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Systematic identification of novel SLE related autoantibodies responsible for type I IFN production in human plasmacytoid dendritic cells

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ABSTRACT

Plasmacytoid dendritic cells [pDC], also known as type I interferon [IFN] producing cells, play a significant role in the pathogenesis of systemic lupus erythematosus [SLE]. The current study was undertaken to identify novel SLE autoantibody specificities associated with interferon-inducing activity in human pDCs. We found that immune complex mixtures from some Interferon signature negative [IFN−] and all interferon signature positive [IFN+] SLE patients could trigger type I IFN production by pDCs. IgGs from IFN− and IFN+ SLE patients were subsequently screened via a high throughput protein microarray to identify novel auto-antibody specifities that mediate type I IFN production by pDCs. This approach identified five novel autoantibodies that may contribute to type I IFN production by pDCs via a nucleic acid dependent mechanism. The newly identified autoantibody specificities function in a myriad of cell processess and, to date, have not been implicated in SLE pathogenesis.

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1. Introduction

Systemic lupus erythematosus [SLE] is a heterogeneous disease with diverse autoimmune manifestations affecting the hematologic, dermatologic, musculoskeletal, and renal organ systems. Many lines of evidence support the notion that type I interferon [IFN] may be a primary pathogenic factor in SLE: Genetic polymorphisms associated with type I interferon pathways, such as those found in the interferon regulatory factor 5 [IRF5] gene, are linked with susceptibility to SLE [1]. Treatment with recombinant IFNα can lead to the development of autoantibodies and manifestations of clinical features of SLE in non-autoimmune patients [2,3]. In addition, elevated IFNα levels and type I interferon activity are associated with greater disease activity in SLE [4,5]. *Ex vivo*, it has been shown that SLE serum can induce human monocytes to differentiate into DCs and that these DCs capture antigens from dying cells and present them to CD4-positive T cells. The capacity of SLE patients' serum to induce DC differentiation correlated with disease activity and depended on the actions of interferon-alpha [IFN-α] [6]. In human DC cultures, it has been shown that IFN-α upregulates expression of B-cell activating factor [BAFF] [7], whereas treatment of SLE patients with an anti-IFN-α mAb downregulates BAFF expression [8].

The above listed clinical observations in humans are supported by data that show a key role for type I interferon in some animal models of SLE. Autoimmune-predisposed mice deficient in the IFNα/β receptor have significantly reduced levels of anti-erythrocyte autoantibodies, hemolytic anemia, anti-DNA autoantibodies, kidney disease, and mortality [9]. Moreover, as in humans, IFNα can induce glomerulonephritis in normal mice and accelerate the onset of the spontaneous autoimmune disease of NZB/W mice [10]. Mouse studies have also shown in both SLE-prone BWF1 mice and non-autoimmune-prone BALB/c mice, circulating BAFF levels increase following IFN-α administration [11].

Type I interferon is the most activated signaling pathway and type I interferon-inducible mRNAs are the most overexpressed mRNAs in whole blood of patients with SLE [12]. Several research groups have confirmed that nearly half of lupus patients display a type I IFN signature and that this gene expression pattern correlates with more severe disease involving the kidneys, hematopoetic cells, and the central nervous system [13,14]. Expression of type I interferon-inducible IP-10/CXCL10 mRNAs is increased in SLE patients with active central nervous system symptoms [12]. Selected type I interferon-inducible mRNAs [IFI44, IFI27, and IFI44L] and type I interferon-inducible proteins [IFI27, STAT1] are overexpressed in the synovium of patients with SLE-related arthritic manifestations [15].

Increased expression of type I IFN regulated genes in PBMCs collected from SLE donors has been associated with specific clinical manifestations, including the presence of anti-Ro, anti-Sm, anti-RNP, and anti-double stranded DNA [anti-dsDNA] antibodies

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[16–18]. A landmark study by Arbuckle et al. revealed that these autoantibodies are present many years before the onset of clinical symptoms in SLE [19]. Reports suggest a mechanistic link between autoantibodies and type I IFN production [20,21]. Specifically, it is believed that autoantibodies bind to self-antigens that are released by apoptotic or necrotic cells and these antigen-antibody immune complexes [ICs] are endocytosed by leukocytes via an FcγRIIa mechanism, wherein they agonize endosomal toll-like receptors and trigger type I IFN production. Immature plasmayctoid dendritic cells [pDCs] are thought to be the main source of continuous type I IFN production in SLE *in vivo* [22,23].

Despite the mechanistic relationship between autoantibodies and type I IFN, most of the autoantibodies identified to date have not been shown to play a pathogenic role in SLE, correlate with disease activity or decrease following high-dose immunosuppressive treatment [24]. Moreover, several studies have shown that SLE-associated autoantibodies are not sufficient to result in high circulating levels of type I IFN in humans [25]. Thus there remains a significant need to identify novel autoantibody biomarkers that correlate with the type I IFN signature and SLE disease activity.

Previous studies have demonstrated that ICs can be generated *in vitro* by combining necrotic or apoptotic cellular material with IgG from SLE donors and that this mixture is capable of inducing type I IFN production in normal healthy human peripheral blood mononuclear cells [PBMCs] and pDCs [22]. In the present study, we generated ICs using IgG from both IFN signature negative and IFN signature positive SLE donors. We found that, in most cases, ICs from both patient populations were able to trigger type I IFN production by pDCs and PBMCs *ex vivo*. A high throughput protein microarray subsequently identified the autoantibodies that may contribute to the observed interferogenic acitvity of the SLE IC mixtures. Using this approach, we identified five novel autoantibodies that may play a role in type I IFN production by pDCs and that may be useful as prognostic biomarkers for SLE.

2. Materials and methods

2.1. Plasma, sera, and preparation of IgG and type I IFN inducers

Plasma and PBMCs from 11 SLE patients with active disease was obtained from a variety of commercial research organizations, including Astarte Biologics [Redmond, WA], Bioreclamation [Hicksville, NY] and used for preparation of IgG. The IgG was purified on a protein A/G column [Sigma–Aldrich] according to the manufacturer's instructions. Plasma from normal healthy donors was used for preparation of normal IgG. To create lysates for IC formation,HEK293T cells were suspended at 5×10^7 cells/ml in 1× phosphate buffered saline [PBS]. Freeze–thawing was performed in 4 cycles of at least 10 min freezing at −80 °C and thawing at 37 °C, except for an initial freezing of at least 30 min; after the freeze–thaw, cell debris was removed by centrifugation [400 g for 5 min] and used in culture at a final concentration of 20% of total culture volume per well of a 96-well u bottom plate. Type I IFN inducers were generated as previously described [22]. IgG was used as at a concentration of 1 mg/ml.

It should be noted that there is a precedent in the literature for using necrotic cell lysates from cell lines. In the original report by Lövgren et al. [22] the authors describe the generation of immune complexes (ICs) using necrotic U937 cell material combined with SLE IgG. There is no universally agreed upon standard for the source of necrotic cell lysates in the generation of ICs. The necrotic cell lysate serves as a source of natural human nucleic acids; there may be a slight bias within the RNA compartment, as different cell types are expected to have different transcriptional profiles. No bias is expected within the DNA compartment. Because we were screening for novel nucleic acid binding proteins in SLE IgG, we wanted to minimize the number of variables in the generation of ICs in our studies. Therefore, we used a constant source of necrotic cell lysates (HEK293T) cells. The necrotic HEK293T cells alone had no interferon inducing activity (data not shown). Addition of SLE IgG conferred interferon-inducing activity to the necrotic cell lysate suggesting that the primary interfrogenic component of ICs lays within the SLE IgG and not the necrotic cell lysate.

The TLR agonists, CpG and Gardiquimod, were purchased from InvivoGen [San Diego, CA] and used at a concentration of 5 μg/ml. For the antigen specific IC mixtures, monoclonal antibody at a concentration of 1 μg/ml was combined with cell culture medium containing 40% necrotic cell supernantant and added in 0.1-ml volumes in 96-well round bottom plates. The following azide free monoclonal antibodies were purchased from Novus Biologicals [Littelton, CO]: SMN antibody [clone 2B1], STAM antibody [clone 2B11-1G1], SSH3 antibody [clone 6F9], SEC24C antibody [clone 1A8], Hsp27 antibody [catalog #NBP1-05013], TIAF1 antibody [catalog #NBP1-04999], CDC37 [Clone 3C7], GCET antibody [clone 1E9], PIM1 [clone 3F5],

2.2. Preparation and culture of pDCs and PBMCs

PBMCs were prepared by Ficoll-Hypaque [GE Healthcare] density gradient centrifugation of buffy coats from healthy donor leukopak preparations [All Cells, Emeryville, CA]. Enrichment of blood dendritic cell antigen 4 [BDCA-4]-positive cells was done using a negative selection strategy via a Plasmacytoid Dendritic Cell Isolation Kit [Miltenyi Biotec, Sunnyvale, CA catalog #130-092-207]. Purity was analyzed by staining with anti-BDCA2 and anti-CD45 monoclonal antibodies, followed by FACS LSRII flow cytometry. A purity of >85% was regularly achieved using this method. SLE PBMCs were isolated using a Vacutainer CPT Cell Preparation tube [BD, catalog #362761]. PBMCs and pDCs were cultured at a density of 2×10^6 - cells/ml and 1×10^6 cells/ml, respectively, in 0.1-ml volumes in 96-well round bottom plates using RPMI, GlutaMAX, medium [Invitrogen] supplemented with HEPES [20 mM], penicillin [60 μg/ml], streptomycin [100 μg/ml], and 10% fetal calf serum [FCS].

2.3. Type I IFN signature and immune activation expression profiling using TaqMan low density array [LDA]

We determined the IFN score using an approach similar to that used by Kirou et al. [17] with slight modifications. Specifically, gene expression analysis was performed using TaqMan immune-profiling/inflammation low-density arrays [Applied Biosystems, Product Number 4322171] or a custom made LDA card containing primers and probes for the following IFIGs including [SEPRING1, IFI27, IFI44, IFI44L, IFIT1, ISG15, MX1, OASL, RSAD2, LY6E, PLSCR1, POLR1C, IFI6, IFIT2, IFIT3, OAS1, OAS2, OAS3, EPSTI1, USP18, HERC5, and RTP4]. Glyceraldehyde-3-phosphate dehydrogenase [GAPDH] was selected as the endogenous control. Real time RT-PCR amplification was performed on an ABI Prism® 7900 H.T. Sequence Detection system [Applied Biosystems, CA, USA] as per manufacturer's instructions. Expression levels for each of the aforementioned IFN related genes was used to calculate the IFNα scores for each of the individual donors as previously described [17]. Briefly, the mean and SD level of each interferon induced gene (IFIG) in the healthy donor group ($mean_{HD}$ and SD_{HD}, respectively) was used to calculate the expression score of each gene for each study subject, defined as the SD_{HD} value above the $mean_{HD}$ value. Cumulative IFNα scores, representing the sum of the scores for each of the 22 genes were derived for each subject. To implement identification of SLE patients with or without activation of the type I IFN pathway, we classified individuals as IFN signature positive if they fulfilled the following criteria: (1) overexpression of at least