

Severe atopic dermatitis is associated with a reduced frequency of IL-10 producing allergen-specific CD4⁺ T cells

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Summary

Background. Several studies have investigated levels of T-cell-derived interleukin (IL)-10 in individuals with atopic dermatitis, with conflicting results.

Aims/Hypothesis. In order to address whether stratification of disease severity may help resolve the different findings, the hypothesis was tested that individuals with severe atopic dermatitis have a lower frequency of circulating IL-10-producing, allergen-specific CD4⁺ T cells than do individuals with mild disease.

Methods. Using peripheral blood mononuclear cells derived from individuals with severe (n = 12) and mild atopic dermatitis (n = 10) and from nonatopic controls (n = 10), we investigated production by CD4⁺ T cells of tumour necrosis factor (TNF)- α , IL-4, IL-5, IL-13 and IL-10 in response to phorbol myristate acetate/ionomycin and Der p1 allergen.

Results. It was observed that there were significantly higher frequencies of allergen-specific circulating CD4⁺ T cells producing TNF- α , IL-4, IL-5 and IL-13, and lower frequencies of these cells producing IL-10 in individuals with severe atopic dermatitis compared with mildly affected individuals and nonatopic controls ($P < 0.01$ for all comparisons). Furthermore, the Der p1-specific CD4⁺ T cells were enriched within the subset of cells positive for cutaneous lymphocyte-associated antigen.

Conclusions. Analysis of levels of allergen-specific CD4⁺ T-cell production of IL-10 in relation to disease severity argues in favour of a role for IL-10 in the control of atopic dermatitis.

Introduction

Evidence is accumulating that interleukin (IL)-10 may play an important role in the control of immune responses associated with atopic disease. For example, IL-10 gene polymorphisms have been differentially associated with asthma phenotypes,^{1,2} including low IL-10-producing promoter genotypes being linked to severe asthma.¹ Mite-specific skin-prick test reactivity

has been observed to inversely associate with schistosomal-specific IL-10 production in Gabon,³ and with mite-specific IL-10 production in Australian children with atopic disease.⁴ Several other studies have documented increased IL-10 levels in individuals with respiratory atopic disease compared with normal controls, but disease-severity stratification reveals potentially important differences.^{5–7} In severe asthmatics, the level of IL-10 in bronchoalveolar lavage fluid has been found to be significantly lower than levels in mildly affected individuals;⁸ and low IL-10 production has been documented after polyclonal stimulation of peripheral blood mononuclear cells (PBMC) derived from severe asthmatics.⁹ Plasma IL-10 levels increase in sensitized but asymptomatic individuals during seasonal pollen-allergen exposure, and increased IL-10

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production has been repeatedly documented during allergen-specific immunotherapy.^{10–12} Furthermore, glucocorticoids may act partly through induction of IL-10, although the dominant target cell population remains controversial.¹³ More recent data suggest that healthy nonsensitized individuals have circulating allergen-specific CD4+ CD25+ or IL-10-producing T cells that are able to respond to antigen challenge.^{14,15}

In contrast, while several studies have documented levels of CD4+ T-cell-derived IL-10 in individuals with atopic dermatitis (AD), the results have been conflicting.^{15–19} Because disease severity stratification has helped reconcile differential findings of IL-10 levels in individuals with respiratory atopic disease, we tested the hypothesis that individuals with severe AD have lower frequencies of circulating IL-10-producing, allergen-specific CD4+ T cells than individuals with mild disease. Through detection of IL-4, IL-5 and IL-13, we firstly confirmed that atopic individuals have higher frequencies of circulating Th2 cytokine-producing mitogen-reactive and allergen-specific T cells than nonatopic people. We next showed that individuals with severe AD had lower frequencies of IL-10-producing allergen-specific CD4+ T cells than do those with mild disease. Furthermore, the Der p1-specific CD4+ T cells were enriched within the cutaneous lymphocyte-associated antigen (CLA)-positive subset, consistent with a relevant role for skin homing Der p1-specific CD4+ T cells in disease pathogenesis. These data add atopic skin disease to the growing evidence supporting a regulatory role for CD4+ T-cell-derived IL-10 in the control of atopic disease severity.

Materials and methods

Subjects

In total, 22 atopic subjects and 10 nonatopic healthy controls were included in the study. The 22 atopic subjects comprised 12 severely symptomatic and 10 mildly affected individuals with AD. The subjects were recruited through the Department of Dermatology, Churchill Hospital, Oxford, under ethics approval from the Oxfordshire Clinical Research Committee. All atopic subjects satisfied the UK AD diagnostic criteria, which are highly specific and sensitive.²⁰ The criteria are an itchy skin condition plus three of more of the following: onset before 2 years of age, history of skin-crease involvement, history of generally dry skin, visible flexural dermatitis, and/or personal history of another atopic disease (or history of atopic disease in a first-degree relative). In addition, all severely symptomatic

atopic subjects had, by definition, a Six Area, Six Sign Atopic Dermatitis (SASSAD) score of >54 (of a possible total of 108).²¹ The mildly affected individuals had SASSAD scores of <10. The SASSAD score is graded 0–3 (absent to severe) for each of the six clinical features (erythema, exudate, excoriation, lichenification, dryness and cracking) at six sites (head/neck, thorax/proximal limbs, mid-upper limbs, mid-lower limbs, hands and feet). All atopic subjects had positive skin-prick test and IgE radioallergosorbent test (RAST) test to house dust mite. The age and sex distribution of all three groups was not statistically different. None of the patients or controls had received systemic immunosuppressive treatments in the preceding 6 months.

Monoclonal antibodies

The following monoclonal antibodies were used in this study: fluorescein isocyanate (FITC)-conjugated rat antihuman CLA, phycoerythrin (PE)-conjugated mouse antihuman IL-4, PE-conjugated rat antihuman IL-13, PE-conjugated rat antihuman IL-5, PE-conjugated mouse antihuman TNF- α , PE-conjugated CD25, PE- and FITC-conjugated mouse IgG1, mouse IgG2b, rat IgG1 and rat IgM, PerCP (peridinin chlorophyll protein)-conjugated CD3, allophycocyanin (APC)-conjugated CD8 (all Becton Dickinson, Oxford, UK), FITC-conjugated rat antihuman IL-4, and PE-conjugated rat antihuman IL-10 (both Caltag, South San Francisco, CA, USA).

Flow-cytometric assays

PBMCs were stimulated using phorbol 12-myristate 13-acetate/ionomycin (PMA/I), or recombinant Der p1 allergen (Indoor Biotechnologies Ltd, Cardiff, UK) used at 10 μ g/mL and 100 μ g/mL concentrations. For the PMA/I experiments, cells were stimulated for 4 h in R-10 medium (RPMI + 10% autologous serum) containing 25 ng/mL PMA and 1 mmol/L ionomycin. Brefeldin A (10 mg/mL) was added for the entire 4 h. In the Der p1 experiments, we found 8 h to be the optimal stimulation time, with brefeldin added for the last 6 h. When cells were stimulated for 4 h (as with PMA/I), numbers of cytokine-positive cells were lower than when measured after 8 h. Incubation for longer than 12 h caused significant cell death. Thus, Der p1 experiments were performed using 8 h of incubation, which, as might be anticipated, is longer than the time interval in peptide-stimulated assays. The cells were then washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA),

incubated in 0.5 mL permeabilizing solution (Becton Dickinson) at room temperature for 10 min, washed with PBS/BSA, and then incubated for 25 min with the relevant monoclonal antibody–fluorochrome conjugates. After staining, the cells were washed and resuspended in 0.5 mL of 1% paraformaldehyde in PBS before flow-cytometric analysis. Negative-control reagents were used to verify staining specificity of experimental monoclonal antibodies, and as guides for setting markers to delineate positive and negative populations. As stimulation with PMA/I causes down-regulation of CD4 from the cell surface, the number of CD3+ CD8- T cells producing cytokines was taken as the number of CD4+ T cells staining positive for the cytokines tested. However, as there was no significant downregulation of CD4 following Der p1 stimulation, we used anti-CD4 monoclonal antibodies to identify CD4+ T cells.

In these experiments, cytokine staining was confirmed to be intracellular, as <0.2% of cells were positive with surface staining (i.e. staining performed prior to permeabilization). In addition, specificity of antibody reactivity was supported by showing that preincubation of the conjugated antibody with 1 µg of the appropriate recombinant cytokine (Peprotech; <http://www.peprotech.com>) for 30 min at 4 °C could abolish staining.

Statistical analysis

After PMA/I or Der p1 stimulation, the mean ± SD percentage (or number) of cells producing different

cytokines was calculated. Statistical analysis of differences between groups of subjects was performed using Student's *t*-test ($P < 0.05$ being accepted as significant).

Results

Cytokine production by CD4+ T cells following PMA/I stimulation

Figure 1(a) shows the mean ± SD percentage of CD4+ T cells positive for IL-4, IL-13, IL-5, IL-10 and TNF-α following PMA/I stimulation. Severely affected subjects had a significantly higher percentage of IL-4-, IL-13- and IL-5-positive CD4+ T cells compared with mildly affected and nonatopic subjects ($P < 0.01$ for all comparisons). In contrast, percentages of TNF-α-positive CD4+ T cells were significantly higher in nonatopic subjects compared with either of the atopic groups ($P < 0.01$ for all comparisons), with severely affected subjects having the lowest percentage. Although mildly affected subjects had a higher percentage of IL-10-positive CD4+ T cells compared with severely affected and nonatopic subjects (mean 2% vs. 1.6% and 1.6%, respectively), this difference did not reach statistical significance.

Cytokine production by CD4+ T cells following recombinant Der p1 stimulation

Mean ± SD percentage of CD4+ T cells positive for IL-4, IL-13, IL-5, IL-10 and TNF-α following Der p1

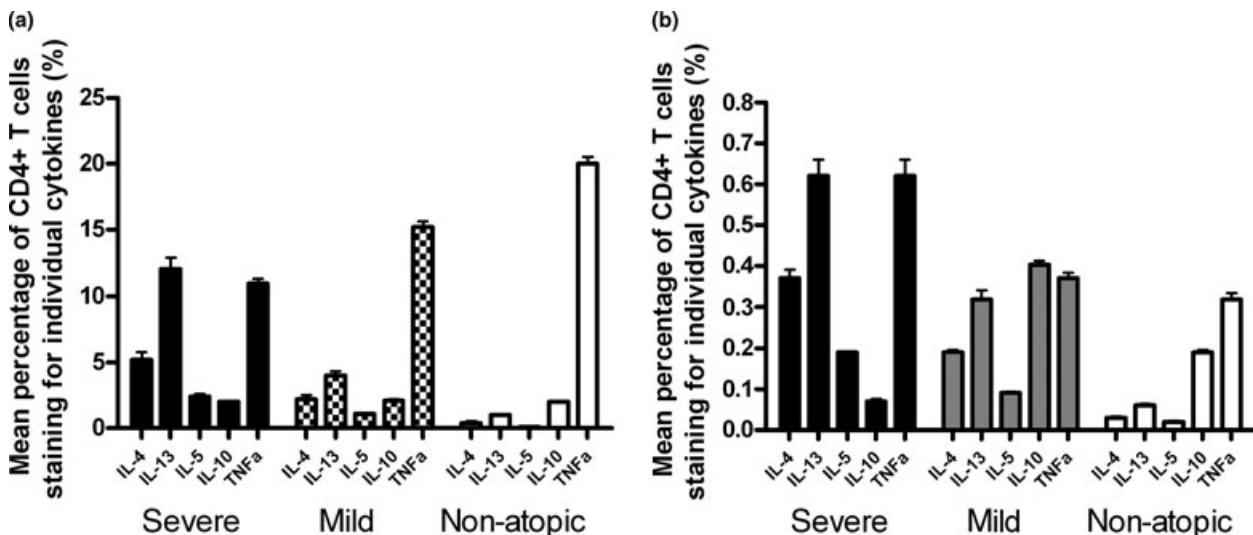


Figure 1 Mean ± SD percentage of CD4+ T cells positive for IL-4, IL-13, IL-5, IL-10 and TNF-α following stimulation of PBMCs with (a) PMA/I or (b) Der p1. Solid bars, severe; hashed/grey bars, mild; clear bars, nonatopic.

stimulation is shown in Fig. 1(b). Frequencies of T cells staining positive for cytokines following Der p1 stimulation were lower than those following PMA/I stimulation. As with PMA/I stimulation, severely affected subjects had significantly higher numbers of CD4+ T cells positive for IL-4, IL-13 and IL-5, compared with mildly affected and nonatopic subjects ($P < 0.01$ for all comparisons). In contrast to the PMA/I findings, the frequency of TNF- α -positive CD4+ T cells was significantly greater in the severely affected subjects compared with the other two groups. Frequencies of IL-10-positive CD4+ T cells were significantly higher among mildly affected subjects compared with severely affected and nonatopic subjects ($P < 0.01$). Furthermore, following Der p1 stimulation, the proportion of IL-10-positive CD4+ T cells staining with the IL-2 receptor α -chain (CD25) derived from severely affected, mildly affected and nonatopic subjects was 18%, 50% and 44%, respectively.

Cytokine production by CLA+ CD4+ T cells

After stimulation with PMA/I, mean $\pm$ SD percentages of CLA+ CD4+ and CLA- CD4+ T cells staining positive for IL-4, IL-13, IL-5, IL-10 and TNF- α are shown in Fig. 2. Atopic subjects had higher percentages of CLA+ T cells positive for IL-4, IL-5 and IL-13 than CLA- CD4+ T cells positive for the same interleukins. Severely affected subjects had the highest percentage of CLA+ CD4+ T cells positive for IL-4, IL-5 and IL-13. Although comparable percentages of CLA+ CD4+ and

CLA- CD4+ T cells from nonatopic subjects stained for TNF- α , a lower percentage of CLA+ CD4+ T cells compared with CLA- CD4+ T cells were positive for this cytokine in subjects with AD. Severely affected subjects had the lowest percentage of IL-10-positive CLA+ CD4+ T cells ($P < 0.01$, Fig. 2) in response to PMA/I.

Mean $\pm$ SD percentages of CLA+ CD4+ and CLA- CD4+ T cells positive for IL-4, IL-13, IL-5, IL-10 and TNF- α following Der p1 stimulation are shown in Fig. 3. Only a small percentage ($< 2\%$) of total CLA+ CD4+ T cells stained positive for cytokines, suggesting that the remainder of the CLA+ CD4+ T cells are possibly specific for other skin-associated antigens. In addition, there was a higher percentage of cytokine-staining cells in the CLA+ subset of T cells compared with the CLA- subset. Der p1-specific CLA+ CD4+ T cells derived from severely affected patients with AD produced significantly less IL-10 than those derived from mildly affected individuals ($P < 0.01$). Significant differences were also observed between CLA- CD4+ cells from severely affected individuals, but the overall levels of responses were considerably lower.

Discussion

We sought to investigate whether stratification of disease severity may help resolve whether levels of T-cell-derived IL-10 are increased or decreased in individuals with cutaneous atopic disease. It was observed that there were significantly higher frequencies of circulating

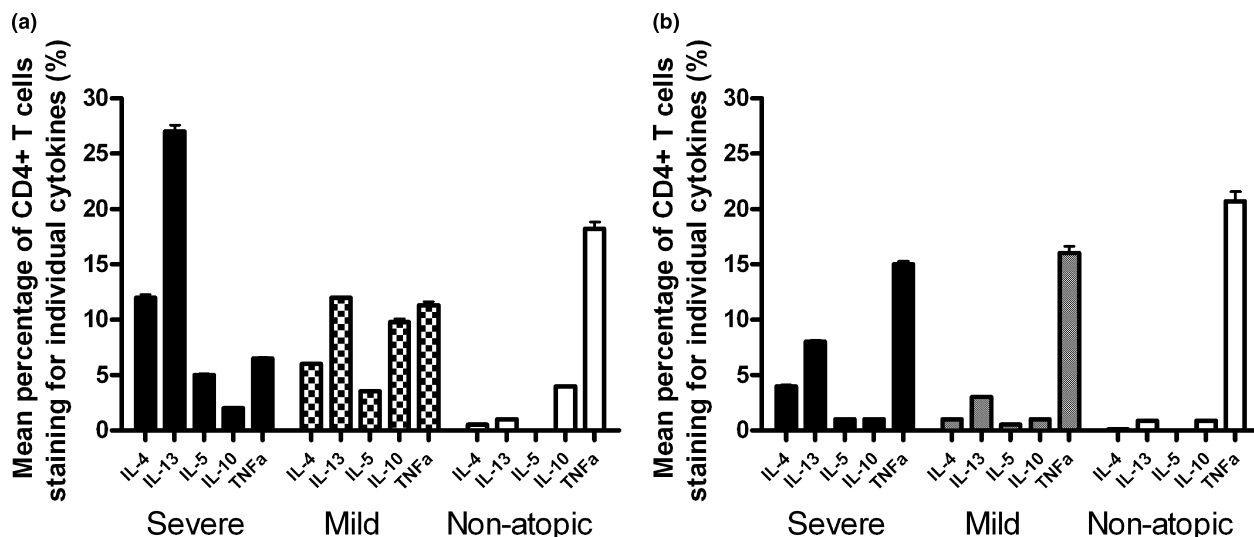


Figure 2 Mean $\pm$ SD percentage of CD4+ T cells positive for IL-4, IL-13, IL-5, IL-10 and TNF- α following stimulation of PBMCs with PMA/I within (a) the CLA+ CD4+ and (b) the CLA- CD4+ subsets. Solid bars, severe; hashed/grey bar, mild; clear bar, nonatopic.

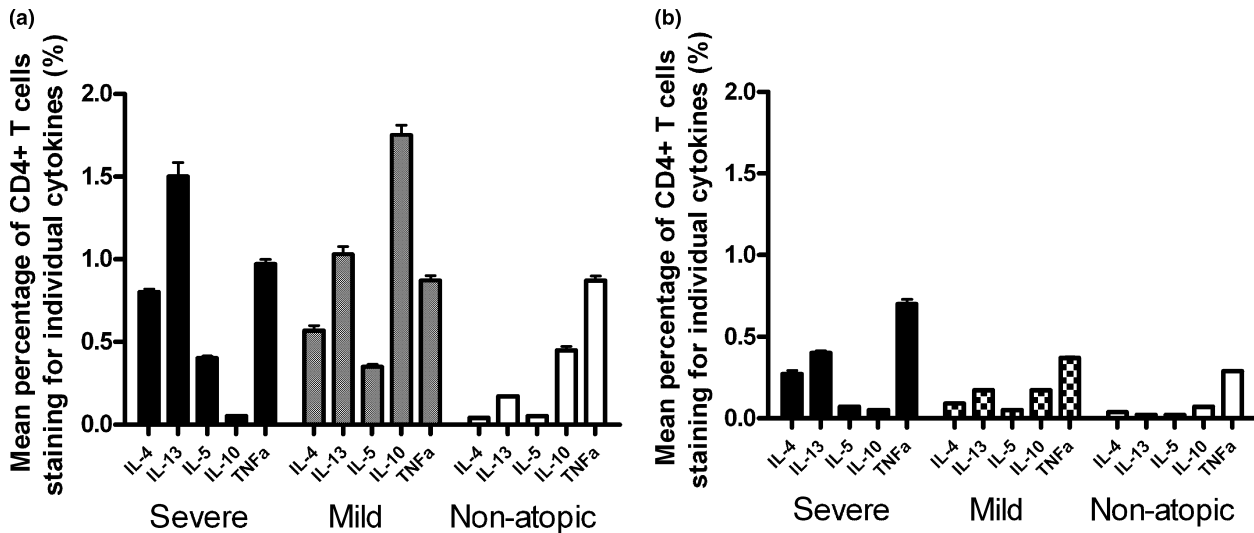


Figure 3 Mean $\pm$ SD percentage of CD4+ T cells positive for IL-4, IL-13, IL-5, IL-10 and TNF- α following stimulation of PBMCs with Der p1 within (a) the CLA+ CD4+ and (b) CLA- CD4+ subsets. Solid bars, severe; hashed/grey bars, mild; clear bars, nonatopic.

allergen-specific CD4+ T cells producing TNF- α , IL-4, IL-5 and IL-13, and lower frequencies of these cells producing IL-10 in individuals with severe AD compared with mildly affected individuals and nonatopic controls. Through analysis of frequencies of allergen-specific CD4+ T-cell production of IL-10 in relation to disease severity, these data argue in favour of a role for IL-10 in the control of atopic disease pathogenesis.

The data confirm that AD is associated with a Th2 production bias in response to both mitogens and allergen stimulation. Furthermore, the frequencies of Th2 cytokines were found to increase with clinical disease severity. TNF- α has been linked to both Th1 and Th2 responses,²² and our data would fit with a role for IL-4, IL-5, IL-13 and TNF- α in contributing to inflammation in AD. However, recent clinical studies suggest mixed benefit from TNF inhibition in individuals with AD.^{23–25}

Der p1 is a ubiquitous environmental allergen, and thus both nonatopic and atopic individuals are likely to be exposed. Through its protease activity, the Der p1 allergen is also capable of disrupting tight junctions, which may contribute to the ability of Der p1 to enter the professional antigen-presenting cell (APC) pathway for processing and presentation to T cells. In atopic individuals, such allergen uptake may be more efficient through IgE-binding to Fc ϵ RI receptors on the surface of APCs.²⁶ However, in nonatopic subjects, where allergen is presumably taken up primarily by non-IgE-mediated mechanisms, the quality and quantity of the allergen-specific T-cell response is different.

IL-10 is known to regulate various effector immune responses.²⁷ Our finding that mildly affected subjects have the highest frequency of CD4+ T cells staining positive for IL-10 may suggest that a process of active suppression could occur during the natural course of the disease. In contrast to previous findings, we were unable to document elevated IL-10 producing allergen-specific CD4+ T-cell responses in nonatopic controls.¹⁵ Thus, resolution of clinical disease may be associated with higher numbers of IL-10-producing T cells.

In summary, among severely symptomatic patients with AD, there is altered production of IL-10 by allergen-specific CD4+ T cells, particularly within the CLA+ subset. Through stratification of affected individuals by disease severity, the data reconcile existing studies of levels of IL-10 associated with AD, and support a role for IL-10 in the control of atopic inflammation.

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