Journal of Autoimmunity 81 (2017) 74–89

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Mass cytometry identifies a distinct monocyte cytokine signature shared by clinically heterogeneous pediatric SLE patients

W.E. O'Gorman, Ph.D. [a, 1, 3], D.S. Kong [b, 3], I.M. Balboni, M.D, Ph.D. [c], P. Rudra, Ph.D. [d], C.R. Bolen, Ph.D. [b, 2], D. Ghosh, Ph.D. [d], M.M. Davis, Ph.D. [a, e, f], G.P. Nolan, Ph.D. [a, e], E.W.Y. Hsieh, M.D. [a, b, c, g, *]

[a] Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA
[b] Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, CO 80045, USA
[c] Department of Pediatrics, Division of Allergy, Immunology and Rheumatology, Stanford University School of Medicine, Stanford, CA 94305, USA
[d] Department of Biostatistics and Informatics, Colorado School of Public Health, Aurora, CO 80045, USA
[e] Institute for Immunity, Transplantation and Infection, Stanford University, Stanford, CA 94305, USA
[f] The Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA
[g] Department of Pediatrics, Division of Allergy and Immunology, University of Colorado School of Medicine, Aurora, CO 80045, USA

ARTICLE INFO

Article history:
Received 30 January 2017
Received in revised form
22 March 2017
Accepted 22 March 2017
Available online 21 April 2017

Keywords:
Pediatric systemic lupus erythematosus
Mass cytometry
Monocytes
Cytokines
MCP1
Plasma circulating factors
Type I interferons
JAK inhibition
Ruxolitinib

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a heterogeneous autoimmune disease with heightened disease severity in children. The incomplete understanding of the precise cellular and molecular events that drive disease activity pose a significant hurdle to the development of targeted therapeutic agents. Here, we performed single-cell phenotypic and functional characterization of pediatric SLE patients and healthy controls blood via mass cytometry. We identified a distinct $CD14^{hi}$ monocyte cytokine signature, with increased levels of monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein-1β (Mip1β), and interleukin-1 receptor antagonist (IL-1RA). This signature was shared by every clinically heterogeneous patient, and reproduced in healthy donors' blood upon *ex-vivo* exposure to plasma from clinically active patients only. This SLE-plasma induced signature was abrogated by JAK1/JAK2 selective inhibition. This study demonstrates the utility of mass cytometry to evaluate immune dysregulation in pediatric autoimmunity, by identification of a multi-parametric immune signature that can be further dissected to delineate the events that drive disease pathogenesis.

1. Introduction

SLE is a highly morbid autoimmune disease characterized by heterogeneous clinical presentation and unpredictable disease activity [1]. Up to 20% of SLE patients are diagnosed as children younger than 16 years old. These pediatric patients typically have more severe disease than adults [2] underscoring the need to better understand immunopathogenesis in this population.

The immunopathogenesis of SLE mirrors its clinical heterogeneity—variably involving multiple cell types and plasma circulating mediators. Neutrophil death results in extrusion of neutrophil extracellular traps, which represent neoantigens for autoantibody formation [3]. Chromatin-containing immune complexes (ICs), free DNA and RNA, and cellular debris engage Toll-like-receptors (TLRs) 7 and 9 on plasmacytoid dendritic cells (pDCs),

Abbreviations: TLR, Toll like receptor; IC, Immune complexes; JAK/STAT, Janus kinase/Signal transducer and activator of transcription; ODN, oligodeoxynucleotide; R848, Resiquimod; MCP1, Monocyte chemotactic protein 1; Mip1β, Macrophage inflammatory protein 1β; IL-1RA, IL-1 receptor antagonist; TNFα, Tumor necrosis factor α; IFNα, Interferon α; mAb, Monoclonal antibody.

* Corresponding author. 12800 East 19th Avenue, Mail Stop 8333, Room P18-8117, Aurora, CO 80045, USA.
E-mail address: elena.hsieh@ucdenver.edu (E.W.Y. Hsieh).
[1] Current address: OMNI Biomarkers, Development Sciences, Genentech, South San Francisco, CA 94080, USA.
[2] Current address: Associate Scientist, Genentech, South San Francisco, CA 94080, USA.
[3] These authors contributed equally.

http://dx.doi.org/10.1016/j.jaut.2017.03.010

resulting in a type I interferon (IFN) signature seen in many pediatric and adult SLE patients [4]. The production of autoantibodies leading to the generation of ICs that co-engage TLRs and the B cell antigen receptor appears to amplify activation of autoreactive B cells [5]. Autoreactive T cells help B cells achieve full activation, differentiation, and isotype switching [6]. Thus, an integrated evaluation of how these apparently dysregulated cellular and molecular processes drive SLE disease activity has the potential to translate into improved therapeutic approaches.

The type I IFN signature has been repeatedly shown to correlate with active SLE [7–10]. This relationship has been suggested primarily by surrogate measures, specifically transcriptomic studies undertaken due to difficulty associated with measuring type I IFN proteins in SLE blood [11]. Findings regarding involvement of other cytokines in SLE have been conflicting. Some studies show that serum TNFα levels are elevated in SLE patients and correlate with disease activity, while others show the opposite, suggesting a protective role for TNFα [12,13]. Similarly, the data on involvement of specific immune cell types in SLE pathogenesis have also been conflicting. For example, in some studies the number of circulating regulatory T cells in SLE patients have been described as decreased, while other studies have shown that the numbers remain the same but the suppressive function is decreased [14,15]. This incomplete picture of SLE pathogenesis may be related to a study design that often focuses on one specific aspect of the immune system (a single cell type or cytokine), or systems-level transcriptomic approaches in the setting of complex cell mixtures. While these studies have been informative, they have provided an assessment of singular cellular and molecular elements, but not within the context of an integrated immune system with single-cell resolution.

To achieve a single-cell systems-level perspective of SLE immunopathogenesis that integrates dysregulated cellular and molecular interactions with clinical outcomes, we leveraged the high-dimensionality of mass cytometry. We simultaneously measured phenotypic and functional (cytokines) perturbations in pediatric SLE whole blood samples to understand how cellular and molecular perturbations may drive SLE disease activity. We applied an unsupervised hierarchical clustering algorithm and regression analysis. The analysis revealed a distinct monocyte cytokine signature shared among clinically heterogeneous pediatric SLE patients. To understand the immune mechanisms underlying this signature, we evaluated *ex vivo* the extent to which this signature was induced by plasma from SLE patients, and abrogated by selective cytokine signaling inhibitors. Plasma from clinically active SLE patients only (and not from those in remission) induced the monocyte cytokine signature in healthy donor peripheral blood, to the same extent as seen in those patients' blood. Selective JAK inhibition fully abrogated the SLE plasma-induced monocyte cytokine signature, but type I IFN receptor blockade did not. This study represents a proof of principle for the application of mass cytometry and complementary computational tools to understand mechanisms of immune dysregulation in pediatric autoimmune disorders, with potential therapeutic applications.

2. Results

2.1. Mass cytometry analysis of newly diagnosed and treatment naive pediatric SLE patients demonstrates a shared distinct monocyte cytokine signature (MCP1/Mip1β/IL1RA)

The underlying immunopathogenesis of SLE involves activation of multiple innate and adaptive cell subsets, and plasma circulating soluble factors such as ICs and pro-inflammatory cytokines, which alter lymphocyte activation and eventually cause organ damage [5,16]. Based on the premise that in SLE immune perturbations involve multiple cell types and cytokines, we used mass cytometry to systematically monitor phenotypic (22 surface markers) and functional (16 cytokines) immune parameters in pediatric SLE patients, with single-cell granularity (Fig. 1).

Peripheral blood samples were collected from 10 newly diagnosed and treatment naïve pediatric SLE patients, and 10 age and gender-matched healthy controls. Every SLE patient met ACR diagnostic criteria [17] (Table 1). Exclusion criteria for SLE patients included prior history of immunosuppression, suspected malignancy or immunodeficiency, and concurrent infection (further details in Methods). Exclusion criteria for healthy controls included chronic medication usage, suspected underlying immunodeficiency, autoimmunity, malignancy, and/or concurrent immunosuppressive therapy (further details in Methods, Table S1).

Peripheral blood was processed within 30 minutes of collection, to remain as close as possible to *in vivo* conditions. To internally control for individual variability, for each study participant, peripheral blood underwent red blood cell (RBC) lysis and fixation either immediately following collection (time zero, T0, Fig. 1A) or after 6 hours of incubation with a protein transport inhibitor cocktail to prevent cytokine secretion (time 6 hours, T6, Fig. 1A). This T6 condition reflects the *in vivo* "baseline" intrinsic cytokine perturbations in SLE patients' blood, as no exogenous stimuli were added. Measurements of cell frequency and cytokine production were analyzed relative to the corresponding participant's time zero measurements (T6-T0), ensuring that each participant served as his/her own control to account for intra- and inter-individual differences. Samples were barcoded using a combination of palladium isotope mass tags to decrease technical variability [18], pooled and stained with antibodies recognizing 22 surface proteins and 16 cytokine proteins, and processed for mass cytometry (Methods, Fig. 1B). Our previous studies validated all of the surface marker and cytokine antibodies used in this assay [19].

Surface markers were chosen to delineate lymphoid and myeloid cell subsets previously described in SLE pathology, such as B and T cell subsets [20], and plasmacytoid dendritic cells (pDCs) [21] (Fig. S1). Evaluated cytokines included those with a pro-inflammatory role, such as the IL-1 family, IL-6, TNFα [22–24], type I and II IFNs [21,25–27], IFN-regulated chemokines [28,29], and IL-17 [30,31]. Data were analyzed via traditional hand-gated strategies (Fig. S1) and with the unsupervised hierarchical clustering algorithm named Citrus (Fig. 1C).

Mass cytometry leverages high-dimensional single-cell analysis, which affords the ability to detect phenotypical and functional disease-relevant cells with minimal bias when applying unsupervised computational data analysis tools [32]. To comprehensively explore SLE-induced immune perturbations across multiple immune cell types and cytokines, we analyzed $CD45^+$ cells from every SLE and healthy control blood sample using Citrus [33] (Fig. 1C). Citrus distilled multidimensional CyTOF data from every patient and healthy control, from T0 and T6 conditions, to a hierarchy of related clusters based on 22 surface markers. It then split the clustered data into individual sample components and calculated features (arcsinh median differences for cytokines at T6-T0 conditions) that describe each cluster on a per-sample basis. Citrus uses a regularized regression model predictive of the experimental endpoint (PAM, Prediction Analysis of Microarrays) to calculate the minimum number of cluster features that best classify the analyzed samples into the correct category (disease vs. control), based on a false discovery rate (FDR) of <1% (Fig. 1C).

Cluster-specific regularized regression analysis of cytokine changes (T6-T0) identified clusters with phenotypic characteristics of activated $CD14^{hi}$ monocytes ($CD66^-CD3^-CD19^-CD7^-CD33^+CD11c^+HLADR^+CD14^{hi}CD16^{lo}CD4^+$) (Fig. 2A, 2B) as those demonstrating cytokine features predictive of the SLE disease category.