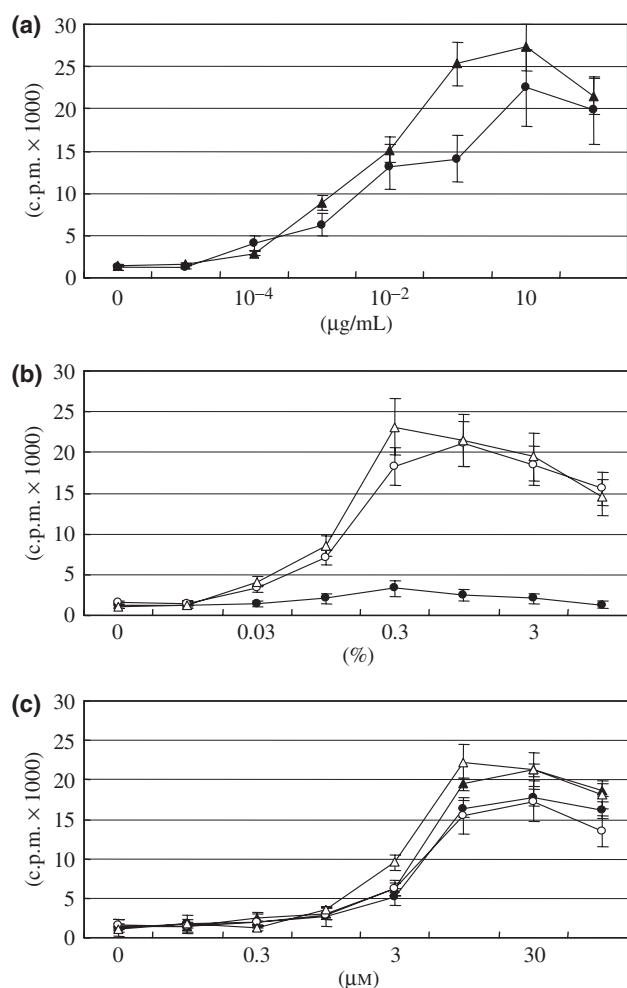


Fig. 2. Specific proliferation of newly established T cell clones and lines in response to their cognate antigens. Dose-dependent specific proliferation of *Dermatophagoides farinae* 2 (Der f 2)-specific T cell clones (a), Bacillus Calmette–Guerin-specific T cell clones (b), and $\gamma\delta$ T cell lines (c). T cell clones and lines generated from two patients with allergic rhinitis (closed circle, patient 1 and closed triangle, patient 2) and two healthy donors (open circle, healthy donor 1 and open triangle, healthy donor 2) were incubated in the presence of varying concentrations of cognate antigens, respectively. The bar lines show the mean and standard deviation of independent conducted experiments three times.

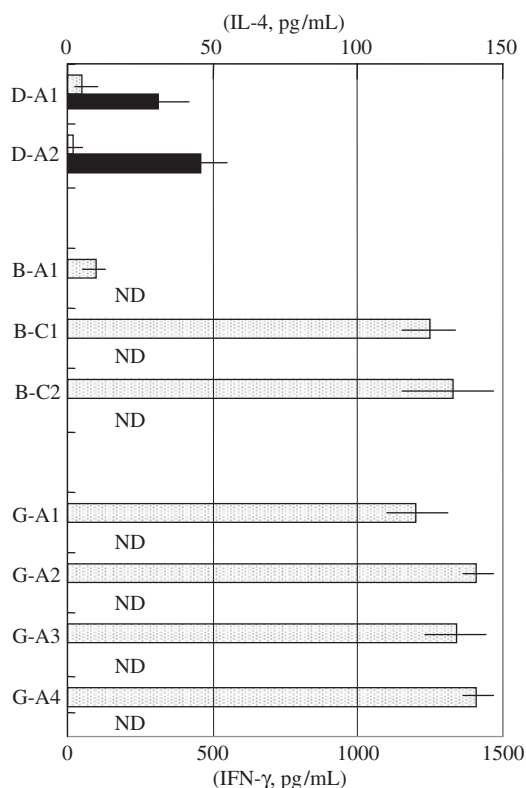


Fig. 3. Cytokine profiles of antigen-specific T cell clones and lines. Medium supernatants of T cell clones and lines were collected to determine IL-4 and IFN- γ by the standard high-sensitivity ELISA (D, *Dermatophagoides farinae*-2-specific T cell clone; B, Bacillus Calmette-Guerin-specific T cell clone; G, $\gamma\delta$ T cell lines; A1, allergic patient 1; A2, allergic patient 2; C1, healthy donor 1; C2, healthy donor 2; black bar, IL-4; and dotted bar, IFN- γ). The bar lines show the mean and standard deviation of independent conducted experiments three times.

(data not shown). When stimulated with the BCG extract, B-C1 and B-C2 generated from healthy donors significantly proliferated in an antigen dose-dependent manner (mean SI, 22.7 and 21.0, respectively), while B-A1 generated from allergic patients exhibited only a weak response to the antigen (mean SI 2.8) as shown in Fig. 2b. All BCG-specific T cell clones exhibited a Th1 character; however, the levels of IFN- γ production (dotted bars) by B-C1 and B-C2 (mean, 133 and 125 pg/mL, respectively) were significantly higher than that by B-A1 (mean, 10 pg/mL) as shown in Fig. 3.

Four $\gamma\delta$ T cell lines expressing CD3⁺, CD4⁻, CD8low⁺, V γ 2⁺, and V δ 2⁺ segments (data not shown) were established from both allergic patients (G-A1, closed circle and G-A2, closed triangle) and healthy donors (G-C1, open circle and G-C2, open triangle). As shown in Fig. 2c, a significant proliferation of both $\gamma\delta$ T cell lines (allergic patients: mean SI, 14.8 and 14.2, healthy controls: mean SI, 20.2 and 10.8, respectively) was noted by stimulation with 2-methyl-3-butenyl-1-pyrophosphate. These $\gamma\delta$ T cell lines produced IFN- γ (dotted bars) equally

(allergic patients: mean, 141.0 and 120.0 pg/mL, healthy donors: mean, 141.0 and 134.0 pg/mL, respectively) as shown in Fig. 3.

Antigen specificity of each T cell in response to distinct antigens

The antigen specificities of the established T cell clones were determined. As shown in Fig. 4a, D-A1 exhibited a proliferative response to both Der f 2 (mean SI, 18.8, closed circle) and the Der f 2 mutant protein C8/119S (mean SI, 41.9, closed triangle), while there was no response to 2-methyl-3-butenyl-1-pyrophosphate (mean SI, 1.1, open circle). On the other hand, a $\gamma\delta$ T cell line, G-A1, exhibited proliferative responses to 2-methyl-3-butenyl-1-pyrophosphate (mean SI, 14.8); however, no proliferation was observed in response to Der f 2 (mean SI, 1.2) nor C8/119S (mean SI, 1.1) as shown in Fig. 4b (closed circles represent native Der f 2 allergen stimulation, closed triangle C8/119S and open circle 2-methyl-3-butenyl-1-pyrophosphate). These findings were confirmed by the experiments with D-A2 and G-A2 (data not shown).

In parallel to the proliferative responses, the Der f 2-reactive T cell clone, D-A1, produced a high level of IL-4 (black bars), IL-5 (stripe bars), and IL-13 (checkered bars), and less amounts of IFN- γ (dotted bars) in response to Der f 2 and C8/119S, while no cytokine production was detected when challenged with 2-methyl-3-butenyl-1-pyrophosphate as shown in Fig. 4c. The $\gamma\delta$ T cell line, G-A1, produced IFN- γ (mean, 1200 pg/mL, dotted bar), whereas no production of IFN- γ was seen in response to mite allergens (Der f 2, mean, 5 pg/mL; C8/119S, mean, 12 pg/mL, respectively) as shown in Fig. 4d.

Modulation by $\gamma\delta$ T cell lines of mite allergen-specific T-helper type 2-skewed immunity

Because Der f 2-specific T cell clones and $\gamma\delta$ T cell lines did not show any antigen cross-reactivity as demonstrated above, the effectiveness of $\gamma\delta$ T cell lines in the function of Der f 2-specific Th2 T cell clones was examined.

When both the T cell clones and lines (D-A1 and G-A1, D-A2 and G-A2) were co-cultured with each other in the presence of a fixed concentration of a mite allergen C8/119S, 10 μ g/mL, only the levels of IL-4 (mean, -87%, black bars) decreased, but not the levels of IL-5 (stripe bars) and IL-13 (checkered bars), with an increasing concentration of $\gamma\delta$ T cell antigen and IFN- γ production (mean, +730%, dotted bars) (Fig. 5a). In the presence of a fixed concentration of 2-methyl-3-butenyl-1-pyrophosphate (10 μ M), the production of IFN- γ increased (mean, +356%) with the dose dependency of C8/119S as shown in Fig. 5b.

Because of its poor survival ability, the BCG-specific T cell clone B-A1 established from allergic patient A1 did

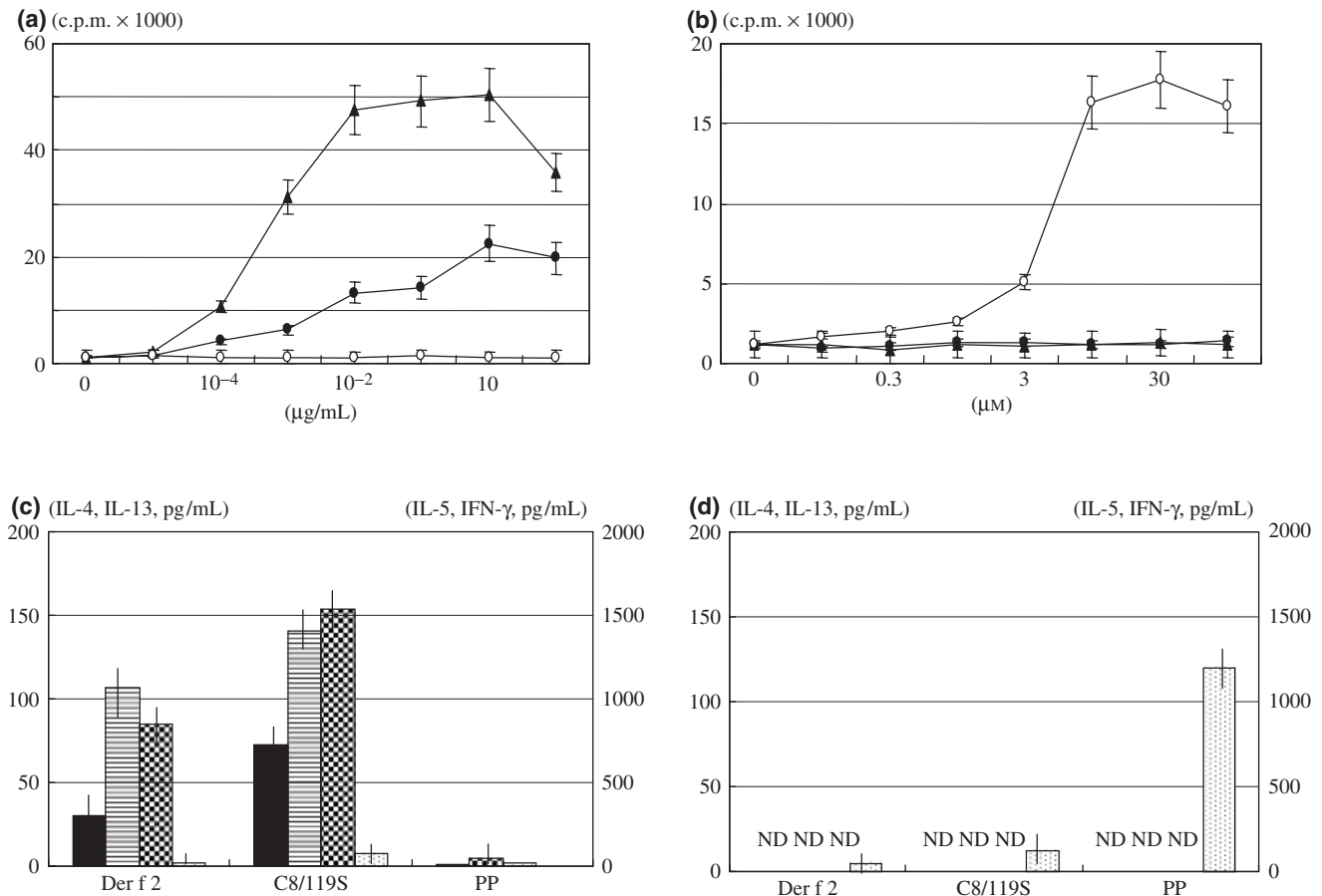


Fig. 4. Antigen specificity of the established T cell clones and lines. *Dermatophagoides farinae* 2 (Der f 2)-reactive T cell clones (a, c) and $\gamma\delta$ T cell lines (c, d) were challenged with native Der f 2 allergen (closed circle), C8/119S (closed triangle), or 2-methyl-3-butenyl-1-pyrophosphate (PP, open circle), respectively. Proliferative responses were determined (a, b), and cytokine profiles in the medium supernatants were examined (c, d, black bars represent IL-4, stripe bars IL-5, checkered bars IL-13 and dotted bars IFN- γ). The bar lines show the mean and standard deviation of independent conducted experiments three times. ND, non-determined.

not show any influence on the levels of IL-4, IL-5, and IL-13 by Der f 2-specific T cell clones (data not shown).

Discussion

In spite of many reports on allergic diseases and their therapeutic strategies, it still remains difficult to control such diseases [1, 3, 23]. Therefore, various kinds of immunotherapy have been conducted [21, 24]. Because allergic diseases are mediated by Th2-type immunity, the development of strategies for augmenting Th1-type immunity and inhibiting Th2 responses to modulate Th1/Th2 imbalance is important. Based on the above observations, mycobacteria-specific Th1T cells seem to be useful tools for allergen immunotherapy, even though BCG-specific Th1T cells have been reported to have a poor efficacy in human allergic patients [7–12]. This might have been caused by the early sensitization through the placenta and breast milk during the fetal and infantile period in human patients. The modulation of sensitized T cells is more

difficult than the modulation of native T cells such as in an animal model. We therefore want to clarify the effectiveness of sensitized Th2 clones, but not native T cells.

Based on the present results, the PBMC from healthy donors responded to BCG extracts, while almost no responses were found in PBMC derived from allergic patients (Fig. 1b). On the other hand, PBMC from healthy donors failed to proliferate in response to Der f 2, while sufficient responses were noted in the PBMC from the patients (Fig. 1a). In human PBMC, IFN- γ is produced not only by BCG-specific Th1 $\alpha\beta$ T cells but also by $\gamma\delta$ T cells. Human $\gamma\delta$ T cells are divided into two different phenotypes. An IL-4-producing phenotype, which possesses V γ 1 and V δ 1 segments, enhances allergic inflammation [17, 18, 25]. In the present study, $\gamma\delta$ T cells, which possess V γ 2, J γ 1.2, and V δ 2 segments have an IFN- γ -producing phenotype [13–15]. In contrast to BCG antigens, pyrophosphomonoester antigens induced the proliferation of $\gamma\delta$ T cells derived from healthy donors as well as allergic patients (Fig. 1c).

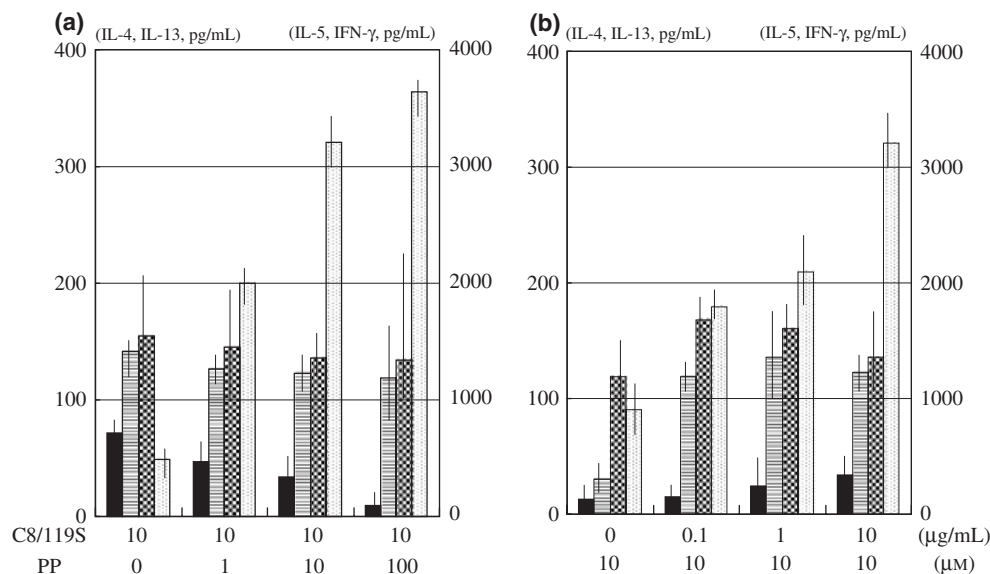


Fig. 5. $\gamma\delta$ T cell lines modulate the levels of IL-4 from *Dermatophagoides farinae* 2 (Der f 2)-specific T cell clones. (a) Dose-dependent modulation of the levels of IL-4 and dose-dependent augmentation of IFN- γ production by $\gamma\delta$ T cell lines reactive to 2-methyl-3-butenyl-1-pyrophosphate (PP). A $\gamma\delta$ T cell line and a Der f 2-specific T cell clone were co-cultured in the presence of a fixed concentration of C8/119S, varying concentrations of PP, and irradiated antigen presenting cells (APC). The medium supernatants were examined for IL-4 (black bar), IL-5 (stripe bar), IL-13 (checkered bar), and IFN- γ (dotted bar). (b) Effect of the presence of $\gamma\delta$ T cells on cytokine profiles of Der f 2-specific T cell clones. A $\gamma\delta$ T cell line and a Der f 2-specific T cell clone were co-cultured in the presence of a fixed concentration of PP, varying concentrations of C8/119S, and irradiated APC. The bar lines show the mean and standard deviation of independent conducted experiments three times.

In addition, the activation of $\gamma\delta$ T cells resulted in the decreased levels of IL-4 but not the levels of IL-5 and IL-13, which are the other crucial cytokines in allergic inflammation [26], from Der f 2-specific Th2 cell clones when co-cultured *in vitro* (Fig. 5a). It is noteworthy that the IFN- γ production by $\gamma\delta$ T cells increased even when the 2-methyl-1-pyrophosphate concentration was fixed accordingly to activate the Der f 2-specific T cells (Fig. 5b), while $\gamma\delta$ T cell lines did not show any proliferation or IFN- γ production in the presence of Der f 2 antigen (Fig. 4). Although whether the mechanism involves IL-4 consumption, inhibition, or cell-to-cell interaction to Th2 cells by $\gamma\delta$ T cells still remains unclear, these results suggested that $\gamma\delta$ T cells have a partial ability to modulate Th2-skewed immunity. Several reports [27] have demonstrated the immune regulatory function of IL-10; however, $\gamma\delta$ T cells were not able to produce this cytokine (data not shown).

$\gamma\delta$ T cells comprise about 4% of all peripheral T cells. Because bisphosphonate compounds are clinically well tolerated, they can be used for $\gamma\delta$ T cell expanders *in vitro* as well as *in vivo* [28]. Recently, $\gamma\delta$ T cells stimulated with these chemical compounds were thus considered to be good candidates for cancer immunotherapy, because of their IFN- γ production and the cytotoxic effect [29–31]. The adoptive transfer of autologous $\gamma\delta$ T cells expanded *in vitro* might therefore be an effective strategy for IL-4-mediated allergy. Taken together, the present study suggests that $\gamma\delta$ T cells may thus help alter the Th2-skewed

immunity in patients with allergic diseases. Further accumulated studies to clarify the ability of $\gamma\delta$ T cells as an allergic immunotherapy candidate are thus called for.

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