Identification of new autoantigens by protein array indicates a role for IL4 neutralization in Autoimmune Hepatitis

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[1]List of Abbreviations:

AIH: Autoimmune Hepatitis

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

HD: Healthy Donor

IL4R: Interleukin-4 receptor

IL4R (FNIII): Interleukin-4 receptor Fibronectin Type III domain

MFI: Mean Fluorescence Intensity

VH: Viral Hepatitis

Summary

Autoimmune Hepatitis (AIH) is an unresolving inflammation of the liver of unknown cause. Diagnosis requires the exclusion of other conditions and the presence of characteristic features such as specific autoantibodies. Presently, these autoantibodies have relatively low sensitivity and specificity and are identified by immunostaining of cells or tissues, therefore there is a diagnostic need for better and easy to assess markers. To identify new AIH-specific autoantigens, we developed a protein microarray comprising 1626 human recombinant proteins, selected *in silico* for being secreted or membrane-associated. We screened sera from AIH patients on this microarray and compared their reactivity with sera from healthy donors and patients with chronic viral hepatitis C. We identified six human proteins that are specifically recognized by AIH sera. Serum reactivity to combination of four of these autoantigens allows identification of AIH patients with high sensitivity (82%) and specificity (92%). Of the six autoantigens, the FNIII domain of IL4 Receptor (CD124), which is expressed on the surface of both lymphocytes and hepatocytes, showed the highest individual sensitivity and specificity for AIH. Remarkably, patients' sera inhibited STAT6 phosphorylation induced by IL4 binding to CD124, demonstrating these autoantibodies are functional and suggesting IL4 neutralisation has a pathogenetic role in AIH.

Introduction

Autoantibodies specific for proteins or non-protein antigens (dsDNA, snRNP, carbohydrates) are often the serological hallmark of autoimmune diseases. Autoantibodies can just be an epiphenomenon secondary to a chronic inflammatory milieu (1) but can also play a direct pathogenetic role, such as anti-thyroglobulin antibodies in Hashimoto's thyroiditis (2).

Autoimmune Hepatitis (AIH) is a chronic necro-inflammatory disease of unknown etiology which affects predominantly women with an incidence of 1-2 per 100,000 per year and a prevalence of 10-20/100,000 (3, 4). AIH is subdivided into two major types on the basis of autoantibodies reactivity (5). Antibodies to nuclei (ANA) and/or to smooth muscle (SMA) characterize type 1 AIH, whereas antibodies to a liver-kidney microsomal constituent (anti-LKM) define patients with type 2 AIH. Since detection of these autoantibodies is done by immunofluorescence on rodents multi-organ sections (liver, kidney, stomach), there are problems such as standardization and interpretation of the immunostaining patterns (6). To overcome these methodological problems, the International Autoimmune Hepatitis Group established an international committee to define guidelines, develop procedures and reference standards for more reliable testing (7, 8). Although ELISA and bead assays with purified or recombinant autoantigens are under development (9), they actually represent a complementary rather than an alternative approach to traditional immunofluorescence. Moreover, serological overlap is frequently observed between AIH and other non-autoimmune liver diseases such as chronic viral hepatitis (10). Therefore new highly specific markers represent an unmet medical need for a more accurate diagnosis and classification of AIH.

Beside the potential diagnostic application, the discovery of novel AIH autoantigens could provide insights on the disease pathogenicity mechanism. Although some AIH

target-autoantigens have been identified and characterized, little is known on their pathogenetic role, and other autoantigens are probably still unknown. Autoantibodies, to be considered pathogenetic, must have at least two features: (i) the target-autoantigen should be either expressed on the plasma membrane of target cells or secreted by cells, i.e. should be exposed to autoantibodies, (ii) binding of the autoantibodies to the target antigen should disturb a cellular function directly or indirectly. A possible pathogenetic role in AIH has been put forward for autoantibodies specific for Cytochrome P450 2D6 (CYP2D6) or Asialoglycoprotein receptor 1 (AGPR-1), which are both present on the hepatocytes cell membrane (10).

Protein microarrays are a powerful technology as they allow the simultaneous screening of thousands of analytes (11). In the present study, to identify new autoantigens with potential diagnostic and/or pathogenetic role in AIH, we printed a microarray with 1626 human proteins whose main features were to be either secreted or membrane associated, i.e., potentially exposed to autoantibody recognition. We used this microarray to screen panels of sera from patients with AIH and identified six new protein antigens that are recognized with high sensitivity and specificity. One of these six autoantigens is FNIII domain of interleukin-4 receptor (CD124) and, interestingly, patients autoantibodies specific for CD124 neutralize IL4 signaling, so suggesting a possible pathogenetic role for IL4 neutralization in AIH.

Experimental Procedures

Serum Samples - Samples used for this study were collected in five different hospitals according to standard operating procedures: i) IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; ii) Hepatology Unit, University Hospital, Pisa, Italy; iii) Sant'Orsola-Malpighi University Hospital, Bologna, Italy; iv) Center for Autoimmune Liver Diseases, IRCCS Istituto Clinico Humanitas, Rozzano, Italy; v) Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), University of Firenze, Italy. For the discovery phase, 203 sera were used (15 AIH, 78 HD, 110 HCV), while for the validation phase 174 sera were used (50 AIH, 50 HD, 50 HCV, 24 HBV). The Institutional Review Board of these Hospital authorized the use of sera for research purposes.

Human proteins - selection, expression and purification - Genes whose translated products carry a secretion signal peptide or at least one transmembrane domain were selected, cloned and expressed in a high throughput system as histidine-tagged products as described (12). A total of 1626 polypeptides were cloned and expressed in *E. coli.* Of these, 1121 were cloned as protein fragments and 505 as full length proteins (Supplementary Table 1-2). The recombinant proteins were affinity-purified from the bacterial insoluble fraction by Immobilized metal ion affinity chromatography (IMAC, GE). Representative SDS-PAGE gels of a panel of purified proteins are shown in Supplemental Fig. 1.

Human, viral or bacterial proteins were used as biological or technical controls in the microarrays (Supplementary Figure 2): HCV Core protein and Non-structural proteins NS3 (from HCV genotype 1), NS3-4a (from HCV genotype 2) NS5b (from HCV genotype 1), Tetanus toxin and H1N1 antigen were produced in house by subcloning the corresponding genes in *E. coli* strain DH5α and expressing them in BL21(DE3); Bovine

Serum Albumin (BSA), Human Serum Albumin, Human Glutathione-S-Transferase and Protein A from Staphylococcus Aureus were purchased from Sigma.

For DELFIA® experiments, plasmids encoding Cytochrome P450 2D6 (CYP2D6) and Asialoglycoprotein Receptor 1 (AGPR-1) were purchased by Invitrogen (Ultimate™ Human ORF Clones), subcloned in *E. coli* strain DH5α and expressed in BL21(DE3). All the corresponding proteins were purified by affinity chromatography on IMAC resin.

Protein quality control - Purified recombinant proteins obtained as described above, were analyzed by SDS-PAGE (Criterion PAGE system Bio-Rad) followed by Coomassie Blue staining of the gel to assess their integrity and purity (Supplementary Fig. 1). Protein purity was assessed by BioRad ChemiDocTM XRS, Quantity One® software. Proteins showing purity levels > 70% were used for protein array preparation.

To further analyse the quality of the purified proteins, we performed western blot analysis on the purified proteins with an anti-His monoclonal antibody (anti-His mAb). More in detail, the proteins were resolved on 4-12% pre-cast SDS-PAGE gradient Tricine gels under reducing conditions, and electroblotted onto nitrocellulose membranes (Bio-Rad), according to manufacturer's instructions. The membranes were blocked with 5% non-fat milk in PBS with 0.1% Tween 20 (TPBS) for 1 h at room temperature, incubated with α-His mAb (GE-Healthcare) diluted 1:1000 in 3% non-fat milk in TPBS for 1 h at room temperature, and washed three times in TPBS. The secondary HRP-conjugated antibody (α-mouse immunoglobulin/HRP, GE-Healthcare) was diluted 1:1000 in 3% non-fat milk in TPBS and incubated for 1 h at room temperature. The proteins were visualized by Enhanced Chemiluminescence (Super Signal West Pico Chemiluminescence Substrate, Thermo Scientific, USA) and detected with LAS-3000 (Fujifilm, USA).

Protein microarray printing - Protein Microarrays were generated by spotting the 1626 affinity-purified recombinant proteins (0.5 mg/ml in 6M Urea) in 4 replicates on nitrocellulose-coated slides (FAST slides, GE-Healthcare) using Stealth SMP3® spotting pins (TeleChem International, Sunnyvale, California) and a Microgrid II microarray contact printer (Biorobotics), resulting in spots with a diameter of approximately 130 µm. As experimental positive control, a curve of human IgG at 11 different concentrations (from 0.001 to 1 mg/ml) was spotted on the arrays in 8 replicates (in 6M Urea) (Supplementary Fig. 3A). Several spots of buffer alone were also printed and used to assess possible non-specific signals due to cross contamination. A quality control of the spotting procedure was performed on 10% of randomly chosen slides. The percentage of proteins successfully spotted on the slides was assessed by hybridizing the arrays with an α-His mAb, followed by an Alexa-647 conjugated α-Human IgG secondary antibody and estimating the number of spots with a MFI value significantly above background. A distance matrix was calculated by TIGR Multiexperiment Viewer (version MeV4.7) software (13) to evaluate the system reproducibility (Supplementary Fig. 3B). The spotted microarrays were allowed to remain at room temperature for 1 h before storage at 4 °C until use.

Incubation and scanning of protein microarray - Incubation was automatically performed with a TECAN Hybridization Station (HS 4800™ Pro; TECAN, Salzburg, Austria). The microarray slides were prewashed 3 min in TPBS and saturated with BlockIt™ Microarray Blocking Buffer (Arrayit Corporation) for 45 min at 25°C under mild agitation. After injection of 105 µl of diluted human serum (1:300 in Blocking Buffer with 0.1% Tween 20), microarrays were incubated at 25°C for 45 min with gentle agitation.

The microarrays were then washed in TPBS at 25°C for three cycles of 1 min (wash time) and 30 sec (soak time).

After that, microarray slides were incubated for 1 h at 25°C with Alexa-647-conjugated α-human IgG (Invitrogen) (1:800 in Blocking Buffer) in the dark. The microarrays were then washed at 25°C two times in TPBS (1 min, wash time; 30 sec, soak time), two times in PBS (1 min, wash time; 30 sec, soak time) and finally one time in milliQ sterile water (15 sec).

The slides were finally dried at 30°C under nitrogen for 2 min, and scanned using a ScanArray Gx PLUS (PerkinElmer, Bridgeport Avenue Shelton, USA). 16-bit images were generated with ScanArrayTM software at 10μm per pixel resolution and analyzed using ImaGene 8.0 software (Biodiscovery Inc, CA, USA). Laser of 635 nm was used to excite Alexa-647 dye. The fluorescence intensity of each spot was measured, signal-to-local-background ratios were calculated by ImaGene, and spot morphology and deviation from the expected spot position were considered using the default ImaGene settings.

Microarray Data analysis - For each sample, the background subtracted Mean Fluorescence Intensity (MFI) of replicated spots was determined, and subsequently normalized on the basis of the human IgG curve to allow comparison of data from different experiments (14). Briefly, the MFIs values of IgG, spotted at different concentrations (Supplementary Fig. 3A), were fitted by a sigmoid curve, using a maximum likelihood estimator (Harris, J. W. and Stocker, H. Handbook of Mathematics and Computational Science. New York: Springer-Verlag, 1998). The experimental average IgG curve of each slide was adjusted on the reference sigmoid IgG curve, and the background-subtracted MFI values of each protein were normalized accordingly. On

the basis of these results, a normalized MFI value of 4.000 (value corresponding to the normalized MFI value of negative controls - BSA, HSA, Hu-GST- plus 2 standard deviations) was chosen as the lowest signal threshold for scoring a protein as positively recognized by human sera. For each protein, a Coefficient of Variation (CV%), was calculated on four replicate spots, for intra-assay reproducibility (14). Each antigen was checked for displaying a CV% correlated to its MFI on the basis of standard IgG curves. If the CV% value was not within the expected range the antigen was not considered for further analysis.

Recognition frequency was defined as the percentage of sera reacting with a particular antigen in protein array with a MFI ≥ 4.000, and it was calculated for each group of sera. TIGR Multiexperiment Viewer (version MeV4.7) software (13) was used to perform an unsupervised bi-dimensional hierarchical clustering.

In-gel enzymatic digestion and MALDI-TOF Mass Spectrometry Analysis **-** The identity of selected candidate autoantigens was further confirmed by tandem mass spectrometry (MS/MS). Protein spots were excised from the gels, destained with 50 mM ammonium bicarbonate (Fluka Chemie AG, Buchs, Switzerland) in 50% acetonitrile (J. T. Baker Inc.), de-hydrated once with pure acetonitrile and air-dried. Dried spots were digested for 2 h at 37 °C in 12 µl of 0.012 µg/µl sequencing grade modified trypsin (Promega, Madison, WI, USA) in 5 mM ammonium bicarbonate. After digestion, 0.6 µl were loaded on a matrix PAC target (Prespotted AnchorChip 96, set for proteomics, Bruker Daltonics, Bremen, Germany) and air-dried. Spots were washed with 0.6 µl of a solution of 70% ethanol, 0.1% trifluoroacetic acid. Analysis was performed using an Ultraflex III matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics). Peptides were selected in the mass range of 900–3500

Da. Also MS/MS spectra were acquired and externally calibrated by using a combination of standards pre-spotted on the target (Bruker Daltonics). MS and MS/MS spectra were analyzed with FlexAnalysis (FlexAnalysis version 3.0, Bruker Daltonics). Search parameters were as follows: variable modifications,Carbamyl (N-term), Oxidation (Met); cleavage by trypsin (cuts C-terminal side of Lys and Arg unless next residue is Pro); mass tolerance, 300 ppm; missed cleavage, 0; mass values, MH+ monoisotopic. Monoisotopic peaks were annotated with FlexAnalysis default parameters and manually revised. The peptide sequence was determined with Mascot software run on a public database (NCBI nr *Homo sapiens*, release 20100616 or SwissProt *Homo sapiens* release 2010_07, # 536789 sequence entries). Accession number, annotation, Mowse score, percentage of protein coverage, number of unique peptides matched, number of masses not matched, are reported in Supplemental Data.

Dissociation-Enhanced Lanthanide Fluoroscence ImmunoAssay (DELFIA®) assays **-** The DELFIA® assay is a time-resolved fluorescence method that can be used to study antibody binding to solid-phase proteins or peptides. The purified recombinant proteins were used at a concentration of 20 μg/ml (15) in 6M Urea to coat DELFIA® plates (PerkinElmer). Plates were then blocked for 1 hour at 37°C with a blocking reagent (PerkinElmer). The serum samples, diluted 1:300 in PBS with 1% BSA (Sigma), and 0.1 %Tween 20 (Sigma) were incubated on the plates for 1 hour at 37°C. Plates were then washed 5 times with washing buffer (PerkinElmer) and then incubated 30 min at room temperature in the dark with Europium-labeled α-human IgG serum (1:500 in diluting buffer, PerkinElmer). After extensive washing, plates were left at room temperature for 10 min and then read on a Infinite F200 PRO instrument (Tecan).

Fluorescence intensity values higher than the mean of buffer plus 3 standard deviations were considered as positive.

Surface staining of IL4R on HeLa cells – To assess recognition of native IL4R by human sera, full length IL4R was overexpressed in HeLa cells. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2mM L-glutamine and 1% penicillin-streptomycin. The human cDNA clone of full length IL4R, transcript variant 1, was purchased from OriGene Technologies. HeLa cells were transfected with Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at 5×10^5 cells/well in 24-well dishes and left overnight in medium without penicillin-streptomycin. 1 µg of plasmid DNA and 2 µl of Lipofectamine 2000 reagent were diluted in 100 µL of Opti-MEM (Invitrogen) and pre-incubated 20 minutes to allow DNA-Lipofectamine complexes to form; cells were then incubated with Opti-MEM (Invitrogen) containing DNA-Lipofectamine complexes, at 37°C for 16 hours. After that, Opti-MEM was replaced with fresh DMEM and, at 24 hours post transfection, cells were assayed for IL4R expression. For surface staining, cells were non-enzymatically detached, harvested and washed in PBS. To assess IL4R expression on the cell surface, 1×10^5 cells were incubated with PE-conjugated anti-CD124 (Beckman-Coulter) and with matched mouse Isotype as control (1:10 in PBS 1% BSA) for 30 minutes at 37°C. Non-transfected cells were used as control. To assess sera reactivity to IL4R expressed on HeLa cells, 1×10^5 transfected cells were incubated with human sera (1:2 in PBS 1% BSA) followed by PE-conjugated anti-Human IgG (1:200 in PBS 1% BSA) for 30 min at 37°C. Cells stained with secondary antibody alone were used as control. IL4R staining was analyzed by Flow cytometry with FACS Canto II

analyzer (Becton Dickinson) and data were processed with the program FlowJo (Flow Cytometry Analysis Software).

STAT6 phosphorylation assay **-** To assess the inhibition of IL4-mediated Stat6 Phosphorylation by patients sera, Peripheral Blood Mononuclear Cells were isolated from healthy donors blood by density gradient centrifugation (Ficoll). CD4 positive T cells were magnetically separated (CD4+T cell Isolation Kit II; Mylteni Biotec), resuspended in RPMI medium (GIBCO) with 10% of fetal calf serum and seeded in 96 well plates at $1x10^6$ cells/ml. Cells were pre-incubated 1 h at 37°C with sera of patients or healthy donors (1:2 in RPMI), or with neutralizing anti-IL4R goat polyclonal antibody (R&D, 100 ug/ml) as control; cells were then stimulated with IL4 (0.2 ng/ml; Mylteni Biotec) for 1 h at 37°C, fixed for 10 min at 4°C (BD Fixation buffer), washed twice in PBS 1% BSA and permeabilized (BD Perm Buffer III) for 30 min at 4°C. Intracellular staining with PE Mouse anti-Stat6(Y641)P antibody (BD) was performed by incubating cells for 30 min at room temperature in the dark. The percentage of Stat6P positive cells was measured by Flow cytometry with FACS Canto II analyzer (Becton Dickinson) and data were processed with the program FlowJo (Flow Cytometry Analysis Software). To confirm that the mechanism was mediated by antibodies and not by other factors present in the serum, patients and healthy donors sera were depleted from anti-IL4R(FNIII) antibodies and tested in Stat6 phosphorylation assay. To do this, 2 mg of purified protein were blotted on nitrocellulose membrane and then incubated with patients or healthy donor sera. After this incubation sera were collected and tested as described above.

Soluble IL4R detection **-** The amount of soluble IL4R was determined with Abcam sIL4R Elisa kit. Briefly, sera were diluted 1:4 in diluent buffer provided by the kit and

incubated in 96 well plates coated with anti-sIL4R. Biotinylated monoclonal antibody specific for sIL4R was added to the wells and plates were incubated 1 h at room temperature. After washing, streptavidin-HRP was added to the wells and plates were incubated for additional 30 min. 3,3',5,5' - tetramethylbenzidine (TMB) substrate was then added and OD at 450 nm was read on a Infinite F200 PRO instrument (Tecan).

Statistical analysis **-** Results of Protein Microarray and DELFIA® experiments were analyzed using the two-tailed $\chi 2$ test, the Student's t-test, the Fisher's exact tests or the ANOVA test. The Benjamini-Hochberg correction for multiple testing was used for the analysis of microarray data. Statistical analysis was carried out with the use of TIGR Multiexperiment Viewer or GraphPad Software Inc. Predictive analysis of microarray (PAM) was performed using the statistical package PAM 1.51 with the statistical tool R (http://wwwstat.stanford.edu/~tibs/PAM/index.html) (16). PAM executes a sample classification training routine from expression data via the nearest shrunken centroid procedure to find markers that discriminate best between AIH patients from HD. Data were log- transformed, mean centered and standard deviaton scaled. After training of the PAM classifier, we performed a 10-fold cross validation, in order to check the accuracy of the model and better select the threshold, as the one giving the lowest misclassification error. To evaluate the performance of autoantigens combinations in discriminating AIH patients from healthy donors, logistic regression analysis was performed with R. We created logistic regression models with signals of 4 autoantigens (IL4R(FNIII), AL137145, C17orf99, APCDD1L) or with signals of two known AIH autoantigens as control (CYP2D6 and AGPR-1). The probabilities were calculated as follows: $p= \exp((\Sigma(b_i x_i)+ c)/(1+\Sigma(b_i x_i)+ c)$, where p is the probability of each case, i= 1 to n; b is the regression coefficient of a given autoantigen, x is signal intensity and c is a

constant generated by the model. ROCR package was used to obtain the ROC curves of the models and the Area Under Curve (AUC) values (17).

Results

Design and construction of a microarray with secreted or membrane associated human proteins - To identify self-antigens recognized by antibodies from patients with AIH, we developed a microarray by printing 1626 recombinant products (Supplementary Table 1-2) that corresponded to 1371 distinct human proteins (Table 1). 1329 of the 1371 proteins were selected through bioinformatic analysis of the whole human genome as hypothetical and/or poorly characterized by the available annotation or published information and either carrying a signal peptide (23% of them) or at least one transmembrane domain (61% of them, 75% of which assigned to the plasmatic cell membrane and the remaining 25% assigned to mitochondrial or endoplasmic reticulum membranes) (12). Fortytwo of the 1371 proteins had a well known immunological function, CD number assigned and were all surface exposed (Supplementary Table 2).

Proteins were cloned as either full length products (31%) or protein domains (69%) with a N-terminal Histidine tag, expressed in *E. coli*, purified by affinity chromatography and checked for purity and integrity (Supplementary Fig. 1). Protein arrays were prepared by printing onto nitrocellulose-covered glass slides four replicates of each protein along with several controls (Supplementary Fig. 2). Replicates were randomly distributed to get optimal signal reproducibility. The final protein microarray layout consisted of 24 grids of 304 spots each, for a total of 7296 spots. Quality of microarrays was assessed by probing 10% of the slides with an anti-His mAb and by determining the number of immobilized proteins with signal intensity significantly above background. About 90% of proteins fulfilled this criterion (data not shown). Moreover, a high correlation among signal intensities of different slides was observed, indicating high experimental reproducibility (Supplementary Fig. 3B). A representative picture of the array is shown in Fig. 1A.

In summary, we obtained high quality microarrays comprising more than 1600 human proteins which can be used as tool for the identification of autoantigens recognized by sera of patients with any disease of interest.

Protein microarray allows identification of new autoantigens recognized by IgG of patients affected by Autoimmune Hepatitis (AIH) - In order to identify a panel of autoantigens differentially recognized by patients with AIH compared to healthy individuals (HD), the protein microarrays were probed with a sample set (defined as Discovery set) comprising 15 sera from patients with AIH and 78 sera from healthy donors. The patients clinical data are summarized in Table 2.

First, we compared autoreactivity of patients sera against healthy donors sera. AIH sera displayed a higher reactivity toward self proteins than HD sera as documented both by the intensity (mean fluorescence intensity, MFI) of recognition signals (Fig. 1B) and by recognition frequencies (Fig. 1C). Autoantigens recognized by AIH sera were then ranked according to (i) the recognition frequency and (ii) the MFI. Self proteins were regarded as potential autoantigens if they were recognized with MFI significantly higher in patients than in healthy donors sera (T test, p val <0.01) and if they were recognized by less than 5% of the healthy donors sera and by more than 50% of patients sera (Fisher test, pval <0.01). In this way we identified 33 proteins that allowed good discrimination of the two populations of sera, as shown in the unsupervised hierarchical clustering analysis in Fig. 2A. We then asked whether sera from patients with HCV liver disease displayed the same autoreactivity pattern. We therefore tested the same microarray with sera from 110 patients with chronic HCV infection (Table 2). Fig. 2B shows the MFIs of the 33 autoantigens with sera from AIH, HD and HCV, and indicates that 16/33 autoantigens react preferentially and significantly with sera from AIH patients (T test, p val <0.01).

In order to confirm the identities of the proteins, the 16 recombinant autoantigens of interest (Supplementary Table 3) were resolved by SDS-PAGE and the prevalent bands were excised from gels, digested with trypsin and subsequelly analyzed by MALDI-TOF mass spectrometry. All antigen identities were confirmed either by Peptide Mass Fingerprint or fragmentation of selected ions (details, Mascot scores and MS spectra are provided as supplemental data).

We therefore conclude that 16 autoantigens, identified by protein microarray, are differentially recognized by AIH patients compared to healthy donors and chronic HCV patients.

Validation of selected autoantigens with an independent sample set of sera confirms six of the sixteen proteins are new potential AIH biomarkers - In order to validate protein microarray results with a different assay and larger panels of patients sera, we used Dissociation-Enhanced Lanthanide Fluorescence ImmunoAssay method (DELFIA®) to screen an independent Validation set of sera comprising 50 AIH patients, 50 healthy donors and 74 patients with chronic viral hepatitis (50 HCV and 24 HBV) (Table 2). DELFIA® assay was therefore used to assess the IgG response both to the 16 autoantigens that were selected with the Discovery set and to CYP2D6 and AGPR-1, two benchmark protein autoantigens in AIH (18, 19).

All sixteen antigens displayed higher mean fluorescence intensity compared to HD and chronic viral hepatitis patients (Supplementary Fig. 4) and six of these sixteen antigens displayed also significantly higher recognition frequency by AIH patients than by healthy donors and viral hepatitis patients (Fig. 3). These six antigens, showed high sensitivity (from 42 to 70% of positive AIH patients) and specificity (from 96 to 100% of negative HD). Interestingly, individual sensitivity was comparable to that obtained in our

assay with CYP2D6 and AGPR-1, two benchmark protein autoantigens in AIH, while individual specificity was higher for our six candidates (Table 3).

We then performed a classification of the validation samples with a nearest shrunken centroid algorithm (Predictive Analysis of Microarrays, PAM), which identified a minimal set of five predictors (Supplementary Fig. 5), and classified AIH samples with an accuracy of 94%. Importantly, these classifiers corresponded to the top antigens previously sorted out on the basis of the sensitivity and specificity values, validating the selection of these proteins as best antigens for further characterization.

We next assessed the discrimination power of combinations of the autoantigens. Fig. 4A shows the seroreactivity of the validation sample set to all six autoantigens and reveals that two of the antigens (LOC646100 and METRNL) have reactivity that overlap with IL4R(FNIII) domain. We therefore assessed whether the combination of only four antigens (IL4R(FNIII), AL137145, C17orf99, APCDD1L) performed better than individual antigens. Fig. 4B shows that the 4 antigens combination achieved 82% sensitivity and 92% specificity, thus performing better than the individual antigens as well as combination of the two known autoantigens AGPR-1 and CYP2D6 (SE=68% and SP=68%). Moreover, Fig. 4C compares the ROC curves of logistic regression models obtained with combination of our four autoantigens and combination of the two known autoantigens, and indicates that our four autoantigen combo is superior to AGPR-1 and CYP2D6 combination.

IL4R is the target of autoantibodies capable of neutralizing IL4 activity - To address the biological significance of the newly identified autoantigens, we checked the public databases for any information regarding their putative function. We found that five of the six autoantigens have poorly known function. Indeed, three of them (AL137145, LOC646100 and C17orf99) are completely uncharacterized secreted proteins; METRNL

is a secreted protein annotated as Meteorin-like protein precursor and APCDD1L is a membrane protein annotated as Protein APCDD1-like precursor (Adenomatosis polyposis coli down-regulated 1 protein-like). One of the proteins is instead a domain of a well known membrane protein, as it corresponds to the fibronectin type III (FNIII) domain of the Interleukin 4 receptor α chain (IL4R or CD124), which is expressed in lymphocytes in association with cytokine receptor common gamma chain (Type I IL4 Receptor) as well as in hepatocytes in association with the Interleukin 13 receptor alpha1 chain (Type II IL4 Receptor) (20). Interestingly, as shown in Table 3, IL4R(FNIII) is the antigen displaying the highest individual sensitivity (70%) and specificity (100%) for AIH. Therefore we decided to focus our attention on the possible functional role of autoantibodies targeting IL4R.

Firstly, we confirmed antibody specificity by titrating sera from four different patients who displayed high reactivity for CD124 and found they were able to recognize the receptor domain up to a 1:800 dilution (Fig. 5A). Secondly, we asked how patients sera compared to commercially available neutralizing anti-CD124 goat polyclonal antibody in their ability to recognize both the human CD124 domain that we used in our protein array, and a recombinant form of the human CD124 expressed in insect cells. Fig. 5B shows that patients sera and the goat anti-CD124 antibodies both recognize the two forms of CD124 in Western blot. Finally, we assessed the capability of human sera to recognize the native IL4R when expressed on mammalian cell surface. We therefore overexpressed full length IL4R in HeLa cells and then incubated cells with sera of AIH patients and HD and with anti-CD124 antibody as control. Fig. 5C shows that sera of patients but not of healthy donors recognize native IL4R on HeLa cell surface.

As the FNIII domain of CD124 is involved in the interaction with IL4, we then asked whether these autoantibodies neutralized the interaction of IL4 with its receptor. As

binding of IL4 to its receptor results in the specific phosphorylation of Stat6, we assessed by flow citometry whether patients sera inhibited IL4-mediated Stat6 phosphorylation. Fig. 6A and 6B show that, when cells expressing CD124 are pre-incubated with sera from AIH patients, but not with control sera, there is a dramatic reduction of the Stat6 phosphorylation that follows exposure to IL4. To confirm that the inhibition of Stat6 phosphorylation was an antibody-mediated mechanism, sera depleted of anti-IL4R (FNIII) antibodies were tested for their ability to block IL4 signaling. Fig. 6A shows that sera depleted of anti IL4R antibodies no longer inhibit IL4 induced Stat6 phosphorylation. Thus we have demonstrated that AIH patients sera neutralize IL4 *in vitro* because of anti-IL4R antibodies. Noteworthy, the inhibition of Stat6 phosphorylation correlated with the signal intensity of the anti-IL4R reactivity we detected in the patient sera by DELFIA® (Supplementary Fig. 6), and it is dilution-dependent (Supplementary Fig. 7). To rule out that the neutralisation of IL4 activity observed with patients sera was due to a competitive effect of circulating soluble form of IL4R (sIL4R) (21), we tested the possible presence of sIL4R by ELISA assay in the sera of 20 AIH patients and 20 healthy donors. No significant differences of sIL4R were observed in the sera of patients and HD (Supplementary Fig. 8). Interestingly, sera from AIH patients under immunosuppressive therapy have a strong reduction of anti-CD124 antibodies as measured by both quantitative titer assessment with DELFIA® (Fig. 6C) and qualitative neutralization of Stat6 phosphorylation (Fig. 6D).

From all the above we conclude that patients with AIH have autoantibodies to CD124 and that these antibodies neutralize IL4 signaling.

Discussion

This study illustrates the steps and outcome of a custom protein array approach to tackle new autoantigens in Autoimmune Hepatitis. Using an array of about 1600 human recombinant products, we report the identification of several human proteins recognized by autoantibodies that are present in sera of patients with AIH. We suggest that these autoantibodies may serve both for the improvement of diagnosis and for the development of new immunotherapeutic agents that could interfere with these autoantibodies. In particular, antibodies to IL4R, the autoantigen recognized with the highest sensitivity and specificity by AIH patients sera, inhibit IL4 signal transduction, demonstrating these autoantibodies are functional, suggesting a pathogenetic role for the inhibition of IL4 signaling and possibly opening new therapeutic perspectives for AIH.

In recent years microarrays have become precious tools for biomedical research as they are very suitable to screen great numbers of samples, with very low amount of biological material, in a very short time (22). A limitation of protein microarrays is that, due to labor intensive protein production processes, they often cover only defined protein families with known relevance to a given scientific question, although important efforts to overcome this limitation have been recently done and arrays with thousands of proteins are now available (11, 23). In this study we printed our custom array with a relatively small group of proteins that are known to play key roles in generation and differentiation of immune responses in health and disease, in combination to a functionally unbiased expression library. Our custom protein microarray is made of a library of more than 1600 recombinant products that were expressed in *E. coli* because of the costs and of the production and purification issues related to the handling of thousands of proteins. Expression of human proteins in bacteria is not ideal for functional studies, because post-translational modifications are generally lost and

because proteins are mostly recovered in denaturing conditions. However, we aimed at identifying polyclonal antibodies specific for linear epitopes of human proteins, as it has been reported that linear epitopes are often recognized by autoantibodies in many autoimmune diseases (24, 25).

The proteins printed in our microarray are recombinant products that are either membrane-associated or secreted proteins, the great majority of which are poorly characterized on the basis of both current annotation in public databases and scientific publications (12). The rationale for the use of this specific protein subset was that we were interested in a functionally unbiased search of new autoantigens among thousands of proteins; on the other hand, we wanted to focus on a functionally well known small group (40) of cell bound proteins that are exposed to the extracellular environment and that play crucial functions in regulating immune responses in health and diseases. It is worth noting that although membrane-associated and secreted proteins play a crucial role in many cell recognition and communication processes (26), most of the autoantigens identified so far in autoimmune diseases are intracellular components (11, 27).

We used this protein microarray to screen a panel of sera from patients with AIH, a disease for which there is an unmet medical need for both new and highly specific biomarkers and for more specific therapies. Indeed, the diagnosis of AIH is a complex process made by exclusion of other factors leading to chronic hepatitis (including viral, toxic, genetic and metabolic causes) and by detecting autoantibodies in indirect immunofluorescence assays on tissue sections from rodents. However, this technique has several limitations, the major of which is the strong dependence on the operator expertise (6). Moreover, serological overlap of AIH with other liver diseases (such as chronic viral hepatitis or drug-induced hepatitis) (10) is frequently observed. The

distinction between autoimmune and viral hepatitis is also important from a therapeutic point of view, since the immunosuppression used in AIH can increase virus replication in chronic viral hepatitis, while treatments used to eradicate viral infection, such as interferon-alpha, may lead to exacerbation of AIH (10).

Here we show that sera from patients with AIH recognize with high specificity and sensitivity five self proteins of unknown function and one known self protein (IL4R or CD124). This recognition pattern was first detected by protein array on fiftteen patients sera and subsequently confirmed in 96-well plate assay with sera from fifty patients with AIH, whereas sera from healthy donors and from patients with chronic viral hepatitis did not show significant recognition of these antigens. This autoreactivity pattern was mainly observed in type 1 AIH. Indeed, the great majority of AIH sera tested (100% of sera used in the discovery phase and 86% of sera used in the validation phase) were type 1 AIH, as reported in Table 2.

Interestingly, while individual performance of our new autoantigens for AIH was comparable to that we obtained with CYP2D6 and AGPR-1, two benchmark protein autoantigens, combination of four of the six autoantigens was superior to sensitivity and specificity of the benchmark autoantigens alone or in combination. Indeed we achieved an accuracy of 87% (SE= 82% and SP%= 92%) in discriminating AIH patients from healthy donors with the new antigens compared to the 68% accuracy of the combined benchmarks (SE=68%, SP=68%). Moreover, we achieved an accuracy of 68% (SE= 82% and SP=61%) in discriminating AIH from viral hepatitis patients compared to the 46% accuracy of the combined benchmarks (SE= 68%, SP= 31%) (data not shown). Finally, compared to the work of Song and colleagues (11), who recently reported the identification by protein array of 3 new AIH autoantigens with similar performances, we

used an ELISA-like approach to validate our candidates, that could be easily translated into the standard laboratory practice.

Therefore, we set the stage for the development of a new serological assay that is easy to perform, is highly specific for AIH, and that could significantly contribute to improve AIH diagnosis.

Of the five proteins with unknown function, one is a membrane protein (APCDD1L) and four are secreted proteins (AL137145, LOC646100, C17orf99, METRNL). The one known autoantigen (IL4R(FNIII)) is the Fibronectin type III domain of the alpha chain of the IL4 Receptor (IL4R or CD124). Obviously we concentrated our functional investigation on autoantibodies targeting this well known receptor, but studies are in progress to address the structure and function of the other five poorly known autoantigens.

IL4 is a cytokine mainly produced by CD4+ Th2 lymphoctes, basophils, mast cells and eosinophils (20). It plays a key role in several aspects of lymphocytes differentiation and function and has been described to be involved in many autoimmune as well as inflammatory diseases (28). In particular, IL4 is reported to inhibit Th1 and Th17 differentiation, which are T cell subsets implicated in many autoimmune diseases including Autoimmune hepatitis (29, 30). Altered IL4 expression has been reported in several liver diseases including chronic hepatitis C, drug induced hepatitis and liver transplantation (31). However its exact pathogenetic role in these diseases is still controversial: for some authors it has a protective effect (32, 33) while others have reported that higher IL4 expression in the liver is detrimental, causing hepatocytes apoptosis (34, 35).

IL4 exerts its action by binding its receptor (CD124), which is present on many cell types including lymphocytes and hepatocytes, (20) and activating specific signaling

cascades. The IL4 Receptor consists of a signaling alpha chain that binds IL4 with high affinity and a trans-activating low affinity chain which can be, according to the cell type, the common gamma chain (immune system cells) or the IL13 receptor alpha1 chain (epithelial cells). After IL4 binding to both immune and epithelial cells, the two chains form a heterodimer and initiate a phosphorylation cascade, which ends up in the specific activation of Stat6. Upon phosphorylation on Tyr641, Stat6 translocates to the nucleus and in turn activates the transcription of specific genes (36).

Interestingly, the FNIII domain of the CD124 alpha chain, which we found to be recognized with very high frequency (70%) by AIH patients sera, contains two binding sites for IL4 (37). Indeed, our data show that not only AIH patients sera recognize IL4R, but also IL4-mediated Stat6 phospohrylation is inhibited *in vitro* by AIH patients sera and not by HD or HCV sera. This finding points to the importance of IL4 pathway in autoimmune hepatitis and to the pathogenetic role that autoantibodies against the IL4 Receptor may play in AIH. Such antibodies might neutralize IL4 activity directly on hepatocytes and thus intefere with a potential anti-inflammatory role of STAT6, which has been put forward to explain the IL4 ability to reduce hepatic ischemia/reperfusion injury (32). Autoantibodies that neutralize IL4 activity might favour liver inflammation also indirectly by favouring development in lymphoid tissues and recruitment to the liver of T cell subsets, such as Th1 and Th17, involved in autoimmunity and inflammation (30). Indeed, IL4 has been shown, in experimental and clinical situations, to be capable of ameliorating the effects of tissue-damaging autoimmunity (20). On the other hand, based on data published in the literature (34, 35), one could also theorize that IL4 is detrimental in AIH and that neutralising anti-CD124 antibodies reflected an attempt of our immune system to buffer a negative role of IL4. Should this be true however, immunosuppressive therapy could be detrimental, as sera of patients after steroids treatment display a strong

reduction of anti-CD124 antibodies as measured by both DELFIA® and neutralization of IL4 activity (Fig. 6).

In conclusion, we describe the identification of a new panel of six autoantigens that are very specific and sensitive biomarkers of AIH. Compared to other similar approaches (11) our strategy based on a protein array enriched in “external” proteins, i.e. proteins physiologically more exposed to the immune system, allowed us to identify autoantigens which not only can be used as diagnostic biomarkers, alone or in combination, but could also give insights into some of the pathogenetic mechanisms involved in this autoimmune disease. Indeed, our results demonstrate that autoantibodies to CD124 (IL4R) have a neutralising effect on IL4 activity and suggest that these antibodies could have a pathogenetic role by favoring an inflammatory mileu leading to liver damage.

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FIGURES LEGEND

Fig. 1. **The microarray made of secreted and membrane proteins highlights a higher immunoreactivity in sera of AIH patients compared to healthy donors.** *A*, Representative image of the protein array containing 1626 His-tagged human recombinant proteins (predicted to be secreted or membrane associated) probed with an anti-His mAb. The microarray included 24 grids (as the one shown in the enlarged box) each one containing 304 proteins spots including also positive (such as viral and bacterial proteins) and negative (such buffer or BSA) controls, as well as IgG calibration curve (HuIgG curve) for data normalization. *B*, Comparison of Mean Fluorescence Intensity (MFI) of all spotted proteins probed with sera of 78 healthy donors (HD) and 15 AIH patients. Each dot represents the MFI of a single protein. A cut-off value ≥ 4.000 was used to score a protein as positive. Asterisk: statistical significance (Student's t-test, pval <0.01). *C*, Bars on the left panel represent the percentage of autoantigens recognized by more than 15% of AIH and HD sera (threshold is determined on the basis of the average HD recognition); bars on the right panel represent the percentage of AIH and HD sera reacting with more than 3% of the array proteins (threshold is determined on the basis of the average HD reactivity). Asterisk: statistical significance (χ^2 test, pval <0.01).

Fig. 2. **Candidate autoantigens specifically recognized by AIH patients compared to healthy donors and HCV patients.** Statistical analyses allowed selection of 33 proteins that underwent unsupervised hierarchical clustering analysis *A,* that shows good discrimination of AIH and HD sera. Sera are arranged in columns and proteins in rows. Red indicates positive immunoreactivity, and blue low or no immunoreactivity. *B,*

MFI of the autoantigens probed with sera from HD, AIH and chronic HCV patients. Significant differences were observed for 33 autoantigens between AIH and HD, and for 16 autoantigens between AIH and HCV (Red box). Asterisks, p val<0.01 (Student's t-test). p(a): p val of AIH versus HCV patients; p(b): p val of AIH patients versus HD. Caret: Protein cloned and expressed as domain

Fig. 3. **Six proteins are confirmed as AIH autoantigens in DELFIA® assay**. Recognition frequency of the best six autoantigens as determined by DELFIA®. Proteins were tested with sera from AIH patients (n. 50), healthy donors (n. 50) and HBV or HCV viral hepatitis (VH) patients (n. 74). Each sera was tested 3 times in independent experiments. Asterisk: statistical significance (Fisher exact test, pval <0.01). Caret: Protein cloned and expressed as domain.

Fig. 4. **Combination of four of the six autoantigens identifies AIH patients with high sensitivity and specificity.** *A,* Recognition of the six identified autoantigens by Validation sera samples. Asterisk indicates protein cloned and expressed as domain. *B*, Ability to discriminate AIH from HD by combination of 4 antigens, IL4R(FNIII), AL137145 and C17orf99, APCDD1L. Numbers in boxes indicate AIH and HD sera that, recognize (positive) or do not recognize (negative) at least one of the four antigens. Sensitivity (SE) and Specificity (SP) with 95% confidence intervals (C.I.) are indicated below the panel. p-values are calculated with χ^2 tests. *C*, Logistic regression models for the combinations of the four autoantigens (red curve) and the two known control proteins (AGPR-1, CYP2D6) (black curve) were calculated and represented as ROC curves. The Area Under the Curve (AUC) values are respectively 0.99 for the 4 new antigens and 0.85 for the known controls.

Fig. 5. **AIH sera recognize IL4 receptor (CD124).** *A,* Sera from 4 AIH Patients (black circles) and 4 HD (white circles) were titrated for recogniton (MFI) of IL4R (FNIII) in DELFIA® assay. Error bars: Standard error. *B,* A representative western blot experiment out of three shows either sera from AIH patients and HD or a neutralizing anti-CD124 polyclonal antibody (α IL4R) reacting with IL4R domain or a recombinant form of CD124 (IL4R) expressed in insect cells. Recombinant IL4R domain, expressed in *E. coli*, migrates as a single band of 11 kDa. The IL4R due to heterogeneous glycosylation appears as a smear ranging from 30 to 35 kDa. *C*, Left panel shows the surface staining of HeLa cells (black line) and HeLa cells overexpressing IL4R (red line) with anti-CD124. Mouse Ig isotype was used as control (solid grey line). Right panel shows a representative result of surface staining of HeLa cells overexpressing IL4R with sera from AIH patients (two patients are shown, solid or dashed red lines) or healthy donor (black line) followed by secondary PE-conjugated anti-HuIgG antibody. Cells stained with secondary antibody alone were used as control (solid grey line).

Fig. 6. **Neutralization of IL4 activity by AIH patients sera.** *A,* a representative experiment shows that patients' sera (left panel) completely inhibit Stat6 phosphorylation induced by recombinant IL4 on human CD4+ T lymphocytes, while HD sera (right panel) do not. The same patient serum when depleted of anti -IL4R(FNIII) antibodies lost its capacity to inhibit Stat6 phosphorylation (left panel, blue line). Black solid line: cells incubated with IL4; grey solid line: cells incubated with medium alone; green line: cells incubated with IL4 and a control neutralizing anti-IL4R polyclonal antibody; red line: cells incubated with IL4 and human sera, PT or HD; blue line: cells incubated with IL4 and human sera, AIH or HD, after depletion of anti-IL4R(FNIII) antibodies. *B,* scatter plot shows percentage of Stat6 phosphorylation induced by IL4 in the presence of sera from

AIH (11 patients) or HD (7 individuals). Each serum was tested twice in duplicate. Statistics: Student's T test (p val <0.01). *C,* DELFIA® assay showing titration of IL4R(FNIII) recognition by two patients sera (● PT-1 and ■ PT-2) collected before (solid lines) and after (dashed lines) immunosuppressive therapy. *D,* The same sera were tested for their ability to inhibit Stat6 phosphorylation. Black solid line: cells incubated with IL4; grey solid line: cells incubated with medium alone; red line: cells incubated with IL4 and PT sera collected before therapy; blue line: cells incubated with IL4 and PT sera collected after therapy.

Table 1: Predicted subcellular localization of the human recombinant proteins used for the microarray construction

Compartment	% of Proteins
Cell membrane	46
Secreted	23
Intracellular Membrane	11
Cytoplasm	11
Mitochondrial Membrane	4
Nucleus	4
Mitochondrion	1

Table 2: Clinical characteristics of the serum samples used in this study

	Group	Abbrev.	Sub group (*n.*)	*n.*	Source[A]	Age Mean ± SD (median)	Sex (*n.*)
Discovery	Healthy Donors	HD	----	78	1	44 ± 10 (45)	f(20) m(58)
	Autoimmune Hepatitis	AIH	Type 1 (15) Type 2 (0)	15	2	50 ± 21 (54)	f(13) m(2)
	Viral hepatitis	VH	HCV	110	2, 3	55 ± 15 (56)	f(41) m(69)
Validation	Healthy Donors	HD	----	50	1	43 ± 8 (43)	f(41) m(9)
	Autoimmune Hepatitis	AIH	Type 1 (43) Type 2 (4) n.c. (3)	50	2, 4	45 ± 21 (49)	f(41) m(9)
	Viral hepatitis	VH	HCV (50) HBV (24)	74	2, 5	51 ± 13 (52)	f(28) m(46)

[A]Origin of samples: (1) IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; (2) Sant'Orsola University Hospital, Bologna; (3) Hepatology Unit, University Hospital, Pisa; (4) Center for Autoimmune Liver Diseases, IRCCS Istituto Clinico Humanitas, Rozzano; (5) Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), Firenze.
n.c.: not classified.

Table 3: Sensitivity (SE) and Specificity (SP) of individual antigens identified as candidate AIH biomarkers. Asterisk: Protein expressed as domain.

Description	Protein ID	Combo[A]	SE %	SP % (HD)	SP % (VH)
Asialoglycoprotein receptor	AGPR-1		56	82	47
Cytochrome P4502D6	CYP2D6		62	72	45
Interleukin-4 receptor domain	IL4R (FNIII)*	●	70	100	86
Putative uncharacterized protein	AL137145*	●	44	96	86
Putative uncharacterized protein	LOC646100		42	98	86
Uncharacterized protein C17orf99	C17orf99*	●	42	96	85
Meteorin-like protein Precursor	METRNL		46	100	78
Protein APCDD1-like Precursor	APCDD1L*	●	58	98	72

HD: healthy donors; VH: Chronic B or C viral hepatitis patients.
[A]Antigens used for combination assay.
SE % = percentage of positive AIH sera; SP % = percentage of negative HD or VH sera.
IL4R (FNIII): Interleukin-4 receptor Fibronectin Type III domain

FIGURE 1

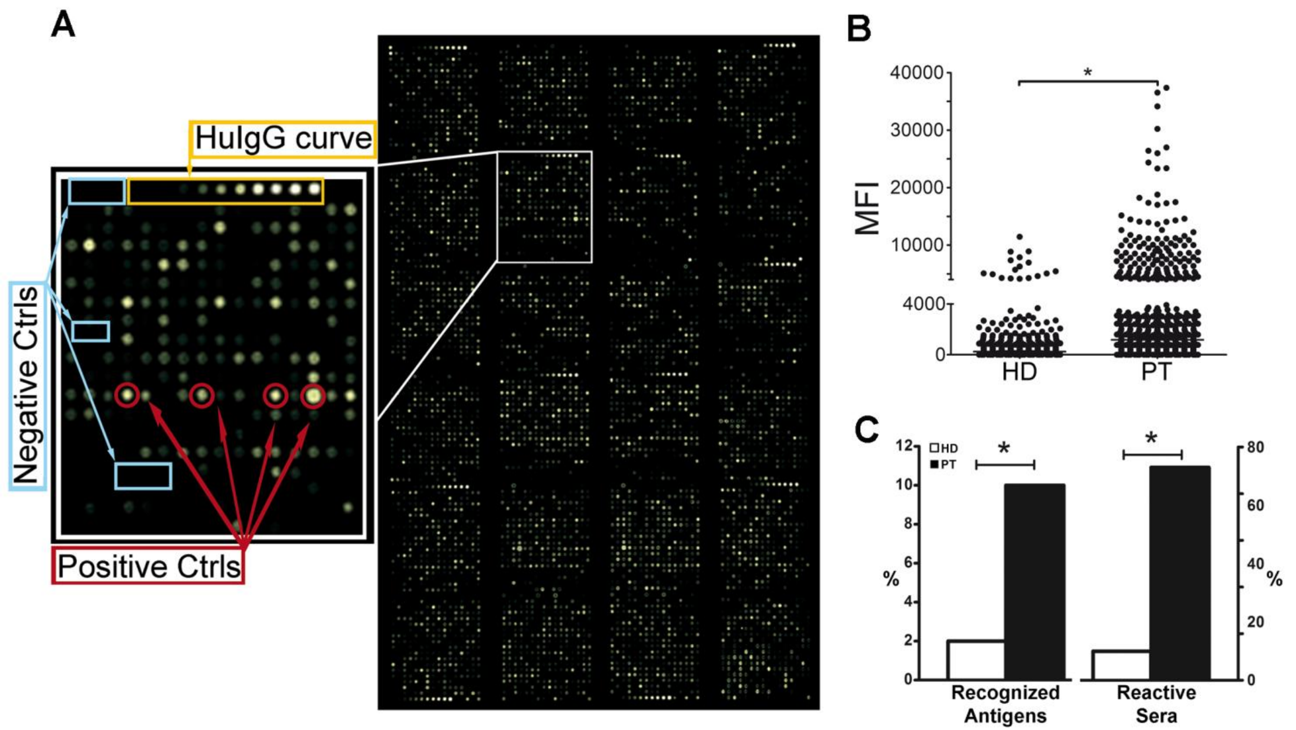
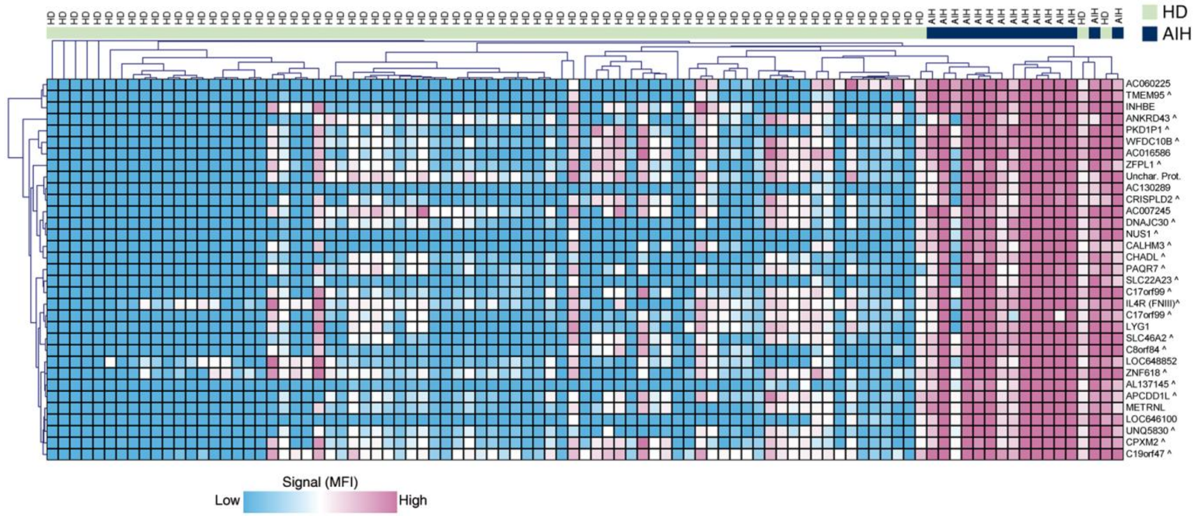


FIGURE 2

A



B

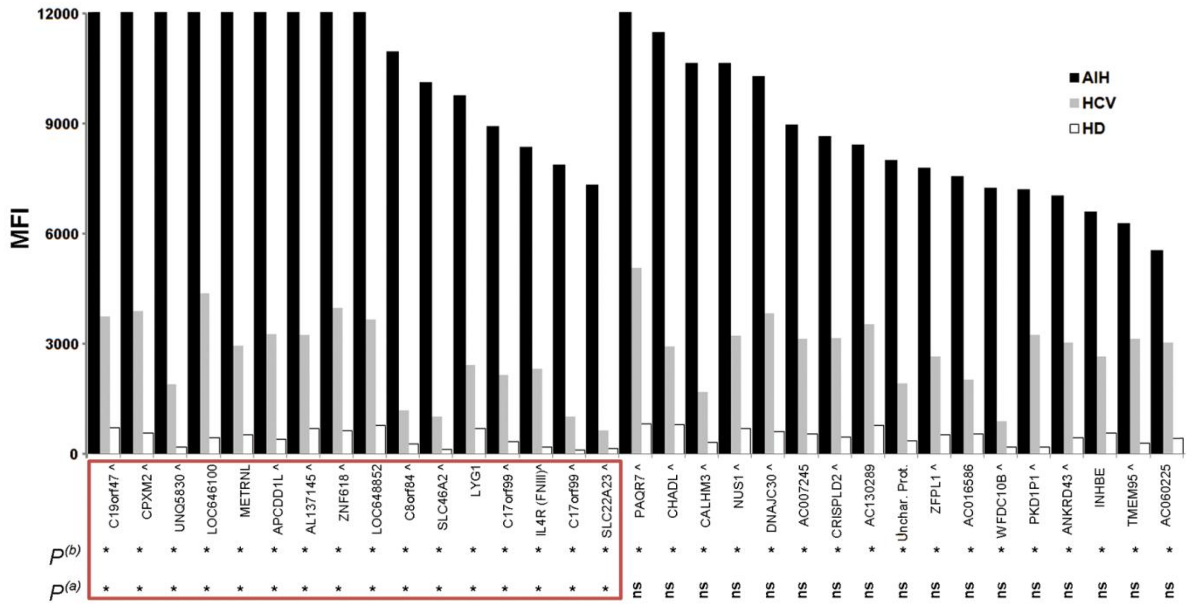


FIGURE 3

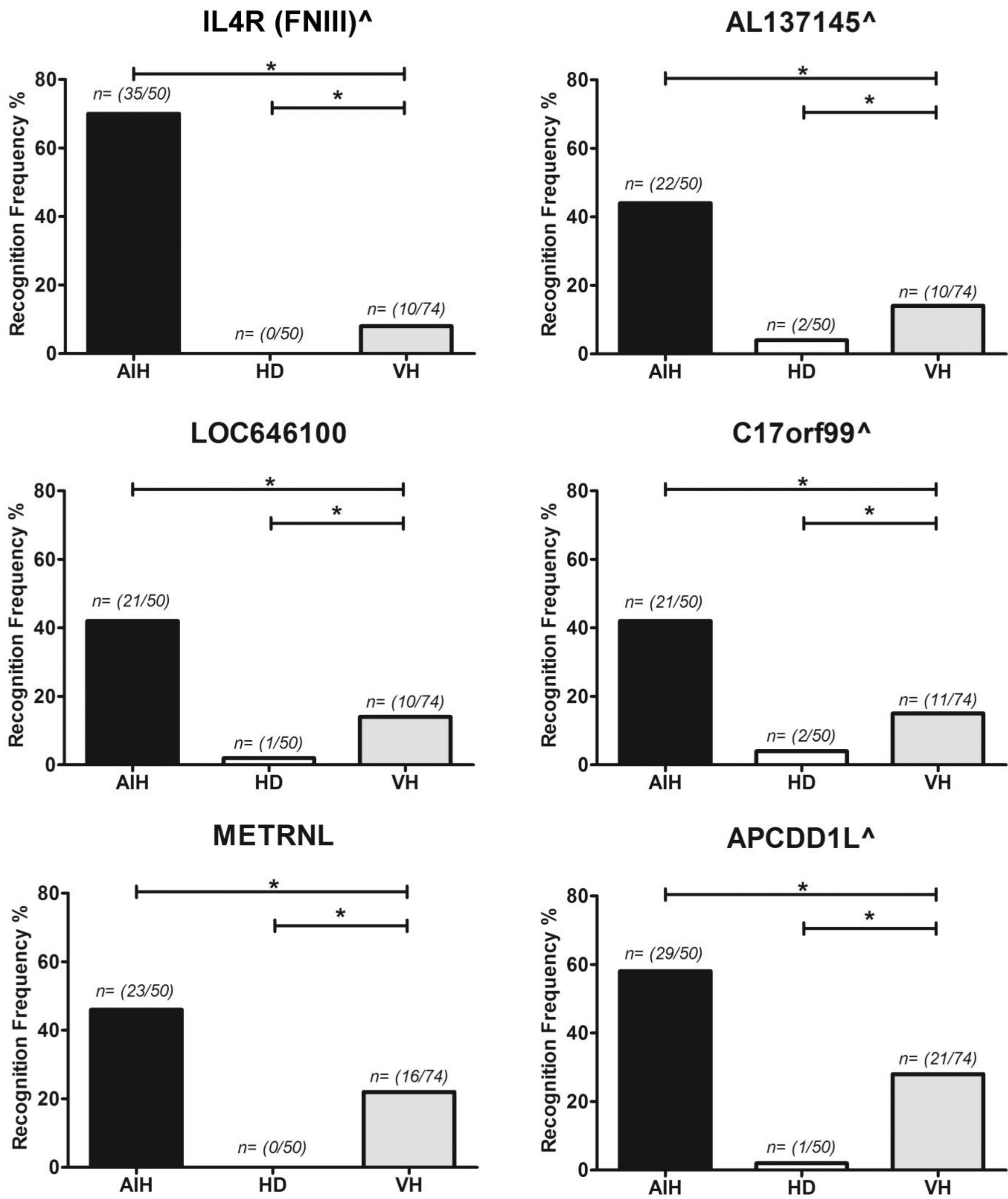
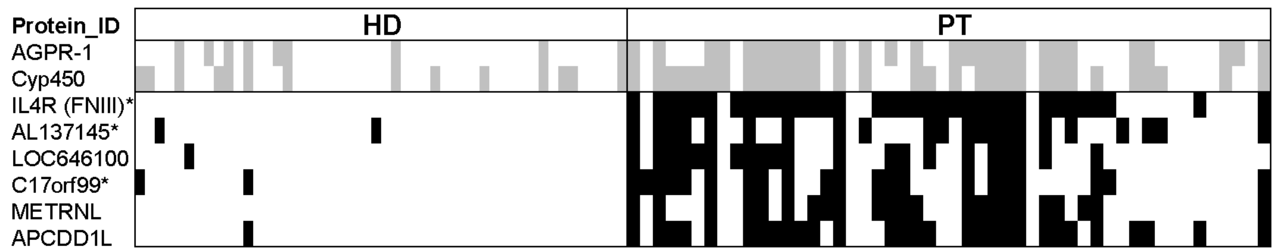


FIGURE 4

A



B

	PT (*n= 50*)	HD (*n= 50*)
Positive	**41**	**4**
Negative	**9**	**46**
SE | SP	82%	92%
C.I.	(0.686 -0.914)	(0.807-0.978)
	P< 0.001	

C

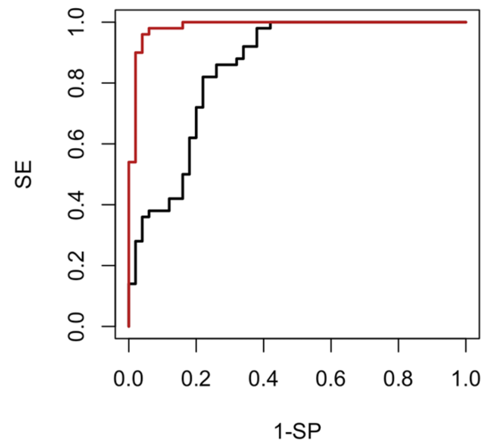


FIGURE 5

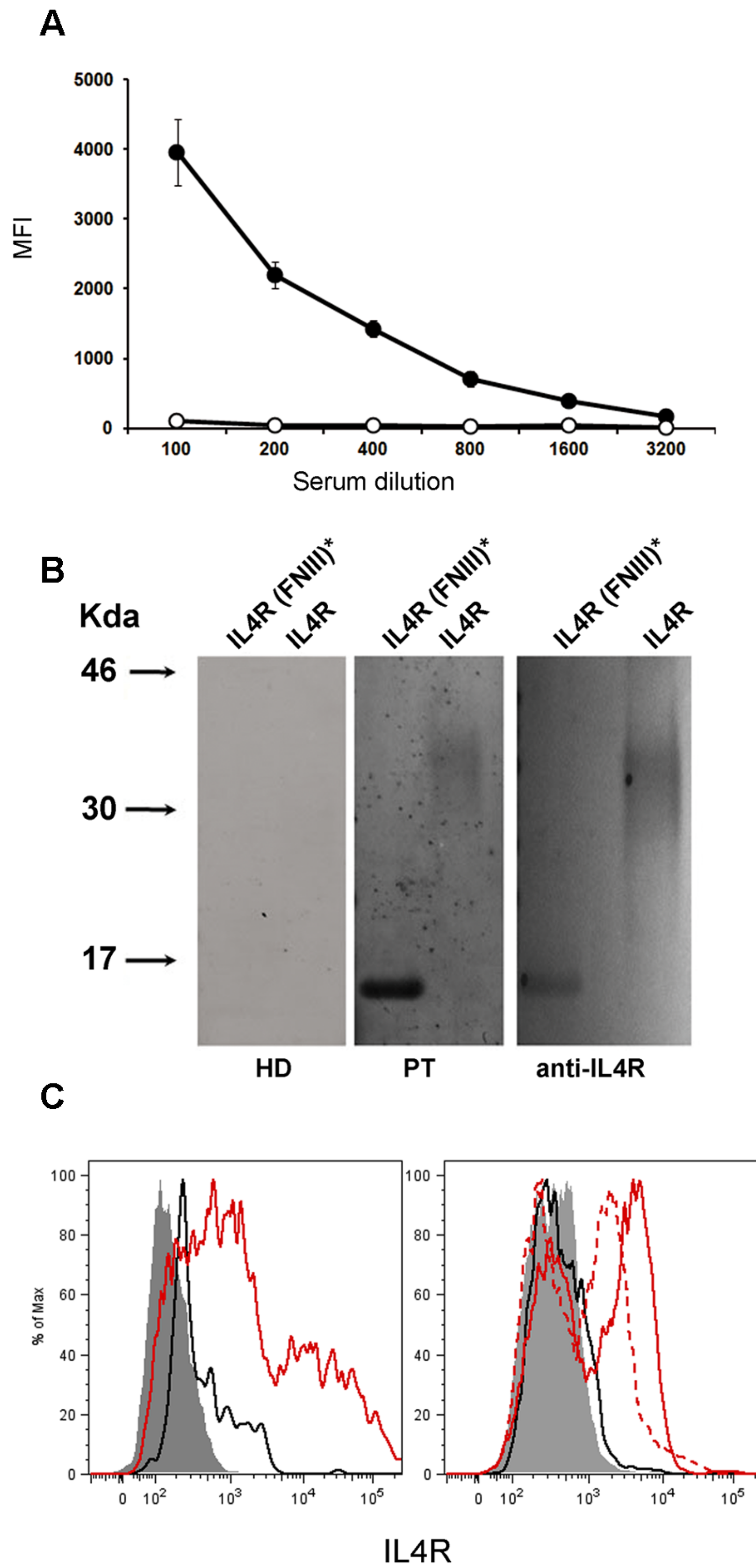


FIGURE 6