Increased expression of TLR7 and TLR9 in alopecia areata

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Running head : Interferons in alopecia areata

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Abbreviations: AA, alopecia areata; IP, immune privilege; MHC, major histocompatibility complex;

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Abstract

Alopecia areata (AA) is thought to be an autoimmune process. In other autoimmune diseases, the innate immune system and Toll-like receptors (TLRs) can play a significant role. Expression of TLR7, TLR9, and associated inducible genes were evaluated by quantitative PCR in peripheral blood mononuclear cells (PBMCs) from 10 healthy individuals and 19 AA patients, categorized according to disease duration, activity, and hair loss extent. Microdissected scalp biopsies from 5 patients and 4 controls were also assessed by quantitative PCR and immunohistology. *TLR9* was significantly upregulated 2.37 fold in AA PBMCs. Notably, *TLR9* was most significantly up regulated in patients with active AA, as shown by a positive hair pull test, compared to stable AA patients. In hair follicle bulbs from AA patients, *IFNG* and *TLR7* exhibited statistically significant 3.85 and 2.70 fold increases in mRNA respectively. Immunohistology revealed TLR7 present in lesional follicles, while TLR9 positive cells were primarily observed peri-bulbar to AA affected hair follicles. The increased expression of TLR7 and TLR9 suggest components of the innate immune system may be active in AA pathogenesis.

BACKGROUND

The pathogenesis of alopecia areata (AA) involves an autoimmune, lymphocyte-mediated attack on hair follicles.[1-6] Peri-bulbar inflammation and hair follicle infiltration by $CD4^+$ and $CD8^+$ lymphocytes is a hallmark feature of AA.[4] However, studies on other autoimmune diseases suggest the innate immune system can also play a significant role in promoting autoimmunity.[7,8] Notably, AA affected skin can also be infiltrated by macrophages, Langerhans cells, dendritic cells, natural killer (NK) cells, mast cells, and eosinophils,[9-13] circumstantially suggesting innate immune system constituents may also be active in AA.

Analysis of AA patient peripheral blood mononuclear cells (PBMCs) has revealed increased expression of mRNA for intracellular toll-like receptors (TLRs).[14] Activation of TLR7 (which senses single stranded RNA) and TLR9 (which senses unmethylated CpG dinucleotides in DNA) is significantly implicated in systemic lupus erythematosus (SLE).[15] Uptake of DNA/RNA-immunocomplexes by plasmacytoid dendritic cells (pDCs) activates intracellular TLRs.[16] TLR signaling initiates the MyD88 signaling pathway.[17,18] Consequently, type I interferons (eg. IFNα, IFNβ) are produced that promote SLE.[19-21]

QUESTIONS ADDRESSED

Here, we explored the hypothesis that TLR7 and TLR9 and associated inducible genes are differentially expressed in AA, consistent with active innate immunity.

EXPERIMENTAL DESIGN

Participants

Written informed consent was obtained from all patients recruited at the Department of Dermatology and Skin Science, University of British Columbia, with University Clinical Research Ethics Board approval. The following exclusion criteria were applied: patients under the age of nineteen and over age 65; a SALT (Severity of Alopecia Tool) score [22] between 30% and 70%; co-existing scalp dermatoses or other hair loss conditions; patients with other autoimmune disorders, viral or bacterial infections; and patients with any treatment history within the three months prior to consultation. Clinical parameters were recorded including age, sex, extent of hair loss, and disease duration. Patients were also evaluated by hair pull test for active or stable AA.[23]

Blood sampling and RNA isolation

Nineteen patients (11 females, 8 males; age range 24–61 years) with AA and 10 healthy controls (4 females, 6 males; age range 28–53 years) provided blood samples. Total RNA was extracted using the QIAamp RNA blood kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's protocols. RNA was eluted with RNase-free water and samples were stored at -80°C before quantitative reverse transcriptase-PCR (qPCR) analysis.

Tissue collection and RNA isolation

For mRNA analysis, AA scalp punch biopsies were obtained from 5 patients (all female; age range 43-57 years). All patients had active, patchy AA (30–50% lesional area) and were not receiving treatment. Specimens were obtained under local anesthesia from the lesional margin (M) of AA patches and externally normal looking perilesional (P) scalp skin at least 2cm from the lesional margin and negative for a hair pull test. Four scalp skin specimens were obtained from normal haired subjects during cosmetic surgery (3 females, 1 male; age range 34-59 years) as controls. All tissue samples were placed in RNA stabilizing reagent (RNAlater; Qiagen). Hair follicles were

isolated from the skin biopsies by microdissection under a stereo microscope as previously described.[24] Telogen stage hair follicles were discarded based on length and absence of a recognizable hair bulb. The hair bulbs of anagen and dystrophic anagen hair follicles were amputated (n>20 per biopsy). Total RNA was extracted using the RNeasy fibrous tissue kit (Qiagen) according to manufacturer's protocols.

Real-time reverse transcription-PCR analysis for gene expression

TLR7, TLR9 and select interferon inducible genes were chosen for qPCR analysis (Supplemental Table 1) and primers designed as described elsewhere.[25] RNA was reverse transcribed into cDNA with the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Real-time qPCR was performed with a SYBR Green Master mix system (Finnzymes, Espoo, Finland) according to the manufacturer's protocols. Universal 18S primers (Ambion, Austin, TX) were used for all analyses described and qPCR data was normalized against 18S expression.

Immunohistology

For immunohistology, AA scalp skin tissue samples were taken from the margin of active lesions of 6 patients (all female; age range 33-52 years). All patients had active, patchy AA (30–50% lesional area) and were not receiving treatment. Scalp tissue derived from 3 normal subjects during cosmetic surgery was utilized as comparative controls (2 females, 1 male; age range 35-59 years). All tissue samples were fixed in 10% formalin and processed routinely to glass slides. Paraffin embedded tissue sections were rehydrated, antigen unmasked with sodium citrate buffer, and hydrogen peroxide was used to block endogenous peroxidase as described elsewhere.[26] Sections were incubated with goat anti-human TLR7 and 9 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and biotinylated secondary antibodies (Jackson ImmunoResearch,

West Grove, PA) Isotype-matched antibodies were utilized as negative controls. The color reaction was produced using the 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlingame, CA).

Statistical analysis

Patients were categorized based on their extent of scalp hair loss (less than 30% versus more than 70% by SALT score calculation), hair loss duration (acute, less than 6 months versus chronic, more than 6 months), and disease progression or stability (positive hair pull test versus a negative result). The qPCR data were analyzed using the comparative cycle threshold method (Ct) ($\Delta\Delta$ Ct) representing the change in mRNA expression relative to control.[27] Results are expressed as the fold change in transcript levels for AA patients compared to controls. The difference in gene expression levels were evaluated by student T-test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Differential gene expression in AA PBMCs

PBMCs from AA patients had significantly higher *TLR9* mRNA levels compared to controls (Supplemental table 2). The mean fold change in mRNA levels for *GBP*, *MDA5*, *IFNA2*, *MX1*, *IFIT*, *TLR7*, and *DDX* were higher in AA patients compared to controls (Figure 1). However the difference did not reach statistical significance, reflecting significant variability in expression between individual samples. Mean mRNA levels for *IFNG*, *IFNGR1*, *IFNGR2*, *IFNA1*, *IFNB1*, *IFI44*, and *IRF1* were lower than controls, but also did not reach statistical significance.

In patients with a SALT score less than 30%, *TLR9* mRNA levels in PBMCs were significantly up regulated compared to controls (Supplemental Figure 1). Though statistical significance was not

achieved, there was a trend for increased *TLR9* in patients presenting with AA of duration greater than 6 months (supplemental Figure 2). In active AA patients, *IFNG* mRNA was significantly down regulated. Notably, *TLR9* was significantly up regulated in active AA compared to stable AA patients (Supplemental Figure 3).

Differential gene expression in follicular bulbs of the lesional AA margin and perilesion

Among genes evaluated in hair follicle bulbs (both lesional margin and perilesion combined), *IFNG* and *TLR7* exhibited statistically significant fold increases. In contrast, *IFNGR2*, *IFNA1*, *IFIT1*, and *GBP* exhibited significant fold decreases in expression (Figure 1, supplemental table 2). When the mRNA expression of the perilesional hair follicles were evaluated against controls, *IFNG* mRNA was significantly upregulated. *TLR7* mRNA showed modest upregulation, but without statistical significance. In contrast, several genes in perilesional hair follicles showed a trend for down regulation in mRNA achieving statistical significance for *IFNGR2*, *IFNA1*, *IFNA2*, *IFIT1*, *IFI44*, and *GBP* (Supplemental Figure 4). Hair follicle bulbs from AA lesional margins showed greater than 2 fold increases in *IFNG*, *IFNB1*, *IFI44*, *IRF1*, *TLR7* and *TLR9*, but none achieved statistical significance.

Toll like receptor expression and immunolocalization in alopecic lesions

TLR7 and TLR9 were evaluated by immunohistochemistry in 6 patients with AA and 3 control scalp biopsies. TLR7 expression was consistently expressed in the outer root sheath and bulb region in AA lesion specimens while no positive cells were seen in control tissues. TLR9 positive cells were variably observed exterior to AA affected hair follicles possibly consistent with expression in inflammatory infiltrate cells (Figure 2).

CONCLUSIONS

TLR7 and TLR9 are located on cell endosomal membranes where they detect virus-derived RNA and DNA, inducing expression of antiviral genes including IFNs.[28] Deregulated production of IFNs is associated with several autoimmune diseases.[29-31] In our analysis, *TLR9* mRNA expression was significantly upregulated in AA patient PBMCs and there was a trend for increased *TLR9* mRNA expression in follicle bulbs of AA patients. *TLR7* expression was significantly increased in preilesional and lesional margin follicular bulbs. We did not conduct any functional studies and as such, it is possible that the expression of TLR7 and TLR9 in AA are secondary and not significant to disease pathogenesis. However, systemic lupus erythematosus and multiple sclerosis studies suggest TLRs are important mediators of the inflammatory response.[32,33] While the functional significance of TLRs in AA remains to be explored, their increased expression may indicate a role in AA. Of note, TLR1 polymorphisms may be related to AA susceptibility.[34]

Type II interferon IFNγ plays an important role in AA;[35-37] IFNγ deficient mice are resistant to AA development.[38,39] Here, *IFNG* mRNA expression was reduced in AA patient PBMCs, perhaps consistent with limited expression during cell transit.[40,41] Other studies have suggested both increases [35] and decreases [42] in IFNγ in AA PBMCs. We observed a significant increase in *IFNG* mRNA expression in AA affected hair follicles consistent with prior observations.[43-45] Previous publications suggest type I IFNs may also be active in AA,[46,47] and AA can develop in subjects receiving IFNα/β treatment.[48-50] However, inconsistent with activation of antiviral pathways, we observed reduced expression of several IFN inducible genes in AA PBMCs and follicle bulbs. Alternatively, TLR expression can promote, and be promoted by, proinflammatory cytokines, including IFNγ,[51-53] which may be the case here.

Our data suggest upregulated TLR7 and TLR9 expression is associated with AA development while multiple associated inducible genes are downregulated. Although our study does not explain the complicated AA pathogenesis, TLR receptors are one candidate category of molecules for further investigation for their potential contribution to the development of AA.

ACKNOWLEDGEMENTS

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Figures

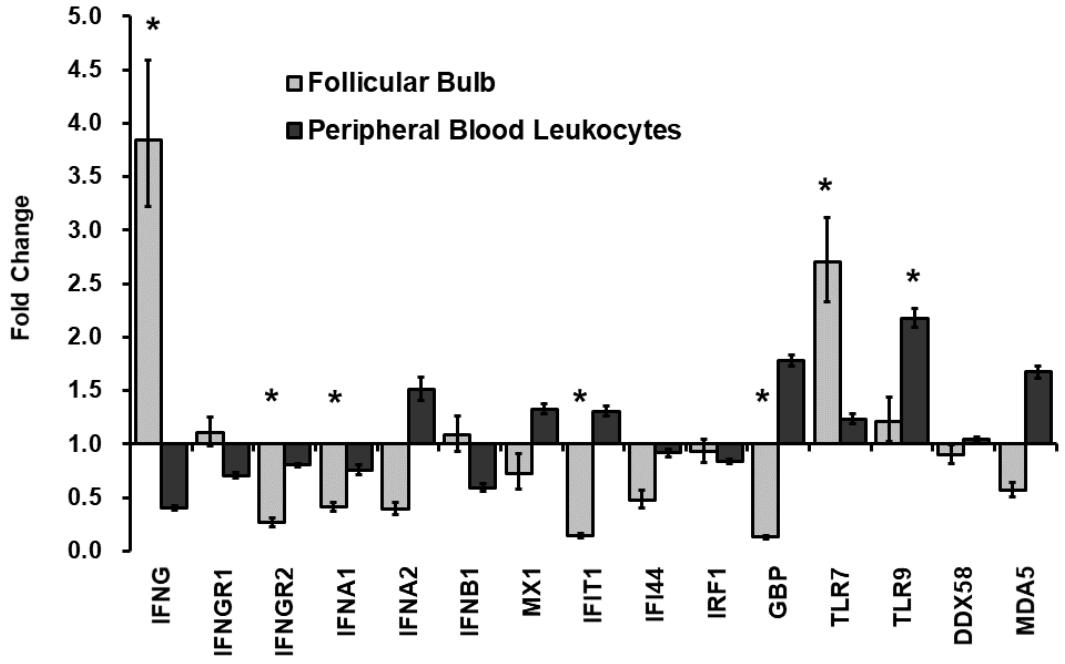

Fig 1. Expression of mRNA in peripheral blood mononuclear cells (PBMC) and follicular bulbs of AA affected scalp skin. QPCR revealed apparent marked increased expression of *TLR9* in PBMC, and *IFNG* and *TLR7* in follicular bulbs (both perilesional and lesional margin combined). Y-axis indicates fold change in mRNA levels of target genes in AA related samples as compared to normal PBMC and hair follicles from controls. Bars represent the mean ± standard error. * indicates P-value ≤ 0.05.

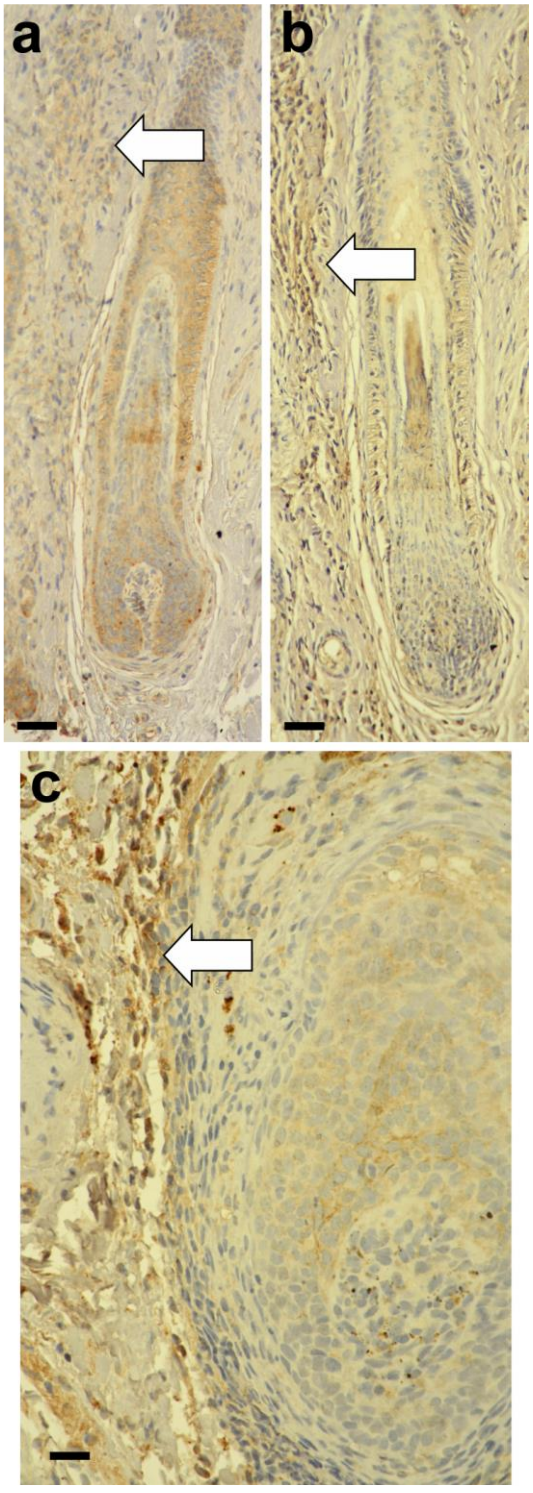

Fig 2. Expression of TLR7 and TLR9 in alopecia areata affected scalp skin.
Immunohistochemistry for TLR7 (a) indicated TLR7 was present in the outer root sheath and hair matrix and possibly slightly expressed in the inflammatory infiltrate (arrow). TLR9 (b, c) expression was present in the inflammatory infiltrate cells (arrows) and possibly in the outer root sheath at a low level (c). Bar = 100μm (a, b); 50μm (c).

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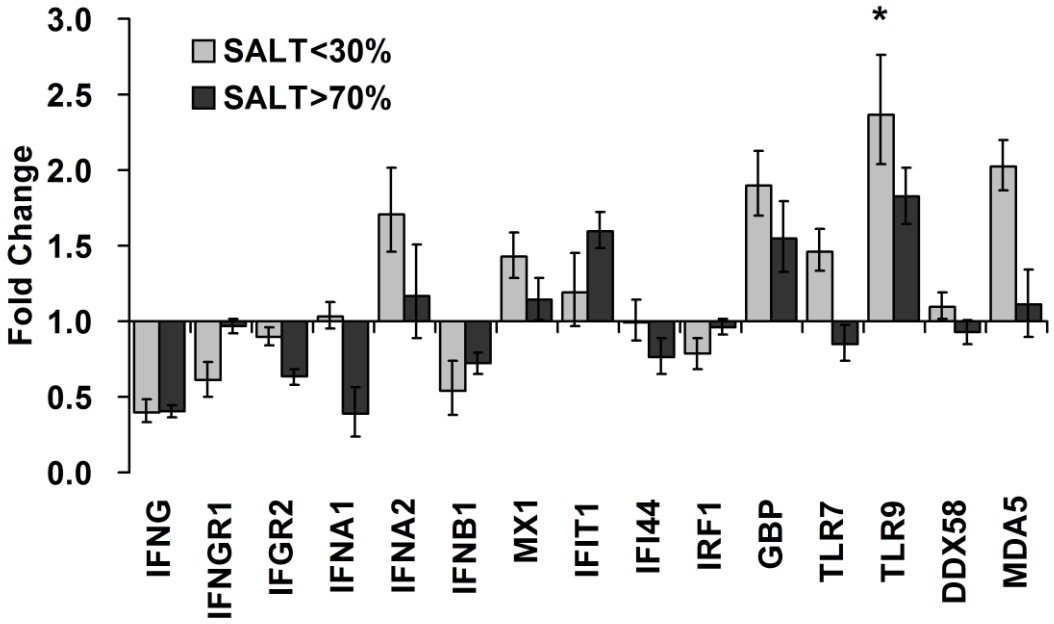
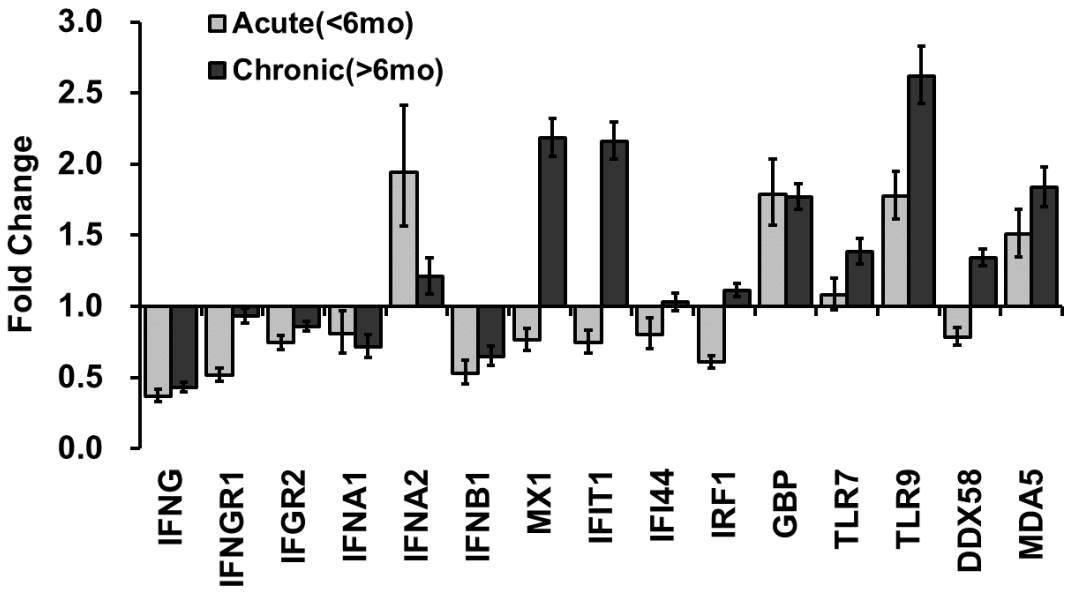
Hoon Kang, Wen-Yu Wu, Mei Yu, Jerry Shapiro, Kevin J. McElwee

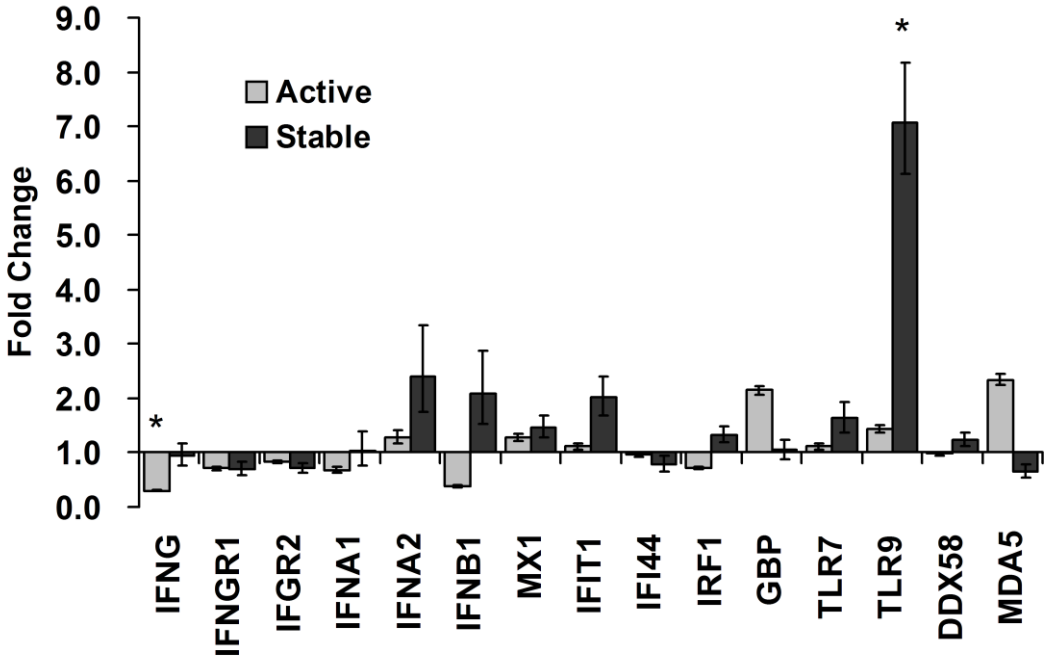
Supplemental figures and table



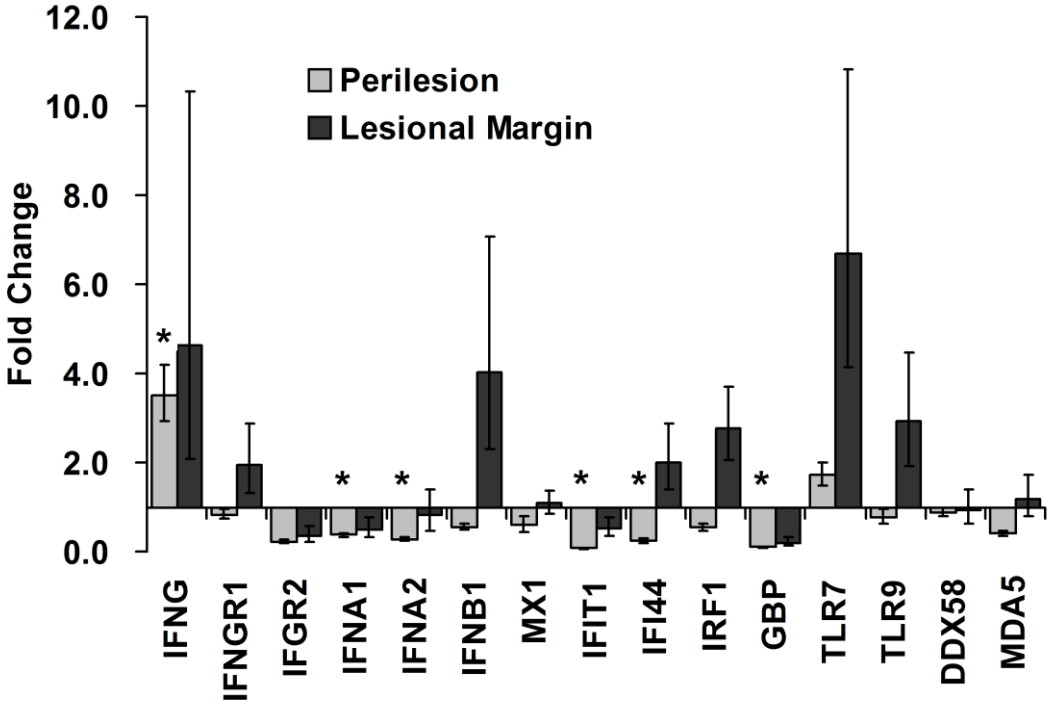
Supplemental Figure 1. Expression of mRNA in peripheral blood mononuclear cells (PBMCs) of patients categorized by extent of AA (SALT score). QPCR revealed apparent marked increased expression of *TLR9* in the SALT score less than 30% group (P=0.024). Y-axis indicates fold change in mRNA levels of target genes in AA related samples as compared to normal PBMCs from controls. Bars represent the mean ± standard error. * indicates P-value ≤ 0.05.



Supplemental Figure 2. Expression of mRNA in peripheral blood mononuclear cells (PBMCs) of patients categorized by time duration of AA. QPCR revealed apparent increased expression, though not statistically significant (P=0.059), of *TLR9* in the more than 6 months hair loss duration group. Y-axis indicates fold change in mRNA levels of target genes in AA related samples as compared to normal PBMCs from controls. Bars represent the mean ± standard error.



Supplemental Figure 3. Expression of mRNA in peripheral blood mononuclear cells (PBMCs) of patients categorized according to AA activity. QPCR revealed apparent marked increased expression of *TLR9* in hair pull negative group (stable AA group; P=0.0009). IFNG exhibited significant decreased expression in the positive hair pull group (active AA group; P=0.014). Y-axis indicates fold change in mRNA levels of target genes in AA related samples as compared to normal PBMCs from controls. Bars represent the mean ± standard error. * indicates P-value ≤ 0.05.



Supplemental Figure 4. Expression of mRNA in lesional margin and perilesional scalp hair follicles. QPCR revealed apparent marked increased expression of *IFNG* in perilesional AA hair follicles (P=0.038). Y-axis indicates fold change in mRNA levels of target genes in AA related samples as compared to normal hair follicles from controls. Bars represent the mean ± standard error. * indicates P-value ≤ 0.05.

Supplemental Table 1. Sequences of primers used in qPCR (from 5' to 3')

target gene	forward primer sequence	reverse primer sequence
IFNG	GGCATTTTGAAGAATTGGAAAG	GCTTTTCGAAGTCATCTCGTTT
IFNGR1	GATGGAAAAATTGGACCACCTA	ACTGGATCTCACTTCCGTTCAT
IFNGR2	TGTTTACAAGTCCAGGCACAAC	ATTTCAGGACCAGGAAGAAACA
IFNA1	CAAAAGATTCATCTGCTGCTTG	CAAGTCATTCAGCTGCTGGTAG
IFNA2	CAAAAGATTCATCTGCTGCTTG	CAAGTCATTCAGCTGCTGGTAG
IFN-β1	TCCACTACAGCTCTTTCCATGA	AGTATTCAAGCCTCCCATTCAA
MX1	CACGAGTTCCACAAATGGAGTA	TTCACGATTGTCTCAAATGTCC
IFIT1	ACTTAAGGATGCAGCAAGAAGG	CCATTTGTAAGTTCTCGAGGCTA
IFI44	TTGGTGGGCACTAATACAACTG	CCCCAGTGAGTCACACAGAATA
IRF1	TGAGGATGAGGAAGGGAAATTA	CTGGCTCCTCCTTACAGCTAAA
GBP	GATTGAAGTGGAACGTGTGAAA	TCGTTCTCCATCTTCTCAGTCA
TLR7	TTGAGAAGCCCTTTCAGAAGTC	CACCTGACTATAGGCCACATGA
TLR9	AGTTCTCTCTCCTGGCTGAATG	GAAGGCCTTGGTTTTAGTGATG
DDX58	CTCTTGATGCGTCAGTGATAGC	CGAGGTCTTTGCAGATTCTCTT
MDA5	GATAAGTGCATGGAGGAGGAAC	TGGACAAGTTCATTGTTTCCTG

Supplemental Table 2. Significance of fold change in mRNA expression observed between AA patients and control samples

Gene	**Follicular bulbs** (both lesional margin and perilesion combined)		**Peripheral blood leukocytes** (all patients combined)	
	Fold Change	**P-value**	**Fold Change**	**P-value**
IFNG	3.85	**0.04**	0.40	0.06
IFNGR1	1.11	0.80	0.70	0.31
IFNGR2	0.26	**0.02**	0.80	0.40
IFNA1	0.41	**0.02**	0.76	0.69
IFNA2	0.39	0.07	1.51	0.60
IFNB1	1.08	0.88	0.59	0.37
MX1	0.72	0.64	1.33	0.50
IFIT1	0.14	**0.00**	1.31	0.54
IFI44	0.48	0.22	0.92	0.87
IRF1	0.93	0.85	0.84	0.55
GBP	0.13	**0.00**	1.78	0.30
TLR7	2.70	**0.05**	1.23	0.59
TLR9	1.21	0.73	2.18	**0.05**
DDX58	0.90	0.76	1.04	0.89
MDA5	0.57	0.19	1.67	0.30