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 2016;137:204-13.)

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,

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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

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.

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