ORIGINAL ARTICLE

Suction blistering the lesional skin of vitiligo patients reveals useful biomarkers of disease activity

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Background: Vitiligo is an autoimmune disease of the skin with limited treatment options; there is an urgent need to identify and validate biomarkers of disease activity to support vitiligo clinical studies.

Objective: To investigate potential biomarkers of disease activity directly in the skin of vitiligo subjects and healthy subjects.

Methods: Patient skin was sampled via a modified suction-blister technique, allowing for minimally invasive, objective assessment of cytokines and T-cell infiltrates in the interstitial skin fluid. Potential biomarkers were first defined and later validated in separate study groups.

Results: In screening and validation, $CD8^+$ T-cell number and C-X-C motif chemokine ligand (CXCL) 9 protein concentration were significantly elevated in active lesional compared to nonlesional skin. CXCL9 protein concentration achieved greater sensitivity and specificity by receiver operating characteristic analysis. Suction blistering also allowed for phenotyping of the T-cell infiltrate, which overwhelmingly expresses C-X-C motif chemokine receptor 3.

Limitations: A small number of patients were enrolled for the study, and only a single patient was used to define the treatment response.

Conclusion: Measuring CXCL9 directly in the skin might be effective in clinical trials as an early marker of treatment response. Additionally, use of the modified suction-blister technique supports investigation of inflammatory skin diseases using powerful tools like flow cytometry and protein quantification. (J Am Acad Dermatol http://dx.doi.org/10.1016/j.jaad.2016.12.021.)

Key words: autoimmunity; biopsy; blisters; biomarkers; CD8; CXCL9; CXCL10; inflammatory skin disease; suction blister; vitiligo.

Vitiligo is a common autoimmune disease of the skin that causes disfiguring depigmented macules and patches.[1] Depigmentation is the result of $CD8^+$ T-cell–mediated killing of melanocytes, the pigment producing cells in the skin.[2] Phototherapy and topical immunosuppressants are used off-label with moderate results that require long

Abbreviations used:

CXCL:	C-X-C motif chemokine ligand
CXCR:	C-X-C motif chemokine receptor
ELISA:	enzyme-linked immunosorbent assay
IFN-γ:	interferon-γ
ROC:	receiver operating characteristic

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treatment courses. Based on studies in our mouse model and human tissues,[3,4] we hypothesized that targeting the interferon-γ (IFN-γ)/C-X-C motif chemokine ligand (CXCL) 10 axis could be an effective new treatment strategy for vitiligo.[5] Recent case reports revealed that Janus kinase inhibitors, which block IFN-γ signaling, are effective at reversing vitiligo,[6,7] supporting our hypothesis. Clinical trials to test new, targeted treatments for vitiligo are on the horizon.

When conducting clinical trials on vitiligo, monitoring disease activity will be critical for measuring the treatment response. A common misconception is that biomarkers of treatment response are unnecessary, since the skin can be observed clinically; however, even the earliest clinical changes in vitiligo take 2-3 months to become apparent because of the time required for melanocyte precursors to proliferate, migrate, differentiate, and produce pigment. Measurable responses require 6 months or more, and current outcome measures are largely subjective and insensitive to small changes.[8,9] Thus, relying only on observable changes in skin pigmentation is problematic.

Recent studies reported that serum levels of IFN-γ–induced chemokines are elevated in patients with active vitiligo[4,10] and correlate with treatment responses.[10] Serum chemokine levels, while reflecting general trends of disease activity in large cohorts, are likely too variable to serve as sensitive and specific markers of disease activity in a single patient. Moreover, patients with other autoimmune diseases exhibit increased inflammatory markers in the serum as well,[11,12] precluding their use as specific markers in vitiligo. Measuring markers directly in the skin could be a more sensitive and specific approach; however, conventional analysis of skin through immunohistochemistry is problematic because lesions have low numbers of infiltrating T cells that are difficult to detect and quantify, unlike more inflammatory diseases. Thus, a more efficient method to sample vitiligo lesional skin and reliably measure treatment responses is needed.

We adapted a minimally invasive, nonscarring skin biopsy technique[13-17] to reliably and accurately sample vitiligo lesions by inducing suction blisters, which contain interstitial skin fluid[18] that can be used for analysis. Lesional blister fluid revealed an infiltrate of $CD8^+$ T cells and CXCL9 protein that was both sensitive and specific for disease activity, in contrast to analysis of patient blood and serum. Suction blistering provides an opportunity to sample lesional skin in patients in a minimally invasive way, while yielding highly useful material to study markers of disease activity for translational studies and treatment responses in clinical trials.

CAPSULE SUMMARY

- Suction blistering is a minimally invasive method to measure immune biomarkers directly in skin.
- C-X-C motif chemokine ligand (CXCL) 9 protein concentration and $CD8^+$ T-cell number in blister fluid are sensitive and specific biomarkers of active disease in vitiligo.
- These markers might support clinical trial investigators in identifying active patients for enrollment and detecting early treatment responses.

METHODS

Study design

Subjects were recruited into 2 sequential groups for this study: the first to define markers of disease activity in vitiligo, and the second to independently validate these markers in a separate, nonoverlapping group of subjects (Table I and Supplemental Table I [available at http://www.jaad.org]). Inclusion/exclusion criteria were as follows: subjects with a diagnosis of vitiligo by clinical exam performed by a dermatologist and off treatment for at least 6 weeks were included in screening and validation groups. Subjects with recent onset of new lesions and objective clinical signs of activity, specifically confetti-like macules or trichrome lesions, were recruited to represent the active subgroup. Subjects with stable disease or no recent change in lesions and no objective clinical signs of disease activity (no confetti, trichrome, or inflammatory lesions and no Koebner phenomenon) were selected on the basis of patient self-reports. Those on treatment, with other inflammatory skin diseases or presenting with the segmental subtype of vitiligo, were excluded from the study. One subject was blistered repeatedly before and after starting treatment to define the treatment response.

Suction-blister skin biopsy and fluid aspiration was performed with internal review board–approved protocols at the University of Massachusetts Medical School and all samples were de-identified before use in experiments. Active subjects with a 1-month history of new or spreading lesions were blistered at sites featuring confetti-like depigmentation or trichrome lesions.[19,20] In subjects with stable disease, sites along the lesional edge consisting of normal and depigmented skin were selected for suction blistering. The nonlesional sites selected were normal-appearing, nondepigmented

Table I. Summary of demographic information

	Active vitiligo defining group	Active vitiligo validation group	Stable vitiligo validation group	Healthy controls defining group	Healthy controls validation group
Sample size, n	8	8	4	8	1
Age, mean (SD), y	40.1 (9.2)	46.3 (17.3)	37.5 (7.2)	29.8 (13.3)	40
Female, n (%)	6 (75)	6 (85.7)	2 (50.0)	5 (62.5)	1 (100.0)
Race, n (%)					
White	5 (62.5)	5 (62.5)	4 (100.0)	6 (75.0)	0 (0)
Black	2 (25.0)	2 (25.0)	0 (0)	2 (25.0)	0 (0)
Asian	1 (12.5)	1 (12.5)	0 (0)	0 (0)	1 (100.0)
Associated autoimmune disease, n (%)	0 (0)	1 (12.5)	0 (0)	0 (0)	0 (0)
FH vitiligo, n (%)	0 (0)	1 (12.5)	0 (0)	1 (12.5)	1 (100.0)
FH other autoimmune, n (%)	0 (0)	1 (12.5)	0 (0)	1 (12.5)	0 (0)
Duration of disease, mean (SD), y	19.6 (4.2)	14.6 (16.1)	19.3 (10.3)	N/A	N/A

FH, Family history; *N/A*, not applicable; *SD*, standard deviation.

skin when examined by Wood's lamp and at least 5 cm from the nearest depigmented macule.

Blister induction and processing

Suction blisters (1 cm in diameter) were induced on the skin by using the Negative Pressure Instrument Model NP-4 (Electronic Diversities, Finksburg, MD). The suction chambers were applied to the skin with 10-15 mm Hg of negative pressure and a constant temperature of 40°C; blisters formed between 30-60 minutes after initiation of the procedure. After blister formation, the blister fluid was aspirated through the roof using a 1 mL insulin syringe (Fig 1). Cells within the blister fluid were pelleted at 330 × *g* for 10 minutes for cell staining and the supernatant was collected and frozen for future analysis by enzyme-linked immunosorbent assays (ELISA). Occasionally (9% of blisters), hemorrhage occurred in the blister: these blisters were excluded from analysis because their infiltrates were contaminated with cells from the peripheral blood and poorly reflected the cells constituting the interstitial fluid of the skin (data not shown).

ELISA and flow cytometry

In brief, the manufacturer's instructions were followed for ELISA and flow cytometry techniques. Further details are provided at http://www.jaad.org in the Supplemental Methods section.

Statistics

Statistical analyses were performed with GraphPad Prism software. Dual comparisons were made with the Wilcoxon's matched-pairs signed rank test for nonparametric data. Groups of ≥3 were analyzed by Kruskall-Wallis with Dunn post tests for nonparametric data. Multiplicity-adjusted *P* values from the Dunn post tests are reported as post test *P* values. Receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity of tests performed on blister samples. *P* values <.05 were considered significant.

RESULTS

T-cell infiltrates are elevated in active vitiligo lesions

As $CD8^+$ T cell recruitment to the skin is required for depigmentation,[4] we examined the T-cell number in the skin as a potential biomarker for active disease. Flow cytometry of blister fluid in the defining group initially revealed that active lesional sites had a larger infiltrate of $CD8^+$ T cells than nonlesional sites did; they also had more $CD8^+$ T cells than the skin of healthy individuals (Fig 2, *A* and Supplemental Fig 1; available at http://www.jaad.org). We and others reported that skin-infiltrating $CD8^+$ T cells express C-X-C motif chemokine receptor (CXCR) 3 when analyzed by immunohistochemistry.[4,10,21] Here we found that $CD8^+$ T cells uniformly express CXCR3 in the skin by flow cytometry (Fig 2, *B*) supporting previous results.

We then sought to quantify the number of T cells in each blister. Lesional sites on average had a 2-fold increased number of $CD8^+$ T cells with a mean of 2727 cells compared with 1305 cells in nonlesional skin after normalization to $CD45^+$ cell count (analysis of variance, all sites, $P = .0009$; t test lesional to nonlesional, post test $P = .0185$; t test lesional to healthy, post test $P = .0015$). No appreciable differences were observed in infiltrates between nonlesional sites of active patients and healthy control skin (Fig 2, *C*). We also found that the total $CD3^+$ T-cell number is significantly higher in active lesional skin with an average difference of 1.4-fold (7651 lesional, 5491

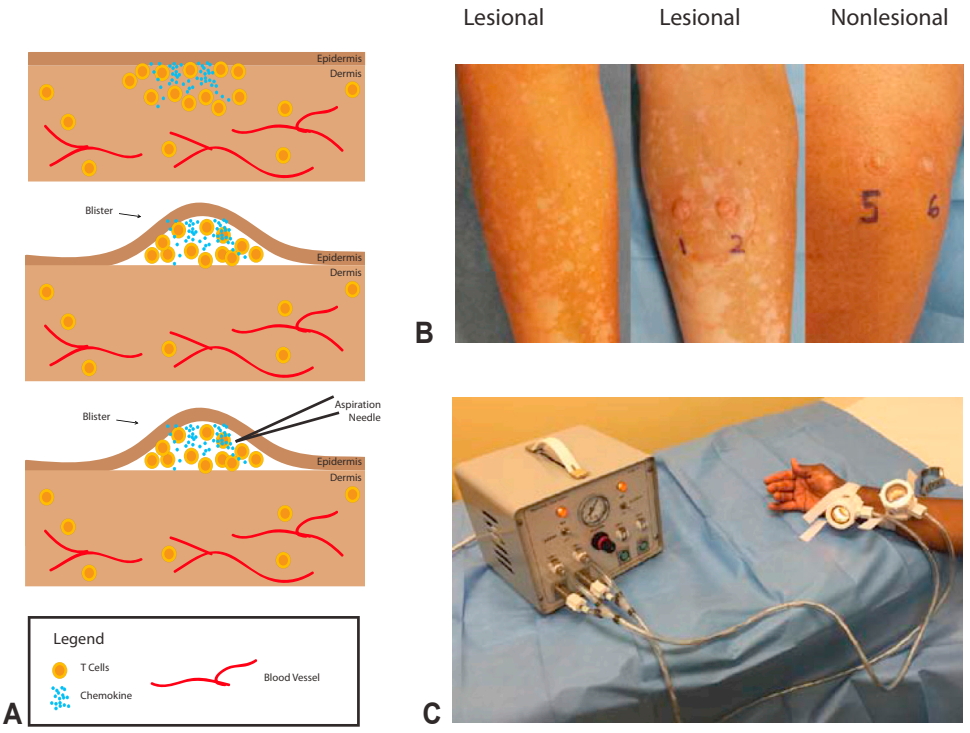

Fig 1. Modified suction-blister technique. **A**, Graphic demonstrating T cells clustering near the epidermis, where the chemokine concentration is highest (top panel). After suction blistering, the epidermis forms the roof (blister), which becomes separated from the dermis (middle panel). T cells and chemokine at the site are present in the blister fluid and can be aspirated by fine needle aspiration (bottom panel). **B**, Representative photos from a patient with active vitiligo before blistering (left panel), the same site after blistering (middle panel), and of a nonlesional site (right panel). **C**, A study subject waits while the suction blisters form.

nonlesional, $P = .0015$). Although the $CD4^+$ T cell number in the skin was elevated in the defining group, comparisons did not reach statistical significance in the validation group or when the total data was compiled (Supplemental Fig 2; available at http://www.jaad.org). We then used a ROC analysis to test whether $CD8^+$ T-cell number could be used to predict disease activity and found it had a reasonable predictive value (Fig 2, *D*): a threshold of 1175 normalized $CD8^+$ T cells achieved a sensitivity of 70% with specificity of 64%.

We next completed the same analyses in a second group of subjects to validate our observations and found similar results: lesional infiltrates contained significantly higher $CD8^+$ T cells than nonlesional sites did (post test $P = .0494$). This group also included 4 subjects with stable vitiligo, and the T-cell number in the skin (lesional and nonlesional) of these individuals were reduced in comparison with that in the lesional skin of persons with active vitiligo (post test $P < .0001$ and post test $P < .0001$, respectively) (Fig 2, *E*). ROC analysis of this group revealed that a threshold of 2766 normalized $CD8^+$ T cells yields 83% sensitivity and 92% specificity (Fig 2, *F*).

Skin CXCL9 protein is a more sensitive and specific marker of disease activity than CXCL10

We previously reported that CXCL9 and CXCL10 RNA transcripts were elevated in lesional skin of vitiligo patients.[4] $CXCR3^+$ $CD8^+$ T cells use CXCL9, CXCL10, and CXCL11 to traffic towards sites of inflammation[22] and CXCL10 is functionally required for the progression and maintenance of vitiligo.[4] By ELISA, we found that CXCL9 was significantly elevated in lesional blister fluid compared with that in nonlesional skin and healthy controls and predicted disease activity better than $CD8^+$ T-cell numbers (Fig 3, *A*). When we examined CXCL10 in the skin of patients with vitiligo, we discovered it was not significantly higher in the active lesional skin and did not have good predictive value for disease activity (Fig 3, *B*). When we evaluated CXCL9 as a candidate biomarker in the second validation group, we detected an average 5.5-fold difference between

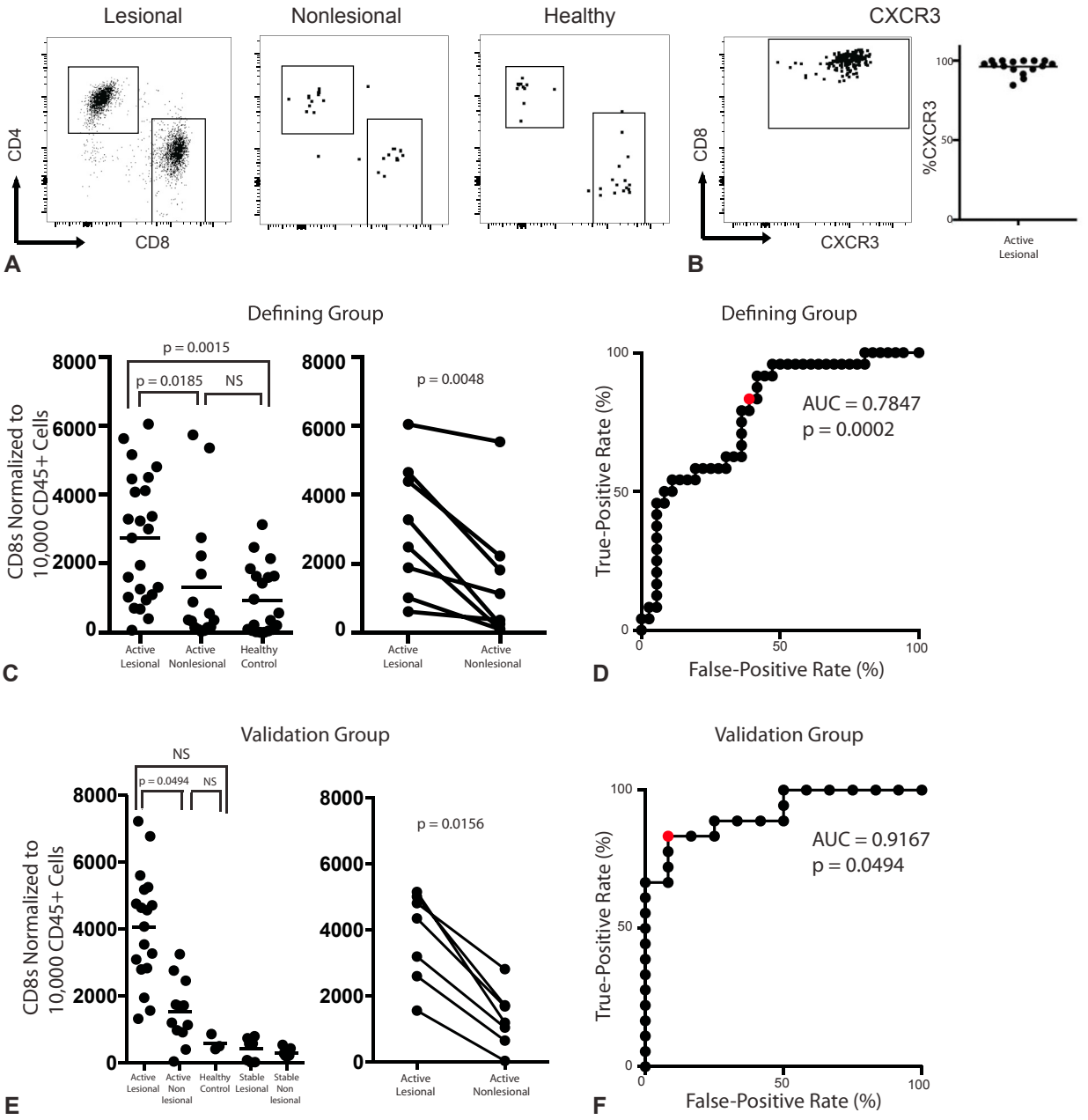

Fig 2. CD8$^+$ T-cell phenotyping and quantification. **A**, Representative flow plots of the T-cell infiltrate isolated from lesional, nonlesional, and healthy control skin. **B**, C-X-C motif chemokine receptor (CXCR) 3 expression on CD8$^+$ T cells in lesional skin. **C**, Quantification of the CD8$^+$ T-cell infiltrate of each sample normalized to 10,000 CD45$^+$ cells for the defining group (left panel) and paired analysis of vitiligo subject samples (lesional vs nonlesional, right panel). **E**, Quantification of the CD8$^+$ T-cell infiltrate of each sample normalized to 10,000 CD45$^+$ cells for the validation group (left panel) and paired analysis of vitiligo subject samples (lesional vs nonlesional, right panel). **D** and **F**, Receiver operating characteristic curve for the defining group (**D**) and the validation group (**F**). Red dots mark threshold values described in the text.

active lesional sites and nonlesional skin (post test $P = .0492$). ROC analysis suggested that CXCL9 is a more sensitive and specific marker of disease activity than T-cell number, achieving a threshold value of 1177 pg/mL with 100% sensitivity and 93% specificity (Fig 3, *C*). We also evaluated CXCL10 in the skin of patients in the validation group and found similar results as the first group, although this time the level of CXCL10 was 3.5-fold higher in lesional skin compared with nonlesional skin (post test

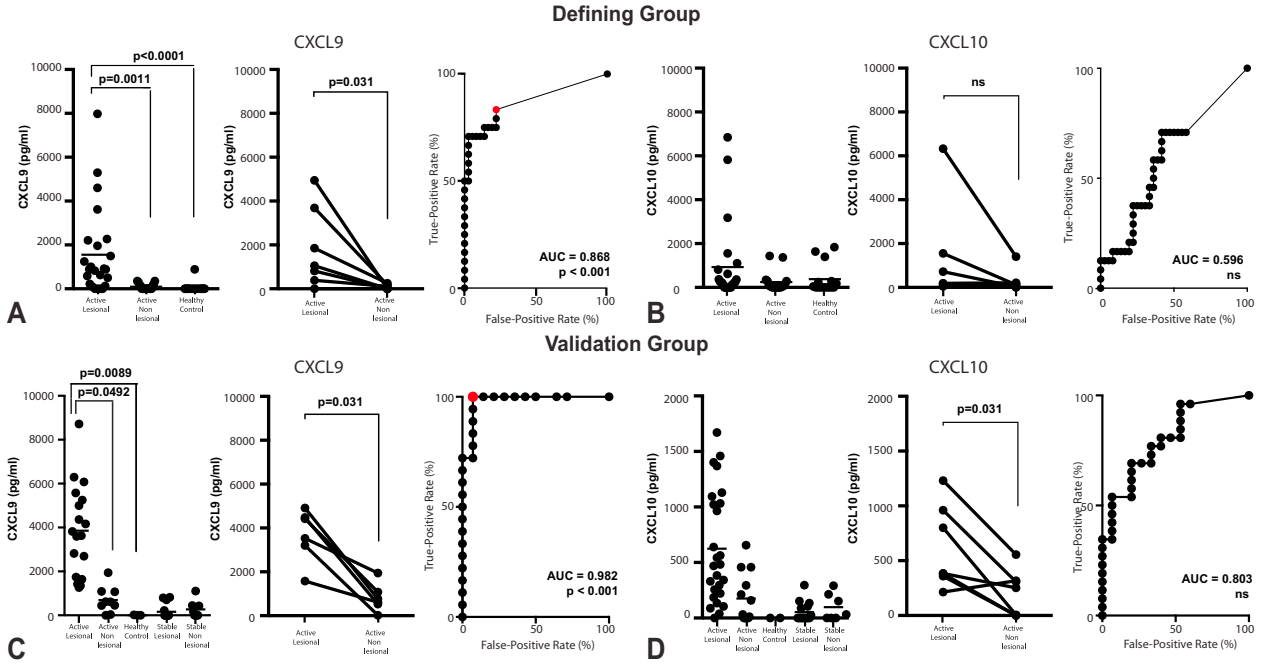

Fig 3. Chemokine protein levels in blister fluid. Quantification of C-X-C motif chemokine ligand (CXCL) 9 (**A** and **C**) or CXCL10 (**B** and **D**) in the blister fluid of the defining group (top panels) and validation group (bottom panels). Each panel is organized in the following manner: quantification of individual samples grouped by active lesional, active nonlesional, and healthy control (left), paired analysis of lesional and nonlesional blisters from vitiligo subjects (middle panel), and receiver operating characteristic curve (right). Red dots mark threshold values described in the text.

P = .0243). Despite detecting elevated levels of CXCL10 in active disease, these differences did not significantly increase the predictive value of CXCL10 (Fig 3, *D*). In initial subjects, CXCL11 and IFN-γ were detected only at low levels, if at all, and levels of these chemokines in lesions were not elevated compared with that in nonlesional sites or healthy control skin, despite having elevated CXCL9 and CXCL10 (Supplemental Fig 3; available at http://www.jaad.org). Thus, we did not measure these cytokines in the remaining subjects.

It was previously reported that serum CXCL9 and CXCL10 were significantly elevated in a large cohort of vitiligo patients compared with healthy controls and that upon intervention, the levels of CXCL10, but not CXCL9, significantly dropped.[10] However, we did not see a correlation between serum chemokines and disease activity in either cohort or in the total group when both cohorts were combined (Fig 4, *A-C*), perhaps because of small sample size or selecting patients with active disease phenotypes. Serum levels of CXCL11 and IFN-γ were either undetectable or not significantly different in sampled patients (Fig 4, *D*).

Case study: CXCL9 protein is most responsive to multimodal treatment at an early time point

We followed one patient who was undergoing treatment for vitiligo with narrow band ultraviolet B phototherapy and alternating topical tacrolimus and clobetasol. We sampled the same locations where the patient exhibited confetti on the first visit and at each subsequent visit. After the first 4 months, the patient reported inconsistent use of treatment, and no treatment for the previous 4 weeks. At this time, her T-cell number remained largely unchanged from her initial visit, yet after an additional 4 months of consistent phototherapy and tacrolimus use, the $CD8^+$ T-cell number in her lesional tissue declined 2-fold. However, her nonlesional $CD8^+$ T-cell count also decreased, leading to no change in the T-cell count ratio between lesional to nonlesional tissue (Fig 5, *A*). Interestingly, lesional CXCL9 was decreased at the second visit and undetectable at the third visit (Fig 5, *B*). Serum CXCL9 was undetectable at the final visit as well (Fig 5, *C*). Lesional and serum CXCL10 were decreased at both visits (Fig 5, *D* and *E*).

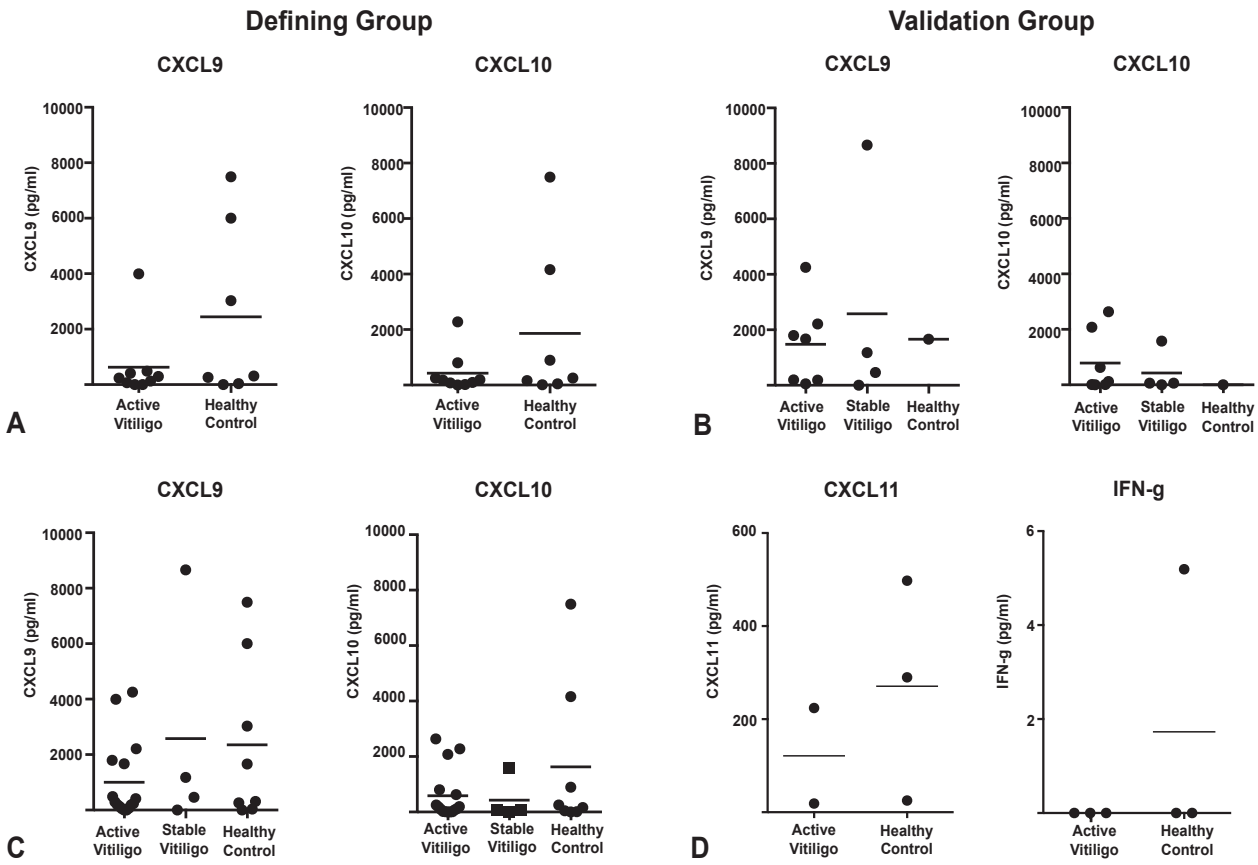

Fig 4. Serum chemokine levels in patients with vitiligo and healthy controls. Serum levels of C-X-C motif chemokine ligand (CXCL) 9 and CXCL10 are reported for the defining group (**A**) and the validation group (**B**). **C**, Serum levels of CXCL9 (left) and CXCL10 (right) are compiled from both groups. **D**, Serum levels of CXCL11 (left) and IFN-γ (right).

DISCUSSION

We adapted a modified suction-blister protocol to sample lesional skin from vitiligo patients and address the need for markers of disease activity and early treatment response. Our testing of potential biomarkers was based on observations we made in our mouse model of vitiligo and in vitiligo patients. Thus, this work was hypothesis-directed rather than a screen of multiple unrelated parameters. While both $CD8^+$ T-cell number and CXCL9 in the blister fluid demonstrated good predictive value in identifying those with active disease, CXCL9 had better sensitivity and specificity, and CXCL10 correlated only weakly.

In contrast to previous studies,[4,10] we could not use serum chemokine levels to distinguish those with active disease from those with stable disease or even healthy controls. This might have resulted from the small number of subjects enrolled in this study compared with other studies or from the patient selection used for this study (requiring highly active confetti or trichrome patterns). Either way, it highlights the limitations of using serum markers to reliably measure disease activity in individual patients, and the advantage of skin-specific analyses.

Our results also indicate that blister fluid $CD8^+$ T-cell infiltration and CXCL9 protein are not induced by the blistering process itself because nonlesional sites showed minimal levels of both. CXCL10 was elevated in the nonlesional skin of some subjects, which could have been caused partially by the blister trauma and might explain why it is a less specific marker when using this method. Alternatively, CXCL9 protein might be more bioavailable in blister fluid and easier to detect by ELISA than CXCL10 on the basis of their different spatial and temporal expression in the skin,[23] binding affinities to structural elements,[24,25] and oligomerization within tissues.[26] We previously reported that both CXCL9 and CXCL10 had functional roles in our mouse model of vitiligo; however, only CXCL10-deficient mice were protected from clinical disease.[4] Although both chemokines are expressed in active disease, they are produced by different cell types with

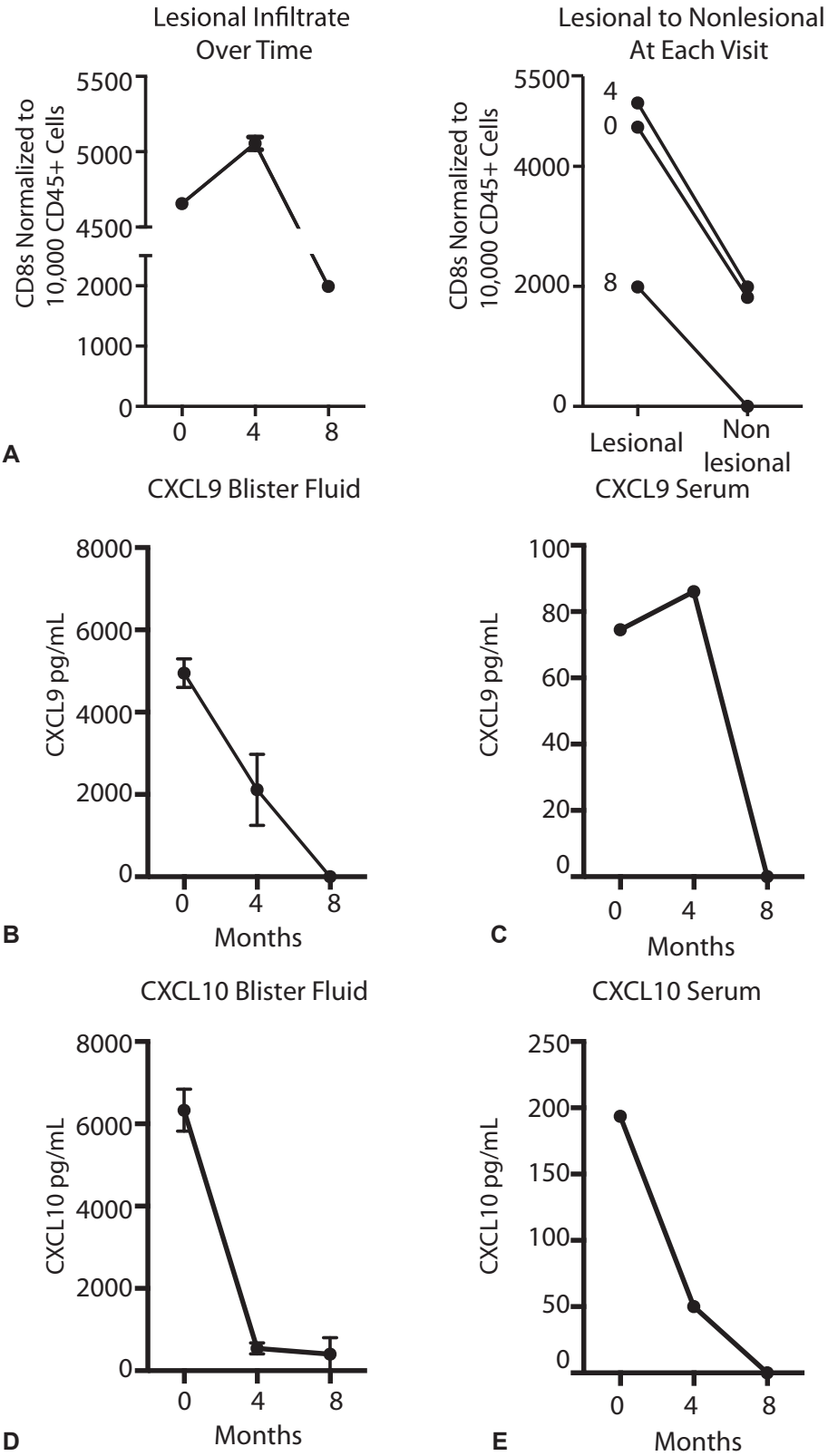

Fig 5. Concentrations of biomarkers CD8, CXCL9, and CXCL10 in a patient following conventional treatment. Subject began treatment at month 0, inconsistently followed treatment up to 4 months, and then consistently followed treatment from month 4 to month 8. **A**, T cells and chemokine protein reported at each visit (left panels). Representative slopes comparing lesional infiltrate to nonlesional infiltrate (right panels). **B** and **C**, CXCL9 concentration measured in the blister fluid (**B**) and serum (**C**) at each visit. **D** and **E**, CXCL10 concentration in the blister fluid (**D**) and serum (**E**) at each visit.

different kinetics during vitiligo progression.[23] CXCL9 is expressed earliest in murine vitiligo lesions and might be a more specific marker of disease activity.[23]

These markers might provide important information to support activities in clinical trials: 1) to help efficiently identify and enroll subjects with active IFN-γ–specific inflammation; 2) to reveal early treatment responses and drug efficacy; and 3) to improve the sensitivity of current, subjective outcome measures. Future studies will further characterize these biomarkers and their sensitivity to change following treatment in a larger patient cohort, where subgroup analysis by disease variants or by subject demographics could provide additional clues for categorizing vitiligo. Future studies could assess the effects of sun exposure on these parameters; our data was gathered primarily in the colder months in the Northeast, where sun exposure is less intense. Finally, this modified suction-blister technique provides an unparalleled opportunity to sample lesions in patients with other inflammatory skin diseases to study the natural history of these diseases and their treatment responses.

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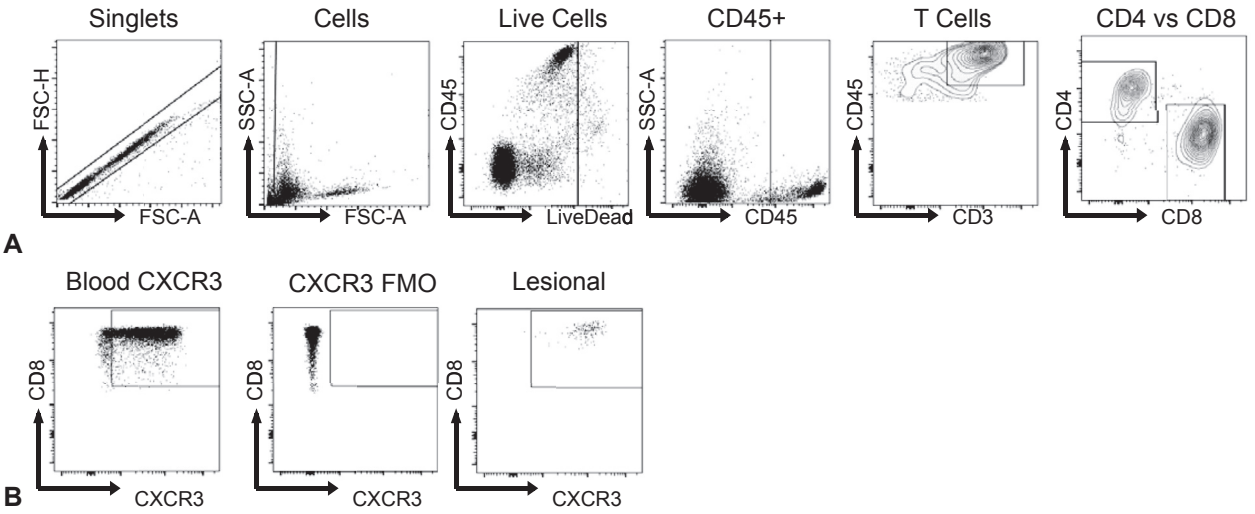
SUPPLEMENTARY METHODS

Enzyme-linked immunosorbent assay (ELISA)

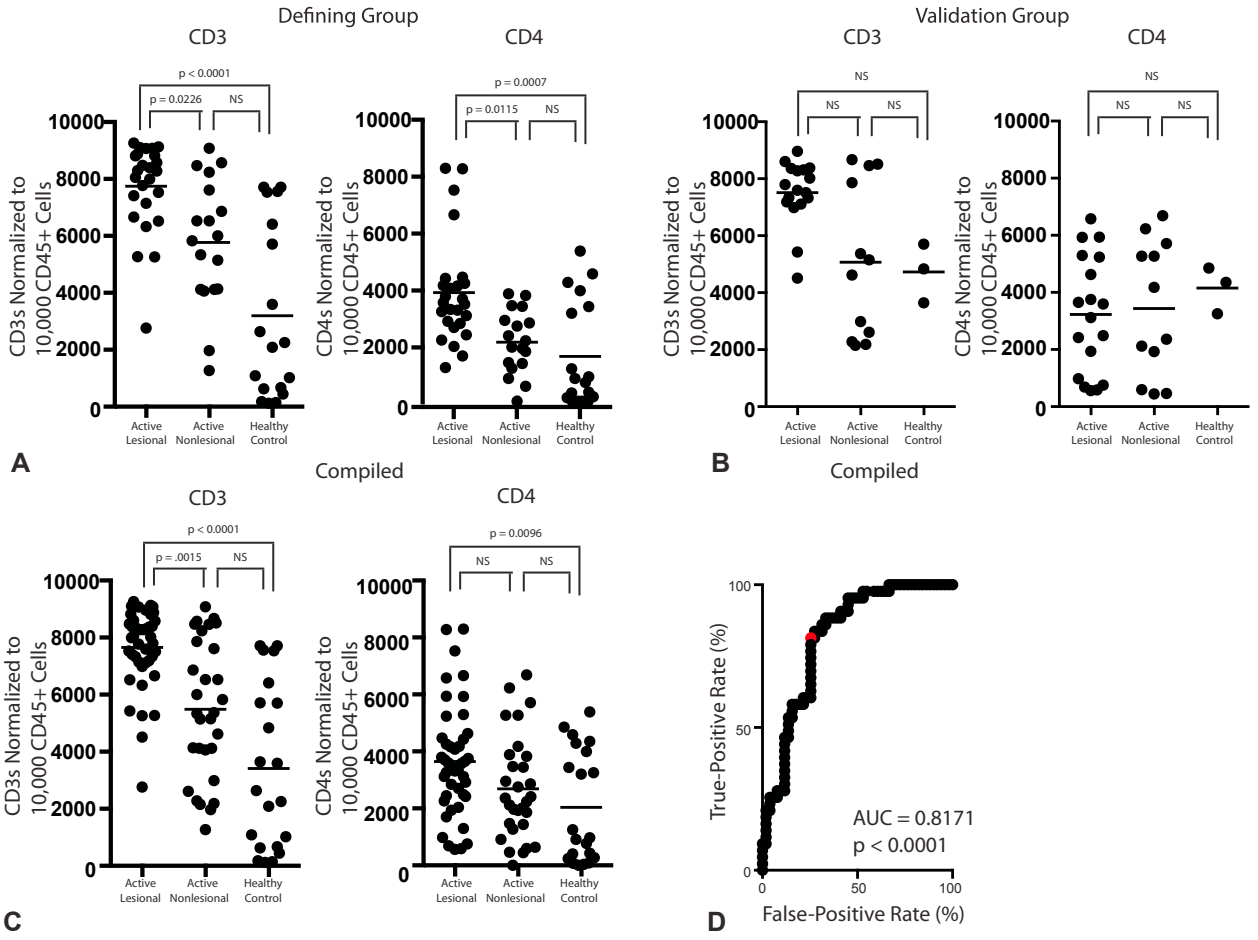
Human IFN-γ, CXCL9, CXCL10 and CXCL11 Duoset ELISA kits (R&D Systems, Minneapolis, MN) were used to measure protein concentrations in blister fluid according to the manufacturer's instructions. Optical densities were measured at 450 nm using a PerkinElmer EnVision 2102 multilabel reader (PerkinElmer, Waltham, MA) and used to calculate concentrations using a 4-parameter logarithmic standard curve with free ELISA analysis software. Based on preliminary observations, we excluded samples from subjects with elevated CXCL9 and CXCL10 in the serum due to the potential for retrograde diffusion of the chemokine into skin fluid, confounding analysis.

Flow cytometry

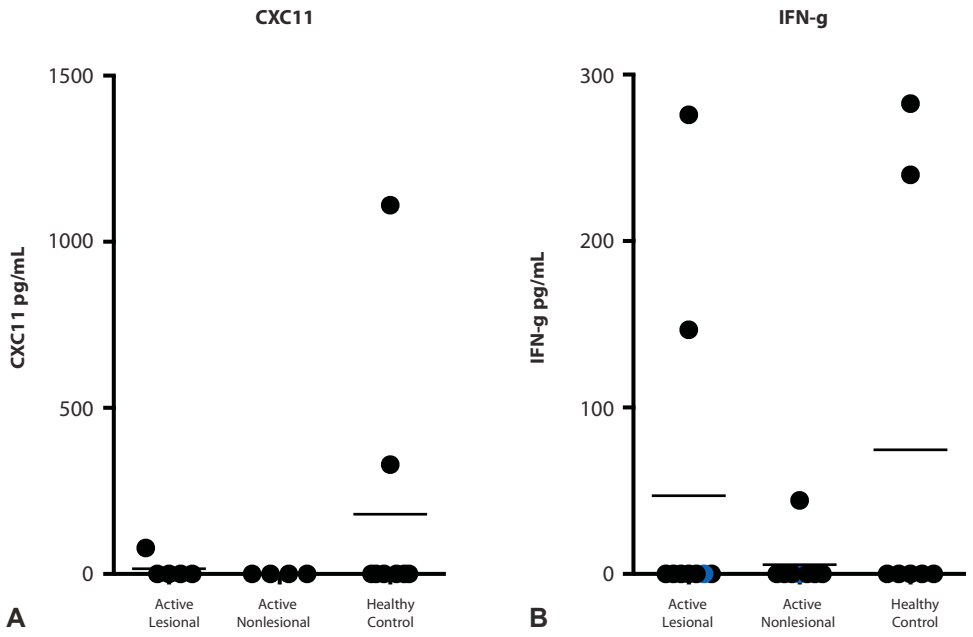
All samples were blocked with Human TruStain FcX (BioLegend, San Diego, CA) and stained with LiveDead Blue (Invitrogen [Carlsbad, CA] 1:1000). The following antibodies were used at a 1:20 dilution: CD45 APC, CD4 PerCP (Tonbo Biosciences, San Diego, CA), CXCR3 PE, and CD8 PE-Cy7 (BioLegend). CD3 Pacific Blue (BioLegend) was used at a 1:200 dilution. Peripheral blood was used for comparison and to assist in gating. Samples were stained and then fixed and lysed using RBC Fixation/Lysis Buffer (Biolegend) per the manufacturer's instructions. Data were collected with an LSR II and analyzed with FlowJo software (FLowJo, LLC, Ashland, Oregon).



Supplemental Fig 1. Gating strategy and flow controls. **A**, $CD8^+$ and $CD4^+$ cells were gated in the following manner: singlets, cell gate, live cells by dead stain exclusion, all CD45+ cells, and all $CD3^+$ T cells. **B**, Stained whole blood was used to make the fluorescence minus one (FMO) controls to draw the C-X-C motif chemokine receptor (CXCR) 3 gate.



Supplemental Fig 2. CD3 and CD4 quantification. **A**-**C**, Quantification of $CD3^+$ and $CD4^+$ cells normalized to $CD45^+$ cells in the skin of vitiligo subjects and healthy controls. Results are shown separately for the defining group (**A**), validation group (**B**), and both data sets combined (**C**). **D**, Receiver operating characteristic analysis of compiled CD3 counts from both groups. Red dot signifies the normalized CD3 T-cell threshold value 6927, which achieves a sensitivity of 81.4% and specificity of 75.5%.



Supplemental Fig 3. Skin C-X-C motif chemokine ligand (CXCL) 11 and interferon-γ (IFN-γ) protein concentration. Quantification of CXCL11 (**A**) and IFN-γ (**B**) protein concentration by enzyme-linked immunosorbent assay of blister fluid in vitiligo subjects and healthy controls.

Supplemental Table I. Subject demographic information

Subject	Group, disease	Age	Sex	Race/Ethnicity	Related FH	Other auto-immune disease	Duration of disease
1	Defining, active	45	F	White	Negative	None	13
2	Defining, active	52	M	Asian	Negative	None	37+
3	Defining, active	30	F	White	Negative	None	23
4	Defining, active	44	F	White/Hispanic	Negative	None	34
5	Defining, active	34	F	White	Negative	None	10+
6	Defining, active	30	F	Black/Hispanic	Negative	None	2
7	Defining, active	34	F	Black/Hispanic	Negative	None	4
8	Defining, active	52	M	White	Negative	None	34+
9	Validation, stable	39	M	White	Negative	None	15+
10	Validation, stable	47	F	White	Negative	None	30+
11	Validation, active	55	F	White	Negative	None	2
12	Validation, stable	31	F	White	Negative	None	25
13	Validation, active	62	F	Black	Negative	Hashimoto thyroiditis	1
14	Validation, active	49	F	White	Negative	None	19
15	Validation, active	54	F	Black	Negative	None	7
16	Validation, active	8	F	Asian	Negative	None	5
17	Validation, active	56	F	White	Negative	None	41
18	Validation, active	51	F	White	Vitiligo	None	37
19	Validation, stable	33	M	White	Negative	None	7
20	Validation, active	55	M	White	RA	None	5
21	Defining, healthy	61	F	White	Type 1 diabetes	None	N/A
22	Defining, healthy	25	F	Black	Negative	None	N/A
23	Defining, healthy	25	F	Black	Negative	None	N/A
24	Defining, healthy	18	F	White/Hispanic	Vitiligo	None	N/A
25	Defining, healthy	24	M	White	Negative	None	N/A
26	Defining, healthy	28	F	White	Negative	None	N/A
27	Defining, healthy	25	M	White	Negative	None	N/A
28	Defining, healthy	33	M	White	Negative	None	N/A
29	Validation, healthy	40	F	Asian	Vitiligo	None	N/A

Plus (+) symbol signifies that the subject had vitiligo for at least the number of years indicated.
FH, Family history; *N/A*, not applicable; *RA*, rheumatoid arthritis.