Altered expression of intracellular Toll-like receptors in peripheral blood mononuclear cells from patients with alopecia areata

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Abdullateef A. Alzolibani [a,1], Zafar Rasheed [b,*,1], Ghada Bin Saif [c],
Mohammed S. Al-Dhubaibi [a], Ahmad A. Al Robaee [a]

[a] Department of Dermatology, College of Medicine, Qassim University, Buraidah, Saudi Arabia
[b] Department of Medical Biochemistry, College of Medicine, Qassim University, Buraidah, Saudi Arabia
[c] Department of Dermatology, College of Medicine, King Saud University, Riyadh, Saudi Arabia

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ABSTRACT

Background: Toll-like receptors (TLRs) are pattern-recognition-receptors that sense a variety of pathogens and initiation of innate and adaptive immune responses. This study was undertaken to investigate the expression of TLRs in peripheral blood-mononuclear cells (PBMCs) of AA patients and to determine whether TLR-mediated inflammatory signals are important for the perspective of AA management.

Methods: Gene expression of TLRs and T-helper (Th) type-1, Th-2, Th-17 and regulatory T-cell cytokines in PBMCs was quantified by TaqMan Assays. Production of these cytokines in serum samples was determined by sandwich ELISAs.

Results: All TLRs (TLRs 1–10) were expressed in PBMCs of AA patients. Importantly intracellular TLRs (TLRs 3, 7, 8 and 9) were significantly up-regulated in AA patients as compared with controls ($p < 0.05$). Interleukin (IL)-2, TNF-α, and IL-17A gene expression in patients' PBMCs and their secretion in patients' sera were significantly higher as compared with their respective controls ($p < 0.05$). Whereas, TGF-β gene expression in patients' PBMCs and TGF-β protein level in patients' sera were significantly lower as compared with their controls ($p < 0.05$).

Conclusion: This is the first report that shows the comprehensive expression profile of TLRs in AA patients. We conclude that up-regulated expression of intracellular TLRs in PBMCs of AA patients may play an active role in abnormal regulation of Th-1, Th-17 and regulatory T-cell cytokines in alopecia areata.

General significance: Targeting of TLRs and their associated inflammatory signaling will open new areas of research; this may lead to the development of novel therapeutic targets for the treatment of AA or other skin disorders.

1. Introduction

Alopecia areata (AA) is a non-scarring, inflammatory skin disease that results in patchy hair loss. AA is unpredictable in its onset, severity, and duration making it potentially very stressful for affected subjects [1,2]. There are many hypotheses for the etiopathogenesis of AA and it is probable that it involves several factors ranging from the genetics of the immune system to autoantigen specificity and expression patterns [1–3]. Studies have shown that it is likely that the time of onset and severity of AA are determined by interaction between patient's genetic predisposition and exposure to environmental triggers [1–3]. Presently, the hypotheses for AA development mostly focus on the damage of immune properties of the hair follicles and the nature of autoimmune reactions that result in the lymphocytes' subsequent attack [1]. Studies are being conducted to look for the autoantigens that may be associated with the development of AA but the exact nature of the inciting antigen epitope(s) remains to be elucidated [1,3]. Now it has been suggested that a T-cell mediated process is stimulated by endogenous or exogenous stimuli with the interaction of several molecules [1,4], where T-helper cells such as Th-1, Th-2 and Th-17 cells and regulatory T-cells have been implicated [5]. In AA, abnormalities in T-cell-mediated immunity and cytokine secretion have been well reported [4–6]. Despite these advancements in modern molecular approaches, still the treatment options for AA are limited and the efficacy of these treatments varies from patient to patient and the exact etiology of AA is still unknown.

Toll-like receptors (TLRs) are key regulators of the innate and adaptive immune response and are activated by discrete molecular components of invading pathogens termed pathogen associated

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* Corresponding author at: Department of Medical Biochemistry, College of Medicine, Qassim University, P.O. Box 6655, Buraidah 51452, Saudi Arabia.
E-mail address: zafarrasheed@qumed.edu.sa (Z. Rasheed).
[1] Authors contributed equally and considered as co-first authors.

molecular patterns (PAMPs) [7–9]. Ten human TLRs and 13 mouse TLRs have been reported till date [7]. TLRs are located either at the cell surface (TLRs 1, 2, 4, 5, 6) or in the intracellular compartment (TLRs 3, 7, 8, 9) primarily on exosomes and endoplasmic reticulum [8]. Activation of TLRs initiates a signaling cascade through the adopter molecules for the induction of variety of cell dependent responses, including expression and production of various inflammatory cytokines, phagocytosis, immune cell recruitment and antigen presentation [7]. In AA patients, impaired apoptosis and invalid cellular debris clearance lead to increased concentration of serum nuclear and cytoplasmic contents, which are well-known ligands for TLRs 3, 7, 8 and 9 [10,11]. Dysfunctionality in the expression of TLRs and their associated effects on the signaling events have been reported in numerous disorders [12,13] and also in few skin diseases [14,15] but the role of TLR expression in AA pathogenesis has never been investigated. Therefore, we hypothesized for the very time that TLR-mediated inflammatory signals contribute to the pathogenesis of AA. To investigate this, we evaluated TLR expressions on PBMCs obtained from AA patients, examined their relations with expression and production of Th-1, Th-2, Th-17 and regulatory T-cell cytokines. Our novel results show that gene expression of intracellular TLRs 3, 7, 8 and 9 were expressed prominently in the PBMCs of AA patients, whereas expression of cell surface TLRs 1, 2, 4, 5 and 6 were comparatively low. Moreover, our results also show that Th-1 cytokines IL-2 and TNF-α gene expression in PBMCs and protein production in serum samples were significantly higher in AA as compared with healthy controls, whereas no significant change was found in gene expression and protein production of Th-2 cytokines IL-5 or IL-4 in AA as compared with healthy controls. Gene expression and protein secretion of Th-17 cytokine IL-17A was found to be higher in AA as compared with controls humans, whereas regulatory T-cell cytokine TGF-β was found to be significantly lower in AA patients as compared with healthy controls. Characterization of TLRs and their role in inflammatory signaling events will definitely open new areas of research which may lead to the development of novel therapeutic targets for the treatment of AA or other skin disorders.

2. Methods

2.1. Patients' recruitment

Present study has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in Tokyo 2004) for humans which was approved by the local ethical committee of College of Medicine, Qassim University, KSA. Study subjects were recruited through the dermatology outpatient clinics of Qassim University, Buraidah, KSA and informed consent was obtained from each subject. Patients were classified according to Alopecia Areata Foundation Clinical Assessment Guidelines [16]. The study group included 25 AA patients with patchy persistent (4 females and 21 males) and their age range was 19–43 years (mean ± SD, 30.2 ± 8.43 years). The severity of the disease was determined using the Severity of Alopecia Toll (SALT) scores and the patients were divided into the following two groups based on the higher versus lower SALT scores: (1) SALT ≥ 25% and (2) SALT < 25%. Venous blood samples from the patients were collected at the time of joining the clinics. Patients did not receive any systemic or topical drug therapy for the last three months, did not have any additional systemic disease, had no history of systemic medication use, and had no smoking and the regular exercising habits except their daily activities. The control group comprised 26 healthy subjects (5 females and 21 males, age range 20–48 years, mean ± SD age 32.3 ± 10.8 years). The mean ages were not significantly different between the groups. The racial/ethnic and sex compositions of the AA groups were comparable with those of

the control group. The demographic and SALT details of patients are summarized in Table 1.

2.2. Preparation of peripheral blood mononuclear cells

Venous blood samples from the control subjects and AA patients were collected during routine laboratory examination. PBMCs were isolated by gradient centrifugation using Histopaque-1077 (cat. # 10,771, Sigma-Aldrich Inc., St. Louis, MO, USA) according to the manufacturer's instruction. Briefly, freshly collected blood was precisely applied on the surface of the gradient and centrifuged at 1600 rpm for 20 min. The obtained buffy coat at the interphase was collected and dispersed in 5 ml of Hank's medium (cat. # H4641, Sigma-Aldrich) and centrifuged at 1600 rpm for 10 min. The supernatant was collected and cells were washed twice with RPMI 1640 medium (cat # SLM-140-B, Millipore, USA) at 1100 rpm for 5 min each time. The PBMCs were then dispersed in phosphate buffered saline (PBS, cat # SH30258.01, HyClone Laboratories, Inc., South Logan, Utah).

2.3. Quantitative real time-PCR

Real time quantitative polymerase chain reaction (qRT-PCR) was used to quantify the expression of mRNA for TLR-1–TLR-10, IL-2, TNF-α, IL-5, IL-4, IL-17A and TGF-β with expression of endogenous control GAPDH as we described previously [17,18]. Total RNA was separated from isolated PBMCs by total RNA isolation kit (catalog # AM1560, Ambion, CA, USA). First strand cDNA was synthesized using 1 μg total RNA and the SuperScript First Strand cDNA synthesis kit (catalog # 75780, Affymetrix Inc., OH, USA). Primers used for PCR assisted amplification are listed in Table 2. PCR amplification was carried out using the core kit for SYBR Green or Taqmann reagent (Applied Biosystem, Foster City, CA) and the Step One Real Time PCR System (Applied Biosystems). Typical profile times used were initial step, 95 °C for 10 min, followed by a second step at 95 °C for 15 s and 60 °C for 60 s for 40 cycles with melting curve analysis. The level of target mRNA was normalized to the level of GAPDH and compared to control (untreated sample). Data was analyzed using ΔΔCT method [19].

2.4. Cytokine ELISA

Levels of IL-2, TNF-α, IL-5, IL-4, IL-17A and TGF-β in the serum samples of AA patients and normal human controls were quantified using commercially available specific sandwich ELISA kits (GenWay Biotech, Inc., CA, USA) according to the instructions of the manufacturer. Plate was read using an automatic microplate reader (Anthos Zenyth 3100 Multimode Detectors, Salzburg, Austria).

2.5. Statistical analysis

All measurements were performed in duplicates and repeated using PBMCs prepared from AA patients and normal human controls. Statistical comparisons were performed by One-way ANOVA analysis followed by Tukey's post-hoc analysis or Two-way ANOVA followed

Table 1
Characterization of enrolled subjects.

Human subjects	Age (years) mean ± SD.	Sex (M/F)	SALT ≥ 25%	SALT < 25%
AA patients (n = 25)	30.2 ± 8.43	21 M/4F	n = 14	n = 18
Healthy controls (n = 26)	32.8 ± 10.8	21 M/5F	–	–

AA, alopecia areata; n, number of samples tested; SALT, severity of alopecia tool scores; M, male; F, female.

Table 2
Details of primers used in gene expression studies.

Gene name	Accession number	Forward primer	Reverse primer
TLR-1	NM_003263.3	5′-TGTGCTGCCAATTGCTCATT-3′	5′-TTTTCCCCATAAGTCTCTCCTAAGAC-3′
TLR-2	NM_003264.3	5′-TCCGTCTTTTTGATGAGAACAATG-3′	5′-ACTCCAGGTAGGTCTTGGTGTTCA-3′
TLR-3	NM_003265.2	5′-TTTGCGAAGAGGAATGTTTAAATCT-3′	5′-CACCTATCCGTTCTTTCTGAACTG-3′
TLR-4	NM_138557.2	5′-GAGCCGCTGGTGTATCTTTGA-3′	5′-CCAGCACGACTGCTCAGAAA-3′
TLR-5	NM_003268.5	5′-CAGCTTTCCTGGCAATTGACT-3′	5′-CGACATCTTCCCTGGATGCT-3′
TLR-6	NM_006,068.4	5′-GGGACTCAGCATGGTAGAAGGTA-3′	5′-CTCCTGTTACTCTGCAAGCTTTCA-3′
TLR-7	NM_016562.3	5′-TCCTTGTGCGCCGTGTAAA-3′	5′-GTCAGCGCATCAAAAGCATTT-3′
TLR-8	NM_016610.3	5′-GCTACGGCAGCGGATCTGT-3′	5′-CCTTCTGCCTTCGGGTTGT-3′
TLR-9	NM_017442.3	5′-GGCAAAGTGGGCGAGATG-3′	5′-GCTCTGCGTTTTGTCGAAGAC-3′
TLR-10	NM_001017388.2	5′-CCGTGTCAACGAATCATCCA-3′	5′-CCCACGGCTTGCACTCTCT-3′
IL-2	NM_000586.3	5′-GTCACAAACAGTGCACCTAC-3′	5′-CCCTGGGTCTTAAGTGAAAG-3′
TNF-α	NM_000594.3	5′-GCAGGTCTACTTTGGGATCATTG-3′	5′-GCGTTTGGGAAGGTTGGA-3′
IL-5	NM_000879.2	5′-TGCCATCCCCACAGAAATTC-3′	5′-AGAAAGCAGTGCCAAGGTCTCT-3′
IL-4	NM_000589.3	5′-ACTTTGAACAGCCTCACAGAG-3′	5′-TTGGAGGCAGCAAAGATGTC-3′
IL-17 A	NM_002190.2	5′-GGAACGTGGACTACCACATGAA-3′	5′-GCGCAGGACCAGGATCTCT-3′
TGF-β	NM_000660.5	5′-CGCGCATCCTAGACCCTTT-3′	5′-CTGTGGCAGGTCGGAGAGA-3′
GAPDH	NM_002046.5	5′-TCGACAGTCAGCCGCATCTTCTTT-3′	5′-ACCAAATCCGTTGACTCCGACCTT-3′

by Bonferroni post-hoc tests using Graph Pad Prism-5 (San Diego, CA, USA) and $p < 0.05$ was considered significant. Values shown are mean $\pm$ SEM unless stated otherwise.

3. Results

3.1. Gene expression profiling of Toll like receptors in PBMCs of alopecia areata patients

In an attempt to understand the role of TLRs signaling in the pathogenesis of AA, we determined the levels of TLR-1 to TLR-10 transcripts in PBMCs of AA patients and the results were statistically compared with age- and sex-matched healthy controls. However, study was performed mainly on male participants as the number of male participants was significantly more compared to female participants ($p < 0.001$). Therefore, results mainly devoted for the male AA patients. We determined the mRNA levels of these TLRs in freshly isolated PBMCs in patients and healthy controls by the highly sensitive and specific quantitative real time-PCR using specific primer sequences described in Table 2. As shown in Fig. 1, the intracellular TLR-3, TLR-7, TLR-8, and TLR-9 mRNA levels in PBMCs of AA patients were significantly higher than those of the healthy controls ($p < 0.05$). However, the differences in the mRNA levels of other TLRs (TLR-1, TLR-2, TLR-4, ILR-5, TLR-6,

IL-10) between the AA patients group and the healthy control group were not significant ($p > 0.05$).

3.2. Age wise distribution of TLRs on PBMCs of alopecia areata patients

To determine whether age of the patients plays a role on gene expression profiling of TLRs in AA, patients and normal humans were divided into two age groups: (i) low age group (age < 20 years) (n = 12) and (ii) high age group (age ≥ 20 years) (n = 13) and the mRNA expression of all studied TLRs were estimated by real time PCR using GAPDH as an endogenous control. As shown in Figs. 2 and 3, age-dependent increase was observed in the mRNA expression of TLR-3 ($p < 0.01$), TLR-7 ($p < 0.001$), TLR-8 ($p < 0.001$) and TLR-9 ($p < 0.05$), whereas other TLRs showed no age-dependent alterations in their gene expression levels in PBMCs of AA patients ($p > 0.05$).

3.3. Gene expression of T-helper and regulatory T-cell cytokines in PBMCs of alopecia areata patients

To validate our hypothesis that dysregulation in the expression of TLRs may be involved in the expression/production of cytokines in AA patients, we assessed mRNA expression levels of Th-1 cytokine (IL-2, TNF-α), Th-2 cytokine (IL-5, IL-4), Th-17 cytokine (IL-17A)
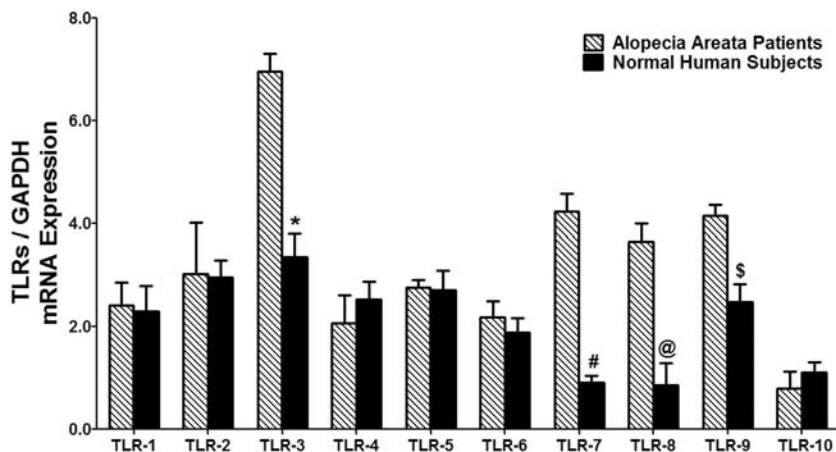

Fig. 1. Gene expression profiling of Toll like receptors in peripheral blood mononuclear cells of alopecia areata patients. The mRNA expressions of TLRs were determined by quantitative RT-PCR using comparative ΔΔCT method. Assays were performed in duplicates and results are presented as Mean ± SEM. *p < 0.0001 versus TLR-3 of alopecia areata patients; #p < 0.001 versus TLR-7 of alopecia areata patients; @p < 0.01 versus TLR-8 of alopecia areata patients; $p < 0.01 versus TLR-9 of alopecia areata patients.
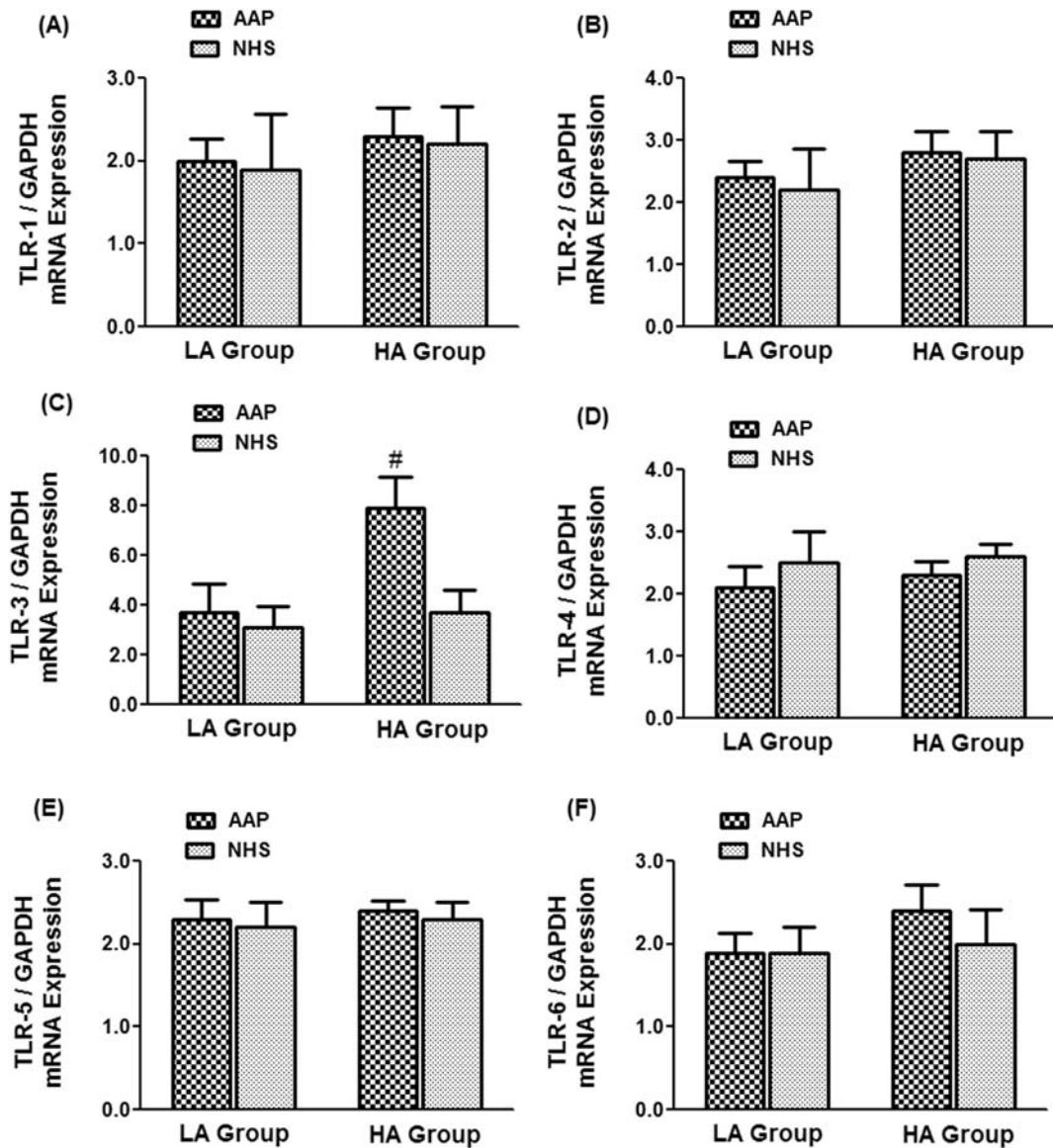
Fig. 2. Age wise distribution of Toll like receptors (TLR-1–TLR-6) mRNA expression in peripheral blood mononuclear cells of alopecia areata patients. The mRNA expression of TLR-1 (A), TLR-2 (B), TLR-3 (C), TLR-4 (D), TLR-5 (E) and TLR-6 (F) were determined by quantitative RT-PCR using GAPDH as an endogenous control. LA group represents low age group (age < 20 years), whereas HA group represents high age group (age ≥ 20 years). AAP, alopecia areata patients; NHS, normal human subjects. Assays were performed in duplicates and results are presented as mean ± SEM. #p < 0.001 versus AAP of LA group.

and T regulatory cytokine (TGF-β) in PBMCs from AA patients and healthy controls and their results were compared. Our quantitative RT-PCR results showed that mRNA levels of IL-2 and TNF-α in patients with AA were significantly higher than those in healthy controls (Fig. 4A & B; p < 0.05), whereas IL-5 and IL-4 mRNA levels in AA patients were found to be almost similar as of healthy controls (Fig. 4C & D; p > 0.05). Moreover, the mRNA levels of IL-17A in PBMCs of AA patients were significantly higher than those in PBMCs of healthy controls (Fig. 4E; p > 0.05). Furthermore, the mRNA levels of T-cell regulatory TGF-β in PBMCs of AA patients were found to be significantly lower as compared with the levels of TGF-β in PBMCs from healthy controls (Fig. 4F; p > 0.05).

3.4. Levels of T-helper and regulatory T-cell cytokines in serum samples of alopecia areata patients

To provide further support to our hypothesis and to assess the contribution of T-helper and regulatory T-cell cytokines in the pathogenesis of AA, we determined the serum levels of IL-2, TNF-α, IL-5, IL-4, IL-17A and TGF-β in patients with AA (n = 25) and their levels were compared with their respective healthy controls (n = 26). As shown in Fig. 5A, the serum levels of IL-2 were significantly higher in AA patients as compared with their respective healthy controls (p < 0.05). The average (± SEM) of IL-2 levels in AA patients' sera and control human sera were 33.2 ± 9.11 and 19.4 ± 5.16 pg/ml, respectively. Similarly, the levels of TNF-α were determined and were found to be significantly higher in AA patients as compared with healthy controls (p < 0.01). The average (± SEM) of TNF-α level in AA patients' sera and control human sera were 27.7 ± 2.4 and 14.7 ± 3.28 pg/ml, respectively (Fig. 5B). Whereas, the level of IL-5 or IL-4 in the serum samples of AA patients was found to be statistically similar as compared with their respective healthy controls (p > 0.05). The average (± SEM) of IL-5 in AA patients' sera and control human sera were 9.10 ± 6.02 and 7.50 ± 4.50 pg/ml, respectively (Fig. 5C), whereas, the average (± SEM) of IL-4 in AA patients' sera and controls' sera were 12.1 ± 2.51 and 10.3 ± 2.1 pg/ml, respectively (Fig. 5D). However, the serum
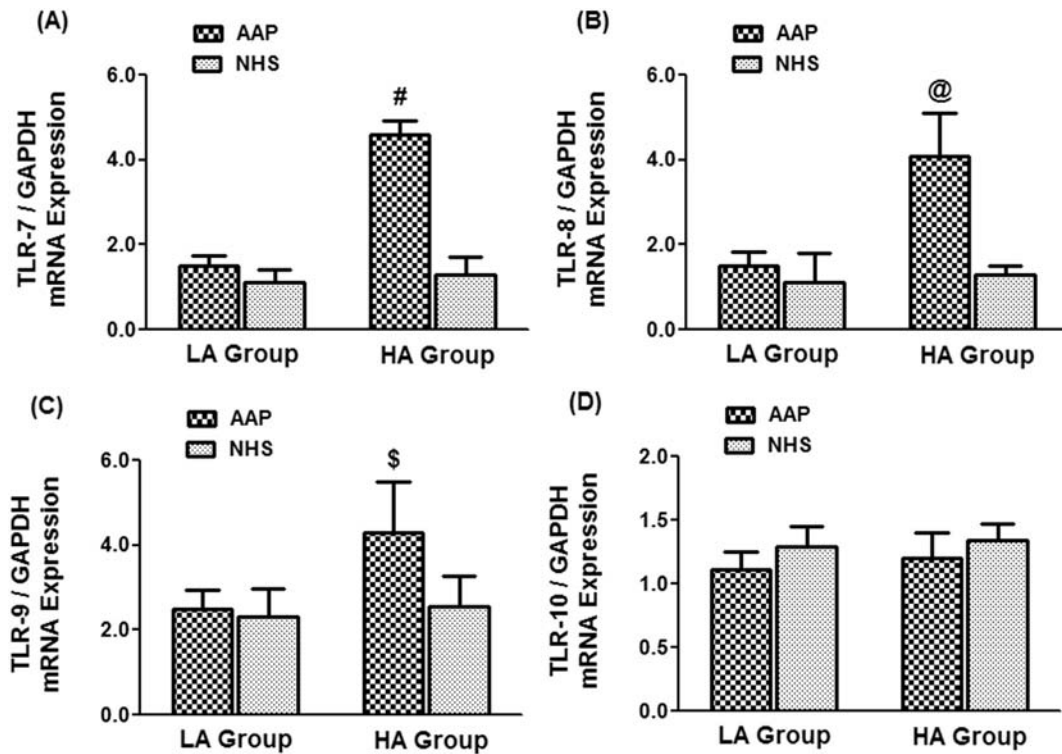
Fig. 3. Age-wise distribution of Toll like receptors (TLR-7–TLR-10) mRNA expression in peripheral blood mononuclear cells of alopecia areata patients. The mRNA expression of TLR-7 (A), TLR-8 (B), TLR-9 (C) and TLR-10 (D) were determined by quantitative RT-PCR using GAPDH as an endogenous control. LA group represents low age group (age < 20 years), whereas HA group represents high age group (age ≥ 20 years). AAP, alopecia areata patients; NHS, normal human subjects. Assays were performed in duplicates and results are presented as mean ± SEM. [#]$p < 0.001$ versus AAP of LA group; [@]$p < 0.001$ versus AAP of LA group; [$]$p < 0.05$ versus AAP of LA group.

levels of IL-17A in AA patients were found to be significantly higher in AA patients ($p < 0.05$), when compared with controls. The average (±SEM) of serum levels of IL-17A in AA patients and healthy controls were $58.2 ± 14.07$ and $21.5 ± 16.32$ pg/ml, respectively (Fig. 5E). Interestingly, the serum levels of TGF-β were found to be significantly lower in AA patients in comparison with healthy controls ($p < 0.05$). The average (±SEM) TGF-β levels in AA patients and control sera were $12.51 ± 5.52$ and $28.49 ± 4.95$ pg/ml, respectively (Fig. 5F).

3.5. The SALT-related changes in the serum levels of T-helper and regulatory T-cell cytokines in alopecia areata patients

To further validate our findings, we assessed the changes in serum levels of T-helper and regulatory T-cell cytokines as a function of the AA disease severity using SALT scoring system. For that patients were divided into 2 groups: (i) higher disease severity (SALT ≥ 25%) (n = 14) and (ii) lower disease severity (SALT < 25%) (n = 18) and the levels of cytokines were estimated. As shown in Fig. 6A, levels of IL-2 were significantly higher among AA patients, whose SALT scores were ≥25% when compared with patients of SALT <25% or with healthy controls ($p < 0.05$). Importantly, patients with SALT <25% showed no significant change in IL-2 levels, when compared with healthy controls ($p > 0.05$). The average (±SEM) IL-2 levels in the patients' sera with SALT ≥25% and SALT <25% were $36.5 ± 4.5$ and $27.5 ± 1.5$ pg/ml, respectively (Fig. 6A). Similarly, the levels of TNF-α were determined and were found to significantly higher in patients with SALT ≥25% as compared to those AA patients with SALT <25% or with healthy controls ($p < 0.05$). The average (±SEM) TNF-α levels in the patients' sera with SALT ≥25% and SALT <25% were $26.3 ± 2.9$ and $17.7 ± 1.9$ pg/ml, respectively. Whereas, the average (±SEM) TNF-α level in the controls' sera was $14.7 ± 3.89$ pg/ml (Fig. 6B). To further strengthen our findings, we also investigated the levels of IL-5 and IL-4 in these patient groups. Interestingly, levels of both IL-5 and IL-4 were found to be almost similar in patients with SALT ≥25% and with SALT <25% and also similar with

healthy controls ($p > 0.05$) (Fig. 6C & D). In addition, we also demonstrated the levels of IL-17A in patients with SALT ≥25%, SALT <25% and in healthy controls. Our data showed that IL-17A levels were significantly increased with the increase of the disease severity ($p < 0.05$). The average (±SEM) IL-17A levels in the patients' sera with SALT ≥25%, SALT <25% groups and healthy controls were $80.5 ± 8.5$, $48.1 ± 4.1$ and $21.5 ± 11.5$ pg/ml, respectively (Fig. 6E). Furthermore, TGF-β levels were also determined in the serum samples of these patient groups and in healthy controls and were found to be significantly lower in patients with SALT ≥25% as compared with patients of SALT <25% or with healthy controls ($p < 0.05$). The average (±SEM) TGF-β levels in the patients sera with SALT ≥25%, SALT <25% groups and healthy controls were $80.5 ± 8.5$, $48.1 ± 4.1$ and $21.5 ± 11.5$ pg/ml, respectively (Fig. 6F). Our data pointed out that the levels of TGF-β were decreased with the increase of disease severity.

4. Discussion

This is the first report to the best of our knowledge that shows the comprehensive characterization of TLR expression in peripheral blood cells of patients with alopecia areata. TLRs are basically a category of receptors that recognize and activate their signaling cascades to defend against the pathogen factors. However, an alteration in the proper activation might occur, resulting in the production of proinflammatory cytokines [20,21]. These improper activations are responsible for the beginning of autoimmune diseases. Studies have also shown that the inhibition of the implicated receptors or their pathways may prevent the onset of autoimmunity [20]. Activation of TLR signaling cascades in peripheral blood cells has been studied to regulate the expression of a range of cytokines [12,21,22]. Since both TLRs and cytokines are linked to the pathogenesis of AA, therefore it can be hypothesized that dysregulation in the expression of TLRs and cytokines after improper activation of TLRs may play an active role in the pathogenesis of AA. To test this hypothesis, we analyzed the gene expression levels of all
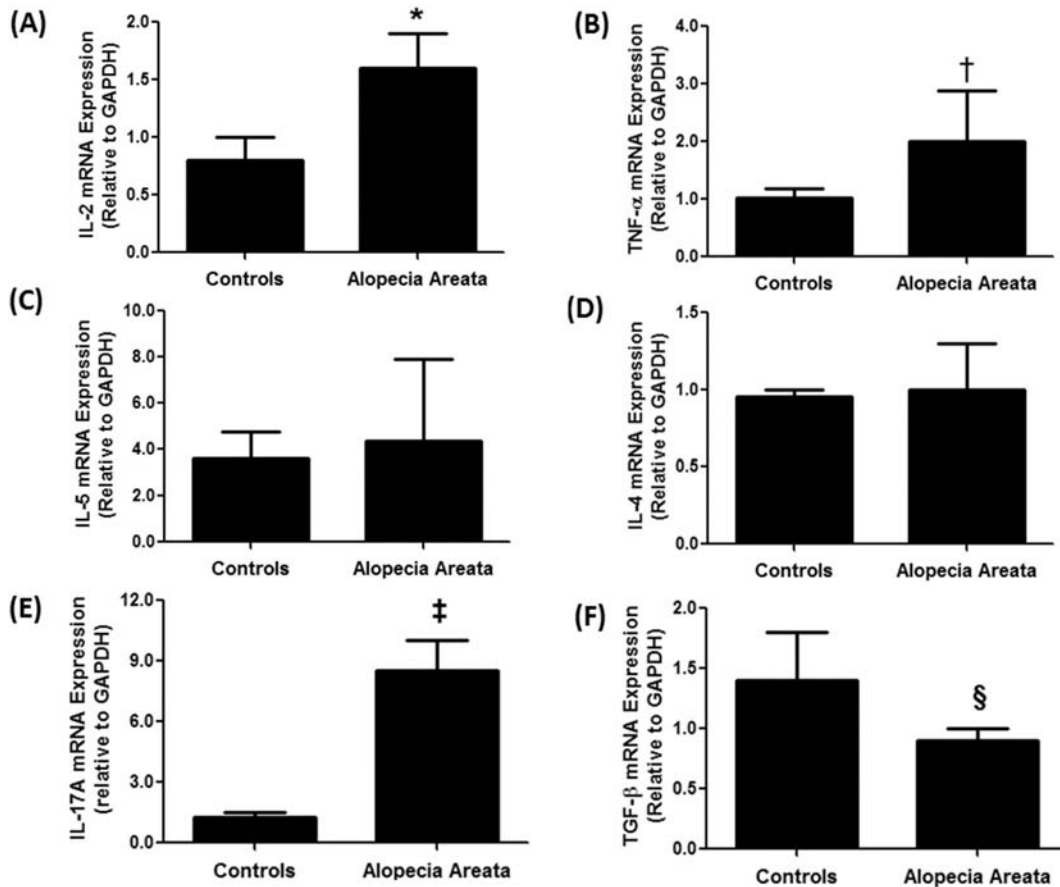
Fig. 4. Gene expression of T-helper and regulatory T-cell cytokines in peripheral blood mononuclear cells of alopecia areata patients. The mRNA expressions of (A) IL-2, (B) TNF-α, (C) IL-5, (D) IL-4, (E) IL-17A and (F) TGF-β in PBMCs from AA patients and healthy controls were determined by quantitative RT-PCR using comparative ΔΔCT method. Assays were performed in duplicates and results are presented as mean ± SEM. *$p < 0.01$ versus IL-2 mRNA of healthy human controls; †$p < 0.01$ versus TNF-α mRNA of healthy human controls; ‡$p < 0.001$ versus IL-17A mRNA of healthy human controls; §$p < 0.01$ versus TGF-β mRNA of healthy human controls.

ten TLRs (TLR-1 to TLR-10) and we also analyzed the gene expression of T-helper (Th) cells type-1, Th-2, Th-17 and regulatory T-cell cytokines in PBMCs of AA patients and their expression levels were compared with PBMCs of healthy human controls. Higher gene expression of intracellular TLRs: TLR-3, TLR-7, TLR-8 and TLR-9 have been found in AA patients, compared to healthy individuals. However, the gene expression levels of other TLRs: TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-10 showed insignificant alterations in AA patients as compared with healthy individuals. Moreover, we also demonstrated that patients' ages play a role on the expression of TLR-3, TLR-7, TLR-8 and TLR-9, as their mRNA levels were found to be higher in patients with higher age group (age ≥ 20 years) compared to the low aged group patients (age < 20 years). However other TLR mRNA expression remains unchanged. Therefore these results may indicate that patients' ages' may play a role in the activation of TLRs 3, 7, 8, and 9 signaling cascades in AA patients. As the study was mainly conducted in male participants, therefore these results are fully symbolized for male AA patients.

The family of TLRs plays a central role in the cutaneous immune defense system. To date, different TLRs have been found on cell populations of skin and blood cells from patients with various dermatological disorders [23]. In AA, correlation of T-cells and their effector cytokines, yet remains to be fully investigated but it is suggested that they may play an active role in the selective loss of hairs [6,24]. In this study, we determined the gene expression of T-helper type 1 cytokine IL-2 in PBMCs of AA patients and healthy individuals. Our novel data showed significant increased levels of IL-2 mRNA in AA patients as compared with healthy controls. To further validate the role of Th-1 cytokines in AA pathogenesis, the level of one of the most well-known Th-1 cytokine, TNF-α was determined at both gene and protein levels in AA patients.

Our data demonstrate higher mRNA levels of TNF-α in PBMCs of AA and higher TNF-α level in the sera of AA patients as compared with their respective healthy controls. The elevated levels of IL-2 and TNF-α in patients' PBMCs and in patients' sera clearly indicate that Th-1 cytokines play an important role in the pathogenesis of the disease. In this study, when AA patients were divided into 2 groups based of their SALT scores (≥25% versus <25%), patients with higher SALT scores (SALT ≥ 25%) showed a remarkable increase in the serum levels of both IL-2 and TNF-α as compared to the patients with lower SALT scores (SALT < 25%). These data clearly indicate that Th-1 cytokines in AA patients may play an active role in the disease progression and in elucidating the mechanisms of disease pathogenesis.

The role of T-helper type-2 cytokines in AA remains unresolved. Different research groups showed different observations on the serum levels of Th-2 cytokines in AA. Therefore the roles of Th-2 cytokines in AA now become controversial [6,25,26]. To the best of our knowledge, the present study is the only one that has demonstrated the role of IL-5 in AA pathogenesis. Our novel data determined mRNA levels of IL-5 in PBMCs of AA and was found to be almost similar as in PBMCs from healthy controls and results were the same in the patients' and controls' sera. It is important to point out that IL-5 is actually not a representative sole marker for Th-2 cytokines; therefore for further validation of IL-5 results, we also investigate the levels of IL-4, which is a well-known Th-2 cytokines. Like IL-5, IL-4 also showed similar mRNA levels in patients' PBMCs and similar levels in the patients' sera as compared with their respective healthy controls. To further validate these results, we also investigated the levels of IL-5 and IL-4 in patient groups with different SALT scores. Interestingly, serum levels of both IL-5 and
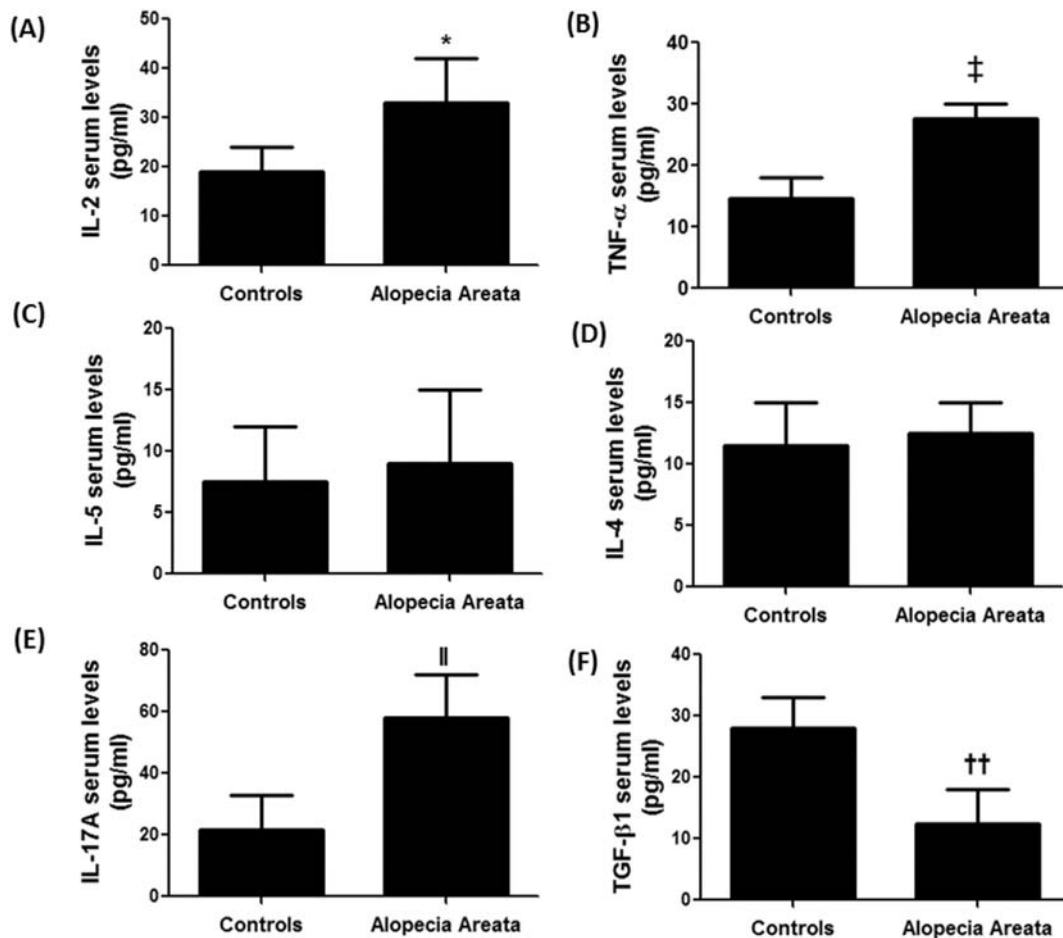
Fig. 5. Serum levels of T-helper and regulatory T-cell cytokines in alopecia areata patients. (A) IL-2 levels in serum samples of patients with AA and healthy human controls. *p < 0.05 versus controls. (B) TNF-α level in serum samples of patients with AA and controls. ‡p < 0.01 versus controls. (C) IL-5 levels in serum samples of patients with AA and healthy human controls. IL-5 levels in AA patients versus controls, p > 0.05. (D) IL-4 levels in serum samples of patients with AA and healthy human controls. IL-4 levels in AA patients versus controls, p > 0.05. (E) IL-17A levels in serum samples of patients with AA and controls. ‖p < 0.01 versus IL-17A serum levels in healthy controls. (F) TGF-β levels in serum samples of patients with AA and controls. ††p < 0.05 versus TGF-β serum levels in controls. Levels of cytokines in the serum samples were determined by specific sandwich ELISAs (GenWay). Assays were performed in triplicates and results are presented as mean ± SEM.

IL-4 were found to be almost similar in patients with SALT ≥25% and with SALT <25% and were also similar with healthy controls. In view of these findings, we conclude that this subclass of cytokines may not have their active role in the pathogenesis of AA, but still further studies are needed to elucidate the detailed mechanistic role in AA. T-helper 17 cells are a distinct subset of T helper cells and their function is mainly mediated by the production of the proinflammatory cytokine IL-17. The role of Th-17 cells has been implicated in the pathogenesis of various autoimmune disorders including animal model [27], but it remains to be identified whether the Th-17 cells play a protective or harmful role in the host immune system. The detailed participation of Th-17 cells in the pathogenesis of AA is still unknown. This study demonstrates higher mRNA levels of IL-17A in PBMCs of AA and higher IL-17A levels in the sera of AA patients as compared with their respective healthy controls, indicating that IL-17A may play a role in the pathogenesis of AA. Not only these, we also provide evidences that serum level of IL-17A in AA patients was significantly increased with the increase of SALT scores as IL-17A: healthy controls < severe (SALT ≥ 25%) patients < mild (SALT < 25%) patients. The increased levels of IL-17A observed in AA patients in the present study provide solid evidence of the involvement of Th-17 cells in the progress of AA. Importantly the increased levels of IL-17A in AA patients, together with significant increases in Th-1 cytokines, IL-2 and TNF-α provide further evidences for the view that AA pathogenesis has a dominant Th-1-mediated component, with potential involvement of Th-17 pathways. However, a fully integrated

view of intersecting cytokine networks that support the autoimmune response in AA is still lacking. Regulatory T-cells are specialized subpopulation of T-cells that play a central role in protection from the onset of autoimmune disorders by inhibiting the responses of different lymphocytes and natural killer cells [6,28]. In this study, we demonstrated the lower levels of TGF-β gene expression in PBMCs and sera of AA patients as compared with their respective healthy controls. Moreover, our results also provide evidences that TGF-β serum level was significantly decreased with the increase of AA activity. AA patients with SALT scores ≥25% showed significantly lower TGF-β level as compared to those patients, whose SALT scores were <25%. Furthermore, patients with SALT <25% showed lower level of TGF-β as compared with the healthy controls. These data clearly indicating dysfunctionality of regulatory T-cells in AA and this may be a reason of the development of autoimmunity in AA patients. However, the study of T-cell subsets and associated cytokines is essential to develop a better understanding of the autoimmune nature of AA. Our findings in this study strongly suggest that functional response of intracellular TLRs 3, 7, 8 and 9 may be involved in the regulation of Th-1, Th-17 cytokines and regulatory T-cell cytokines in AA patients. However, this study has a few limitations; the most obvious limitation of the study is the sample size and region of sample collection. It would have been better to include 50–75 patients, and not to confine the sample collection to only one region. In addition, study on more female AA patients would further strengthen our findings.
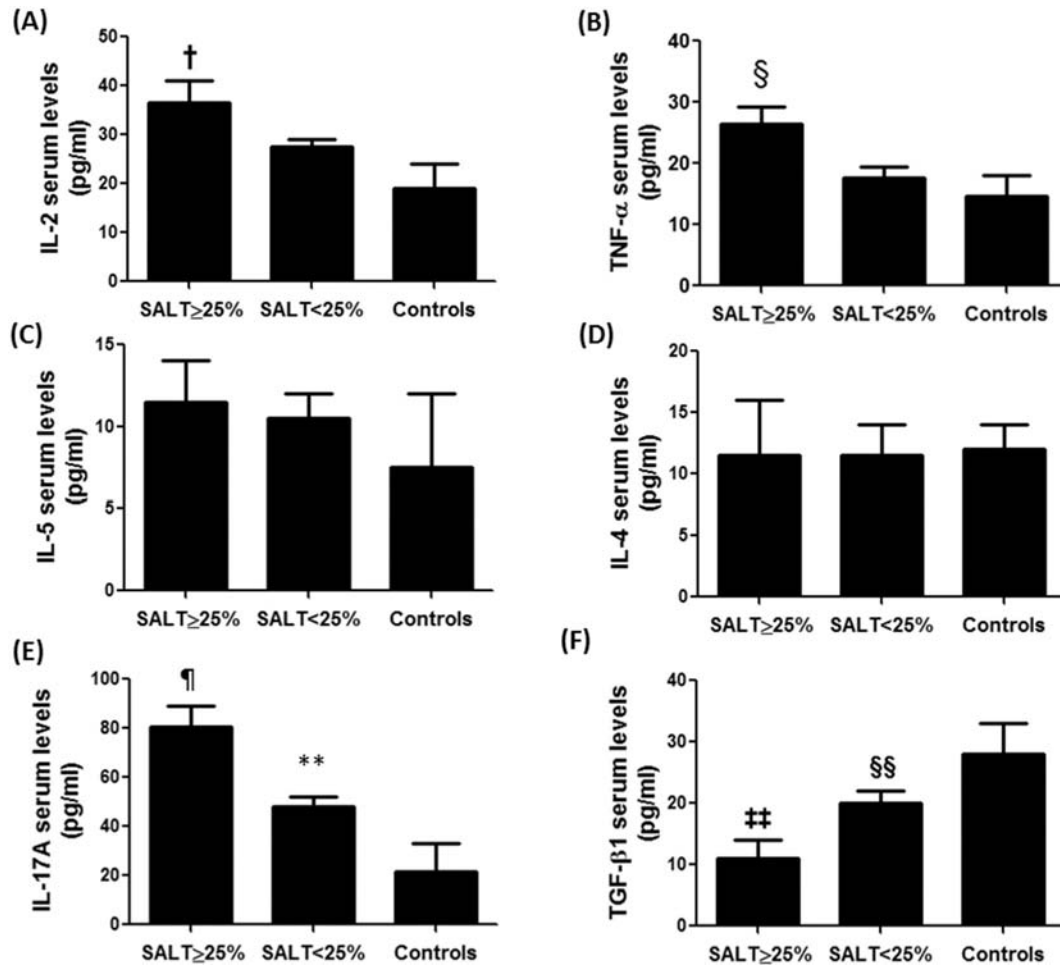
Fig. 6. SALT-related alterations in serum levels of T-helper and regulatory T-cell cytokines in alopecia areata patients. (A) Serum levels of IL-2 in AA patients with severity of alopecia tool (SALT) score ≥ 25% and those with SALT score < 25% compared with normal human controls. †p < 0.05 versus controls. (B) Serum levels of TNF-α in AA patients with SALT score ≥ 25% and those with SALT score < 25% compared with controls. §p < 0.05 versus controls. (C) Serum levels of IL-5 in AA patients with SALT ≥25% and those with SALT score < 25% compared with controls. (D) Serum levels of IL-4 in AA patients with SALT ≥25% and those with SALT score < 25% compared with controls. (E) Serum levels of IL-17 A in AA patients with SALT score ≥ 25% and with SALT score < 25% compared to controls. ¶p < 0.05 versus SALT < 25%, ¶p < 0.001 versus controls, **p < 0.05 versus controls. (F) Serum levels of TGF-β in AA patients with SALT score ≥ 25% and with SALT score < 25% compared to controls. ‡‡p < 0.05 versus SALT < 25%, ‡‡p < 0.01 versus controls, §§p < 0.05 versus controls. Levels of cytokines in the serum samples were determined by specific sandwich ELISAs (GenWay). Assays were performed in triplicates and results are presented as mean ± SEM.

5. Conclusions

This is the first report that shows the comprehensive expression profiles of all ten Toll-like receptors in the peripheral blood cells of alopecia areata. Our novel data conclude that all intracellular TLRs: TLR-3, TLR-7, TLR-8 and TLR-9 are overexpressed in PBMCs of AA patients. Our results support an idea that unregulated expression of TLR-3, TLR-7, TLR-8 and TLR-9 in peripheral blood cells of AA patients may be involved in their signaling cascade to dysregulation of Th-1, Th-17 and regulatory T-cell cytokines. Moreover, study also suggested that these TLRs can be targeted for the development of novel treatment regimens for better management of alopecia areata patients. Even so, more targeted studies concerning the biology and function of TLRs are warranted and may lead to the development of a new class of drugs.

Authors' contributions

AAA, GBS, MSA, AAR carried out the experimental work, data collection and manuscript drafting. ZR conceived of the study, its design, experimentation, coordination, data collection, data interpretation and manuscript drafting. All authors have read and approved the final manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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