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Cutaneous CCL13 mRNA and protein expression in vitiligo; a new insight to its immunopathogenesis

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Abstract

Background: **Vitiligo is an acquired emotionally distressing skin depigmenting immune related disorder yielded by selective melanocyte death through type 1 immune response and an emerging participation of type 2 immune response. CCL13 is a hallmark in type 2 helper T cells immune-related skin diseases. Our previous study showed that CCL13 expression, a type 2 immune response marker, was higher in vitiligo patients than controls.** ***Aim:*** **To investigate the relationship between mRNA and protein expressions of CCL13 in vitiligo lesions.** ***Subjects and methods:*** **This cross-sectional study comprised 30 non-segmental marginal recent vitiligo lesions. Immunohistochemistry, and real-time polymerase chain reaction were used to assess CCL13 protein expression, and relative CCL13 mRNA gene expression, respectively.** ***Results:*** **CCL13 is expressed at both genetic and tissue levels as there is relation between mRNA and protein expressions of CCL13 in vitiligo lesions.** ***Conclusion:*** **Relation between mRNA and protein expressions of CCL13 in vitiligo lesions may suggest its positive participation in vitiligo immune pathogenesis.**

Keywords: Vitiligo; CCL13; autoimmune response.

Introduction

Vitiligo is an acquired psychologically devastating skin depigmenting immune related disorder caused by the selective melanocyte death [(1)]. It affects people of diverse skin types, with an estimated prevalence of 0.18% in Egypt [(2)]**.** It has been known that type 1 immune response is the fundamental event in vitiligo immunepathogenesis [(3)]. The axis of interferon (IFN)-γ induced CXCL9/10 and their receptor C-X-C motif chemokine receptor type 3 (CXCR3) enhances the chemotaxis of $CXCR3^+$ anti-melanocyte cytotoxic T cells, resulting in melanocyte death [(4)]. Many recent studies explore the participation of type 2 immune response in type 1 highlighted dermatological disorders as vitiligo [(5)].

C-C motif chemokine ligand 13 (CCL13) is one of the CC chemokines. Its gene is found on chromosome 17q11.2 in a CC

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chemokine gene cluster. It is typically expressed in several tissues and plays a crucial function in leukocyte circulation [6].
It has been proven that during inflammation, epithelial cells are activated by pathogen-associated molecular patterns, IFN-γ, and IL-1, 4, 6, 13 via toll like receptors (TLRs) especially TLR 2, 3, 4 and 5, leading to the release CCL13 through nuclear factor kappa B (NF-κB) stimulation [7].
Our previous research found that CCL13 relative gene expression, as well as immunohistochemical protein expression were higher in vitiligo patients' skin lesions than controls [8]. Therefore, this study was conducted to investigate if there is a relation between mRNA and tissue expressions of CCL13 in vitiligo lesions.

Materials and methods:

Study design:

We began our cross-sectional study in January 2022 to February 2024 after approval of the Research Ethics Committee, Faculty of Medicine, Suez Canal University (no. 4686), following the items of Declaration of Helsinki and the STROBE guidelines. Thirty vitiligo participants (8 males and 22 females) were enrolled in the study. Each participant had an informed written consent before enrolling in the study.
Patients with non-segmental vitiligo, aged 18 years or more, and of both genders who attended the Dermatology Outpatient Clinic, Suez Canal University Hospital, Ismailia, Egypt were included. Vitiligo was diagnosed clinically by the presence of well-defined non-scaly depigmented or chalky- white macules and patches on the skin and mucous membranes. Wood' s lamp examination was done for confirmation.
Patients with autoimmune, allergic or inflammatory disorders, cancers, infections, and patients on systemic treatment, including phototherapy, within 3 months or topical medications for vitiligo in the previous 2 weeks, were excluded. Molecular and biochemical analyses were performed at the Molecular Biology unit of center of excellence in Molecular and Cellular Medicine, Faculty of Medicine, Suez Canal University.
Detailed history was obtained from enrolled patients: age of patient, age at disease onset, sex, vitiligo duration, course, site and pattern of lesions, duration of the most recent vitiligo lesion or expansion of old lesions, and positive family history. Skin was examined to identify non-segmental vitiligo clinical type including generalized, acrofacial, focal, and universal vitiligo. Vitiligo Disease Activity Index (VIDA) [9], and vitiligo activity signs (hypochromic ill-defined areas, recent Koebner phenomenon, confetti-like depigmentation, and inflammatory lesions / itching) were used to assess vitiligo activity [10]. Vitiligo Extent Score (VES) was utilized to assess severity of vitiligo through an online calculator **(https://www.vitiligo-calculator.com)** [11].

CCL13 gene expression:

Four mm punch skin biopsies were excised from the margin of newly developed vitiligo lesions, splited into two equal parts, one of which was fixed in 10% neutral formalin and preserved in paraffin blocks for immunohistochemical examination, while the other was frozen for RNA extraction for quantitative real-time polymerase chain reaction (RT-PCR).

RNeasy Mini Kit (cat no 74104, Qiagen, Hilden, Germany) was utilized to extract whole RNA from biopsies. The extracted RNA purity and concentration were determined using the ratio of (OD260 and OD260/280) on a NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA) followed by storing at -80 ℃. RevertAid H Minus First Strand cDNA Synthesis kit (Cat#K1632; Thermo Scientific Fermentas, St. Leon-Ro, Germany) was used to reversely transcribe the extracted RNA to cDNA.

Polymerase chain reaction reaction mix was primed by mixing 20 ng of cDNA to QuantiTect SYBR-Green PCR master mix kit (QuantiTect SYBR Green PCR Kit cat no.204141, Qiagen GmbH, Hilden, Germany) and 0.5 mM of the reverse and forwards of CCL13 primer pairs. The internal reference used was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers utilized for the qRT-PCR assays listed at **table 1**.

Table (1): Primers used for real time polymerase reaction assay	
GAPDH: (internal reference)	forward: ACAGCCTCAAGATCATCAGC reverse: GGTCATGAGTCCTTCCACGAT
CCL13:	forward: TCTGGCCTCCTCT TCTATGCT reverse: GGGCTTAGAGACAGCAACCT

CCL13: C-C motif chemokine ligand 13, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Then, Step One instrument (Applied Biosystems, Foster City, CA, USA) was used in the following steps: initial denaturation for 15 minutes at 95 ℃, then 40 cycles for 15 seconds with denaturation at 95 ℃, then annealing for 30 seconds at 60 ℃, followed by extension for 20 seconds at 72 ℃. To calculate the relative expression of CCL13 messenger RNA (mRNA), the comparative cycle threshold (ΔΔCt) method was applied, and GAPDH was used as a housekeeping [12].

CCL13 immunohistochemical expression:

The primary antibody was anti-CCL13 rabbit, polyclonal antibody, (product Code: CSB-PA08939A0Rb), 50Mg (CUSABIO technology LLC, Houston, TX 77054, USA) in a dilution 1:200 by phosphate buffer saline. The second antibody was anti-mouse/rabbit immunoglobulin conjugated to Horseradish Peroxidase, (cataloge NO. BSB0205), 100ml each (Bio SB products and technology for molecular pathology, USA). Both were applied according to manufacturers' instructions.

CCL13 tissue expression level was evaluated by CCL13 intensity score (negative, weak, intermediate, or strong), as well as the percentage of stained cells were assessed. The distribution of CCL13 expression whether basal, both basal and suprabasal or full thickness, as well as CCL13 expression at cellular level whether nuclear, cytoplasmic, membranous, or both cytoplasmic and membranous, and immunereactivity of inflammatory cells were also assessed.

Statistical Analysis

We use IBM SPSS software version 20.0 (Armonk, NY: IBM Corp) to analyze the data. To verify the normality of data distribution we use Shapiro-Wilk. We use percentage and numbers to display qualitative data. In addition to, we use median, inter-quartile range, standard deviation, mean, and range (minimum to maximum values) to display the quantitative data. For parametric data, two-sided Chi-square, Monte Carlo correction, and Student-t tests were applied, while Mann-Whitney U and Kruskal Wallis tests were applied for non-parametric data. The results were significant at the 5% level (P value ≤ 0.05).

Results

Clinical and demographic data of vitiligo participants:

Vitiligo patients were 8 (26.7 %) males and 22 (73.3%) females, with a mean age of 32.13 ± 9.76 years (range 18 – 55 years). The mean of age of vitiligo onset was 25.0 ± 10.90 years. The mean duration of the disease was 7.13 ± 4.64years. 8 (26.7%) patients had a positive family history. Regarding VIDA score, 19 (63.3%) patients were VIDA +4, 3 (10.0%) patients were VIDA +3, 1 (3.3%) patient was VIDA +2, 4 (13.3%) patients were VIDA= +1 and 3 (10.0%) patients were VIDA= 0.

Relative mRNA expression of CCL13 in skin biopsies of vitiligo patients (n=30) sub groups (stable and unstable groups)

No statistically significant difference between the mean relative mRNA of CCL13 between stable (VIDA 0 and VIDA -1) and active vitiligo patients (VIDA +1,+2,+3,+4) **(Table 2).**

Table (2): Relative CCL13 mRNA gene expression in skin biopsies of vitiligo patients (n=30) subgroups (stable and unstable groups according to VIDA score)

PCR fold change	Vitiligo			$^{U}p_1$
	Total (n = 30)	Stable (n= 3)	Active (n= 27)	
Mean ± SD.	3.30 ± 3.45	5.06 ± 6.13	3.10 ± 3.16	0.795
Median (Min. – Max.)	1.87 (1.09 – 15.42)	1.88 (1.17 – 12.13)	1.87 (1.09 – 15.42)	

U: Mann-whitney test. P1: between stable and active vitiligo groups. VIDA: Vitiligo Disease Activity Index

CCL13 immunohistochemical staining features of vitiligo lesions (n=30)

CCL13 was positively expressed in all vitiligo skin biopsies: 5(16.7%) patients had mild degree of intensity, 11(36.7%) patients had moderate degree of intensity and 14(46.7%) patients had strong degree of intensity. The percentage of positive cells range was 15.0 – 95.0 with a median of 90.0 cells. They were diffusely distributed at both cytoplasmic and membranous level in the basal cell layer in 3(10.0%) biopsies, in both basal and supra basal cell layers in 9(30.0%) biopsies and full thickness in 18 (60.0%) biopsies. The inflammatory infiltrate was immune-reactive in about 27 (90.0 %) vitiligo skin biopsies **(Table 3) (Figure 1)**

Relation between relative mRNA CCL13 gene expression and different clinical and

immunohistochemical data of vitiligo patients (n=30).

Relative mRNA CCL13 gene expression was varying significantly by different degrees of CCL13 immunohistochemical stain intensity among vitiligo patients (P value=0.041). Otherwise, relative mRNA CCL13 gene expression was not influenced by influence by different clinical and immunohistochemical data of vitiligo patients **(Table 4) (Figure 2).**

Table (3): CCL13 immunohistochemical staining features of vitiligo lesions (n=30)

	Vitiligo patients (n = 30)	
	No.	%
Degree of Intensity		
Negative	0	0.0
Mild	5	16.7
Moderate	11	36.7
Strong	14	46.7
Percentage of positive cells		
Min. – Max.	15.0 – 95.0	
Mean ± Standard deviation.	78.50 ± 20.26	
Median (Inter quartile range)	90.0 (70.0 – 90.0)	
Field distribution (vertical)		
Negative	0	0.0
Basal cell layer	3	10.0
Both Basel &Supra basal layers	9	30.0
Full thickness	18	60.0
Field distribution (horizontal)		
Negative	0	0.0
Focal	0	0.0
Diffuse	30	100.0
Expression at cellular level		
Negative	0	0.0
Nuclear	0	0.0
Cytoplasmic	0	0.0
Membranous	0	0.0
Both Cytoplasmic and membranous	30	100.0
Immune reactivity of inflammatory cells		
Negative	3	10.0
Positive	27	90.0

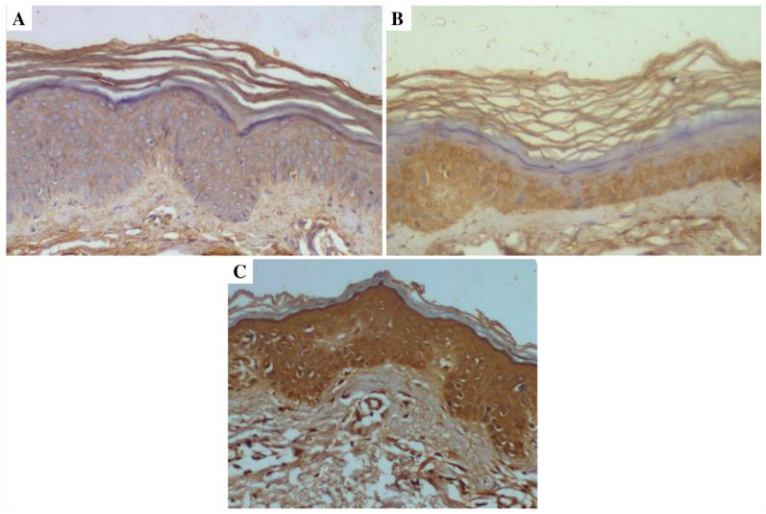

Figure (1): Immunohistochemical staining intensity of CCL13 in vitiligo skin lesions show **(A)** mild staining intensity **(B)** moderate staining intensity **(C)** strong staining intensity

Table (4): Relation between relative mRNA CCL13 gene expression and various clinical and immunohistochemical data of vitiligo patients (n=30).

	N	Relative mRNA CCL13 gene expression		Test of Sig.	P
		Mean ± Standard deviation.	Median (Min. – Max.)		
Gender					
Male	8	2.25 ± 1.53	1.65 (1.11 – 5.68)	U= 79.50	0.696
Female	22	3.68 ± 3.88	2.08 (1.09 – 15.42)		
Family history of vitiligo					
Yes	8	2.21 ± 0.94	2.09 (1.12 – 3.76)	U= 87.50	0.982
No	22	3.69 ± 3.94	1.65 (1.09 – 15.42)		
Clinical variant					
Generalized	26	3.16 ± 3.44	1.65 (1.09 – 15.42)	U= 41.00	0.536
Focal	4	4.21 ± 3.87	2.97 (1.17 – 9.71)		
Last activity according to VIDA score: (+4) Activity in the last 6 weeks or less (+3) Activity in the last 6 weeks to 3 months (+2) Activity in the last 3-6 months (+1) Activity in the last 6-12 months (0) Stable for 1 year or more	 19 3 1# 4 3	 3.49 ±3.58 3.62 ±2.13 1.21# 1.33 ± 0.06 5.06 ±6.13	 2.41 (1.09 –15.42) 3.76 (1.42 –5.68) 1.35 (1.25 –1.39) 1.88 (1.17 –12.13)	2.719	0.437
Degree of Intensity Mild Moderate Strong	 5 11 14	 1.49 ± 0.54 4.86 ± 4.29 2.71 ± 2.93	 1.42 (1.09 – 2.41) 3.76 (1.25 – 15.42) 1.60 (1.09 – 12.13)	H= 6.388*	0.041*

H: H for Kruskal Wallis test**, U:** Mann Whitney test, **VIDA**: vitiligo disease activity,
P value < 0.05 was considered as statistical significant

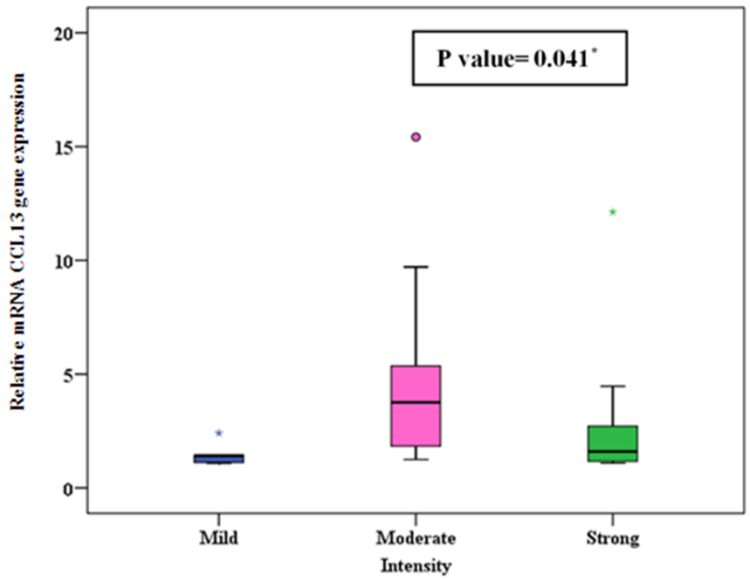

Figure (2): Relation between relative mRNA CCL13 gene expression and different degrees of CCL13 immunohistochemical stain intensity among vitiligo patients (n=30): relative mRNA CCL13 gene expression was varying significantly by different degrees of CCL13 immunohistochemical stain intensity among vitiligo patients (P value=0.041).

Correlation between relative mRNA CCL13 gene expression and different clinical and immunohistochemical data of vitiligo patients (n=30).

Relative mRNA CCL13 gene expression was not correlated with different clinical and immunohistochemical data of vitiligo participants **(Table 5)**

Table (5): Correlation between relative mRNA CCL13 gene expression and different clinical and immunohistochemical features of vitiligo patients (n=30).

	Relative mRNA CCL13 gene expression	
	r_s	P
Age (years)	-0.276	0.140
Age of onset (years)	-0.201	0.286
Disease duration (years)	-0.075	0.694
VES	-0.171	0.366
Percentage of positive IHC staining cells	-0.132	0.487

r_s: Spearman coefficient, VES: vitiligo extent score

Discussion

It has been known that vitiligo is mainly a type 1 skewed immune response [3]. The axis of interferon (IFN)-γ induced CXCL9/10 and CXCR3 enhances the chemotaxis of CXCR3+ anti-melanocyte cytotoxic T cells, resulting in melanocyte death [4]. Interestingly, by mapping upregulated and downregulated genes in AA patients into the STRING database, CCL13 gene has been revealed to be connected with CXCL10, CXCL9, and CXCR3 genes, which were all upregulated in patients with AA [13].

Our previous study revealed that the relative mRNA and immunohistochemical

expressions of CCL13 were greater in vitiligo participants than in controls [8]. This study revealed that CCL13 is expressed at both genetic and tissue levels as there is significant relation between mRNA and protein expressions of CCL13 in vitiligo lesions. Moderate CCL13 immunohistochemical stain intensity vitiligo lesions had the highest mRNA CCL13 gene expression that may be due to different CCL13 gene polymorphisms leading to different mRNA CCL13 gene half life, therefore different amount of CCL13 protein expression.

Concerning the implication of type 2 immune response in vitiligo, Martins et al. [14] noticed that the expression of Th2 response-related genes (e.g., CCL18, CCL5, and IL-13) was increased in vitiligo lesional skin. Also, Jin et al. [15] found that, in vitiligo lesional skin, IFN-γ can stimulate dermal fibroblast to express CCL8 and CCL2, which are members of type 2 cytokines. In addition, Czarnowicki et al. [16] mentioned that IL-4, 13 had the ability to restrain melanogenesis. Zhang et al. [17] found that Th1 cells had the upper hand in active vitiligo, but during stability, Th9, Th17, and Th2 cells were prominent. Interestingly, both Li et al. [6] and Yamaguchi et al. [18] have confirmed that CCL13 is one of Th2/ type 2 cytotoxic T lymphocytes hallmarkers. Moreover, Rojahn et al. [19] and Yu et al. [21]t) reported a marked elevation of CCL13 serum level in atopic patients, where Th2 cells play a significant role. Doran et al. [21] declared that the increased levels of CCL13 were correlated with upregulation of IL-13. Thus, the increased mRNA and protein expression of CCL13 in vitiligo patients might play a role in inducing Th2 cells in vitiligo skin lesions.

It has been proven that during inflammation, epithelial cells are activated by pathogen-associated molecular patterns, IFN-γ, and IL-1, 4, 6, 13 via toll like receptors (TLRs) especially TLR 2, 3, 4 and 5, leading to the release CCL13 through nuclear factor kappa B (NF-κB) stimulation [7]. Notably, oxidative stress in vitiligo promotes the secretion of the high mobility group box1. This causes NF-κB phosphorylation, resulting in the release of multiple mediators such as macrophage inflammatory protein-1α, IL-1β, IL-6, transforming growth factor-β, and tumor necrosis factor-α from macrophages, fibroblasts, monocytes, endothelial, T, and B cells [22]. It has been found that CCL13 is released through NF-κB activation [6], which may explain its increased mRNA and tissue expression in vitiligo patients recruited in this study.

During inflammation, CCL13 induces upregulation of adhesion molecules in endothelial cells, leading to T and immature dendritic cells chemotaxis, and eosinophils chemotaxis and degranulation beside basophil histamine release. CCL13 also provokes fibroblasts and smooth muscle proliferation [7]. Interestingly, Le et al. [23] revealed that in vitiligo, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1are upregulated in endothelial cells, which facilitates neutrophils chemotaxis and reactive oxygen species production, leading to melanocytes apoptosis. Concerning the role of eosinophils in vitiligo, Weissmann et al. [24] emphasized that in vitiligo, the interplay between IL-17F, IL-17A, and eosinophils enhances Th2 response. Moreover, cytotoxic granules and proteins emerged from eosinophils can induce tissue injury. Also, in severe vitiligo patients, eosinophil to either lymphocyte,

neutrophils or monocytes ratios were higher.

Xu et al. [25] showed that dermal fibroblasts enhance accumulation of autoreactive CD8+ T cells via CXCL9/CXCL10–CXCR3 axis upon IFN-γ stimulation. Additionally, Jin et al. [15] showed that upon IFN-γ stimulation of vitiligo lesional fibroblasts, CCL2 and CCL8 were upregulated. Also, Abdel-Malek et al. [26] revealed that fibroblasts enhanced the secretion of hepatocyte growth factor and Dickkopf1 in response to the pro-oxidant state in vitiligo, resulting in decreased E-cadherin expression and subsequent melanocytes detachment. Thus, elevated expression of CCL13 in vitiligo skin biopsies may highlight its role in eosinophils chemotaxis and degranulation, as well as fibroblasts proliferation and activation.

In fact, CCL13 has been studied in many comorbidities of vitiligo such as AA, AD, ocular uveitis, rheumatoid arthritis (RA), and systemic sclerosis [6]. Abu El-Asrar et al. [27] showed that patients with human leukocyte antigen-B27-associated non-granulomatous uveitis have greater CCL13 values in their aqueous humor than controls. Iwamoto et al. [28] elicited that in RA patients, CCL13 mRNA and protein levels were greatly high in synovial fibroblast and chondrocytes, and it enhances synovial fibroblasts formation and proliferation.

Moreover, Wang et al. [13] revealed that in AA patients' tissue and serum, CCL13 mRNA and protein levels were greatly elevated, with the highest levels in alopecia totalis and universalis, and its immunohistochemical expression was positively correlated with the immune reactivity of the immune cell infiltrate. Also, Renert-Yuval et al. [29] found that the clinical improvement of AA during the use of dupilumab was related to a decrease in the inflammatory infiltrates and the pre-treatment elevated Th2 chemokines (CCL13, CCL18, CCL26, CCL24).

In addition, Rojahn et al. [19] and Yu et al. [20] showed that in AD patients, especially upon TLR2 stimulation, CCL13 mRNA and protein levels were upregulated in peripheral blood monocytes, macrophages, dendritic cells, and lesional skin, with upregulation of IL-13, which enhances CCL13 release from epithelia [21].

Our limitations include a small sample size and a lack assessment of CCL13 in vitiligo patients' serum. Further researches with a larger sample size are recommended. Furthermore, the assessment of CCL13 in serum and tissue of vitiligo patients before and after treatment is recommended. Moreover, the correlation between CCL13 serum and tissue levels with other vitiligo activity biomarkers is warranted.

Conclusion

Relation between mRNA and protein expressions of CCL13 in vitiligo lesions may indicate its potential role in the autoimmune mechanisms implicated in vitiligo pathogenesis.

Abbreviations:

CCL: C-C motif chemokine ligand; IL: Interleukin; CXCR3: C-X-C motif chemokine receptor type 3; mRNA: messenger RNA; VIDA: Vitiligo disease of activity index; VES: Vitiligo extent score; RT-PCR: Real time polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; AD: Atopic dermatitis; RA: Rheumatoid arthritis; AA: Alopecia areata; IFN-γ: Interferon γ; Th: T helper cells; TLR:

Toll like receptor; NF-κB: Nuclear factor kappa B.

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