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T lymphocyte abnormalities in juvenile systemic sclerosis patients

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Abstract Multi-center evaluations of pediatric patients with juvenile systemic sclerosis (jSSc) have suggested that the pathogenesis of jSSc may differ from that of systemic sclerosis (SSc) in adult patients. Therefore, we undertook to identify abnormalities in the T lymphocytes of jSSc patients and to determine if they differed from the abnormalities reported in the T lymphocytes of adult SSc patients. We identified decreases in the frequency of resting regulatory T lymphocytes and an increased frequency of CD45RA expressing effector memory (EMRA) CD4 T lymphocytes, which were characterized by an increased frequency of CCR7 protein expressing cells. Neither the increases in the EMRA subpopulation nor the increased CCR7 protein expression have been reported in adult SSc patients. The decrease in resting regulatory T lymphocytes in jSSc patients may permit the expansion of the disease initiating CD4 T lymphocytes present in the CCR7 expressing EMRA CD4 T lymphocyte subpopulation.

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1. Introduction

Recent multicenter cross-sectional evaluations of juvenile systemic sclerosis (jSSc) in pediatric patients have identified clinical differences between jSSc patients and adult patients with systemic sclerosis (SSc) [1–4]. These clinical differences suggest that the pathogenesis of the two diseases (jSSc and SSc) may differ. No studies on the pathogenesis of jSSc have been reported; however, studies into the pathogenesis of adult SSc have focused on three areas: abnormalities in regulatory T lymphocytes (Treg), the characterization of the autoreactive, disease initiating CD4 T lymphocytes, and the mechanism of the increased collagen synthesis and deposition. We undertook to determine whether abnormalities in the T lymphocytes were present in jSSc patients.

The results of the evaluations of Treg lymphocytes from adult SSc patients have varied with most reports demonstrating an increased frequency of Treg lymphocytes while others reported decreases [5–10]. Most reports are complicated by the fact that they used the expression of FoxP3 to identify the Treg lymphocytes. Unfortunately, activated conventional T lymphocytes (aTcon) also express FoxP3, making the interpretation of the initial reports difficult [11]. Recently the assessment of the Treg lymphocytes of SSc patients using the Miyara classification, which permits the identification of both resting (rTreg) and activated (aTreg) Treg lymphocytes, has been reported [12,13]. The Miyara classification uses the expression of CD45RA and FoxP3 to identify resting Treg lymphocytes [rTreg; CD45RA+, FoxP3+; I], activated Treg lymphocytes [aTreg; CD45RA−, FoxP3+++; II], and activated conventional T lymphocytes [aTcon; CD45RA−, Fox P3+; III] (Figs. 1a and b). In the Miyara classification, Treg lymphocyte function is associated with both rTreg and aTreg lymphocytes (I and II), but not with the FoxP3 expressing aTcon lymphocytes (III) [12]. In the SSc patients aTreg lymphocytes were initially decreased and followed by a disease duration dependent decrease in their rTreg lymphocytes.

Other studies have characterized the disease initiating, autoreactive CD4 T lymphocytes, especially their T lymphocyte clonality, which has been detected in both their peripheral circulation and skin. However, the same T lymphocyte clones were not detected in both the peripheral circulation and skin of the SSc patients, making the interpretation of the data difficult [14–16]. Evaluation of the increased collagen synthesis and deposition has centered on the cytokines involved in stimulating the collagen synthesis (TGF-β, IL-4, INF-γ, etc.) and the intracellular control of collagen synthesis (connective tissue growth factor) in attempts to identify new targets for anti-fibrotic therapy [17–20].

Our assessment of the T lymphocyte from jSSc patients focused on 1) whether there were abnormalities in the CD4 T lymphocytes of jSSc patients and 2) whether the T lymphocyte abnormalities in the jSSc patients were similar to or differed from those reported in adult SSc patients.

2. Materials and methods

2.1. Study subjects

All studies were performed on patient samples obtained on clinical protocols and after signing informed consent documents approved by the Committee on Clinical Investigations (IRB) of the Children's Hospital Los Angeles (CHLA).

jSSc patients were diagnosed based upon the classification system proposed by the Committee on Classification Criteria for jSSc [21]. In the classification system the diagnosis of jSSc requires the presence of proximal skin sclerosis/induration and at least two of twenty minor criteria including among others pulmonary fibrosis, sclerodactyly, gastro-esophageal reflux, and the presence of either anti-nuclear or anti-topoisomerase I antibodies. Antibody assays were performed in the CLIA certified laboratories of CHLA. Control individuals were normal pediatric donors for hematopoietic stem cell transplantation between the ages of 6 and 18 years old.

2.2. Immunophenotypic analysis

Human peripheral blood was collected in heparin, and mononuclear cells isolated on Ficoll-Hypaque gradients within 24 h of collection and cryopreserved in the vapor phase of liquid nitrogen. Thawed mononuclear cells were stained with anti-CD4 [-FITC from BD Pharmingen (BDP)], anti-CD127 (-Alexa Fluorescent 647, BDP), anti-CD25 (-PE Cy7, BDP), CD45RA (-PE Cy5, BDP), and anti-FoxP3 (-PE, clone 236A/E7, e-Bioscience). Intracellular antigen detection was performed on fixed and permeabilized cells with Cytofix/Cytoperm (e-Bioscience). Regulatory T lymphocyte (Treg) subpopulations were identified using a sequential gating strategy: FCS/CD4 → CD127/CD25 → CD45RA/FoxP3 (Fig. 1a). rTreg lymphocytes (I) were defined as CD45RA+ cells with FoxP3 staining >99% of the isotype control while aTreg lymphocytes (II) were defined as CD45RA− cells with FoxP3 staining >99% of the rTreg lymphocytes. CD4 T lymphocyte subpopulations were identified by staining with anti-CD45RA (-APC, BDP) and anti-CD62L (-FITC, BDP), and the surface expression of CCR7 by staining with anti-CCR7 (-PE, R&D Systems). CD62L and CCR7 expressions were the same on fresh and cryopreserved leukocytes. Recent thymic emigrants (RTEs) were identified as CD4+, CD45RA+, and CD31+ T lymphocytes. Data was acquired on a FACSCanto II (Becton Dickinson) and analyzed with BD FACSDIVA software.

2.3. Exon gene array analysis

The peripheral blood of jSSc and normal individuals was collected in PAXgene tubes (Becton Dickinson). Total RNAs (coding and non-coding) were extracted using Qiagen columns. The integrity of the RNA was determined in the Agilent 2100 BioAnalyzer, and the RNA was labeled using Ambion WT target labeling kits. The cRNA fragments were then hybridized to Affymetrix GeneChip Human Exon 1.0 ST arrays and scanned, which generated a CEL file with 1.9×10^6 transcript expression values that covered the entire expressed genome. The six raw CEL files (3 patients and 3 controls) were analyzed as a group. The group of six was quantile normalized and assessed for any outlier values. All six files passed quality control metrics. The two groups were compared using two-dimensional hierarchical clustering with expectation maximum ordering of the cluster results. Expression values were included that both showed greater than 2-fold expression level changes and were