



Deciphering the Molecular Mechanism of Flares in Patients with Systemic Lupus Erythematosus through Single-Cell Transcriptome Analysis of the Peripheral Blood

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ABSTRACT

A remarkable, yet poorly explained feature of Systemic Lupus Erythematosus (SLE) is the propensity to flare following a preceding period of disease inactivity. The clinical burden of lupus flares is substantial since they often tend to involve multiple or major organs, and carry a near two-fold increased risk for accrual of irreversible organ damage. The cellular and molecular mechanisms underlying the progression of SLE from inactive to active state remain ill-defined. Application of novel sequencing technologies together with cellular immunology assays, have illustrated the important role of multiple types of both innate and adaptive cells and associated pathways. We have previously described significant differences in the blood transcriptome of SLE patients at active versus inactive disease, and we have also defined genome regions (domains) with co-ordinated expression of genes implicated in the disease. In the present study, we aim to decipher the cellular and molecular basis of SLE exacerbations by utilising novel single-cell sequencing approaches, which allow us to characterise the transcriptional and epigenetic landscapes of thousands of cells in the peripheral blood of patients. The significance of the study lies in the detailed characterisation of the molecular and regulatory program of immune cell subpopulations that underlie progression from inactive to active SLE. Accordingly, our results may be exploited to identify biomarkers for disease monitoring and novel therapeutic targets.

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BACKGROUND AND STUDY RATIONALE

One of the intriguing characteristics of Systemic Lupus Erythematosus (SLE) is its tendency for increases in disease activity, also known as flares, which are often unpredictable. Indeed, in various patient cohorts, the frequency of SLE

flares (or relapses) is approximately 0.30–0.50 per patient-year, and despite available treatments, long-term disease quiescence occurs only in a small fraction of patients.¹ The clinical burden of lupus flares is remarkable, since about 30–40% of exacerbations affect multiple or major organs (for instance, kidneys),

they necessitate treatment escalation or switch (including administration of high-dose glucocorticoids or other potent immunosuppressives), and are linked to almost 2-fold increased risk for irreversible organ damage.^{2,3} Importantly, treatment of SLE relapses remains empirical due to lack of randomised evidence and personalised options based on the underlying pathophysiology.

The cellular and molecular mechanisms implicated in SLE re-activation following a previous period of quiescence remain elusive.⁴ During recent decades, the advent of next-generation genome sequencing technologies coupled with focused cellular immunology studies, have provided unique insights regarding the role of specific cell types of both innate (eg, neutrophils) and adaptive (eg, follicular helper T-cells, long-lived plasmablasts) immunity as well as of pertinent pathways (eg, interferon-alpha, autoantibodies) in SLE pathogenesis.⁵⁻¹⁰

To this end, the gene expression and functional state of cells may be altered under the effect of exogenous or other (for instance, inflammatory cytokines) factors through epigenetic changes including histone and chromatin modifications.^{11,12} In fact, changes in the epigenetic and transcriptional program of tumour-surveilling immune cells have been linked to resistance to chemotherapy and progression of malignant disease.^{13,14}

In a previous work using RNA-sequencing (RNA-seq), we identified widespread perturbations in gene expression in the peripheral blood of active SLE patients as compared to their counterparts at remission or low disease activity state.¹⁵ A total of 693 genes showed differential expression in patients with active versus inactive or low disease activity, and these genes were enriched in pathways such as type I interferon, proteasome, and oxidative phosphorylation. Further analysis revealed that genomes of SLE patients are organised into distinct regions of co-expressed genes (known as *Domains of Coordinated gene Expression [DCEs]*), which seem to regulate important pathways, such as interferon.¹⁶ These DCEs varied according to the level of SLE activity and correlated with changes in chromatin accessibility. Notably, the genomes of patients at low disease activity or remission displayed co-expression of genes implicated in kidney disease and neutrophil activation, which suggests the persistence of genomic aberrations contributing to disease flare-up.

Furthermore, other research groups have described alterations in chromatin accessibility and/or activation of gene enhancers in SLE patient-derived monocytes,^{17,18} neutrophils¹⁹ and B-cells.^{20,21} Altogether, these data underscore a possible role of the chromatin environment and epigenetic factors on determining the transcriptional program and function of immune cells contributing to lupus. Still, the time-dynamics of the aforementioned molecular cues and the interaction between various immune cell types have not been studied in the context of SLE transition from inactive to active state.

AIMS OF THE STUDY

In this research study, we aim to investigate the cellular and molecular basis of flares in patients with SLE. Considering the complexity of the disease, we will utilise state-of-the-art single-cell sequencing technologies to obtain a detailed map of the transcriptional and epigenetic profiles in multiple subtypes of peripheral blood immune cells. Through a prospective biosampling protocol, we plan to simultaneously characterise gene expression and chromatin accessibility at single-cell resolution in the peripheral blood mononuclear cells (at least 3,000 to 5,000 cells per patient sample) from two (2) SLE patients assayed at three (3) consecutive time points: disease remission, flare and post-flare (after treatment modification). Our hypothesis is that specific epigenetic modifications (chromatin accessibility) direct the molecular reprogramming of specific immune cells thus contributing to SLE relapse.

METHODS

This is a prospective, non-interventional, clinical-translational study with the following design and implementation plan.

Patient recruitment

Patients diagnosed with SLE will be screened consecutively and recruited from the Connective Tissue Disease outpatient clinic of the Rheumatology Department, University Hospital of Heraklion, Greece. Inclusion criteria will include: a) fulfilment of the 2019 EULAR/ACR (European Alliance of Associations for Rheumatology / American College of Rheumatology) classification criteria,²² b) age 18–40 years, c) history of positive anti-dsDNA autoantibodies, d) relapsing-remitting disease pattern,²³ and e) low disease activity or remission during the past 3 months according to the LLDAS²⁴ and DORIS²⁵ definitions, respectively. Exclusion criteria will include: a) coexistence of other systemic autoimmune or inflammatory disease, b) pregnancy or planning for pregnancy, c) chronic infection, d) history of malignancy, e) treatment with cyclophosphamide during the previous 3 months or with rituximab during the previous 6 months. The study will receive approval by the Institutional Review Boards of the University Hospital of Heraklion and the University of Crete, and all participants will provide informed consent form.

Sample size

Considering that single-cell technologies enable the profiling of thousands of individual cells (up to 10,000 cells per sample) from peripheral blood samples, a relatively small number of patients is adequate. Since this is a pilot study, only two (2) patients will be analysed, each patient sampled on three consecutive time points: during remission or low disease activity, at the time of flare, 3 months post-flare and after treatment administration.

Patient samples of each time point will be combined and profiled in a separate 10x Multiome library. To identify two eligible patients, a total 20 SLE patients who meet the aforementioned inclusion criteria will be monitored clinically over a period of 12 months.

Clinical monitoring and bio-sampling protocol

Enrolled patients will be monitored every 3-4 months for a total period of 12 months. On each visit, routine laboratory, serological (C3/C4, anti-dsDNA) and urine tests will be performed, and disease activity will be quantified by the validated indices: a) SLE Disease Activity Index-2000 (SLEDAI-2K)²⁶ and b) Physician Global Assessment (PhGA; scale 0–3).²⁷ For the detection and quantification of flares, the SELENA-SLEDAI Flare Index (SFI) will be employed,²⁷ following minor modifications as outlined in **Table 1**. Patient history, physical examination and any relevant laboratory or imaging tests will be considered to exclude any flare-mimics such as infection or drug adverse effect. Administered medications and dosages will be recorded.

Upon inclusion to the study, all patients will donate a first blood sample (15 ml) obtained by venipuncture (low disease activity/remission stage). During follow-up, a second blood sample will be obtained from two SLE patients who will develop moderate or severe flare according to the SFI (flare stage). Those patients will be managed for active disease at the discretion of the treating physician and in line with the EULAR recommendations.²⁸ Three months after therapeutic intervention of the flare, a third blood sample will be collected (post-flare stage).

Isolation of peripheral blood mononuclear cells and single-cell RNA-seq/ATAC-seq

We will assay blood as a relevant and easily accessible tissue to define complex inflammatory signatures.^{29,30} Venous samples (2 patients x 3 time-points, 6 in total) will undergo Ficoll density centrifugation for isolation of peripheral blood mononuclear cells (PBMCs). In SLE, the PBMCs fraction also includes circulating neutrophils,³¹ which are relevant to disease pathogenesis. Single-cell suspensions will be prepared on microplates through the robotic Fluidigm C1 system, followed by purification of RNA and chromatin from the cell nuclei.³² Genomic material will be processed for library synthesis (DNA for chromatin, cDNA for gene expression) and analysis by the chromium-based (10x) single-cell multiome ATAC (Assay for Transposase-Accessible Chromatin) & gene expression.³²⁻³⁴ Next-generation sequencing will be carried out at the Genomic Facility, IMBB-FORTH.

Bioinformatics and data analysis

We will create a single-cell transcriptomic and epigenomic atlas followed by modularity optimisation to detect immune cell clusters across patient groups and

Table 1. Modified SELENA-SLEDAI Flare Index.²⁷

Moderate flare	Severe flare
Increase in SLEDAI by ≥ 3 points (but not to >12)	Increase in SLEDAI to >12 points
New or worse: discoid, photosensitive, profundus, cutaneous vasculitis or bullous lupus rash, nasopharyngeal ulcers, serositis, arthritis, fever	New/worse: neurological lupus, vasculitis, nephritis, myositis, platelet $<60.000/\mu\text{L}$, haemolytic anaemia (Hb <7 g/L or \downarrow Hb > 3 g/L)
Increase or added prednisone, but to a dose <30 mg/day	Increase or added prednisone to a dose ≥ 30 mg/day, or pulses of intravenous methylprednisolone
Added NSAID or antimalarials (for SLE activity)	Added cyclophosphamide, azathioprine, methotrexate or mycophenolate, or new biological drugs (rituximab, belimumab) for SLE activity, or hospitalization
Increase in PhGA by ≥ 1 (but not to >2.5)	Increase in PhGA to >2.5

SLEDAI, SLE disease activity index; NSAID, non-steroid anti-inflammatory drugs; PhGA, physician global assessment; Hb, haemoglobin.

time-points.^{35,36} Differential gene expression between pertinent immune cell clusters, coupled with functional enrichment analysis, will characterise the cellular states and dedicated transcriptional programs predisposing to flares. Chromatin accessibility data will help to disentangle the regulatory programs governing the transition to active SLE, guided by the cell-to-cell linkage between modalities (scRNA-seq, scATAC-seq) and the cell-state annotations of the transcriptome atlas. Using the scATAC-seq data, we will characterise our immune cell clusters, and determine high resolution cell sub-clusters with unique chromatin accessibility profiles. Open chromatin regions (OCRs) will be subjected to functional enrichment analysis (LOLA, GREAT tools) to infer their putative regulatory roles.^{37,38} Also, mapping of transcription factor (TF) binding sites (TFBS) motifs³⁹ will reveal enrichment of specific TFs involved in the acquisition of a primed state of immune cells. Correlation between gene expression, TF expression and motif accessibility will identify flares-predisposing Gene Regulatory Networks

(TF-to-gene promoter/enhancer relationships). Finally, reconstruction of immune cell lineage differentiation and cell maturation trajectories will delineate the cellular dynamics and underlying states during SLE re-activation.^{35,40} Collectively, our analysis will decipher how gene regulatory reprogramming in immune cells triggers flare initiation. We will also test the reversibility of these molecular/regulatory programs in the post-flare samples.

ANTICIPATED RESULTS AND PROJECT SIGNIFICANCE

Patients with SLE often manifest flares (relapses) of their disease, which contribute to substantial clinical, financial,⁴¹ and societal burden and tend to trigger treatment intensification including administration of glucocorticoids. The cellular and molecular mechanisms underlying transition of SLE from inactive to active state remain incompletely understood. In view of the biological complexity of the disease, we hereby propose the utilisation of novel, high-resolution genomic technologies which enable the simultaneous profiling of gene expression and chromatin state (accessibility) in circulating blood cells of the lupus patients both during disease quiescence, flare-up, and following introduction of therapy. Through this approach, a detailed atlas of the genome will be obtained in thousands of cells assayed across consecutive time points. We will be able to characterise specific immune cell subpopulations that are altered in terms of frequency and/or functional state during SLE flare. In addition, chromatin analysis will provide novel insights into regulatory/epigenetic drivers of perturbed transcriptomes associated with disease re-activation. Altogether, our analysis could uncover novel and biologically-relevant biomarkers for disease remission and exacerbation, as well as putative targetable genes and pathways for flare prevention and treatment.

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CONFLICT OF INTEREST

The declare no conflict of interest.

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