Reduction of CRKL expression in patients with partial DiGeorge syndrome is associated with impairment of T-cell functions

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Background: Partial DiGeorge syndrome (pDGS) is caused by deletion of the 22q11.2 region. Within this region lies CrK-like *(CRKL)*, a gene encoding an adapter protein belonging to the Crk family that is involved in the signaling cascade of IL-2, stromal cell–derived factor 1α, and type I interferon. Although recurrent infections can be observed in patients with deletion of chromosome 22 syndrome, the immune pathogenesis of this condition is yet not fully understood.

Objective: We aimed to investigate the role of CRKL in T-cell functional responses in patients affected with pDGS.

Methods: Protein expression levels and phosphorylation of CRKL were evaluated in patients with pDGS. T-cell functional assays *in vitro* and gene-silencing experiments were also performed.

Results: CRKL protein expression, as well as its phosphorylation, were reduced in all patients with pDGS, especially on IL-2 stimulation. Moreover, T cells presented impaired proliferation and reduced IL-2 production on anti-CD3/CD28 stimulation and decreased c-Fos expression. Finally, *CRKL* silencing in Jurkat T cells resulted in impaired T-cell proliferation and reduced c-Fos expression.

Conclusions: The impaired T-cell proliferation and reduction of CRKL, phosphorylated CRKL, and c-Fos levels suggest a possible role of CRKL in functional deficiencies of T cells in patients with pDGS. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *CRKL, DiGeorge syndrome, c-Fos, proliferation, T-cell receptor activation, IL-2, signal transducer and activator of transcription 5*

Abbreviations used

AP-1:	Activator protein 1
APC:	Allophycocyanin
CFSE:	Carboxyfluorescein succinimidyl ester
CRKL:	Crk-like
del22q11:	Deletion of chromosome 22
DGL:	DiGeorge-like
DGS:	DiGeorge syndrome
ERK:	Extracellular signal-regulated kinase
FITC:	Fluorescein isothiocyanate
pDGS:	Partial DiGeorge syndrome
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
SDF-1α:	Stromal cell–derived factor 1α
siRNA:	Small interfering RNA
STAT5:	Signal transducer and activator of transcription 5
TCR:	T-cell receptor
TBS:	Tris-buffered saline

Crk-like (CRKL) is a 39-kDa adapter protein that belongs to the Crk family. It is involved in signaling processes of various growth factors and cytokines[1,2] and plays an important role in many cellular functions, including cell migration, adhesion, and immune response. Its activity is modulated by many factors, which include stromal cell–derived factor 1α (SDF-1α), thrombopoietin, interferon type I, and IL-2.[3-6] In particular, interferon type I, thrombopoietin, and IL-2 stimulate CRKL to form complexes with the transcription factor signal transducer and activator of transcription 5 (STAT5). The CRKL-STAT5 complex translocates to the nucleus, where it binds the STAT5 consensus sequence, thereby inducing gene transcription.[3-6] In this context CRKL also functions as a regulator protein that can influence STAT5 binding to regulatory regions of DNA.[7] Moreover, CRKL has been reported to be involved in the Raf-1/MEK/Erk pathway[3,8,9] and in activation of the transcription factor activator protein 1 (AP-1). AP-1 is a heterodimer comprising c-*Fos* and c-*Jun* that is involved in the mechanism of T-cell proliferation and regulation of IL-2 expression[10] and cell-cycle factors, such as cyclin D1.[11-15]

Recently, it has been reported that CRKL is expressed at high levels by tumor-derived cell lines and can play an important role in the proliferation and growth of many tumors.[16,17] Indeed, CRKL downregulation in these tumors induces exit of cells from the cell cycle and drastically decreases their cyclin-dependent proliferation.[18,19]

CRKL is expressed at high levels in the neural crest and pharynx region.[20] Null mutations of CrkL in mice show a phenotype resembling DiGeorge syndrome (DGS). In fact, this

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condition is characterized by defects that affect tissues and organs derived from the neural crest, such as the thymus, parathyroid, craniofacial structures, and heart outflow tract. Histologic examination of these mice has shown a hypoplastic, malpositioned, malformed, or completely missing thymus in 7 of 20 mutant embryos.[20,21] Similarly, compound heterozygous mutations in *CrkL/Tbx1* result in the development of hypoplastic thymus with higher penetrance compared with single heterozygous mutations of the same genes.[22] However, these observations do not fully clarify the role of Crlk in the immune defects of DiGeorge syndrome.

DiGeorge syndrome is caused by heterozygous deletion of a portion of chromosome 22 (del22q11). In 90% of patients, the deleted region includes the *CRKL* gene locus.[20] The main clinical manifestations include congenital heart diseases, velopharyngeal insufficiency, hypocalcemia, delayed speech, and immune disorders.[23] Nevertheless, these features can vary within the same familial group or among patients with DGS with the same deletion. The type of heart defects are well characterized,[24-26] but the mechanisms leading to immune dysregulation and increased susceptibility to infections in patients with DGS are not yet fully understood.

DGS can present as partial DiGeorge syndrome (pDGS), which is observed in the majority of patients with del22q11 and is characterized by a normal or slightly reduced number of T-lymphocytes and a broad spectrum of clinical manifestations. However, it can present in about 1% to 4% of cases as complete DGS with severe lymphopenia and invasive infections.[27-29] The most common infections observed in patients with pDGS include bronchopneumonia in 20%, otitis in 22%, bronchitis in 20%, and pharyngotonsillitis in 16% of cases, whereas only 8% of patients have sepsis or autoimmune manifestations.[23]

In this study we investigated the role of CRKL in the pathogenesis of pDGS. In particular, we evaluated whether the heterozygous deletion of *CRKL* might account for T-cell defects and susceptibility to infections in patients with del22q11. To address this question, we analyzed the expression of CRKL protein and its phosphorylation in T cells from patients with pDGS. In these patients we observed impaired CRKL expression and phosphorylation together with a partial defect of proliferative response and reduced production of IL-2. In addition, we found that *CRKL* silencing in the Jurkat T-cell line leads to impaired proliferation and reduced levels of c-Fos expression.

METHODS

Patients

Seven patients with pDGS together with 2 patients with DGS-like clinical features but normal chromosome 22 diagnosed by using the fluorescence *in situ* hybridization technique (HIRA Probe; Abbott Molecular, Abbott Park, Ill) and 9 age-matched healthy subjects were analyzed in this study. The HIRA Probe recognizes the typical deleted region of 22q11, which also includes the *CRKL* gene.[25] The study was approved by the institutional review board. Written informed consent was obtained for all subjects.

The patients presented with heart defects, facial anomalies, hypocalcemia, and infections, as described in Table I. In 6 patients (PT1-PT3 and PT5-PT7) the 22q11 deletion was identified at birth because of the typical manifestations of the disease, whereas in PT4 DGS was diagnosed in adulthood based on family history. Orthopedic alterations, including scoliosis, scapula alata, muscular hypotonia, and genu valgum, were observed in 3 patients (PT1-PT3).

Cardiac malformations leading to systemic hypoperfusion and metabolic cyanosis at birth were observed in PT2, PT5, and PT7. These patients with cardiac defects, including patency of the ductus arteriosus, bicuspid aortic valve defects, intra-atrial and intraventricular defects, and ectasia of the pulmonary duct, were treated with heart surgery within the first year of life. In patients PT1, PT3, and PT6 the disease was suspected after observation of hypocalcemia, craniofacial defects, or skeletal malformations of the hands and feet.

During the first decade of life, patients with pDGS presented with respiratory tract infections. In particular, PT1, PT2, PT3, PT5, PT6, and PT7 had bronchopneumonia or pharyngotonsillitis caused by *Haemophilus* or *Streptococcus* species. Cutaneous infections by *Staphylococcus aureus* or gastrointestinal infections were observed in PT1 and PT3. None of the patients had autoimmune diseases. PT3 showed moderate lymphopenia, whereas others had normal lymphocyte counts (Table II). Immunoglobulin levels were normal in all subjects. The proliferative response of PBMCs to mitogens, as evaluated by using tritiated thymidine incorporation, was normal in 5 patients (PT1, PT3, PT5, PT6, and PT7) and not evaluated in patients PT2 and PT4 (Table II).

Details on the materials and methods used in this study can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Decreased levels of CRKL and phosphorylated CRKL in patients with pDGS

To assess levels of CRKL expression in patients with pDGS and DiGeorge-like (DGL) syndrome, results of Western blot analysis in PHA-activated T cells were evaluated. CRKL immunoblotting showed a significant reduction of protein levels in patients with pDGS ($P < .01$) compared with those in control subjects (Fig 1, *A* and *B*), whereas patients with DGL syndrome had CRKL levels comparable with those of control subjects (Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org).

Because IL-2 and SDF-1α can induce CRKL activation in various cells types,[3,5] we assessed CRKL phosphorylation (pY207) in response to these cytokines. Western blot analysis showed that both IL-2 and SDF-1α induced CRKL phosphorylation in T cells from healthy donors or from patients with DGL syndrome (Fig E1, *C*). In contrast, CRKL phosphorylation in response to IL-2 was significantly reduced ($P < .01$) in cells from patients with pDGS (Fig 1, *C* and *D*). Next, we evaluated *CRKL* copy numbers in patients with pDGS, healthy control subjects, and 2 patients with DGL syndrome. Using real-time PCR analysis, we observed that a single allele of *CRKL* was detectable in patients with pDGS, whereas 2 alleles were present in healthy subjects and patients with DGL syndrome (Fig 1, *E*).

Impaired proliferation in patients with pDGS

To investigate T-cell functional activities in patients with pDGS, we studied T-cell proliferation, induction of T-cell activation markers, cytokine production, and apoptosis. We observed that the proliferative response to CD3/CD28 cross-linking or PHA stimulation was abnormal in patients with pDGS. Both the division index and proliferation index were decreased in the patients. Despite the broad variability observed among patients, T cells from patients PT2, PT4, and PT5 showed a severe defect of response to both αCD3/αCD28 and PHA stimulation compared with those from the other patients (Fig 2, *A*). Analysis of the proliferation index and division index revealed a significant difference ($P < .01$) between patients and control subjects that was more evident in T cells activated with αCD3/αCD28 compared with cells activated with PHA (Fig 2, *C* and *D*). Similarly, the proliferative response defect to αCD3/αCD28