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Novel molecular signatures in mononuclear cell populations from patients with systemic lupus erythematosus

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ABSTRACT

To gain novel insights into the immunopathogenesis of systemic lupus erythematosus we have analyzed gene expression data from isolated $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD56^+$ NK-cell enriched peripheral blood cell fractions from patients and healthy donors. As predicted, type I interferon-inducible gene transcripts are overexpressed in all populations. Transcripts preferentially expressed in SLE $CD4^+$ and $CD8^+$ T cells include those associated with Tregulatory and Th17 effector cell programs, respectively, but in each case additional transcripts predicted to limit differentiation of those effector cells are detected. Evidence for involvement of the Wnt/β-catenin pathway was observed in both B and T cell fractions, and novel transcripts were identified in each cell population. These data point to disrupted T effector cell differentiation and the Wnt/β-catenin pathway as contributors to immune dysfunction in SLE while further supporting a central role for the type I interferon pathway in lupus.

1. Introduction

Systemic lupus erythematosus (SLE) is often considered the prototypic systemic autoimmune disease, in part due to its multisystem clinical manifestations. From the perspective of those studying disease mechanisms and pathophysiology, lupus provides lessons relevant to many diseases based in the immune system. Virtually all immune system cells and many soluble mediators contribute to development of the self-directed autoimmunity and inflammation that result in the systemic manifestations of disease, including its debilitating fatigue, cognitive dysfunction and arthralgias, and organ-targeted tissue damage, including cutaneous lesions, nephritis, serositis, vasculopathy and cardiovascular disease. In view of the protean immunologic alterations that characterize the disease, peripheral blood provides an informative window into characterization of the cellular and molecular basis of the specific immune alterations that contribute to lupus pathogenesis [1]. However, study of peripheral blood mononuclear cells or whole blood can mask significant signals that might be revealed through study of isolated cell populations.

Investigation of peripheral blood cells from lupus patients has documented a broad signature of type I interferon (IFN-I)-inducible gene (IFIG) transcripts, an observation that is supported by additional data at the tissue level [2–6]. Gene expression analysis, along with confirmatory studies from murine lupus models and more recently from clinical trials of agents targeting the IFN-I pathway, points to the IFN-Is as central pathogenic mediators in SLE [7,8]. IFNα is largely responsible for the IFN-I signature detected in peripheral blood, and additional IFN-Is, such as IFNβ and IFNω, may be produced in tissue and contribute to activation of the IFN pathway. Extensive investigation of murine models of virus infection and in vitro studies have defined many of the immunomodulatory properties of IFN-I [9–13]. The sustained production of IFN over time in many lupus patients and its association with disease activity in some patients suggest that the inducers, signaling pathways and genes regulated by this cytokine family could be therapeutic targets [14]. Well documented is the contribution of nucleic acid-containing immune complexes that drive induction of IFN-I after accessing endosomal Toll-like receptors (TLR) [15]. Mutations in enzymes that degrade cytoplasmic nucleic acids, receptors that sense those nucleic acids or components of the signaling pathways activated by those receptors are rare among those diagnosed with lupus, but the elevated IFN-I associated with those mutations suggests the potential for cytoplasmic nucleic acids to play a role in lupus more generally [16].

The strong and dominant IFN-I signature in SLE blood cells has been confirmed in many studies, and additional molecular signatures, particularly those reflecting granulocyte- and plasmablast-derived transcripts, have been described in unfractionated blood cells [4,17–19]. More limited data are available that identify additional gene signatures or suggest novel functions of cell populations based on study of mononuclear cell fractions [20–26]. For example, a study of CD8 T cells isolated from lupus patients described a transcript profile that was associated

Abbreviations: DEG, differentially expressed genes; IFN-I, type I interferon; IFIG, interferon-induced gene; SLE, systemic lupus erythematosus.

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with a relatively benign course and was described as consistent with an "exhausted" phenotype previously studied in murine models of virus infection [20,25]. None of those studies has analyzed data from $CD8^+$ T cells and B cell fractions in relation to each other and described potentially relevant molecular pathways revealed through their gene expression profiles. In order to gain new insight into underlying mechanisms of immune dysfunction that contribute to lupus pathogenesis we have characterized those gene transcripts differentially expressed between patients with SLE and healthy donors (HD) in highly purified $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells, as well as in $CD56^+$ NK cell-enriched fractions and unfractionated PBMC. Data have been analyzed with the goal of identifying novel transcripts that are not broadly expressed across all cell populations but rather are characteristic of one or two of the cell populations. Transcripts identified provide new insights into the molecular pathways and immune system alterations that characterize patients with SLE and suggest potential avenues for study as well as candidate therapeutic targets.

2. Materials and methods

2.1. Study subjects

The clinical diagnosis of SLE was established for all 15 SLE patients who participated in the study. Ten HD subjects were also enrolled. All subjects were female. General characteristics of SLE patients are summarized in Table 1. Patients who had received high dose pulse glucocorticoid therapy, intravenous cyclophosphamide or rituximab therapy in the prior 6 months were excluded from participation in the study. The number of classification criteria for diagnosis of SLE, disease activity based on the SLEDAI 2000 score and SELENA flare status were recorded. The study was approved by the Institutional Review Board of Hospital for Special Surgery and all subjects agreed to participation in the study and signed the approved consent form.

2.2. Biologic sample collection

Blood samples from HD ($n = 10$) and SLE patients ($n = 15$) were processed within 30 min of phlebotomy. The blood composition was determined immediately using an Advia 120 automatic haemocytometer. PBMC were purified using Ficoll-Paque™Plus (GE Healthcare Life Sciences, Piscataway, NJ) gradient centrifugation and preserved in RNeasy lysis buffer (QIAGEN, Inc., Valencia, CA). Samples were stored at −70 °C until RNA extraction.

Table 1
SLE study subjects.

Patient code	Age	RACE	Disease duration (yr)	ACR-score	SLEDAI-2000	Flare SELENA flare
S01	41	Black	4	5	0	0
S02	54	Caucasian	7	4	6	0
S03	24	Caucasian	1	5	2	0
S04	34	Caucasian	1	5	2	0
S05	50	Caucasian	N/A	5	2	0
S06	25	Black	1	5	2	Mild/Moderate
S07	39	Caucasian	16	5	4	0
S08	27	Caucasian	11	7	4	0
S09	57	Black	7	4	0	0
S10	22	Asian	12	8	4	0
S11	28	Caucasian	1	7	4	0
S12	43	Black	15	4	8	Mild/Moderate
S13	54	Caucasian	12	4	6	Mild/Moderate
S14	21	Caucasian	3	4	4	0
S15	20	Caucasian	3	8	8?	Severe

2.3. Positive selection of $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ fractions

$CD4^+$, $CD8^+$, and $CD19^+$ fractions were obtained using MACS Whole blood MicroBeads from Miltenyi (CD4: cat # 130-090-877, CD8: cat # 130-090-878, CD19: cat # 130-090-880) according to the manufacturer's instructions. Unbound fractions after $CD4^+$ and $CD19^+$ positive selection were used to enrich $CD56^+$ NK cells. The unbound fractions were first purified and concentrated using Ficoll-Paque gradient centrifugation. Positive selection was then performed using CD56 Microbeads Human (cat #130-050-401) according to manufacturer's instructions. The cellular composition of each fraction was analyzed by flow cytometry. An equal number of cells was used for RNA isolation from each of the fractions. RNA was extracted simultaneously from all samples using the Trizol (Invitrogen) protocol, quantified and after quality control assessment using the Agilent 2100 Bioanalyzer was used for the microarray hybridization protocol.

2.4. Flow cytometry

The following antibodies were used for flow cytometry: CD14 FITC cat #11-0149-42, CD20 eFluor450 cat #48-0209-42, CD38 FITC cat #11-0389-42, CD27 APC cat #17-0279-42, CD45 PE-eFluor710 cat #46-0459-42, CD56 APC cat #17-0567-42 from eBioscience; CD4 APC cat #130-091-232, and CD8a FITC cat #130-080-601 from Miltenyi. Prior to labeling the samples were incubated with the FcγR binding inhibitor (#14-9161 eBioscience) to reduce non-specific binding of mouse monoclonal antibodies used for flow cytometric analysis. PBMC samples or mononuclear cells obtained by magnetic positive selection were labeled with antibodies conjugated with different fluorochromes. Before analysis 7-amino-actinomycin D was added to exclude dead cells (BD Bioscience). To identify monocytes, side scatter properties were incorporated with cell staining to assign the percent positive. The fluorescence was acquired with a FACSCanto II flow cytometer and analyzed with the FlowJo software. Purity of the isolated cell fractions is shown in Supplemental Table 1.

2.5. RNA isolation, amplification, and hybridization

Fifty nanograms of total RNA were used to prepare targets with the Two-Cycle Target labeling kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions and hybridized onto Human Genome U133 Plus 2.0 GeneChips® (Affymetrix) at 45 °C overnight. Chips were scanned in a GeneChip® scanner 3000 (Affymetrix). Data from the arrays (CEL files) were uploaded to GeneSpring GX11 software (Agilent Technologies, Santa Clara, CA) and processed using Affymetrix Power Tools software. The Robust Multi-array Analysis (RMA) algorithm was used for sample normalization and linear transformation. Only probe-sets with a detection p-value < 0.05 in >90% of the samples per group were included in the subsequent analysis. The major variation in the signals was associated with the various cell fractions based on principal component analysis, and the effect of disease was less important. As all samples were obtained from female donors of different demographics and clinical manifestations we did not observe any other major variability from our samples.

2.6. Data analysis

Transcripts differentially expressed in SLE blood for $CD4^+$, $CD8^+$ and $CD19^+$ fractions, containing relatively pure populations of Thelper (Th), Tcytotoxic (Tc) and B cells, as well as in PBMC were identified using the bioconductor R package limma. The limma package can handle multilevel experimental design with the estimates for correlation between measurements made on the same subject (random effect). The fold-change cutoff value of 2 and false detection rate corrected p value of <0.05 has been chosen as a cut-off for selecting differentially expressed transcripts.