Protein microarrays identify disease-specific anti-cytokine autoantibody profiles in the landscape of immunodeficiency

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Background: Anti-cytokine autoantibodies (ACAAs) are pathogenic in a handful of rare immunodeficiencies. However, the prevalence and significance of other ACAAs across immunodeficiencies have not yet been described.
Objective: We profiled ACAAs in a diverse cohort of serum samples from patients with immunodeficiency and assessed the sensitivity and specificity of protein microarrays for ACAA identification and discovery.
Methods: Highly multiplexed protein microarrays were designed and fabricated. Blinded serum samples from a cohort of 58 immunodeficiency patients and healthy control subjects were used to probe microarrays. Unsupervised hierarchical clustering was used to identify clusters of reactivity, and after unblinding, significance analysis of microarrays was used to identify disease-specific autoantibodies. A bead-based assay was used to validate protein microarray results. Blocking activity of serum containing ACAAs was measured *in vitro*.
Results: Protein microarrays were highly sensitive and specific for the detection of ACAAs in patients with autoimmune polyendocrine syndrome type I and pulmonary alveolar proteinosis, detecting ACAA levels consistent with those reported in the published literature. Protein microarray results were validated by using an independent bead-based assay. To confirm the functional significance of these ACAAs, we tested and confirmed the blocking activity of select ACAAs *in vitro*.
Conclusion: Protein microarrays are a powerful tool for ACAA detection and discovery, and they hold promise as a diagnostic for the evaluation and monitoring of clinical immunodeficiency. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Anti-cytokine autoantibodies, immunodeficiency, autoimmune polyendocrine syndrome type I, pulmonary alveolar proteinosis, thymoma, protein microarray*

In patients with a handful of immunodeficiencies, recent work has shown that the presence of serum autoantibodies against endogenous cytokines accurately distinguishes discrete disease entities.[1,2] Important examples include autoimmune polyendocrine syndrome type I (APS-1) associated with antibodies against type I interferons and T_H17-related cytokines,[3-5] pulmonary alveolar proteinosis (PAP) caused by antibodies against GM-CSF,[6-9] and acquired susceptibility to mycobacterial infection associated with antibodies to IFN-γ.[10-12] These anti-cytokine autoantibodies (ACAAs) are thought to be an integral component of disease pathogenesis because of 3 strong lines of evidence: epidemiologic specificity, *in vitro* blocking activity, and biologic plausibility. ACAAs against other cytokines have also been described in the sera of healthy subjects.[13] These observations raise the possibility that ACAAs might be a ubiquitous component of immune homeostasis in health and disease.

Current tools to measure ACAAs include ELISA-based[4,14] and bead-based[5] assays. Additionally, new tools, such as a liquid-phase luciferase assay, allow for detection of 10s of ACAAs simultaneously from a single sample.[15,16] However, key questions in the field remain regarding the landscape of ACAAs in immune homeostasis and whether ACAAs contribute to the pathophysiology of other immunodeficiencies. New proteomic tools are needed to query for autoantibodies against hundreds of cytokines, chemokines, and growth factors in health and disease.[17,18] Previously, our laboratory has developed protein microarrays for the detection of ACAAs in patients with the autoimmune disease systemic lupus erythematosus and identified enrichment of ACAAs against B cell–activating factor in patients.[19] We also showed that arrays could detect ACAAs against IFN-α in patients with APS-1 and ACAAs against IFN-γ in patients with atypical mycobacterial infection.[19] Here, using a cohort of patients referred to the immunodeficiency service at Cambridge University Hospital, we describe the further development and validation of a highly multiplexed tool for the detection and discovery of ACAAs using microliter quantities of serum or plasma.

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Abbreviations used
ACAA: Anti-cytokine autoantibody
AIRE: Autoimmune regulator
APS-1: Autoimmune polyendocrine syndrome type I
MFI: Mean fluorescence intensity
MIP-1α: Macrophage inflammatory protein 1α
PAP: Pulmonary alveolar proteinosis
PBST: PBS plus Tween
SAM: Significance analysis of microarrays

METHODS

Protein microarrays

Protein microarrays were printed using a Bio-Rad ChipWriter Compact robotic microarrayer and ChipWriter Pro software (Bio-Rad Laboratories, Hercules, Calif), as described previously.[20] Briefly, 104 purified biomolecules were purchased from multiple vendors and printed in triplicate at dilutions of 200 μg/mL onto nitrocellulose-coated glass slides (Maine Manufacturing, Sanford, Me). A complete list of molecules and their vendors can be found in Fig E1 in this article's Online Repository at www.jacionline.org.

Arrays were first blocked in 5% milk (Bio-Rad Laboratories) in PBS plus 0.1% Tween (PBST; Sigma-Aldrich, St Louis, Mo) for 1 hour and then washed 3 times in PBST. Arrays were probed with serum diluted 1:150 in 10% FCS in PBST. Arrays were subjected to three 5-minute washes in PBST. Serum reactivity was detected by using an Alexa Fluor 647–conjugated goat anti-human IgG secondary antibody (Jackson Laboratory, Bar Harbor, Me) diluted to 0.375 μg/mL in PBST for 45 minutes. Arrays were washed 3 times in PBST and dried under negative pressure.

Arrays were scanned with an Axon microarray scanner and processed with GenePix 6 software (Molecular Devices, Sunnyvale, Calif). Local background (defined as the mean of pixels surrounding each unique circular feature of interest) was subtracted from mean fluorescence intensity (MFI). Means were calculated across replicates. Those values that were negative because of background subtraction were set to zero.

Statistics

Unsupervised hierarchical clustering was performed by using a Pearson correlation with average linkage clustering with the program Multiple Experiment Viewer.[21] Significance analysis of microarrays (SAM) was performed as previously described, with significantly different reactivities defined by a false discovery rate of less than 0.1% after 10,000 permutations of the data.[22] Pearson correlation coefficients, receiver operating characteristic curves, and linear regression were generated with Prism 6 software (GraphPad Software, San Diego, Calif). For blocking experiments, unpaired *t* tests of stimulation indices were calculated by using Prism 6 software (GraphPad Software).

Multiplex particle-based flow cytometry

Recombinant human cytokines (IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-17A, IL-17F, IL-18, IL-22, IL-23, IL-26 monomer, IL-26 dimer, IFN-α, IFN-β, IFN-γ, TNF-α, and GM-CSF; R&D Systems, Minneapolis, Minn) were covalently coupled to carboxylated beads (Bio-Plex; Bio-Rad Laboratories). Beads were first activated with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (Thermo Fisher Scientific, Waltham, Mass) in the presence of N-hydroxysuccinimide (Thermo Fisher Scientific), according to the manufacturer's instructions, to form amine-reactive intermediates. The activated beads were incubated with the corresponding cytokines at a concentration of 20 μg/mL in the reaction mixture for 3 hours at 37°C on a rotator. Beads were washed and stored in blocking buffer (PBS, 1% BSA, and 0.05% NaN_3). Cytokine-coupled beads were incubated with plasma or serum from patients for 1 hour in 96-well filter plates (Multi Screen HTS; Millipore, Temecula, Calif) at 37°C in the dark on a horizontal shaker. Fluids were aspirated with a vacuum manifold, and beads were washed 3 times with PBS/0.05% Tween 20. Beads were incubated for 30 minutes with a phycoerythrin-labeled anti-human IgG-Fc antibody (Leinco/Biotrend, St Louis, Mo), washed as described, and resuspended in 100 μL of PBS/Tween. Samples were then analyzed on a Bio-Plex platform by using Bio-Plex Manager 6.1 software (Bio-Rad Laboratories). Successful coupling of the cytokines to their respective bead sets was verified with specific mAbs.

IFN-α neutralization assay

The ability of IFN-α to upregulate LPS-induced production of TNF-α by PBMCs was used to test for the neutralizing activity of sera containing high-titer blocking antibodies to IFN-α.

Anti–IFN-α–positive sera, negative control sera, or FBS were preincubated for 30 minutes in a 96-well plate (Corning, Corning, NY) at a dilution of 1:5 in RPMI 1640. IFN-α (2000 IU per well, INTRON A; Merck, Kenilworth, NJ) or LPS (1 μg/mL; LIST Biologicals, Campbell, Calif) alone or both stimuli were added. Human PBMCs (separated from blood of a healthy control subject by using Ficoll-Hypaque gradient centrifugation) were then added at a concentration of 1×10^5 cells per well and incubated for 24 hours (37°C in a 5% CO_2 atmosphere).

Supernatants were obtained and cytokines were measured on a Luminex analyzer (TNF-α, R&D Systems Fluorokine MAP and Bio-Plex, Bio-Rad Laboratories), according to the manufacturer's recommendations.

GM-CSF neutralization assay

GM-CSF–mediated upregulation of LPS-induced production of IL-6 by U937 cells was used to test for the neutralizing activity of sera containing high-titer blocking antibodies to GM-CSF.

Anti–GM-CSF–positive sera, negative control sera, or FBS were preincubated for 30 minutes in a 96-well plate without stimulus or in the presence of GM-CSF (100 ng/mL; ImmunoTools, Friesoythe, Germany), IFN-γ (2×10^4 IU/mL; Immukin; Boehringer Ingelheim, Ingelheim am Rhein, Germany), or LPS (1 μg/mL, BioLabs) alone or in combination as indicated at a dilution of 1:5 in complete Dulbecco modified Eagle medium (Gibco, Carlsbad, Calif). U937 cells (ATCC CRL1593.2) were added at 1×10^5 cells per well and incubated for 24 hours (37°C in a 5% CO_2 atmosphere).

Supernatants were obtained, and IL-6 levels were measured with a Luminex analyzer (R&D Systems Fluorokinemap and Bio-Plex, Bio-Rad Laboratories), according to the manufacturer's recommendations.

IL-12 neutralization assay

The ability of IL-12 to synergistically upregulate the production of IFN-γ from PBMCs on costimulation with IL-18 was used to measure the neutralizing activity of sera containing high-titer blocking antibodies to IL-12.

Anti–IL-12–positive sera, negative control sera, or FBS were preincubated for 30 minutes in a 96-well plate without stimulus or in the presence of IL-12 (20 ng/mL, R&D Systems) or IL-18 (25 ng/mL, R&D Systems) or in combination, as indicated, at a dilution of 1:5 in RPMI 1640 in 96-well F plates (Corning). Human PBMCs (separated from blood of a healthy control subject by means of Ficoll-Hypaque gradient centrifugation) were then added at a concentration of 1×10^5 cells per well and incubated for 24 hours (37°C in a 5% CO_2 atmosphere).

Supernatants were obtained, and IFN-γ concentrations were measured with a standard ELISA (IFN-γ; Pelikine; Sanquin, Amsterdam, The Netherlands), according to the manufacturer's recommendations.

Accession numbers

Protein microarray data were deposited into the Gene Expression Omnibus and are freely available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62599.

Human samples and study approval

Serum samples were collected from patients with immunodeficiency and control subjects after obtaining verbal consent and anonymized for subsequent

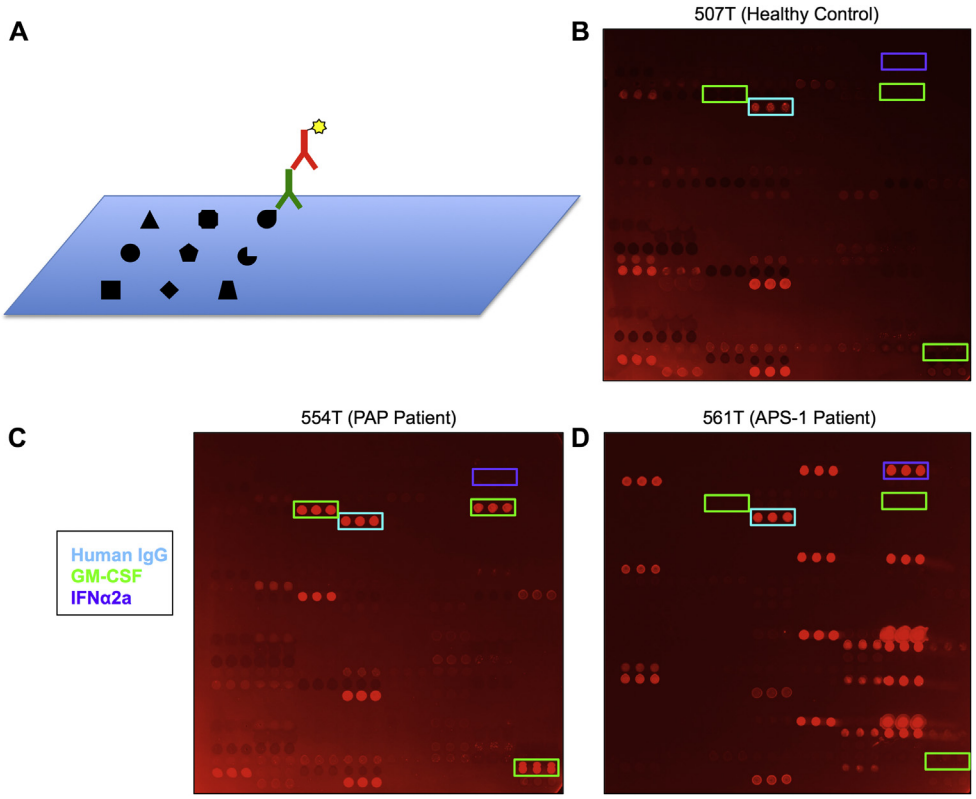

FIG 1. Protein microarrays for ACAA detection. **A,** Schematic representation of cytokine microarrays. Cytokines, chemokines, growth factors, and traditional autoantigens are printed onto a nitrocellulose-coated microscope slide. Arrays are probed with serum *(green)* and detected by using fluorophore-conjugated secondary antibody *(red)*. **B-D,** Representative images of protein microarrays probed with serum from a healthy control subject (Fig 1, *B*), a patient with PAP (Fig 1, *C*), and a patient with APS-1 (Fig 1, *D*). A control IgG feature printed on the array is boxed in *cyan*, GM-CSF features are boxed in *green*, and IFN-α2a features are boxed in *purple*.

investigation at the Immunodeficiency Service at Addenbrooke's Hospital, Cambridge University Hospitals, Cambridge, United Kingdom.

RESULTS

Protein microarrays identify distinct clusters of anti-cytokine autoantibody reactivity in a blinded cohort of patients with immunodeficiency disorders

Protein microarrays were fabricated by using a robotic microarray printer and printed on nitrocellulose-coated glass microscope slides. One hundred four purified biomolecules, including known autoantigens, as well as cytokines, chemokines, growth factors, and receptors, were printed on the array, forming discrete spot features (Fig 1, *A*, and see Fig E1, *A*). All targets were printed in triplicate.

To determine whether protein microarrays could accurately detect known or novel autoantibodies in serum, we probed arrays with serum samples from an immunodeficiency cohort collected at Cambridge University Hospital. This cohort consists of patients with immunodeficiency disorders of diverse cause referred from throughout Europe and includes patients with the diseases PAP and APS-1 and susceptibility to mycobacterial infection, each of which are conditions with described ACAAs (Table I and see Fig E2 in this article's Online Repository at www.jacionline.org). Sera were anonymized and provided with barcoded identification, and protein microarrays were then probed and analyzed. Only after data analysis was complete was unblinding to clinical presentation performed.

TABLE I. Clinical characteristics of the cohort

Disease	No. of patients (n = 58)
APS-1	5
APS-1 phenotype without known *AIRE* mutation	2
Atypical mycobacterial infection	6
Candidiasis	6
Thymoma	3
PAP	11
Suspected PAP	4
Other (see Fig E2 for a complete listing of other immunodeficiencies)	15
Healthy control	6

Protein microarrays showed a diversity of reactivity to various autoantigens, cytokines, and chemokines across samples (Figs 1, *B-D*, and 2). After unsupervised hierarchical clustering, distinct patterns of reactivity were clearly observed in at least 2 groups by means of clustering linkage. The first group demonstrated a marked reactivity to GM-CSF purchased from multiple vendors (Fig 2, green box), which led us to hypothesize that these patients had PAP.

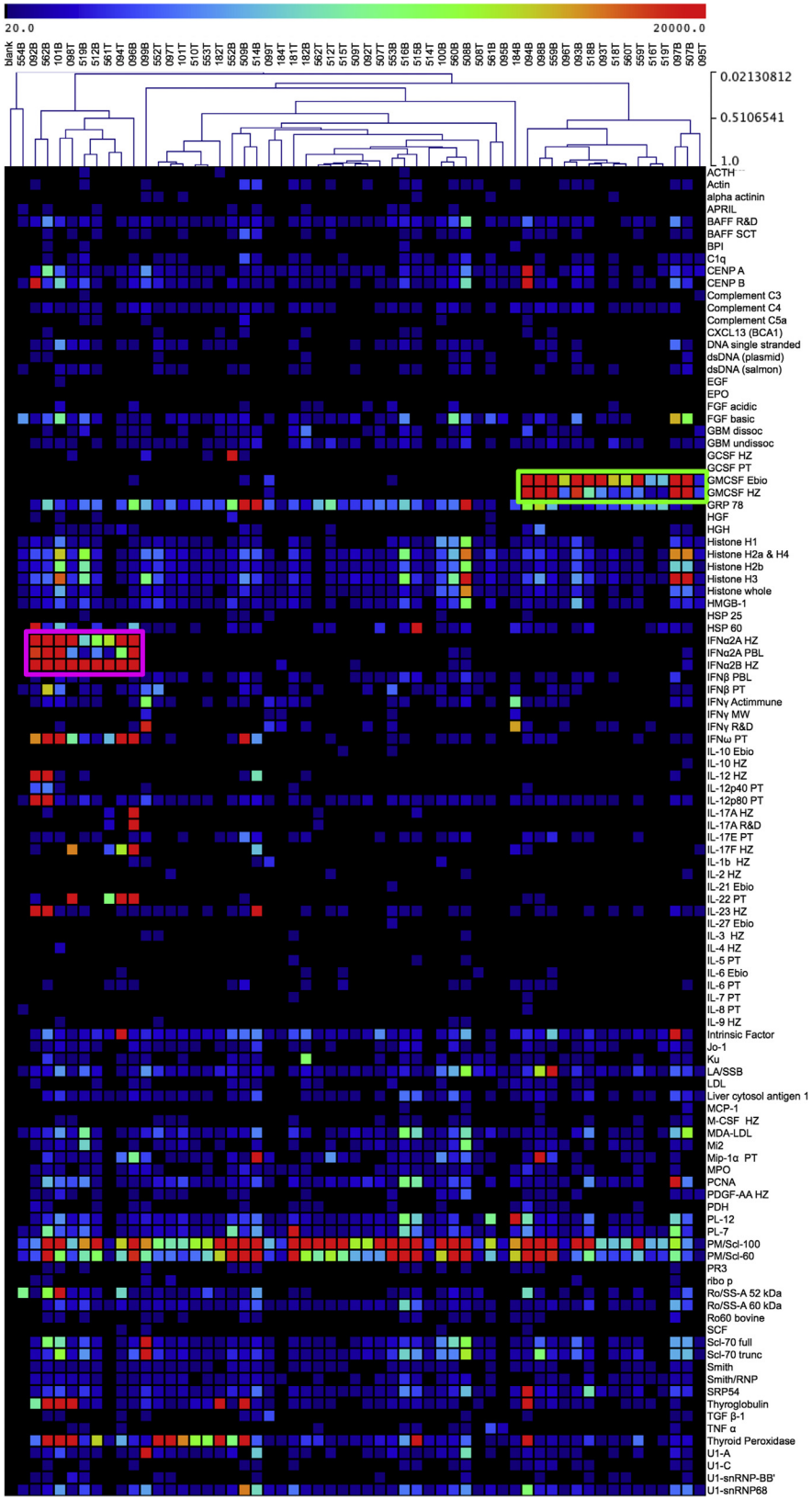

FIG 2. Unsupervised hierarchical clustering demonstrates 2 distinct groups of cytokine reactivity. Fifty-eight serum samples from patients with immunodeficiency and healthy control subjects were probed on protein microarrays. Samples were hierarchically clustered in an unsupervised fashion by using a Pearson correlation and plotted with samples along the *x-axis*, and autoantigens, cytokines, and chemokines were plotted along the *y-axis*. Clusters of high reactivity to GM-CSF *(green box)* and type I interferons *(purple box)* were identified.

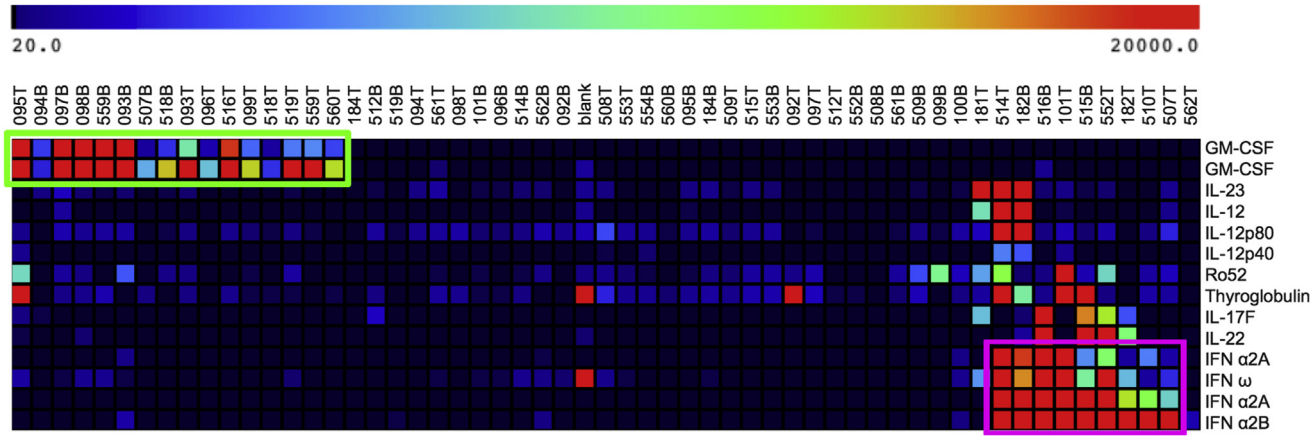

FIG 3. SAM identifies reactivities associated with ACAAs against type I interferons and GM-CSF. Three-class SAM was applied based on cytokine reactivity to type I interferons, GM-CSF, or neither. SAM identified 14 statistically significant differences. These targets include cytokines purchased from multiple vendors. GM-CSF reactivity and type I interferon–associated reactivity are boxed in *green* and *purple*, respectively, for emphasis.

The second group identified by means of unsupervised hierarchical clustering demonstrated marked reactivity to type I interferons, such as IFN-α2A, IFN-α2B, and IFN-ω, which were also purchased from multiple commercial vendors (Fig 2, purple box). This led us to hypothesize that these samples were derived from patients with APS-1 or thymoma. Intriguingly, we observed 2 distinct subgroups within this reactivity group. The first subgroup demonstrated reactivity to the T_H1-associated cytokines IL-12 and IL-23, whereas the second subgroup demonstrated reactivity to the T_H17-associated cytokines IL-17A, IL-17F, and IL-22.

In addition, we observed reactivity to a number of traditional autoantigens. Autoantigen reactivity was broadly distributed throughout the cohort, although we observed nonstatistically significant clustering of reactivities to features, such as histone components; nuclear autoantigens, such as the U1 small nuclear ribonucleoprotein complex (U1-snRNP); and endocrine autoantigens, such as thyroglobulin, thyroid peroxidase, and intrinsic factor. Reactivity to endocrine autoantigens was enriched in samples with reactivity to type I interferons. The finding of endocrine-associated autoantibodies supported the designation of these samples as derived from patients with APS-1.

Protein microarray ACAA profiling accurately assigns clinical disease

Once data analysis was complete, we were unblinded to the clinical history of individual subjects within the cohort. Of 16 patients with PAP, the arrays correctly identified all 16 as those samples with the highest reactivity against GM-CSF (Fig 2, boxed in green). Protein microarrays also identified 9 samples with high type I interferon reactivity (boxed in purple). We observed that all 5 patients with APS-1 in the cohort (101B, 096B, 094T, 561T, and 098T) fell within this assignment. The other 4 patients with high type I interferon reactivity all had chronic candidiasis, a key clinical feature of APS-1, which in patients with APS-1 has been proposed to be caused by neutralizing ACAAs to the T_H17 cytokines IL-17A, IL-17F, and IL-22.[3,5] Two of these 4 patients (092B and 512B) had coincident thymoma, for which antibodies against type I interferons and T_H1 and T_H17 cytokines have been described.[16,23] The other 2 patients were clinically suspected of having APS-1; however, a mutation in autoimmune regulator *(AIRE)* was not identified by means of gene sequencing, and imaging results for thymoma were negative. Given the specificity of type I interferon ACAAs for APS-1 and the clinical similarity of these patients with those with APS-1, we speculate that these patients might harbor an undescribed mutation in *AIRE* or a mutation in a gene that might phenocopy APS-1.

To identify additional autoantibodies that might be associated with either APS-1 or PAP, we performed 3-class SAM, a permutation-based algorithm designed for analysis of large data sets.[22] We assigned groupings based on the presence of ACAAs against IFN-α, GM-CSF, or neither. With a false discovery rate of less than 0.1%, SAM identified the ACAAs discussed previously and additionally identified autoantibodies against the autoantigens Ro52 and thyroglobulin as enriched in patients with APS-1 (Fig 3). Reactivity to thyroglobulin, a thyroid autoantigen, is consistent with the systemic endocrine autoimmunity described in patients with APS-1. The presence of Ro52 autoantibodies, which are found prototypically in patients with systemic lupus erythematosus and Sjögren syndrome, suggests a possible shared autoimmune pathophysiology with APS-1 and identifies Ro52 as a candidate biomarker worthy of further study in patients with APS-1.[24,25]

Validation of protein microarray profiling results on a second platform

To validate our results, we compared protein microarray reactivities with results from a bead-based assay performed independently in the laboratory of one of the authors (R.D.). We compared ACAAs against GM-CSF and IFN-α between the 2 platforms. Pearson correlation coefficients were calculated (r = 0.888 and r = 0.9778, respectively) (Fig 4). Both correlations had P values of less than .005.

To more precisely determine whether protein microarrays could sensitively and specifically identify samples with ACAAs, we built receiver operating characteristic curves for each of the

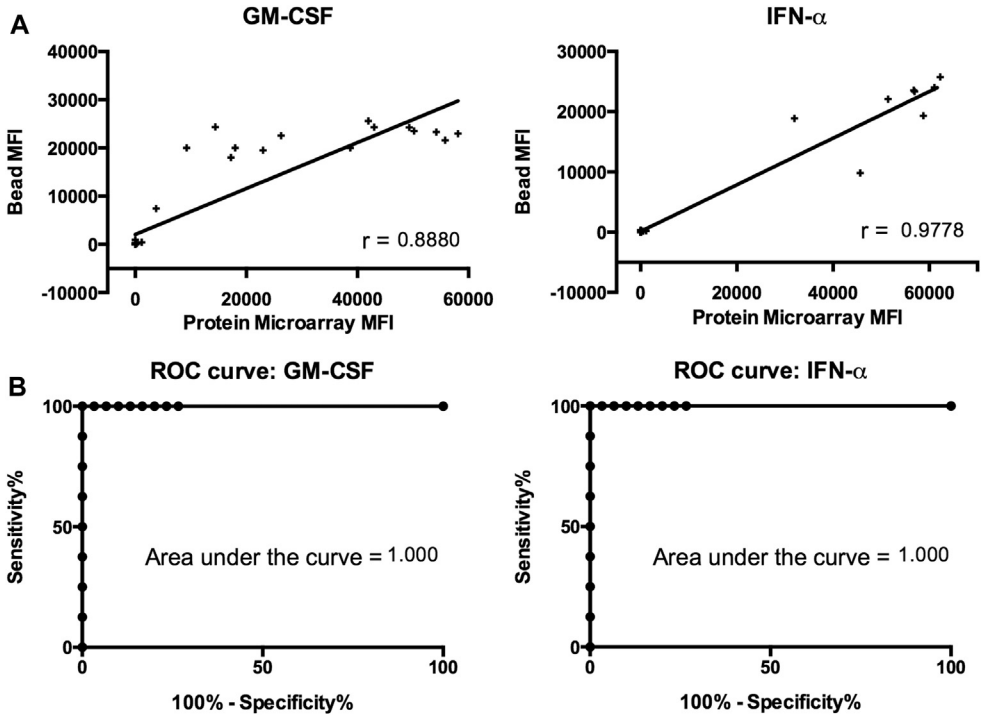

FIG 4. Comparison of protein microarray performance with an established bead-based assay. **A,** Corresponding MFI values from protein microarrays and a bead-based assay were plotted for the targets GM-CSF and IFN-α, and Pearson correlation coefficients were determined. All *P* values were less than .005. **B,** Receiver operating characteristic *(ROC)* curves were generated to assess the performance of protein microarrays, with a bead-based assay used as the gold standard. *P* values were less than .05 for all areas under the curve.

ACAAs listed above using the bead-based assay as the gold standard. Receiver operating characteristic curves had areas under the curve of 1.000 for both GM-CSF and IFN-α.[19] This high level of concordance between protein microarrays and an independently performed bead-based assay demonstrate that protein microarrays can detect ACAAs with a sensitivity and specificity comparable with those of current gold standard assays.

Sera containing select ACAAs block cytokine signaling *in vitro*

To test the functional activity of identified ACAAs, we performed *in vitro* blocking assays using sera from patients with ACAAs and healthy control subjects. We tested the ability of sera with ACAAs against GM-CSF, IFN-α, and IL-12 to block downstream cytokine production from heterologous PBMCs or U937 cells, as described below.

To test GM-CSF blocking activity, we incubated U937 cells with sera from either patients with PAP or healthy control subjects. Cultures were stimulated with LPS alone or LPS and GM-CSF. IL-6 release was measured from the supernatants by using ELISA as a readout for signaling activity. After stimulation with LPS alone, low IL-6 levels were detected in the supernatants of all samples. However, when LPS and GM-CSF were added together, we observed a synergistic upregulation of IL-6 release in healthy control but not PAP cultures (Fig 5, *A*). To quantify this effect, we plotted the ratio of IL-6 released after stimulation with LPS and GM-CSF and divided by IL-6 released after LPS stimulation alone. The difference between healthy control and PAP indices was statistically significant ($P = .0015$). These results demonstrate that sera containing ACAAs against GM-CSF but not sera from healthy control subjects have the ability to block GM-CSF signaling *in vitro*.

To test the blocking activity of IFN-α ACAA-containing sera, we measured supernatant levels of TNF-α released from heterologous PBMCs. It is known that LPS induces production of TNF-α from PBMCs.[26] We found that stimulation by the combination of LPS and IFN-α resulted in a synergistic increase in TNF-α production above that induced by LPS alone. In the presence of sera containing IFN-α ACAAs but not the presence of sera from healthy control subjects, this synergistic increase in TNF-α levels was blocked (Fig 5, *B*). To quantify this effect, we plotted the ratio of TNF-α released after stimulation with LPS and IFN-α and divided by TNF-α released after LPS stimulation alone ($P = .0029$). These data show that sera containing IFN-α ACAAs but not those from healthy control subjects have the ability to block IFN-α signaling *in vitro*.

Finally, we tested the ability of IL-12 ACAA–containing sera to block the activity of IL-12. To do so, we used the ability of IL-12 to synergistically upregulate the production of IFN-γ from heterologous PBMCs when costimulated with IL-18.[27] This effect was blocked by the single IL-12 ACAA–containing serum sample available for testing (Fig 5, *E*). Given the small sample size, statistical analysis was not performed. To test whether blocking activity was restricted to the serum or whether an additional cell-intrinsic defect was present, we performed a mixing experiment. Washed PBMCs from an IL-12 ACAA–positive patient regained their ability to upregulate IFN-γ secretion in the presence of serum from a healthy control subject, and washed PBMCs from a healthy control subject lost their ability to upregulate IFN-γ in

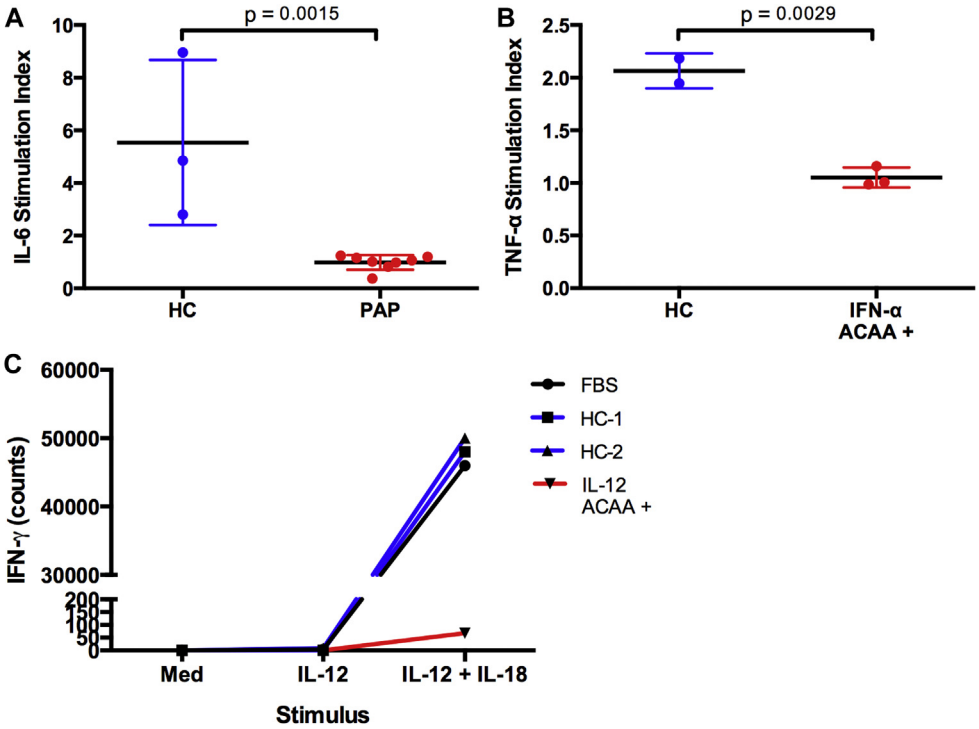

FIG 5. Sera from patients with ACAAs block cytokine signaling *in vitro*. **A,** U937 cells were incubated in the presence of sera from healthy control subjects or from patients with PAP and stimulated with LPS alone or LPS and GM-CSF. IL-6 concentrations were measured by the using Luminex analyzer. An IL-6 stimulation index was calculated as described in the text. **B,** Heterologous PBMCs were incubated in the presence of LPS alone or LPS and IFN-α. TNF-α concentration was measured by using a Luminex analyzer. A TNF-α stimulation index was calculated as described in the text. **C,** Heterologous PBMCs were incubated in the presence of medium, IL-12, or IL-12 and IL-18. IFN-γ levels in the supernatant were measured by ELISA and plotted. Given the limited sample size, statistical analysis of a stimulation index was not performed.

the presence of patient serum (data not shown). These experiments demonstrate that in the single patient available for testing, IL-12 ACAA–containing serum can block IL-12 signaling *in vitro* and that this IL-12 signaling defect is intrinsic to the serum component in the conditions tested.

Protein microarrays detect novel ACAAs

Protein microarrays also identified individual samples with interesting or novel ACAA reactivities by using a threshold of reactivity of 2 SDs greater than the mean of all features. For example, sample 552B, a patient with multiple sclerosis and borderline neutropenia receiving IFN-β treatment, demonstrated high reactivity to granulocyte colony-stimulating factor, a critical cytokine in granulopoiesis.[28] ACAAs to granulocyte colony-stimulating factor have been described in a cohort of patients with Felty syndrome, which is characterized by the triad of rheumatoid arthritis, neutropenia, and splenomegaly,[29] but have not been described in patients with multiple sclerosis. Two other patients with PAP demonstrated high reactivity to basic fibroblast growth factor, an important growth factor involved in angiogenesis, wound healing, and tissue repair.[30] To our knowledge, basic fibroblast growth factor autoantibodies have not been previously described, although we identified sporadic reactivity against this cytokine in cohorts of previously screened patients with lupus.[19]

Additionally, we identified ACAA reactivities not previously described in the literature. Serum samples 092B and 562B demonstrated prominent reactivity to IL-23. Sample 092B was derived from a patient with thymoma and chronic candidiasis, and sample 562B was derived from a patient with a congenital immunodeficiency of unknown cause with normal *AIRE* sequencing and chest imaging results. To confirm these results, we tested 39 samples for IL-23 ACAAs using our bead-based assay. Samples 092B and 562B were the only 2 samples with MFIs 2 SDs greater than the mean. Comparing the bead-based MFIs of these 2 samples against all others yielded a *P* value of less than .0001, confirming our array-based results (see Fig E3 in this article's Online Repository at www.jacionline.org).

IL-23 is composed of p19 and p40 subunits, the latter of which is shared with IL-12. Indeed, on both our protein microarrays and bead-based assay, serum samples 092B and 562B were reactive against IL-12 in addition to IL-23. Given the known association of IL-12 ACAAs in patients with thymoma and a recent case report in a patient with *Burkholderia* species infection, we hypothesized that these IL-23 ACAAs bind the shared p40 subunit.[16,23,31] Using both protein microarrays and our bead-based assay, we successfully detected ACAAs in samples 562B and 092B against the shared IL-12p40 subunit (Fig 2). These results suggest that IL-12 ACAAs might cross-react against the related cytokine IL-23 through recognition of the shared p40 subunit.

Novel reactivity was also observed against macrophage inflammatory protein 1α (MIP-1α). Serum samples 514B and 098B both demonstrated reactivity to MIP-1α, which was defined as greater than 2 SDs above the mean of all feature reactivities. After

unblinding, we found that sample 514B was derived from a healthy control subject; however, this sample also demonstrated autoantibodies against the autoantigens PM/Scl-60, PM/Scl-100, and GRP-78, suggesting possible undiagnosed or not yet clinically apparent autoimmunity. This patient also harbored moderate ACAAs against IL-23 and IL-12, although at lower levels than the patients discussed above. The other MIP-1α–reactive sample (098B) was obtained from a patient with PAP and contained autoantibodies against GM-CSF and the autoantigens PM/Scl-60 and PM/Scl-100. These data demonstrate that protein microarrays can detect novel ACAAs, although further studies will be required to determine their clinical significance.

DISCUSSION

The burgeoning study of ACAAs has already provided key contributions to our understanding of human immunity and immunodeficiency. Studies of diseases, such as PAP, APS-1, and acquired susceptibility to nontuberculous mycobacterial infection, have demonstrated the fundamental roles in immunity of their ACAA targets: GM-CSF; IL-17A, IL17-F, and IL-22; and IFN-γ, respectively. These insights arose from careful clinical observation of "experiments of nature" combined with a rich knowledge of cytokine biology.[32]

A complementary strategy for the discovery of novel ACAAs is an unbiased multiplexed approach. Using protein microarray technology, we have developed a tool that allows for the screening of an order of magnitude more ACAAs than current technologies permit, probing for hundreds of ACAAs simultaneously by using only a few microliters of serum. Protein microarrays are a powerful tool for the discovery of novel ACAAs in patients with immunodeficiency and autoimmunity and can be applied to any disease context in which serum is available.[17] Additionally, ACAAs have been described in healthy subjects, and protein microarrays provide the opportunity to study how ACAAs can regulate immune homeostasis by using a proteomic approach.

Here we have shown that protein microarrays can accurately detect ACAAs in patients with PAP and APS-1 from a cohort of patients with complex immunodeficiencies. We included cytokine antigens on the array from multiple vendors to ensure the accuracy of our platform. Although MFI values from different vendors can vary to some degree because of discrepancies, such as differences in expression systems, there remains a high degree of correlation between different vendors with accurate classification of disease throughout. We then validated our platform by correlating our results to an established bead-based assay.

Because of their high-throughput nature and ease of use, protein microarrays also hold potential as a diagnostic tool in the clinic, allowing screening for multiple ACCAs simultaneously with a single platform. Given the high coincidence of autoimmunity and immunodeficiency demonstrated in this cohort, protein microarray diagnostics could simultaneously measure ACAA and traditional autoantibody levels, alerting clinicians to organ-specific autoimmunity and monitoring ACAA-disease progression and response.[33,34]

The results described here provide the broadest view to date of the landscape of ACAAs in patients with immunodeficiency, and they identify targets worthy of further investigation. Our cohort consists of a diverse group of patients with immunodeficiency. One of the most striking observations is the robust nature of the reactivity signal in patients with APS-1 and PAP, which is in agreement with previously published work.[4,6] In almost all samples from patients with APS-1 and PAP, the MFI of type I interferon and GM-CSF ACAAs, respectively, approached the upper limits of detection on our instrument. Although MFI is not designed here as a precise quantitative measurement, the MFIs of these reactivities were also markedly higher than those of other well-studied clinical autoantibodies, such as the anti-thyroglobulin and anti-thyroid peroxidase autoantibodies found in patients with autoimmune thyroiditis.

A second observation is that unlike APS-1 and PAP, we do not find groups of high-titer ACAAs to be of a uniform pattern across all immunodeficiency states. Although this cohort lacks statistical power to rule out ACAAs as enriched in many of the individual immunodeficiencies tested, the extensive list of cytokines tested here across many different immunodeficiencies suggests that the ACAAs tested are not a general phenomenon of immunodeficiency. Although our arrays screened for ACAAs against 50 cytokines, chemokines, and other secreted proteins, it is possible that some ACAA targets were not included in our arrays. Furthermore, it is also possible that with increased sample size of individual immunodeficiencies, other unique ACAA patterns might emerge. We are currently developing expanded protein microarrays to test both possibilities.

A third conclusion from our results is the description of previously undescribed high-titer ACAAs. We found ACAAs against IL-23 in 2 patients with type I interferon reactivity and have shown that sera from these patients also react against the shared p40 subunit of both IL-12 and IL-23. We have not excluded the possibility that these ACAAs additionally bind IL-23p19, although a previous study of patients with thymoma assayed for but did not identify IL-23p19 ACAAs.[16] We also observed high levels of traditional autoantibodies in IL-12/IL-23–reactive samples. Our observations hold potential clinical relevance because an mAb directed against IL-12p40 is approved for the treatment of psoriasis and has demonstrated efficacy in the treatment of Crohn disease.[35,36]

We also identified previously undescribed ACAAs against MIP-1α in the sera of 2 subjects, although a clinical presentation could not be correlated to these ACAAs. The finding of ACAAs against MIP-1α in serum from a healthy control subject parallels the finding of sporadic classical autoantibodies in healthy subjects.[37] Whether such ACAAs have physiologic or diagnostic importance cannot be determined from our experiments.

Lastly, we tested the functional activity of ACAAs by assaying the capacity of serum from patients to block cytokine signaling. We designed our assays for IFN-α, GM-CSF, and IL-12 blocking activity to measure the ability of serum to block downstream cytokine secretion from heterologous cells. Previous studies have used downstream signal transducer and activator of transcription phosphorylation as a readout for blocking activity.[5,16,38] The consistency of our results with published findings that used other assays is reassuring.

Pressing questions still remain about the cause and function of ACAAs.[3] First, what gives rise to the autoantibody patterns found in patients with diseases such as APS-1 and PAP? Possible causes of ACAAs include autoimmunization events or specific defects in tolerance. Second, what is the function of ACAAs? In patients with PAP, passive transfer experiments in nonhuman primates, as well as the clinical phenotype both of human subjects and mice with defects in the GM-CSF receptor, offer convincing evidence in support of the pathophysiology of GM-CSF

ACAAs.[7,39-42] Additionally, in the case of PAP and susceptibility to atypical mycobacterial infection associated with ACAAs against IFN-γ, pilot studies have shown efficacy for B-cell depletion therapy with rituximab.[43-45] Such studies provide supporting evidence toward a pathophysiologic role for these ACAAs; however, a functional role for many high-titer ACAAs in health and disease is still lacking.

In summary, we have developed and validated a tool for the discovery and study of ACAAs in patients with immunodeficiency. We have demonstrated a high level of accuracy of the platform and used it to profile a cohort of patients with diverse immunodeficiency. In doing so, we have generated a broad view of the landscape of ACAAs and traditional autoantibodies in immunodeficiency to serve as a resource for future investigation and have identified novel ACAAs worthy of further study. Future efforts focused on screening cohorts of specific diseases for ACAAs with this technology are needed to better understand the complicated role of ACAAs in both health and disease.

Key messages

- **We have profiled anti-cytokine, anti-chemokine, and traditional autoantibodies in patients with diverse immunodeficiencies at the deepest level to date.**
- **Protein microarrays accurately detect ACAAs and will be an important tool for the discovery of novel ACAAs.**
- **Sera from patients with ACAAs against IFN-α and GM-CSF block signaling of their respective cytokines *in vitro*.**

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Autoantigen	Vendor	Catalog #	Notes
Actin	Sigma	A2522	From rabbit muscle
alpha actinin	Sigma	A9776	Chicken gizzard
BPI	The Binding Site/Arotec	ATB01-02	Bactericidal/permeability increasing protein,from human neutrophils
C1q	Biodesign	A90150H	Human serum
CENPA	Diarect	16901	Recombinant human
CENP B	Diarect	12501	Recombinant human
dsDNA (plasmid)	Diarect	12301	Double-stranded DNA 3.4 kb plasmid, from E. coli
dsDNA (salmon)	Sigma	D1626	Double-stranded DNA, from salmon
GBM-dissoc	Diarect	16801	Glomerular basement membrane, dissociated, human alpha-3 chain of collagen IV, recombinant human
ssDNA	Sigma	D7656	Single-stranded DNA (587-831 nt), from salmon
GBM-undissoc	Diarect	15901	Glomerular basement membrane, undissociated, human alpha-3 chain of collagen IV, recombinant human
Grp78	Assay Designs/Stressge	SPP-765F	Immunoglobulin heavy chain binding protein (BiP), recombinant hamster
HIS 1000 (whole hist	Immunovision	His-1000	Whole histones, from chicken red blood cells
HIS 1001	Immunovision	His-1001	Histone H1, from bovine tissue
HIS 1002	Immunovision	His-1002	Histone H1, from bovine thymus
HIS 1003	Immunovision	His-1003	Histone H2b, from bovine tissue
HIS 1004	Immunovision	His-1004	Histone H3 and H4, from bovine tissue
Hsp60	Assay Designs/Stressge	NSP-540	Heat shock protein 60kDa, recombinant human
Hsp25	Assay Designs/Stressge	NSP-510F	Heat shock protein 25kDa, recombinant murine
IF	Diarect	16701	Intrinsic factor, recombinant human
Jo-1	Diarect	12900	Histidyl-tRNA synthetase, recombinant human
Ku	Diarect	17300	p70/p80, recombinant human
La/SS-B	Diarect	12800	Recombinant human
LC1	Diarect	13700	Formiminotransferase Cyclodeaminase (liver cytosol type 1 antigen), recombinant human
mda ldl	Academy Bio-medical C	20P-MD-L110	Malondialdehyde modified human low density lipoprotein
HDL	Academy Bio-medical	80P-HD101	
Mi-2	Diarect	18100	Mi-2protein, recombinant human
MPO -BD	Biodesign	A3C181H	Human neutrophils
PCNA	Diarect	15400	Proliferating cell nuclear antigen, recombinant human
PDH	Sigma	P7032	Pyruvate dehydrogenase, from porcine heart
PL-12	Diarect	15701	Alanyl-tRNA synthetase, recombinant human
PL-7	Diarect	15601	Threonyl-tRNA synthetase, recombinant human
PR3	ImmunoVision	PR3-3000	Proteinase 3 antigen (cANCA antigen), from human promyelocytic cell line
ribo p	Diarect	14101	Ribosomal Phosphoprotein PO, recombinant human
Ro/SS-A 52 kDa	Diarect	12701	Ro-52 ribonucleoprotein, recombinant human
Ro/SS-A 60 kDa	Diarect	17401	Ro-60 ribonucleoprotein, recombinant human
bRo/SS-A 60kDa	Diarect	15501	Ro-60 Ribonucleoprotein, from calf thymus
Scl-70 (full)	Diarect	12401	DNA topoisomerase I full-length 100k0a form, recombinant human
Scl-70 (trunc)	Diarect	14501	DNA topoisomerase I truncated 70kDa processed antigenic form, recombinant human
SMA 3000 (Smith)	ImmunoVision	SMA-3000	Smith antigen (small nuclear ribonuclearproteins B, B', D), from bovine spleen or thymus
SRC 3000 (Sm/RRP	ImmunoVision	SRC-3000	Small nuclear ribonuclearprotein complex (A. B. B', C. D. E/F, G). from calf spleen, thymus and/or rabbit thymus
SRP54	SurModics	A18401	Signal recognition particle 54, recombinant human
TG	Diarect	12201	Thyroglobulin, from human thyroid glands
TPO	Diarect	12101	Thyroperoxidase, recombinant human
U-snRNP-BB'	Diarect	13301	Small nuclear ribonuclearprotein 88', recombinant human
U1-A	Diarect	13101	Small nuclear ribonuclearprotein A, recombinant human
U1-C	Diarect	13201	Small nuclear ribonuclearprotein C, recombinant human
U1-snRNP68	Diarect	13001	Small nuclear ribonuclearprotein 68 (U1-70), recombinant human

FIG E1. Protein microarray composition. Purified biomolecules, including cytokines, chemokines, growth factors, and autoantigens, are listed with their respective vendors.

Disease Code	Number of Patients with Finding
Pulmonary alveolar proteinosis	11
Candidiasis	6
Healthy controls	6
APS-1	5
Hypothyroidism	5
Suspected pulmonary alveolar proteinosis	4
TNF-inhibitor treated	3
Undiagnosed immunodeficiency	3
APS-1 phenotype with no AIRE mutation on sequencing	2
Rheumatoid arthritis	2
Non-tuberculous mycobacterial disease	2
T-cell cytopenia	2
Multi-organ autoimmunity	2
Thymoma	2
Acanthosis	1
Bronchiectasis	1
Cholangitis	1
Colitis	1
Chronic salmonella	1
Cutaneous lupus	1
Common variable immunodeficiency	1
Granulomatous lung disease	1
Good´s syndrome	1
HHV6 infection	1
Hyperkeratosis	1
Interferon gamma treated	1
IL-12 receptor deficiency	1
Klebsiella infection	1
Late onset progressive multifocal leukoencephalopathy	1
Systemic lupus erythematosus	1
Mucor mycosis	1
Pulmonary fibrosis	1
Pneumococcal infection	1
Recurrent tuberculosis	1
Shingles and persistent warts	1
Systemic sarcoidosis	1

FIG E2. Clinical characteristics of the cohort. The clinical diseases and phenotypes of patients and healthy control subjects are listed. Patients with more than 1 disease or disorder are listed more than once under each applicable category.

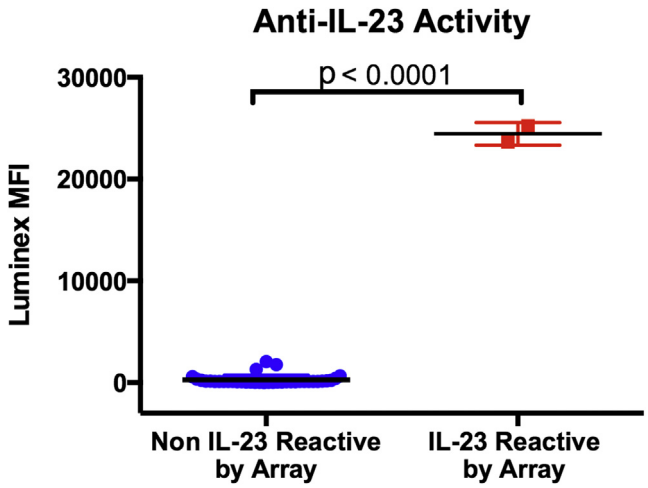

FIG E3. IL-23 ACAA validation. IL-23 binding was also measured by means of a Luminex analyzer, as described in the Methods section, to confirm IL-23 reactivity observed by using a cytokine microarray. MFI of samples 092B and 562B were compared with all others by using the unpaired *t* test.