

The quantitative and qualitative defect of CD4⁺ CD45RO⁺ memory-type T cells are involved in the abnormality of TH1 immunity in atopic dermatitis patients

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Summary

Peripheral blood mononuclear cells (PBMCs) obtained from atopic dermatitis (AD) patients produced low levels of IFN- γ in response to *Dermatophagoides farinae* antigen (Der f Ag) plus IL-2 or OKT3 MoAb in contrast with PBMCs obtained from healthy donors. The reduced IFN- γ production in AD patients' T cells appeared to be derived from the defect of CD4⁺ T cells but not CD8⁺ T cells. Indeed, from the cytoplasmic staining analysis of cytokines, it was demonstrated that the frequency of IFN- γ producing CD4⁺ T cells (TH1 cells) in AD patients was markedly lower than that of healthy donors. From the phenotypic analysis using flow cytometry, it was also found that the number of CD4⁺ CD45RO⁺ memory type T cells was significantly reduced in AD patients compared with that of healthy donors. In addition to quantitative defect of memory type CD4⁺ T cells, functional defect of CD4⁺ CD45RO⁺ memory type T cells was also demonstrated in AD patients. Enriched CD4⁺ CD45RO⁺ T cells obtained from AD patients, who exhibited greatly reduced delayed-type hypersensitivity (DTH) response in tuberculin test, showed no significant TH1 immunity in terms of IFN- γ production by stimulation with OKT3 MoAb or purified protein derivative (PPD). Thus, the immunological abnormality of TH1 immunity in AD patients appeared to be induced in concomitant with both the quantitative and qualitative defect of memory type CD4⁺ T cells.

Keywords: Atopic dermatitis, CD45RO, human, TH1/TH2

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder associated with abnormalities of the immune system [1,2]. The most prominent immunological abnormality is B-cell IgE overproduction [3,4]. Although the precise mechanisms of the elevation of serum IgE levels in AD patients has not been clarified, recent works have suggested that TH1/TH2 imbalance is one of the important causes [5–7]. IL-12 and IFN- γ -driven TH1 immunity inhibits IgE production, while IL-4 produced by TH2 cells is an essential cytokine for the class switch of IgE [8,9].

Therefore, the abnormal activation of TH2-dominant immunity has been considered to be a major cause for overproduction of IgE in AD patients. Indeed, it has been shown that lesional skin of AD patients clearly contained TH2 cells and high percentage of TH2 clones were established from lesional AD skin [10–12].

Because IL-4 inhibits TH1 immunity and IFN- γ reversely inhibits TH2 immunity [8,13], the decreased TH1 immunity may also become the cause of the polarized TH2 immunity in AD patients. It has been demonstrated that AD patients showed an increased susceptibility to viral and fungal infection [14,15]. Moreover, AD patients exhibited greatly reduced delayed-type hypersensitivity (DTH) and over 50% of the patients changed to misleading negative in tuberculin test [16,17]. Actually, recently, Shirakawa *et al.* clearly

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showed inverse association between tuberculin responses and atopic disorder [18]. It has been also demonstrated that polyclonally activated IFN- γ production is decreased in AD patients [19,20]. Such phenomena can be explained by the decreased TH1-dominant immunity which is important for the protection of infectious diseases and DTH responses. Therefore, the defect of TH1-dominant immunity may be also involved in the induction of TH1/TH2 imbalance in AD patients. However, little has clarified the mechanisms concerning the defect of TH1-dependent cellular immunity in AD.

To evaluate the cellular mechanisms underlying the abnormality of TH1-dominant immunity in AD patients, we investigated the function of CD4⁺ T cells obtained from AD patients to compare with that of healthy donors. In the present paper, we initially proposed that the defect of TH1-dependent IFN- γ production and PPD-specific immune responses may be derived from both the quantitative and qualitative defect of CD4⁺ CD45RO⁺ memory type T cells in AD patients.

Materials and methods

Patients

PBMCs were prepared from 42 patients with AD. All the patients showed a high titre of serum IgE specific to *Dermatophagoides farinae* (Der F) Ag (class to Der p > 5) by CAP RAST system (Pharmacia, Tokyo, Japan). In some experiments (Fig. 5), tuberculin test was carried out in all patients to detect their DTH responses and PBMCs obtained from patients who showed negative tuberculin responses were used in the experiment.

PBMCs

PBMCs were isolated from healthy donors or AD patients by Ficoll-Hypaque gradient centrifugation. PBMCs (2×10^5 /well), which were suspended in AIM-V medium (GIBCO BRL, New York NY, USA) supplemented with pooled human AB serum, were cultured with 50 μ g/mL of Der f Ag (kindly donated by Torii Pharmaceutical Co. Ltd, Tokyo, Japan) plus 20 U/mL of IL-2 (kind gift of Shionogi Pharmaceutical Co. Ltd, Osaka, Japan). A marked proliferative response and IFN- γ production of PBMCs was not detected by stimulation with Der f Ag or IL-2 alone, but greatly enhanced responses were observed by addition of Der f and IL-2 at initial of the culture. In some experiments, PBMCs were stimulated with immobilized OKT3 MoAb (PharMingen, San Diego CA, USA), PMA (10 ng/mL; Sigma, St Louis MO, USA) plus A23187 (100 ng/mL; Sigma) or PPD (50 μ g/mL; Japan BCG Institute Co. Ltd). The OKT3 MoAb purchased from PharMingen was coated

on the flat-bottomed Costar 96 well plate by incubation of 50 μ g/mL of OKT3 MoAb suspended in PBS for 2 h at 37°C. Generally, culture supernatants were harvested after 4-day culture.

Phenotypic analysis and separation of lymphocytes by flow cytometry

The analysis of cell surface markers was carried out by FACScan (Japan Becton Dickinson Company, Ltd, Tokyo, Japan) using the Consort 30 program. FITC-conjugated anti CD45RA MoAb, FITC-conjugated anti CD45RO MoAb and PE-conjugated anti-CD4 MoAb were purchased from PharMingen. The detailed procedure for staining was described in previous paper [21]. Fluorescence data were collected with logarithmic amplification. For each sample, data from 10 000 volume-gated viable cells were collected. The ratio of CD45RA⁺/CD45RA⁻ (or CD45RO⁺) in CD4⁺ T cells was calculated by the following formula:

$$\text{Ratio} = \frac{\% \text{positive CD4}^+ \text{CD45RA}^+ \text{T cells}}{\% \text{positive CD4}^+ \text{CD45RA}^- \text{T cells}}$$

Although CD4⁺ T cells contained CD45RA⁺ RO⁺ T cells, most (over 90%) of CD4⁺ T cells exclusively express CD45RA or CD45RO antigens. The CD4⁺ T cells and CD8⁺ T cells were isolated from PBMCs by sorting with FACStar (Japan Becton Dickinson, Tokyo, Japan). The purity of each cells was over 98%.

Cytokine production

Unfractionated PBMCs or FACStar-sorted T cells were stimulated with various antigen for 4 days. In the case of isolated CD4⁺ T cells or CD8⁺ T cells, the cells were cultured with Ag in the presence of mitomycin C-treated PBMCs as a source of APC or they were stimulated with immobilized OKT3 MoAb in the absence of APC [22]. After incubation, culture supernatants were harvested and their IFN- γ activity or IL-4 activity was determined by RPN2757 human IFN- γ ELISA kits or RPN2753 human IL-4 ELISA kits, respectively (Amersham, Tokyo, Japan).

Intracellular staining of IFN- γ and IL-4

Cytoplasmic contents of IFN- γ and IL-4 were determined by FastImmune IFN- γ FITC/IL-4 PE kits (Japan Becton Dickinson Company). In brief, freshly isolated PBMCs (10^6 cells) were cultured in serum-free RPMI medium in the presence of PMA (10 ng/mL) plus A23187 (100 ng/mL) and 10 μ g/mL of brefeldin-A (Sigma) for 4 h. Then, the cells were harvested, stained with per CP-conjugated anti CD4 MoAb (Japan Becton Dickinson Company) and reacted with FITC-conjugated anti IFN- γ MoAb plus PE-conjugated anti

IL-4 MoAb after fixation and permeabilization of the cells using lysing solution (Japan Becton Dickinson Company) and permeabilizing solution (Japan Becton Dickinson Company).

Results

Reduced IFN- γ production of CD4⁺ T cells in AD patients

When PBMCs obtained from healthy donors were cultured with Der f, small amounts (40 pg/mL) of IFN- γ was produced. However, addition of IL-2 markedly synergized with Der f antigen to induce IFN- γ production (2300 pg/mL) though IL-2 alone induced low levels (400 pg/mL) of IFN- γ production. This enhanced IFN- γ production by IL-2 might be because of expansion of a small Ag-specific population of T cells. Stimulation of PBMCs obtained from healthy donors with immobilized OKT3 MoAb also resulted in the production of high levels of IFN- γ . In contrast, PBMCs in AD patients showed greatly decreased IFN- γ production when they were stimulated with Der f plus IL-2 or immobilized OKT3 MoAb (Fig. 1a). To elucidate which subpopulations of T cells was defect in terms of OKT3 MoAb-induced IFN- γ production, both CD4⁺ T cells and CD8⁺ T cells were isolated from AD patients or healthy donors by FACStar and their ability to produce IFN- γ was determined by culturing them with OKT3 MoAb-coated plate in the absence of APC. As shown in Fig. 1(b), the reduced IFN- γ production of T cells was demonstrated only in CD4⁺ T

cells but not in CD8⁺ T cells. The proliferative response of CD4⁺ T cells to immobilized anti CD3 MoAb was also reduced (data not shown).

To confirm the defect of IFN- γ production in CD4⁺ T cells in AD patients, the frequency of IFN- γ and IL-4-producing CD4⁺ T cells in PBMCs was examined by carrying out intracellular staining using Fast Immune cytokine system (Japan Becton Dickinson). The typical staining profile of intracellular IL-4/IFN- γ in CD4⁺ T cells obtained from healthy donors or AD patients was shown in Fig. 2(a, b). Such staining profile was obtained only when PBMCs were stimulated with PMA plus A23187 for 4 h before the assay. From the accumulated data (Fig. 2c), it was clearly demonstrated that the IFN- γ -producing ability of freshly isolated CD4⁺ T cells was greatly reduced in AD patients compared with that of healthy donors. The CD4⁺ T cells obtained from healthy donors contained 8.7% IFN- γ -producing cells, while AD patient's CD4⁺ T cells contained lower frequency (2.7%) of IFN- γ -producing cells.

We also tried to determine the intracellular expression of IL-4 in CD4⁺ T cells in AD patients (Fig. 2d). Unexpectedly, the percentage of IL-4-producing CD4⁺ T cells in AD patients was very low (0.53%) and no significant difference between AD patients and healthy donors (0.35%) was demonstrated.

Quantitative and qualitative defect of CD4⁺CD45RA⁻ T cells in AD patients

To determine the cellular mechanisms of the impaired TH1 immunity in AD patients, we next analysed the subsets of CD4⁺ T cells in both healthy donors and AD patients. It has been demonstrated that CD4⁺ T cells exclusively expressed either CD45RA Ag or CD45RO Ag [23,24]. The former is the marker for naive T cells and the latter is the marker for antigen-primed memory T cells [24,25]. We also confirmed that most (over 92%) of CD4⁺ T cells exclusively expressed either CD45RA or CD45RO markers though about 6.2% of CD4⁺ T cells were CD45RA⁺RO⁺ cells (Fig. 3a). As shown in Fig. 3(b–e), we found that the percentage of CD4⁺CD45RA⁻ (or CD4⁺CD45RO⁺) memory type T cells were greatly decreased in AD patients compared with that of healthy donors. The accumulated data (Fig. 3f) using 25 AD patients and 25 healthy donors demonstrated that this result was statistically significant and suggested that the quantitative defect of peripheral blood CD4⁺CD45RA⁻ T cells is a generalized phenomenon in AD patients.

We next examined whether CD4⁺CD45RA⁻ T cells obtained from AD patients possessed functional defects in addition to the quantitative defect using FACStar-sorted CD4⁺ T cell subsets (Fig. 4). Although CD4⁺CD45RA⁻ memory-type T cells of healthy donors produced high levels

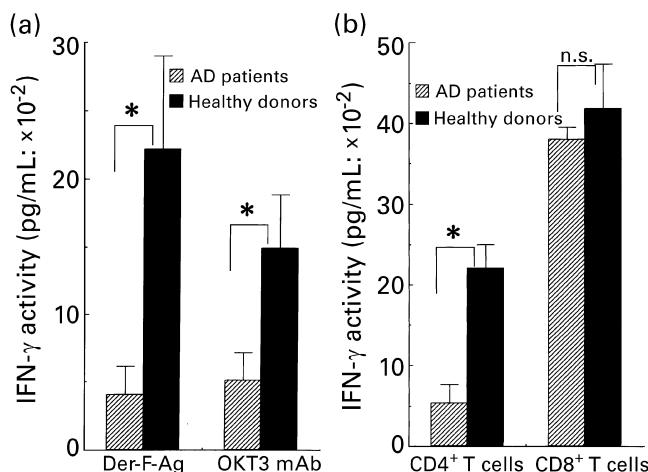


Fig. 1. The reduced IFN- γ -producing ability of CD4⁺ T cells in AD patients. (a) PBMCs (2×10^5 cells) obtained from healthy donors or AD patients were stimulated with Der f Ag (50 μ g/mL) plus IL-2 (20 U/mL) or immobilized OKT3 MoAb. (b) CD4⁺ or CD8⁺ T cells were isolated from PBMCs of either healthy donors or AD patients by FACStar. The bars represent mean $\pm$ SE of five donors. * $P < 0.05$. n.s. = not significant.

of IFN- γ by stimulation with immobilized OKT3 MoAb, the significant decrease of IFN- γ production was demonstrated in CD4⁺CD45RA⁻ memory T cells in AD patients as well as CD4⁺T cells. The OKT3 MoAb-induced proliferative response was also reduced in CD4⁺CD45RA⁻ memory T cells in AD patients (data not shown). However, both CD4⁺T cells and memory type CD4⁺T cells from AD patients exhibited the same levels of IFN- γ production as those from healthy donors when the cells were stimulated with polyclonal activator, PMA plus A23187. These data strongly suggested that the defect of in AD CD4⁺ memory T cells appeared to be restricted to the CD3 or T cell-receptor activation pathway.

The qualitative and quantitative defect of CD4⁺CD45RA⁻ memory type T cells are involved in an impaired DTH response to recall antigen in AD patients

We next examined whether the quantitative and/or qualitative defect of CD4⁺CD45RA⁻ T cells were involved in the impaired DTH response of AD patients against recall Ag such as PPD. PBMCs were obtained from five AD patients, who revealed high serum IgE levels (Fig. 5a) and were to be negative in tuberculin test (<2 mm). In contrast, all the healthy donors showed negligible serum IgE levels and they were positive in tuberculin test. Using these

PBMCs, CD45RA/CD45RO ratio and PPD-induced IFN- γ -producing ability of CD4⁺T cells were analysed. It was clearly demonstrated that the percentage of CD4⁺CD45RA⁻ memory-type T cells was greatly reduced in AD patients compared with healthy donors (Fig. 5b).

In PBMCs obtained from healthy donors, it became obvious that CD4⁺CD45RA⁻ memory T cells, but not CD4⁺CD45RA⁺ naive T cells, were major responsible cells for IFN- γ production induced by recall PPD Ag even when the number of cells (2×10^5 cells/well) were adjusted to the same number of those from healthy donors. Therefore, these results indicated that the abnormality of DTH responses against recall PPD Ag in AD patients may be derived from both the lower frequency and functional defect of IFN- γ -producing CD4⁺CD45RA⁻ memory type T cells.

Discussion

In this paper, we initially demonstrated that both the quantitative and qualitative defect of CD4⁺CD45RA⁻ (or CD4⁺CD45RO⁺) memory-type T cells may be involved in the abnormality of TH1 immunity including an impaired DTH response in AD patients. It has been demonstrated that PBMCs obtained from AD patients showed markedly decreased IFN- γ production when they were polyclonally stimulated with lectin or PMA plus ionophore [19–21].

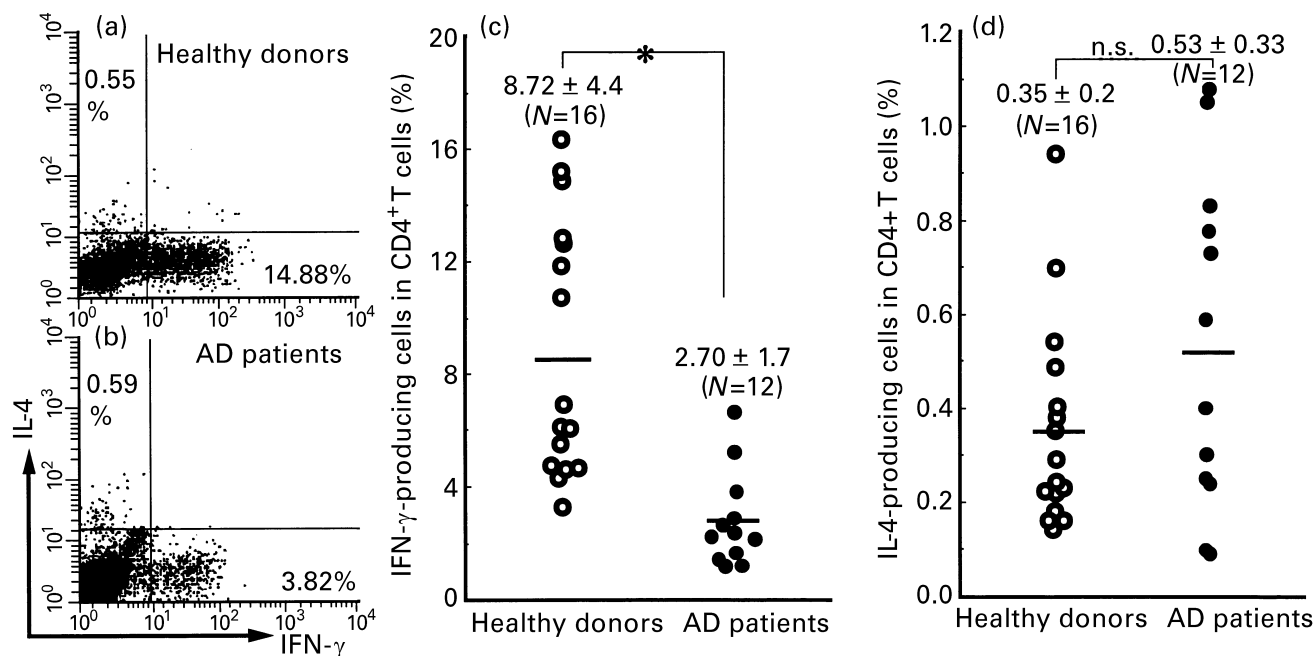


Fig. 2. Decreased frequency of IFN- γ producing CD4⁺ T cells in AD patients. Intracellular staining of IL-4 and IFN- γ was carried out using PBMCs obtained from healthy donors (a) or AD patients (b) by the method described in materials and methods. The accumulated data for IFN- γ or IL-4 is summarized in (c) or (d), respectively. In both experiments, PBMCs obtained from 10 healthy donors and 12 AD patients were used. The bars show mean of the positive percentage in each group. * $P < 0.05$. n.s. = not significant.

Consistent with these results, we also confirm that IFN- γ -production is reduced in PBMCs, especially in CD4⁺ T cells, obtained from AD patients compared with those from healthy donors (Fig. 1a, b). The defect of IFN- γ -producing ability of CD4⁺ T cells in AD patients is also demonstrated by cytoplasmic staining of IFN- γ in freshly isolated CD4⁺ T cells (Fig. 2c). Thus, it is a prominent phenomenon that IFN- γ -producing CD4⁺ T cells, which play an important role in TH1-dominant cellular immunity, are defect in peripheral blood of AD patients.

It has been demonstrated that naive CD45RA⁺ T cells develop into CD45RO⁺ memory type T cells after priming with Ag [26]. In contrast with specific pathogen-free experimental animals, PBMCs obtained from humans suffering with various environmental infections, contain high percentage of memory type CD4⁺CD45RA⁻RO⁺ T cells. These memory type T cells play a pivotal role in the protection of host from infectious diseases through the production of cytokines [27,28]. Therefore, it is strongly suggested that the defect of cellular immunity to recall antigen such as PPD might be derived from the abnormality of memory type CD4⁺ T cells. We find that the percentage of CD4⁺ memory type T cells is greatly decreased in AD patients compared with that of healthy donors (Fig. 2). This result is not inconsistent with previous report by Schauer *et al.* [29]

who suggested the imbalance of CD4⁺CD45RA⁺ and CD4⁺CD29⁺ T cells in AD patients. In addition to the quantitative abnormality of memory type T cells, we also demonstrate the functional defect of CD4⁺CD45RA⁻ T cells obtained from AD patients (Fig. 3). CD4⁺CD45RA⁻ memory type T cells showed markedly decreased response to immobilized OKT3 MoAb. Moreover, CD4⁺CD45RA⁻ memory T cells of AD patients show no significant IFN- γ production (TH1 immunity) in response to recall PPD Ag, though the patients were once primed with tuberculin. It is a well-accepted phenomenon that tuberculin test, which is mediated by the TH1-dependent DTH response, changes from positive to negative during the course of AD disease [16,17]. Moreover, it is demonstrated that there is an inverse association between tuberculin responses and atopic disorder [18]. Therefore, our results strongly suggest that the abnormality of DTH responses including negative tuberculin test in AD patients, may be derived from not only the quantitative defect but also qualitative defect of CD4⁺CD45RA⁻ memory type T cells which can produce large amounts of IFN- γ .

The experiments were carried out using PBMCs obtained from patients with high Six Area, Six Sign, Atopic Dermatitis (SASSAD) severity score [30] and high IgE serum titre (> 4000). Moreover, most AD patients were treated with

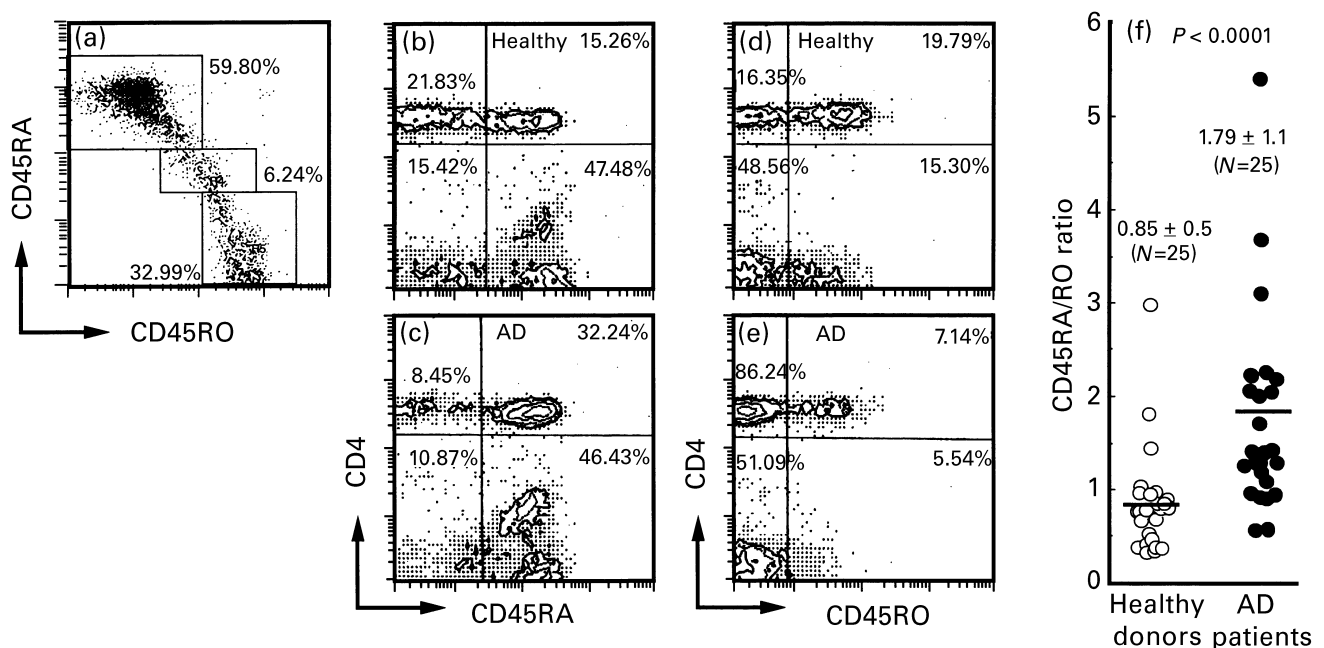


Fig. 3. The quantitative defect of CD4⁺CD45RA⁻RO⁺ memory type T cells in AD patients. (a), triple staining pattern of PBMCs obtained from AD patients using perCP-anti CD4 MoAb, PE-CD45RA MoAb and FITC-anti CD45RO MoAb. PBMCs obtained from healthy donors (b, d) or AD patients (c, e) were stained with PE-conjugated anti CD4 MoAb plus FITC-conjugated anti CD45RA MoAb or FITC-conjugated anti-CD45RO MoAb. The typical staining profile of CD4/CD45RA (b, c) and CD4/CD45RO (d, e) was shown. The CD45RA/CD45RO ratio in CD4⁺ T cells was calculated by the formula described in materials and methods. The accumulated data for CD45RA/CD45RO ratio was summarized in (f). The bars represent mean of the ratio in 25 healthy donors or 25 AD patients.

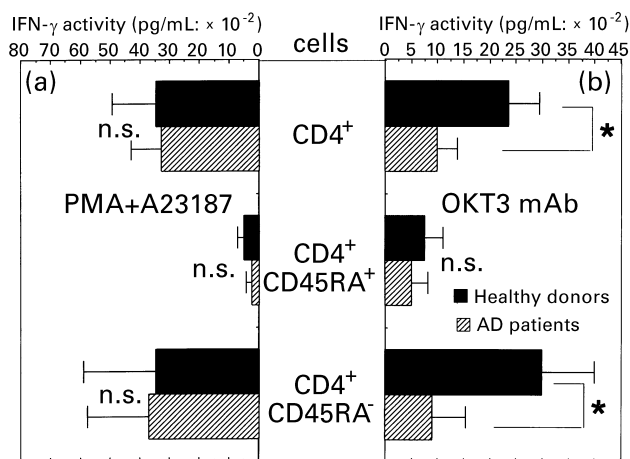


Fig. 4. The qualitative defect of CD4⁺ CD45RA⁻ RO⁺ memory type T cells in AD patients. CD4⁺ T cells, CD4⁺ CD45RA⁺ T cells, and CD4⁺ CD45RA⁻ T cells were isolated from PBMCs of healthy donors or AD patients by FACStar. Then the isolated cells were stimulated with PMA plus A23187 (a) or immobilized OKT3 MoAb (b). The bars represent mean $\pm$ SE of five donors. * $P < 0.05$. n.s. = not significant.

potent topical steroid cream (betamethasone dipropionate 0.05%, 2–8 g/day) for long term. Therefore, we cannot exclude the possibility that the abnormal immune responses of AD patients described in this paper might be magnified by these factors as compared with active AD patients.

However, some immunological causes have been considered to induce such immunological abnormality of AD patients as described previously. It has been demonstrated that overproduction of prostaglandin E, which inhibits TH1 immunity but not TH2 immunity, from monocytes is one of the mechanisms explaining the functional abnormality of CD4⁺ T cells in AD patients [31,32]. However, the isolated CD4⁺ CD45RA⁻ memory type T cells also show markedly reduced IFN- γ production as well as unseparated PBMCs after stimulation with immobilized OKT3 MoAb in the

absence of monocytes. Therefore, peripherally circulated CD4⁺ T cells by themselves are demonstrated to show functionally defect in AD patients. Because if CD4⁺ T cells are stimulated with PMA plus A23187, they produced the same levels of IFN- γ as those of healthy donors (Fig. 3). We also have the evidence that the activation of PBMCs of AD patients with Der f and IL-12 caused the recovery of IFN- γ production (900 pg/mL) though the stimulation of PBMCs with Der f alone induced less than 50 pg/mL of IFN- γ production. Thus the reduced TH1 immunity in AD patients appeared to be recovered by addition of IL-12 into the culture consistent with the previous report by Manetti *et al.* [8]. These findings are further suggesting that CD4⁺ CD45RA⁻ memory type T cells of AD patients might be defect in T cell-mediated signalling [33].

The detailed mechanism for the decreased number of memory T cells in peripheral blood in AD patients remains unclear. However, recently, it was demonstrated that IFN- γ is a key cytokine for the maintenance for memory T cells [34]. Therefore, the maintenance of a memory T-cell pool might decline in parallel with the decrease of TH1-dominant cellular immunity. Alternatively, cytokines produced by abnormally activated TH2 cells in lesional skin of AD patients actively inhibits the long survival of memory T cells *in vivo* through its direct or indirect effect. Indeed, IL-4 have been shown to inhibit the development of CD4⁺ CD45RA⁻ memory type T cells from CD4⁺ CD45RA⁺ naive T cells [35].

As described in Fig. 2(d), we cannot detect abnormality in IL-4-producing TH2 cells in PBMCs of AD patients. Therefore, IL-4-producing TH2 cells infiltrating in lesional skin [36], or other IL-4-producing cells such as eosinophils and mast cells [5,37] might be responsible cells for the induction of imbalanced immunity, the abnormality of TH1 immunity, which may explain the high susceptibility to infectious disease and an impaired DTH responsiveness in AD patients [1,2,14–17], appeared to be induced in accordance with the both the quantitative and qualitative defect of

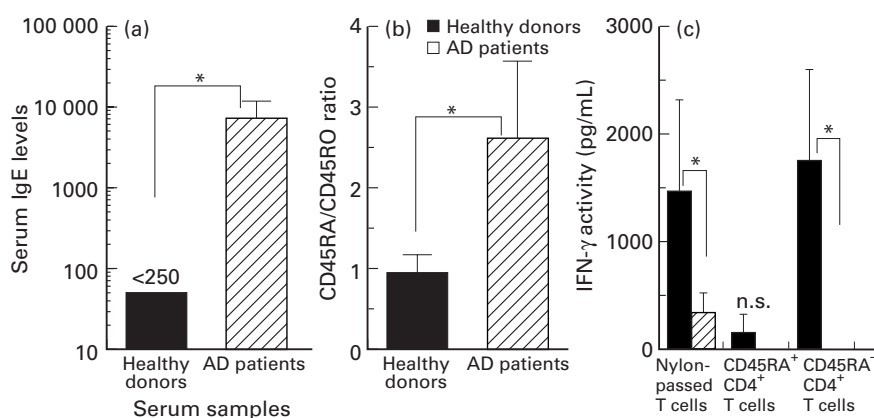


Fig. 5. Both the quantitative and qualitative defect of CD4⁺ CD45RA⁻ RO⁺ memory type T cells was involved in the abnormality of TH1-dependent DTH response in AD patients. (a) Serum total IgE levels in healthy donors or AD patients. (b) CD45RA/CD45RO ratio in CD4⁺ T cells obtained from healthy donors or AD patients. (c) Nylon-passed T cells, CD45RA⁺ T cells or CD45RA⁻ T cells were prepared from healthy donors or AD patients. The bars represent mean $\pm$ SE of five donors. * $P < 0.05$. n.s. = not significant.

memory type CD4⁺ T cells. It has been suggested that both genetic and environmental factors were involved in the induction of imbalanced TH1/TH2 immunity [29,38–42]. Therefore, it will be of great interest to pursue the factors inducing abnormal TH1 and/or TH2 immunity in AD patients.

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