

T-helper and regulatory T-cell cytokines in the peripheral blood of patients with active alopecia areata

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Summary

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Conflicts of interest

None declared.

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Background Alteration in T-lymphocyte function and cytokines secreted by T-cell subsets has been proposed in the immunopathogenesis of alopecia areata (AA). The role of T-helper and regulatory T-cell cytokines in the pathogenesis of active AA has not been established.

Objectives To assess the role of hallmark cytokines of T-helper cells (Th1, Th2 and Th17) and regulatory T cells (Tregs) in the pathogenesis of AA, and its clinical correlation.

Methods Fifty-one patients with AA and 45 age- and sex-matched healthy control subjects were included in the study. Serum interleukin (IL)-2, interferon (IFN)- γ , IL-10, IL-13, IL-17A and transforming growth factor (TGF)- β 1 were measured by enzyme-linked immunosorbent assay in both groups. Correlation of serum cytokine levels with age, sex, disease subtype and duration, number of patches on the scalp, associated autoimmune disorders and atopy was studied.

Results The serum cytokine levels of IL-2, IFN- γ , IL-13 and IL-17A were significantly increased, and serum TGF- β 1 levels were significantly decreased ($P < 0.05$) in patients with AA compared with controls. Serum IL-2 levels were significantly different among AA subgroups ($P < 0.05$). IL-2 levels were positively correlated with the total disease duration and the number of patches on the scalp.

Conclusions The increased levels of serum IL-2, IFN- γ , IL-13 and IL-17A suggested altered T-helper cell function, and reduced serum TGF- β 1 levels suggested a defect in Treg function. Therefore, enhanced T-cell-mediated immunity and breakdown of immune tolerance due to deficiency in Tregs may facilitate the occurrence of AA.

What's already known about this topic?

- Alopecia areata (AA) is an organ-specific autoimmune disorder.
- CD4⁺ T-cell subsets are implicated in the pathogenesis of AA.
- Cytokines may facilitate the development and/or progression of AA.

What does this study add?

- The present study revealed the role of hallmark cytokines of T-helper and regulatory T cells in the pathogenesis of AA, and their clinical correlations.
- These cytokines can be targeted to develop therapeutics in the management of AA.

Alopecia areata (AA) is considered an autoimmune disorder characterized by patchy loss of hair from the scalp and other body parts. Alteration in the specialized subpopulation of CD4⁺ T cells, namely T-helper (Th)1, Th2 and Th17 cells

and regulatory T cells (Tregs), has been implicated in autoimmune disorders.^{1–3} Evidence has suggested the role of altered T-cell-mediated immunity and cytokines in the pathogenesis of AA.^{4–7} Th1 cells predominantly produce interferon (IFN)- γ ,

tumour necrosis factor (TNF)- β and interleukin (IL)-2; Th2 cells produce IL-4, IL-5, IL-10 and IL-13; Th17 cells produce IL-17, IL-23 and IL-6; and Tregs synthesize transforming growth factor (TGF)- β and IL-10.^{8–10} The role of Th1 and Th2 cytokines in the peripheral blood has been studied in AA, but the role of Th17 and Treg cytokines has not been studied yet. Moreover, previous studies on these cytokines are few and the results are often contradictory. To the best of our knowledge, the role of IL-13 and IL-17A in AA has not been investigated. The purpose of this study was to elucidate the role of major cytokines secreted by this specialized subpopulation of T cells in the pathogenesis of AA.

Patients and methods

Patient and control groups

Fifty-one patients with AA and 45 age- and sex-matched healthy controls were enrolled in the study. The patient group included 12 female (24%) and 39 male (76%) patients and the control group included 10 female (22%) and 35 male (78%) subjects. The mean ages of the patients with AA and the controls were 24.19 ± 6.69 years and 26.42 ± 4.35 years, respectively. Age, sex, duration of disease, site and age of onset, total number of patches, progression of disease, family history of AA, any systemic or autoimmune diseases and nail changes were recorded. The patients had not used any medications (topical or systemic steroids) in the preceding 4 weeks and all patients had active AA. The course of AA was defined as active when there was an appearance of new lesions or progression of old lesions within 3 months, and stable when there was no appearance of new lesions or progression of old lesions within 1 year. In the present study we recruited patients with AA undergoing only their first episode of attacks, and all patients had active disease. We excluded patients with multiple episodes of attacks in the current study to develop a better understanding of cytokine status during the induction/development phase of the disease. The total duration was defined as the time from when the patient had first experienced the symptoms of the disease to the day of sample collection during the first episode of attacks. We also excluded patients and control subjects having any past or current history of tobacco, alcohol or drug use to avoid the influence of any such factors on serum cytokines, and control subjects with any past or current history of atopy were also excluded from the study.

The patients were classified into the following three groups: (i) AA (patients with fewer than 10 patches of hair loss); (ii) extensive AA (EAA; defined as hair loss involving > 40% of the scalp and/or more than 10 patches on the scalp, arms, legs or other sites in the past 3 months); (iii) alopecia totalis (AT) or alopecia universalis (AU) (patients who experienced near or complete baldness were categorized as having AT or AU, respectively). In the present study, 39 patients had AA, eight had EAA and four had AT or AU. Of the patients with AA, three (6%) had a personal history of associated

autoimmune diseases (one each with vitiligo, thyroiditis and diabetes) and a positive family history was recorded for other associated autoimmune diseases in seven patients with AA (14% of the total). Nine patients (18%) had nail involvement (four in the AA subgroup, three with EAA and two with AU/AT), in the form of discoloration, nail pitting, frayed ep-onychium or longitudinal striations. There were five patients (10%) with AA with a history of atopy (atopic dermatitis, $n = 3$; allergic asthma, $n = 2$; but none with allergic rhino-conjunctivitis). Written informed consent from all the patients and control subjects, and institutional ethics committee approval were obtained.

Methods

Peripheral venous blood samples were collected from patients with AA and healthy volunteer controls. Sera were isolated, and the concentrations of serum cytokines, namely IL-2, IFN- γ , IL-10, IL-13, IL-17A and TGF- β 1 (eBiosciences Inc., San Diego, CA, USA), were detected quantitatively in freshly collected serum samples by the enzyme-linked immunosorbent assay (ELISA) method in both patients and controls. ELISA tests were performed according to the manufacturer's instructions. The serum cytokine levels were compared between both groups and among the AA subtypes. The correlation of serum cytokine levels with age, sex, duration of AA, number of patches on the scalp and associated autoimmune disorders and atopy was studied. The ELISA test was performed in triplicate for each sample to ensure minimal variation between the wells.

Statistical analysis

The Mann–Whitney U-test (or Wilcoxon rank-sum test) was used to compare the median serum cytokine levels between the AA and control groups. The nonparametric test, Kruskal–Wallis one-way analysis of variance, was used to compare median serum cytokine levels between the AA subgroups, and logarithmic transformation of the data was applied. The Spearman correlation test was used for the study of correlation between quantitative parameters. The significance level was set at $P < 0.05$. Statistical analyses were performed using a software package (Stata version 9 for Windows; StataCorp, College Station, TX, USA).

Results

The serum IL-2, IFN- γ , IL-13 and IL-17A levels were significantly increased and TGF- β 1 levels were significantly decreased in patients with active AA compared with control subjects (Mann–Whitney U-test, $P < 0.001$ for IL-2, IFN- γ , IL-13, IL-17A and TGF- β 1) (Table 1). The serum IL-10 levels were comparable between the AA and control groups ($P = 0.453$). No significant difference was observed between the serum cytokine levels of IFN- γ , IL-10, IL-13, IL-17A and TGF- β 1 when compared between the AA subgroups

Table 1 Comparison of median serum cytokine levels in patients with alopecia areata (AA) and controls

Cytokine (pg mL ⁻¹)	Controls (n = 45)	AA (n = 51)	P-value
IL-2	10.8 (3.6–32.6)	18.6 (7.2–53.2)	0.0008
IFN- γ	11.53 (2.57–27.84)	19.94 (2.05–50.47)	0.001
IL-10	10.47 (3.11–14.11)	9.47 (3.11–34.16)	0.453
IL-13	5.34 (2.07–10.75)	13.11 (2.12–26.29)	0.001
IL-17A	9.16 (2.71–20.77)	18.19 (4.43–67)	0.001
TGF- β 1	5223.4 (1086.4–6987.4)	1121.4 (190.4–2801.4)	0.001

Data are represented as median (minimum–maximum). Wilcoxon rank-sum (Mann–Whitney U-test) was used and $P < 0.05$ is significant. IL, interleukin; IFN, interferon; TGF, transforming growth factor.

(Table 2); however, for IL-2 a significant difference was found among the disease subgroups (overall $P = 0.001$; AA vs. EAA, $P = 0.002$; AA vs. AU/AT, $P = 0.001$; EAA vs. AU/AT, $P = 0.027$). However, the differences in median serum levels of IL-17A ($P = 0.06$) and TGF- β 1 ($P = 0.059$) were of borderline significance among the AA subgroups. Furthermore, no correlation was found between serum cytokine levels and the total duration of AA, except for serum IL-2, where levels were found to be significantly increased when the total duration of AA was increased (Spearman correlation, $\rho = 0.3155$, $P = 0.024$; Table 3). In addition, serum IL-2 levels were positively correlated with the number of patches on the scalp (Spearman correlation, $\rho = 0.6677$, $P = 0.001$; Table 4). However, no correlation was found between serum cytokine levels and age, sex, family history of AA, or other associated autoimmune diseases. Finally, we found no significant difference in serum cytokine levels between patients with AA with and without atopy (Table 5).

Discussion

AA is a site-specific autoimmune disease of hair follicles, although the precise aetiology is not known. Evidence suggests that T cells and their effector cytokines play an important role in the pathogenesis of autoimmune diseases and may play a role in the selective loss of hair in AA.^{11–16}

The Th1 cytokine IL-2 is a lymphokine produced by activated T cells, and IL-2 receptor (IL-2R) is expressed on T

cells.^{17,18} The role of IL-2/IL-2R is implicated in the pathogenesis of AA. A previous study reported significantly elevated serum levels of IL-2 in patients with the extensive form of AA compared with those with localized AA.¹² Valsecchi *et al.*¹⁹ detected significantly higher concentrations of soluble IL-2R in patients in the active phase of AA compared with those in the stable phase, and in controls. Barahmani *et al.*²⁰ found higher serum IL-2 levels in patients with AA. The current study also reported higher levels of serum IL-2 in patients with active AA compared with the control group. In addition, a significant difference was found in serum IL-2 levels when compared among the AA subgroups, and a positive correlation was found between serum IL-2 levels and total duration of AA. When comparing serum IL-2 levels with the number of lesional patches on the scalp, a significant positive correlation was found, suggesting the important role of IL-2 in the induction and progression phases of AA.

The Th1 cytokine IFN- γ is produced by the perifollicular or follicular antigen-presenting cells and interferes with anagen hair growth.²¹ This notion is supported by the present study, which found significantly higher circulating levels of IFN- γ in patients with active AA compared with the control group. Increased serum IL-2 and IFN- γ levels suggest an overall impairment in the Th1-mediated immune response in AA. A previous study also suggested higher serum levels of IFN- γ in patients with AT or AU compared with controls, but no significant difference was found in levels of IFN- γ between patients with localized AA and those with more extensive

Table 2 Comparison of median serum cytokine levels in alopecia areata (AA) subgroups

Cytokine (pg mL ⁻¹)	AA subgroup			Overall P-value
	AA (n = 39)	AU/AT (n = 4)	EAA (n = 8)	
IL-2	15.4 (7.2–37.6)	43.4 (38.2–53.2)	26.6 (19.6–42.2)	0.001
IFN- γ	19.4 (2.05–50.47)	26.26 (21.5–33.6)	21.2 (8.3–44.6)	0.286
IL-10	13.74 (2.55–26.29)	9.17 (2.13–17.57)	13.42 (7.14–17.57)	0.574
IL-13	13.2 (5.8–28.9)	13.73 (8.78–18.78)	13.21 (8.29–18.78)	0.823
IL-17A	14.96 (4–38.83)	33.03 (15.93–43.67)	20.77 (4.32–36.90)	0.060
TGF- β 1	1030.4 (190.4–2801.4)	1275.4 (792.4–1758.4)	1446.9 (1037.4–2773.4)	0.059

Data are represented as median (minimum–maximum). Kruskal–Wallis (Mann–Whitney U-test) was used, $P < 0.05$ is significant. IL, interleukin; IFN, interferon; TGF, transforming growth factor; AU, alopecia universalis; AT, alopecia totalis; EAA, extensive AA.

Table 3 Correlation between serum cytokine levels and total disease duration

Cytokine (pg mL ⁻¹)	Spearman ρ	P-value
IL-2	0.3155	0.024
IFN- γ	-0.075	0.597
IL-10	0.163	0.254
IL-13	-0.052	0.719
IL-17A	-0.064	0.653
TGF- β 1	0.132	0.355

Spearman's rank correlation test was performed, $P < 0.05$ is significant. IL, interleukin; IFN, interferon; TGF, transforming growth factor.

Table 4 Correlation between serum cytokine levels and number of patches on scalp

Cytokine (pg mL ⁻¹)	Spearman ρ	P-value
IL-2	0.6677	0.001
IFN- γ	0.068	0.638
IL-10	-0.186	0.192
IL-13	0.090	0.529
IL-17A	0.216	0.126
TGF- β 1	0.227	0.109

Spearman's rank correlation test was performed, $P < 0.05$ is significant. IL, interleukin; IFN, interferon; TGF, transforming growth factor.

forms.^{12,15} Contrary to this, levels of IFN- γ mRNA were found to be lower in patients with AA than in healthy controls,¹⁶ and they were comparable in both the AA and control groups.²⁰

The Th2 cytokines IL-10 and IL-13 are anti-inflammatory cytokines, and their role has been implicated in the pathogenesis of AA.²²⁻²⁵ In the previous study, peripheral blood IL-10 mRNA levels were found to be comparable,¹⁶ and serum IL-10 levels²⁰ were found to be higher in patients with AA compared with control subjects. The present study found comparable serum IL-10 levels in the active AA and control groups. IL-13 levels were found to be significantly higher in patients with active AA compared with control subjects. No

statistically significant difference was found in serum IL-10 and IL-13 levels when compared among AA phenotypes. Furthermore, no correlation was found between serum IL-10 and IL-13 levels when comparing the total duration of AA and the number of patches on the scalp.

The role of IL-13 in the pathogenesis of AA is not known, and the current study is the first to report the participation of IL-13 in active AA. Recently, Jagielska *et al.*²⁶ reported the association of IL-13 and KIAA0350 with AA. Their genome-wide association study was carried out in two separate groups of patients with AA with and without atopy, and reported that IL-13 and KIAA0350 are associated with AA irrespective of the presence or absence of atopy. An expression study of IL-13 and KIAA0350 also confirmed the presence of these genes in hair and skin milieu.²⁶ The above findings supported our study concerning the involvement of IL-13 in the pathogenesis of active AA. However, the study was performed in a different ethnic population (Central European), and further genetic study is necessary in an Indian population to confirm the involvement of IL-13 as one of the susceptibility loci for AA in Indians. Furthermore, Barahmani *et al.*²⁰ found no significant difference for IL-13 in AA and control groups, and also no correlation was reported with associated atopy for IL-13. However, in that study higher levels of TNF- α , IL-1 α , IFN- γ and IL-12 were reported in patients with AA with atopy. The percentage of patients with AA with associated atopy was 54% (14 with transient patchy AA, 51 with persistent patchy AA and 79 with AT/AU). Moreover, patients in the control group also had atopy in eight out of 18 controls (44%), and comparison of cytokine levels and associated atopy in controls was lacking.²⁰ The present study had only five patients (10%) with AA with a history of atopy, and control subjects did not have any associated atopy. Moreover, cytokine levels can be affected by several factors such as age and ethnicity (Stowe *et al.*).²⁷ Barahmani *et al.*²⁰ performed their study on a good sample size of patients with AA ($n = 269$), but approximately 90% of patients with AA were white, and were compared with only 18 controls (low statistical power), only 56% of whom were white. Further, the mean ages were 39 ± 18.8 years for patients and 44 ± 13.6 years for controls. The present study was performed in an Indian population (different ethnicity) and the mean ages of the patients with AA and controls were

Table 5 Comparison of median serum cytokine levels in patients with alopecia areata (AA) with and without associated atopy

Cytokine (pg mL ⁻¹)	AA without atopy ($n = 46$)	AA with atopy ($n = 5$)	P-value
IL-2	17.8 (7.2-53.2)	21.6 (17.4-37.6)	0.248
IFN- γ	19.94 (2.1-50.5)	19.42 (9.94-41.0)	0.812
IL-10	12.96 (5.8-28.9)	15.01 (9.7-22.2)	0.715
IL-13	13.85 (2.12-26.2)	9.06 (4.6-20.12)	0.326
IL-17A	18.67 (4-43.6)	13.6 (7.54-18.83)	0.281
TGF- β 1	1131.9 (190.4-2801.4)	1121.4 (190.4-2703.4)	0.466

Data are represented as median (minimum-maximum). Wilcoxon rank-sum (Mann-Whitney U-test) was used, $P < 0.05$ is significant. IL, interleukin; IFN, interferon; TGF, transforming growth factor.

24.19 ± 6.69 years and 26.42 ± 4.35 years, respectively. The abovementioned facts collectively may have influenced the cytokine levels and may be the reason for differences between the two studies.

Th17 cells are a distinct subset of Th cells and their function is mediated by the production of the proinflammatory cytokines IL-17 and IL-22.^{28,29} The role of Th17 cells has been illustrated in the pathogenesis of many autoimmune disorders in human and animal models,^{1,30–32} but it remains elusive whether the Th17 cells play a protective or harmful role in the host immune system. Details of the participation of Th17 cells in the pathogenesis of AA are not known. To the best of our knowledge, the present study is the only one that has demonstrated higher serum IL-17A levels in patients with active AA, and IL-17A levels were also found to be varied among AA phenotypes, but the difference was of borderline significance. However, serum IL-17A levels were not correlated with the total duration of AA and the number of patches on the scalp. The data suggest that IL-17A may play a role in the induction or active phase of AA, indicating the involvement of IL-17A and hence Th17 cells in facilitating the selective damage to hair follicles in AA.

Tregs form a specialized subpopulation of CD4 + T cells that play a central role in protection from the development of autoimmune disorders. The role of Tregs has been suggested in several autoimmune disorders.^{33–39} Tregs are important in the prevention of autoimmune diseases by inhibiting the responses of CD8 + T cells, natural killer cells and CD4 + T cells.⁴⁰ The suppression function of Tregs is mediated by cell-to-cell contact or through secretion of soluble factors such as TGF-β and IL-10.^{1,9,40} TGF-β is secreted predominantly by Tregs, and TGF-β-deficient mice demonstrated reduced numbers of peripheral Tregs.^{1,41} The compartment of the hair follicle is considered to be a site of immune privilege, chiefly characterized by a very low level of expression of major histocompatibility complex class I antigens and by the local production of potent immunosuppressive agents, such as TGF-β1 and α-melanocyte-stimulating hormone.⁴¹ The collapse of the anagen hair bulb immune privilege due to altered function of Tregs or TGF-β may play a crucial role in the pathogenesis of AA.^{41,42} Therefore, autoimmune diseases may be exacerbated in the absence of Tregs, or due to their malfunction. In the present investigation we reported reduced serum TGF-β1 levels in patients with active AA, and serum TGF-β1 levels were also found to be varied among AA subgroups, but the difference is of borderline significance.

The data support the notion that the altered function of Tregs may lead to decreased serum TGF-β1 levels in patients with active AA. However, no correlation was found between serum TGF-β1 levels, total duration of AA and the number of patches on the scalp, suggesting that the Tregs are severely compromised in the active phase of AA. Thus, breakdown of peripheral immune tolerance and enhanced T-cell-mediated immunity together may lead to the selective destruction of hair follicles in patients with AA. Furthermore, no correlation was found between serum TGF-β1 levels and associated

autoimmune diseases and atopy. Katagiri *et al.*¹⁶ reported low TGF-β1 mRNA levels in both AA and atopic dermatitis. The association of AA and atopy has been reported in many studies,^{16,20} but there is no concrete and direct evidence explaining the effect of atopic attributes on cytokine levels in AA. However, there is a possibility that atopy and AA may share some common effector molecules and pathways in the onset of disease.

The study of T-cell subsets and associated cytokines is essential to develop a better understanding of the autoimmune nature of AA. However, circulating levels of cytokines could be affected by several factors, and these operate in complex networks. In the present study it is not clear whether AA is dominated by Th1, Th2 or Th17; however, the overall increased level of T-helper cytokines suggests a heightened immune response due to deficiency of the regulatory cytokine TGF-β1. Further study of comparative levels of serum and tissue cytokines is required. These cytokines can be targeted to develop therapeutics and can be combined with current treatment regimens for better management of AA.

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