

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 1 α , 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-1 α :	Stromal cell-derived factor 1 α
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 1 α (SDF-1 α), 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 expression¹⁰ 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 *Crkl* 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-1 α 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-1 α 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 α CD3/ α CD28 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 α CD3/ α CD28 compared with cells activated with PHA (Fig 2, C and D). Similarly, the proliferative response defect to α CD3/ α CD28

TABLE I. Clinical and immunologic features of patients with pDGS

	PT1	PT2	PT3	PT4	PT5	PT6	PT7
Age (y)/sex	4/male	5/female	6/male	46/male	5/female	4/male	11/male
Age at diagnosis	2 mo	2 mo	2 mo	46 y	2 mo	2 mo	2 mo
Heart or outflow tract malformations	Yes	Yes	Yes	No	Yes	Yes	Yes
Facial anomalies	Yes	Yes	Yes	Yes	No	Yes	Yes
Skeletal abnormalities	Yes	Yes	Yes	No	No	No	No
Hypocalcemia	Yes	No	Yes	No	No	No	No
Skin infections	Yes	No	No	No	No	No	No
Sinusitis*	No	No	2	No	No	Yes	No
Pneumonia*	1	1	No	No	Common	No	Common
Pharyngotonsillitis	No	Common	Common	No	Common	Common	Common
Other infections	Gastrointestinal	No	Gastrointestinal/urinary tract	No	No	No	No
Language retardation	Yes	Yes	Yes	No	No	Yes	Yes

Yes/no, Presence/absence of the symptoms listed.

*Number of episodes reported.

was more striking when the percentage of dividing cells at each generation was calculated, especially in late generations ($P < .01$ for third, fourth, and fifth generations; Fig 2, F). However, the proliferative response of the 2 patients with DGL syndrome was comparable with that of the control subjects (Fig 2, A). In addition, analysis of T-cell proliferation in response to mitogens by using the carboxyfluorescein succinimidyl ester (CFSE) dilution assay in the presence of increasing concentrations of neutralizing anti-IL-2 antibodies in 1 healthy subject showed dose-dependent reduction of T-cell divisions, suggesting that this assay can detect changes in IL-2-dependent T-cell proliferation (Fig 2, B).

T-cell activation markers, apoptosis, and IL-2 production in patients with pDGS

We analyzed upregulation of the activation markers CD69 and CD25 in T cells stimulated for 24 hours with IL-2 and α CD3/ α CD28 in patients with pDGS and control subjects. Evaluation of these markers showed that cells from patients with pDGS presented a lower increment of CD69⁺/CD25⁺ cell percentage after activation ($P < .01$) than those from control subjects (Fig 3, A). Moreover, levels of IL-2 released in the supernatants by activated T cells were also reduced in patients with pDGS compared with those in control subjects ($P < .01$; Fig 3, B).

Next, we evaluated T-lymphocyte apoptosis in PBMCs from patients and control subjects after serum deprivation for 96 hours or after stimulation with LPS or culture with medium alone. Patients with pDGS were less susceptible ($P < .01$) to apoptosis after serum deprivation (Fig 3, C), whereas apoptosis was normal in patients with the other experimental conditions (Fig 3, D).

Decreased c-Fos expression and reduced STAT5 phosphorylation in patients with pDGS

Cyclins, extracellular signal-regulated kinase (ERK) 1/2, and STAT5 are key molecules involved in the signaling pathways associated with proliferation of T cells after IL-2 stimulation or T-cell receptor (TCR) cross-linking. In particular, cyclins D1, D2, and D3 are activated by STAT5³⁰⁻³³ and Map/Ras pathway proteins.³⁴⁻³⁸ Hence we analyzed the protein expression levels of these signaling molecules in PHA-activated T cells from patients.

Western blot analysis of T cells cultured overnight with α CD3/ α CD28 showed that c-Fos ($P < .01$) and cyclin D3 ($P < .05$) levels were significantly lower in patients with pDGS, whereas the expression of cyclin D1 and D2 was normal (Fig 4). Moreover, STAT5 phosphorylation in response to IL-2 in PHA-activated T cells derived from patients with pDGS was reduced ($P < .05$), whereas ERK1/2 phosphorylation was normal (Fig 5, A-C).

We also investigated STAT5 phosphorylation in response to IL-2 in whole blood from 4 patients with pDGS and 4 control subjects. Flow cytometric analysis of STAT5 phosphorylation of CD3⁺ lymphocytes showed that a lower percentage of T cells from patients with pDGS are responsive to IL-2 when stimulated at lower cytokine concentrations (6 or 60 U/mL) compared with that seen in control subjects (Fig 5, D), whereas higher concentrations of IL-2 showed normal STAT5 phosphorylation. However, STAT5 phosphorylation and expression of c-Fos and cyclin D3 were normal in patients with DGL syndrome (Fig E1, D-H).

Next, we evaluated whether the impaired T-cell activation in patients with pDGS is associated with reduced STAT5 binding to its target genes using chromatin immunoprecipitation. We observed that STAT5 binding to IL-2 receptor α (IL2RA) and B-cell CLL/lymphoma 2 (BCL2) in T cells activated with α CD3/ α CD28 or IL-2 for 1 hour was normal in patient PT2 (Fig 5, E). This observation suggests that the reduction of STAT5 phosphorylation observed in patients with pDGS does not completely account for the impaired activation of T cells.

Reduced levels of c-Fos and impaired proliferation in CRKL-specific small interfering RNA transfected cells

To define the role of CRKL in the proliferative response, we investigated the proliferation and expression of both CRKL and c-Fos protein after silencing CRKL in Jurkat cells. The cells transfected with CRKL-specific small interfering RNA (siRNA) showed a significant reduction in CRKL mRNA levels (Fig 6, A) accompanied by diminished protein levels of both CRKL and c-Fos (Fig 6, B-D). In addition, proliferation was reduced in these cells compared with that seen in cells transfected with nontarget siRNA (Fig 6, E). This observation suggests that CRKL expression might be relevant for T-cell proliferation.

TABLE II. Lymphocyte subsets, immunoglobulin levels, and proliferation assays in patients with pDGS

	PT1	PT2	PT3	PT4	PT5
Age/sex	4/male	5/female	6/male	46/male	5/female
T cells CD3 ⁺	2227 (53.5%)	1671 (52.2%)	744 (69.5%)	1350 (54.9%)	1558 (53.8%)
CD3 ⁺ CD4 ⁺	1349 (32.4%)	747 (23.4%)	336 (31.4%)	1017 (41.4%)	834 (28.8%)
Naive (CD4 ⁺)	894 (66.2%)	358 (48%)	134 (39.8%)	339 (33.3%)	276 (33.1%)
CD45RA ⁺ CCR7 ⁺					
RTE	632 (46.9%)	316 (42.3%)	108 (32.0%)	229 (22.5%)	200 (23.9%)
CD45RA ⁺ CCR7 ⁺					
CD31 ⁺					
Centr. Mem.	296 (21.9%)	289 (38.7%)	134 (39.8%)	445 (43.7%)	352 (42.1%)
CD45RA ⁺ CCR7 ⁺					
Eff. Mem.	139 (10.3%)	79 (10.5%)	60 (17.8%)	238 (23.4%)	175 (21.0%)
CD45RA ⁺ CCR7 ⁺					
Ter. Diff.	21 (1.6%)	21 (2.8%)	9 (2.6%)	4 (0.4%)	31 (3.7%)
CD45RA ⁺ CCR7 ⁺					
CD3 ⁺ CD8 ⁺	590 (14.2%)	533 (16.6%)	256 (23.93%)	250 (10.2%)	523 (18.0%)
Naive (CD8 ⁺)	375 (63.6%)	352 (66.0%)	118 (46.1%)	99 (39.5%)	150 (28.6%)
CD45RA ⁺ CCR7 ⁺					
Centr. Mem.	21 (3.5%)	28 (5.2%)	12 (4.6%)	24 (9.5%)	24 (4.7%)
CD45RA ⁺ CCR7 ⁺					
Eff. Mem.	75 (12.7%)	124 (23.3%)	83 (32.5%)	110 (43.9%)	223 (42.6%)
CD45RA ⁺ CCR7 ⁺					
Ter. Diff.	120 (20.3%)	28 (5.3%)	43 (16.6%)	18 (7.2%)	126 (24.1%)
CD45RA ⁺ CCR7 ⁺					
B cells	1114 (26.8%)	814 (25.4%)	220 (20.6%)	397 (16.1%)	613 (21.2%)
CD19 ⁺					
NK cells	692 (16.6%)	658 (20.6%)	82 (7.6%)	661 (26.9%)	658 (22.7%)
CD3 ⁺ CD16 ⁺ CD56 ⁺					
Lymphocyte counts (cells/ μ L)	4160	3200	1070	2460	2900
IgA (mg/dL)	93	110	182	—	183
IgG (mg/dL)	759	1360	1030	—	1370
IgM (mg/dL)	52	92	43	—	82
Proliferation α CD3	346×10^3	—	74×10^3	—	168×10^3
Proliferation PHA [†]	326×10^3	—	342×10^3	—	61×10^3
Proliferation PMA [†]	140×10^3	—	155×10^3	—	349×10^3
DI α CD3/ α CD28	0.90	0.20	0.30	0.15	0.18
PI α CD3/ α CD28	1.65	1.37	1.68	1.40	1.20
DI PHA	0.75	0.22	0.30	0.50	0.20
PI PHA	1.70	1.33	1.56	1.10	1.50

Centr. Mem., Central memory; DI, division index; Eff. Mem., effector memory; NK, natural killer; PI, proliferation index; PMA, phorbol 12-myristate 13-acetate; RTE, recent thymic emigrants; Ter. Diff., terminally differentiated.

*The reference value is obtained from a sample of healthy control subjects evaluated at our immunology department.

[†]For tritiated thymidine proliferation assays, PBMCs were cultured at a cell density of 100,000 cells/mL for 72 hours. Incorporation of tritiated thymidine was made during the last 6 hours of cell growth. Radioactive signal was measured by using a scintillation counter, and proliferation was expressed as follows: Stimulation index (SI) = Net counts for minute (cpm).

DISCUSSION

We report that T cells from patients with pDGS display reduced levels of CRKL protein and impaired phosphorylation after TCR triggering. The impaired activation of CRKL is associated with decreased levels of the AP-1 complex component c-Fos, which is known to be involved in T-cell proliferation and regulation of cyclins and IL-2 expression.¹⁰⁻¹⁵ Moreover, we showed that CRKL inhibition in Jurkat T cells by CRKL-specific siRNA leads to reduction of the transcription factor c-Fos and reduced proliferation. This is in accordance with the recent observation that overexpression of CRKL in the MDA-MB-435 cell line and in other cell types promotes cell proliferation associated with

advanced tumor-node-metastasis stage and increased tumor size.^{18,39} In addition, a recent study by Zheng et al⁴⁰ has highlighted the relevance of CRKL in patients with DGS, revealing a link between CRKL haploinsufficiency and impairment of natural killer cell cytolytic activity caused by abnormal polarization of lytic granules.

CRKL silencing in the MDA-MB-453 cell line, which expresses CRKL at high levels, results in impaired proliferation and decreased cyclin D1 levels.¹⁸ In contrast to these observations, cyclin D1 levels were not affected in T cells of patients with pDGS, suggesting that the role of cyclins is strictly cell type dependent. Nonetheless, there is evidence that CRKL

TABLE II. (Continued)

PT6	PT7	Range (control value* [4-6 y old])	Range (control value* [10-16 y old])	Range (control value* [adults])
4/male	11/male			
2037 (64.4%)	1632 (75.9%)	1966-3810 (58.1% to 78.6%)	1250-2400 (60.5% to 79.8%)	991-2071 (66.6% to 86.1%)
1087 (34.4%)	789 (36.7%)	1166-2158 (27.3% to 48.5%)	775-1330 (30.3% to 48.3%)	389-1441 (31.7% to 60.2%)
488 (44.9%)	286 (36.2%)	656-1638 (53.6% to 81.4%)	370-830 (34.3% to 74.6%)	159-495 (17.2% to 61.0%)
324 (29.9%)	174 (22.0%)	574-1464 (41.7% to 72.8%)	230-680 (21.1% to 63.5%)	139-395 (9.5% to 51.9%)
410 (37.7%)	334 (42.3%)	178-381 (12.1% to 24.5%)	110-450 (13.0% to 43.5%)	89-525 (14.2% to 43.1%)
170 (15.6%)	151 (19.1%)	103-262 (5.1% to 17.9%)	100-230 (8.5% to 28.1%)	26-306 (15.3% to 58.2%)
20 (1.8%)	16 (2.0%)	14-78 (1.0% to 9.5%)	6-50 (0.7% to 6.6%)	3-46 (0.4% to 7.0%)
508 (16.1%)	734 (34.2%)	601-1608 (15.0% to 32.7%)	380-1050 (13.8% to 37.5%)	164-687 (14.7% to 39.4%)
212 (41.8%)	207 (28.2%)	121-768 (23.0% to 85.1%)	120-450 (26.7% to 72.9%)	65-318 (4.1% to 63.0%)
53 (10.5%)	26 (3.5%)	3-75 (0.2% to 7.6%)	8-85 (1.2% to 11.6%)	7-93 (0.2% to 18.4%)
84 (16.6%)	226 (30.8%)	80-342 (7.0% to 37.8%)	40-530 (6.0% to 53.6%)	28-340 (7.8% to 52.8%)
158 (31.1%)	275 (37.5%)	40-906 (4.0% to 71.1%)	13-700 (3.9% to 72.0%)	16-287 (16.8% to 64.0%)
893 (28.2%)	197 (9.2%)	409-1344 (9.8% to 28.0%)	130-530 (5.7% to 19.7%)	87-283 (4.2% to 11.7%)
160 (5.1%)	284 (13.2%)	155-1099 (5.0% to 28.4%)	150-540 (4.6% to 27.8%)	95-634 (4.4% to 23.6%)
3160	2150	1500-8500	1500-6500	900-4000
208	149	37-257	60-270	85-410
711	1390	528-1959	707-1919	690-1500
53	56	49-292	61-276	40-240
508×10^3	249×10^3	25-580 cpm	34-476 cpm	96-535 cpm
545×10^3	176×10^3	57-626 cpm	74-458 cpm	25-376 cpm
73×10^3	376×10^3	29-506 cpm	29-626 cpm	22-390 cpm
0.30	0.36	0.7-2.09	0.8-1.30	1.02-1.41
1.59	1.58	1.54-2.2	1.5-2.08	1.08-2.04
0.55	0.60	0.53-1.25	0.99-1.3	1.34-1.85
1.80	1.70	1.7-2.21	1.59-2.62	1.9-2.6

overexpression is associated with c-Fos upregulation and increased AP-1 transcriptional activity sustained by ERK activation and to increased levels of matrix metalloproteinase 9 in HBE and H1299 cells.⁴¹ The link between CRKL and c-Fos is also supported by previous studies showing that the CRKL-C3G complex plays a key role in c-Fos gene transcription through Elk-1 activation in hematopoietic cells stimulated with IL-3 or erythropoietin.⁴²

T-cell proliferation in response to IL-2 is mediated by IL-2 receptor β subunit activation and phosphorylation of tyrosines 338 and 510. This results in activation of the adapter protein Shc, which in turn activates the Grb2/Sos complex and Ras kinases. IL-

2 receptor β activation also induces STAT5 phosphorylation.⁴³⁻⁵⁰ Furthermore, overexpression of CRKL in NOD mice carrying mutations in the region encoding the DNA-binding domain of STAT5 results in increased DNA-binding ability of STAT5. However, analysis of STAT5 binding to IL2RA or BCL2 has shown normal binding of this transcription factor to its target genes, suggesting that the reduced proliferative response of T cells in patients with pDGS is linked to other pathways involving CRKL. In particular, we have shown that reduced T-cell proliferation is associated with lower IL-2 production and decreased expression of the AP-1 component c-Fos. Because AP-1 activation involves recruitment of the ERK pathway and is required for IL-2

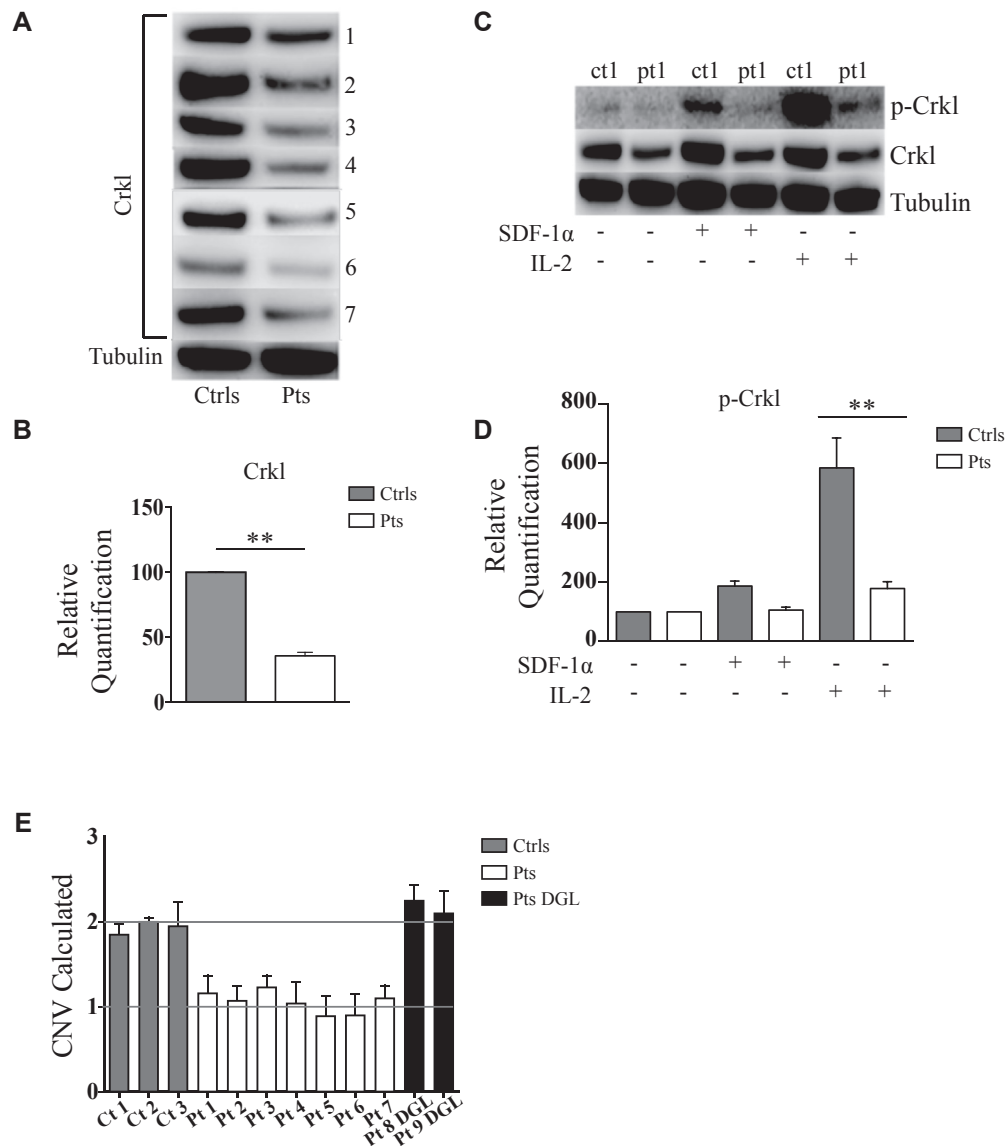


FIG 1. Patients with pDGS display significantly reduced levels of CRKL and p-CRKL compared with healthy control subjects. **A**, CRKL protein expression was investigated by Western blot analysis in PHA-activated T cells from patients with pDGS and control subjects. Tubulin expression was analyzed as a control. **B**, Expression levels of CRKL protein in patients with pDGS and control subjects. **C**, CRKL phosphorylation in response to IL-2 or SDF-1 α was investigated by means of immunoblotting with a specific anti-p-CRKL mAb in activated T cells from patients with pDGS or control subjects. Expression levels of total CRKL protein and p-CRKL in a representative patient (pt1) and a representative healthy control subject (ct1) are shown. **D**, The extent of CRKL phosphorylation in PHA-activated T cells after stimulation with IL-2 or SDF-1 α was assessed by means of Western blot analysis in 7 patients with pDGS and 7 healthy control subjects. **E**, Copy numbers of the *CRKL* gene were investigated by using real-time PCR with TaqMan Gene Expression Assays. The number of *CRKL* copies in DNA from 3 control subjects, 7 patients with pDGS, and 2 patients with DGL syndrome is reported on the y-axis as the mean $\pm$ SD value of quadruplicate data. Statistical analysis was performed by using the Kruskal-Wallis comparison test. ** $P < .01$.

production by activated T cells,¹⁰⁻⁴¹ it can be speculated that the reduced expression of c-Fos can be linked to the impaired IL-2 production observed in patients with pDGS.

CRKL interactions are pivotal for many cellular activities, including proliferation, apoptosis, and adhesion. Crk family proteins can bind other cytoplasmic proteins either through the

SH2 domain of the protein, which recognizes the phosphorylated residues of tyrosine, or through the SH3 domain, which interacts with the proline-rich consensus sequences because of their hydrophobic regions.² CRKL can interact by these means with the signaling proteins ζ chain-associated protein of 70 kDa and Cbl or phosphoinositide 3-kinase, which are quickly activated

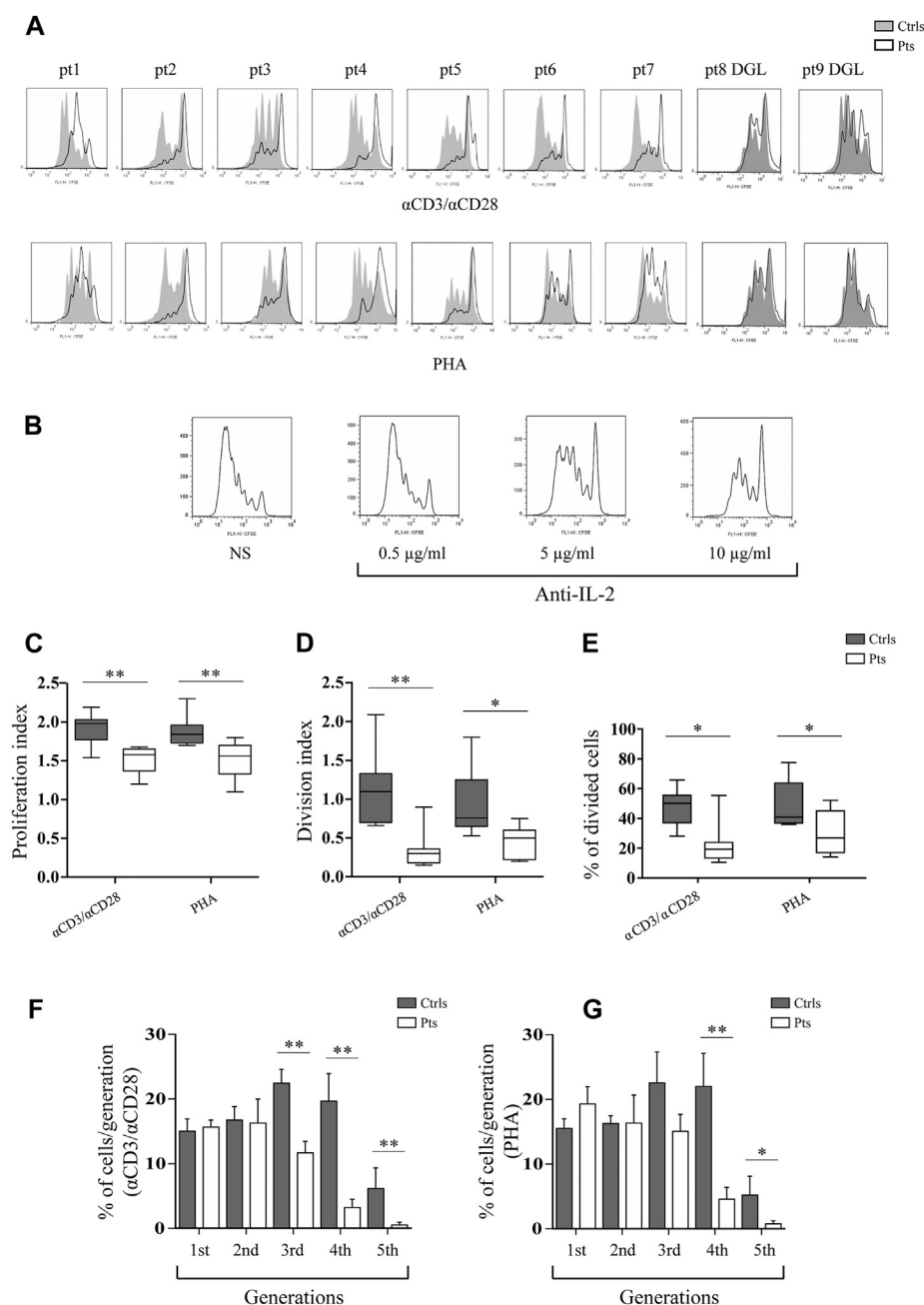


FIG 2. Impaired T-cell proliferation in patients with pDGS. **A**, T-cell proliferation in response to α CD3/ α CD28 (upper panels) or PHA (lower panels) was investigated by using flow cytometry with CFSE in 7 patients with pDGS (black lines), 7 healthy donors (gray pattern), 2 patients with DGL syndrome, and matched control subjects. **B**, Proliferative response of T cells in response to α CD3/ α CD28 in the presence of increasing amounts of anti-IL-2. Flow cytometric analysis of CFSE incorporation in a control subject is shown. **C-E**, Proliferation index (Fig 2, C), division index (Fig 2, D), and percentage of proliferating T cells in response to α CD3/ α CD28 or PHA (Fig 2, E) are displayed as gray bars (control subjects) or white bars (patients with pDGS). In the box plot the internal horizontal line represents the median value, and the upper and lower lines represent the first and third quartiles. **F** and **G**, Percentage of proliferating T cells in response to α CD3/ α CD28 (Fig 2, F) or PHA (Fig 2, G) at each generation in 7 patients with pDGS (white bars) and 7 healthy subjects (gray bars) is shown. Each bar represents the mean $\pm$ SD of cells proliferating at each division cycle. Statistical analysis was performed by using the Kruskal-Wallis test. * $P < .05$ and ** $P < .01$.

after TCR triggering.⁵¹⁻⁵³ Therefore it is likely that the haploinsufficiency of *CRKL* might play a role in the pathogenesis of the immunologic dysfunctions observed in patients with pDGS. For example, it is known that *CRKL* is involved in the adhesion

process through Rap1 after engagement of the TCR. In fact, *CRKL* silencing in primary human T cells reduces cell adhesion and production of IL-2.⁵³ This finding is in agreement with the observation that impaired T-cell proliferation in patients with

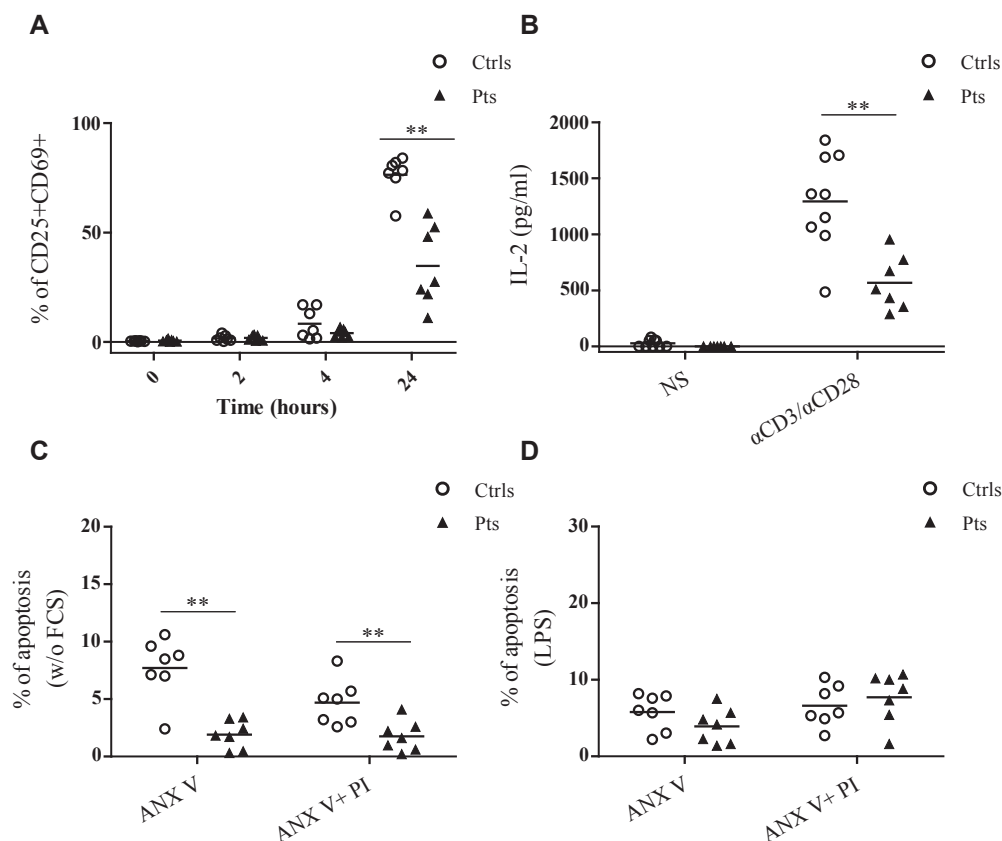


FIG 3. Impaired expression of the activation markers CD25 and CD69, IL-2 production, and apoptosis analysis in patients with pDGS. **A**, Percentage of CD4⁺ lymphocytes expressing the activation markers CD25 and CD69 after αCD3/αCD28 activation for 24 hours. **B**, IL-2 production by PBMCs stimulated for 3 days with αCD3/αCD28 in patients with pDGS, as measured in supernatants. **C** and **D**, Induction of apoptosis in PBMCs of patients with pDGS in comparison with healthy subjects after serum deprivation for 96 hours (Fig 3, **C**) or stimulation with LPS (Fig 3, **D**). The extent of apoptosis was measured as the percentage of Annexin V⁺ cells and Annexin V⁺/propidium iodide⁺ cells. In the scatter plots the *internal horizontal lines* indicate the mean value. Statistical analysis was performed by using the Kruskal-Wallis test. ***P* < .01.

pDGS is associated with reduced IL-2 production. Likewise, the impairment of T-cell proliferation that is observed in NOD mice is associated with lower responsiveness under crosslinking of the TCR and decreased secretion of IL-2⁵⁴⁻⁵⁶ and thus can be due to the low level of CRKL.⁷

Autoimmune diseases, such as thrombocytopenic purpura, hemolytic anemia, diabetes, and psoriasis, are frequently observed in patients with DGS. Because the Crk protein family is also involved in apoptosis,^{8,57} this observation might account for the increased susceptibility of patients with pDGS to autoimmune manifestations.

The observation that patients with pDGS display reduced apoptosis after serum deprivation seems to contradict the impaired proliferative response. However, we have observed abnormal T-cell apoptosis in patients with pDGS only when cells were challenged in serum-free culture conditions, whereas apoptosis was normal in response to LPS, suggesting that CRKL activities are probably strictly specific to the activation pathway. Moreover, investigation of NOD mice has revealed that these animals display low CRKL expression,⁷ which could be associated with a refractory response to proapoptotic stimuli⁵⁸ and possibly to increased susceptibility to diabetes development.

Indeed, peripheral T lymphocytes and thymic cells of NOD mice showed a proliferative hyporesponsiveness after TCR activation.⁵⁴⁻⁵⁶ In sharp contrast, other authors have reported that CRKL neutralization in tumors was accompanied by increased apoptosis,⁵⁹ suggesting that the role of CRKL on apoptosis is regulated in T cells at multiple levels.

Despite previous studies that have reported normal T-cell proliferation in the majority of patients with pDGS, we found that T-cell proliferation, as measured by means of analysis of CFSE dilution, was reduced in 7 patients with pDGS. In contrast, analysis of T-cell proliferation by tritiated thymidine incorporation was normal in the same patients.^{60,61} This discrepancy might be related to the type of method used. In fact, the CFSE assay can easily detect differences between generations of proliferating T cells and discriminate vital from necrotic cells, whereas analysis of tritiated thymidine incorporation cannot distinguish among these conditions, suggesting that analysis of T-cell proliferation by using CFSE is probably more sensitive than tritiated thymidine incorporation.

Despite the reduced proliferative response of T cells in patients with pDGS, most of the patients do not present with lymphopenia. This difference might be related to the partial defect of

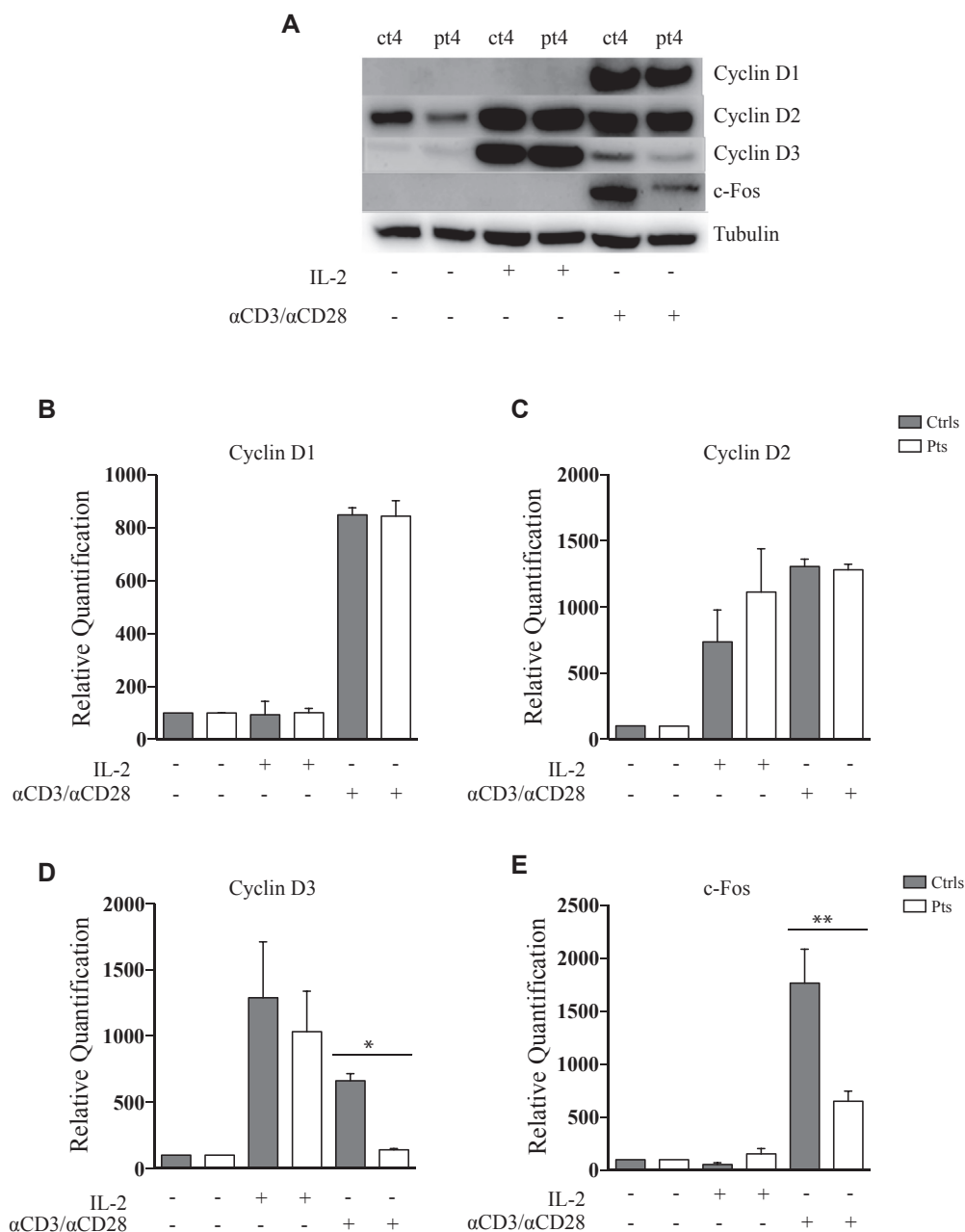


FIG 4. Regulation of cyclins D1, D2, and D3 and c-Fos expression in PHA-activated T cells of patients with pDGS. **A**, Immunoblot analysis of cyclins D1, D2, and D3 and c-Fos expression in PHA-activated T cells from a representative patient (pt4) and a representative healthy control subject (ct4). **B-E**, Expression levels of cyclins D1, D2, and D3 and c-Fos in 4 patients and 4 healthy subjects. Protein expression was measured by using immunoblot analysis followed by quantitative densitometry and normalized with the tubulin level. In the graphics the *column* indicates the mean $\pm$ SD value. Statistical analysis was performed by using the Kruskal-Wallis test. * $P < .05$ and ** $P < .01$.

proliferative response that affected T cells of patients with pDGS only after 3 or 4 division cycles, suggesting that the contribution of this defect to lymphocyte homeostasis is probably modest. In addition, lymphocyte counts are also affected by thymus output and lymphocyte homing to lymphoid organs that might contribute to maintain nearly normal values of T cells in patients with pDGS.

In conclusion, we observed an association between CRKL expression and defective proliferation of T lymphocytes in

patients with pDGS, which associated with a significant decrease in levels of c-Fos and reduced IL-2 production. To the best of our knowledge, this represents the first study suggesting that CRKL deficiency in patients with pDGS is involved in the mechanisms leading to impaired T-cell proliferation. In spite of this observation, the full pathogenesis of the immunologic defects observed in patients with pDGS remains elusive. In particular, the analysis of other intracellular proteins, such as C3G or cyclin-dependent

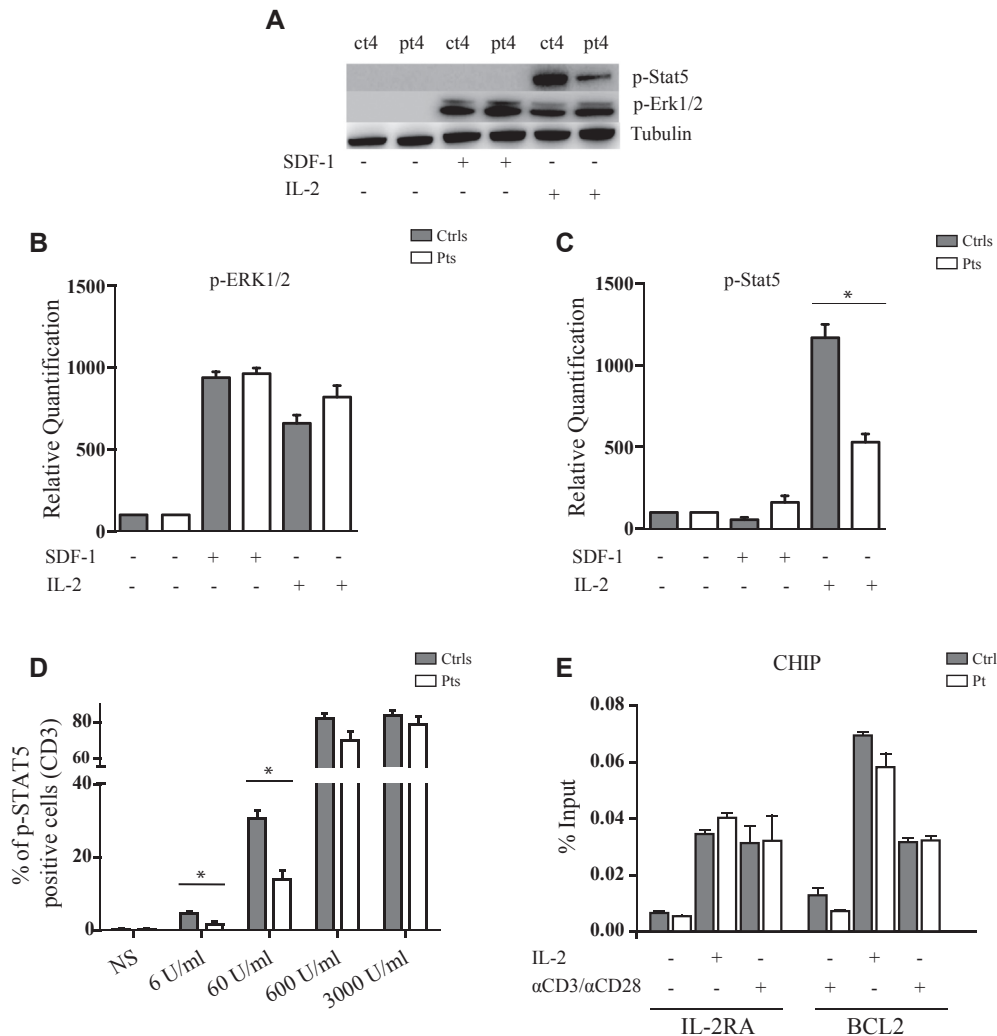


FIG 5. Immunoblot analysis of STAT5, ERK1/2 phosphorylation, and STAT5 signaling in patients with pDGS. **A**, Immunoblot analysis of p-ERK1/2 and p-STAT5 expression in PHA-activated T cells from a representative patient (pt4) and healthy control subject (ct4). **B** and **C**, Expression levels of p-ERK1/2 (Fig 5, **B**) and p-STAT5 (Fig 5, **C**) in 4 patients with pDGS and 4 healthy subjects. p-ERK1/2 and p-STAT5 expression were measured by using immunoblot analysis and quantitative densitometry and normalized with tubulin levels. **D**, Extent of STAT5 phosphorylation in response to increasing concentrations of IL-2 is shown as the percentage of CD3⁺ lymphocytes after intracellular staining with anti-p-STAT5 mAb. Each bar represents the mean $\pm$ SD values from 4 patients with pDGS and 4 healthy control subjects. **E**, Graph shows the analysis of STAT5 binding at 2 target genes (*IL2RA* and *BCL2*) carried out by the using chromatin immunoprecipitation method on PT2 and a related healthy control subject in PHA-activated T cells. In the graph the column indicates the mean $\pm$ SD value. Statistical analysis was performed by using the Kruskal-Wallis test. * $P < .05$.

kinases or other components of the AP-1 complex, might shed some light on the mechanisms leading to impairment of T-cell proliferation in these patients. These future studies will be important to define the role of CRKL in pDGS pathogenesis and to identify potential targets for therapeutic intervention.

We thank the patients and their families for their participation in the study.

Clinical implications: Haploinsufficiency of the CRKL adaptor protein is observed in patients with del22q.11.2. This is accompanied by reduction of CRKL phosphorylation and altered proliferation in T cells in patients with pDGS.

REFERENCES

1. Feller SM. Crk family adaptors-signalling complex formation and biological roles. *Oncogene* 2001;20:6348-71.
2. Birge RB, Kalodimos C, Inagaki F, Tanaka S. Crk and CrkL adaptor proteins: networks for physiological and pathological signaling. *Cell Commun Signal* 2009;7:1-23.
3. Arai A, Aoki M, Weihua Y, Jin A, Miura O. CrkL plays a role in SDF-1 α -induced activation of the Raf-1/MEK/Erk pathway through Ras and Rac to mediate chemotactic signaling in hematopoietic cells. *Cell Signal* 2006;18:2162-71.
4. Schulze H, Ballmaier M, Welte K, Germeshausen M. Thrombopoietin induces the generation of distinct Stat1, Stat3, Stat5a and Stat5b homo- and heterodimeric complexes with different kinetics in human platelets. *Exp Hematol* 2000;28:294-304.
5. Oda A, Wakao H, Fujihara M, Ozaki K, Komatsu N, Tanaka S, et al. Thrombopoietin and interleukin-2 induce association of CRK with STAT5. *Biochem Biophys Res Commun* 2000;278:299-305.

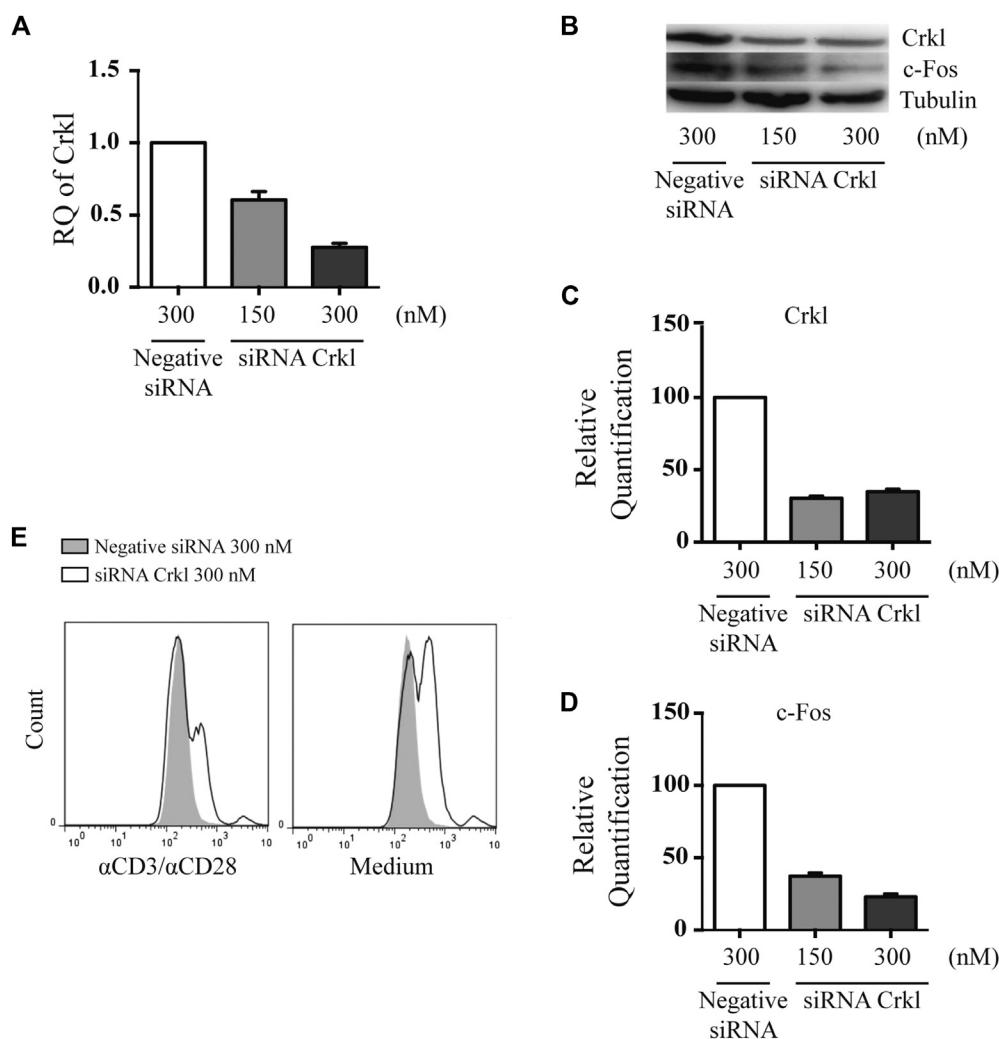


FIG 6. *CRKL* silencing in Jurkat cells. **A**, Effect of *CRKL*-specific siRNA (150 or 300 nmol/L) on mRNA expression levels, as measured by using quantitative real-time PCR. **B**, Immunoblot analysis of *CRKL* and *c-Fos* expression levels after treatment with *CRKL* siRNA. **C** and **D**, Protein expression levels of *CRKL* and *c-Fos*, respectively, after normalization with tubulin levels. **E**, Flow cytometric analysis of cell division by means of dilution of CFSE in Jurkat cells stimulated with α CD3/ α CD28 or medium alone after transfection with siRNA for *CRKL*.

- Fish EN, Uddin S, Korkmaz M, Majchrzak B, Druker BJ, Platanias LC. Activation of a CrkL-stat5 signaling complex by type I interferons. *J Biol Chem* 1999;274:571-3.
- Laloraya M, Davoodi-Semiromi A, Kumar GP, McDuffie M, She JX. Impaired Crkl expression contributes to the defective DNA binding of Stat5b in nonobese diabetic mice. *Diabetes* 2006;55:734-41.
- Smith JJ, Richardson DA, Kopf J, Yoshida M, Hollingsworth RE, Kornbluth S. Apoptotic regulation by the Crk adaptor protein mediated by interactions with Wee1 and Crm1/exportin. *Mol Cell Biol* 2002;22:1412-23.
- Smith JJ, Evans EK, Murakami M, Moyer MB, Moseley MA, Vande Woude G, et al. Wee1-regulated apoptosis mediated by the crk adaptor protein in *Xenopus* egg extracts. *J Cell Biol* 2000;151:1391-400.
- Granelli-Piperno A, Nolan P. Nuclear transcription factors that bind to elements of the IL-2 promoter. Induction requirements in primary human T cells. *J Immunol* 1991;147:2734-9.
- Pai SR, Bird RC. *c-fos* expression is required during all phases of the cell cycle during exponential cell proliferation. *Anticancer Res* 1994;14:985-94.
- Güller M, Toualbi-Abed K, Legrand A, Michel L, Mauviel A, Bernuau D, et al. *c-Fos* overexpression increases the proliferation of human hepatocytes by stabilizing nuclear cyclin D1. *World J Gastroenterol* 2008;14:6339-46.
- Parralles A, Palma-Nicolás JP, López E, López-Colomé AM. Thrombin stimulates RPE cell proliferation by promoting *c-Fos*-mediated cyclin D1 expression. *J Cell Physiol* 2010;222:302-12.
- Abate C, Curran T. Encounters with Fos and Jun on the road to AP-1. *Semin Cancer Biol* 1990;1:19-26.
- Resnitzky D, Gossen M, Bujard H, Reed SI. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol* 1994;14:1669-79.
- Bell ES, Park M. Models of crk adaptor proteins in cancer. *Genes Cancer* 2012;3:341-52.
- Yeung CL, Ngo VN, Grohar PJ, Arnaldez FI, Asante A, Wan X, et al. Loss-of-function screen in rhabdomyosarcoma identifies CRKL-YES as a critical signal for tumor growth. *Oncogene* 2013;32:5429-38.
- Zhao T, Miao Z, Wang Z, Xu Y, Wu J, Liu X, et al. Overexpression of CRKL correlates with malignant cell proliferation in breast cancer. *Tumour Biol* 2013;34:2891-7.
- Wang Y, Dong QZ, Fu L, Stoecker M, Wang E, Wang EH. Overexpression of CRKL correlates with poor prognosis and cell proliferation in non-small cell lung cancer. *Mol Carcinog* 2013;52:890-9.

20. Guris DL, Fantes J, Tara D, Druker BJ, Imamoto A. Mice lacking the homologue of the human 22q11.2 gene CRKL phenocopy neurocristopathies of DiGeorge syndrome. *Nat Genet* 2001;27:293-8.
21. Moon AM, Guris DL, Seo JH, Li L, Hammond J, Talbot A, et al. Crkl deficiency disrupts Fgfr3 signaling in a mouse model of 22q11 deletion syndromes. *Dev Cell* 2006;10:71-80.
22. Guris DL, Duester G, Papaioannou VE, Imamoto A. Dose-dependent interaction of Tbx1 and Crkl and locally aberrant RA signaling in a model of partial DiGeorge syndrome. *Dev Cell* 2006;10:81-92.
23. Cancrini C, Puliafito P, Digilio MC, Soresina A, Martino S, Rondelli R, et al. Clinical features and follow-up in patients with 22q11.2 deletion syndrome. *J Pediatr* 2014;164:1475-80.
24. Scambler PJ. The 22q11 deletion syndromes. *Hum Mol Genet* 2000;9:2421-6.
25. Yamagishi H, Srivastava D. Unraveling the genetic and developmental mysteries of 22q11 deletion syndrome. *Trends Mol Med* 2003;9:383-9.
26. Keyte A, Hutson MR. The neural crest in cardiac congenital anomalies. *Differentiation* 2012;84:25-40.
27. Müller W, Peter HH, Kallfelz HC, Franz A, Rieger CH. The DiGeorge sequence. II. Immunologic findings in partial and complete forms of the disorder. *Eur J Pediatr* 1989;149:96-103.
28. Sullivan KE, Jawad AF, Randall P, Driscoll DA, Emanuel BS, McDonald-McGinn DM, et al. Lack of correlation between impaired T cell production, immunodeficiency, and other phenotypic features in chromosome 22q11.2 deletion syndromes. *Clin Immunol Immunopathol* 1998;86:141-6.
29. Markert ML, Hummel DS, Rosenblatt HM, Schiff SE, Harville TO, Williams LW, et al. Complete DiGeorge syndrome: persistence of profound immunodeficiency. *J Pediatr* 1998;132:15-21.
30. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, et al. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* 1999;10:249-59.
31. Martino A, Holmes JH. 4th, Lord JD, Moon JJ, Nelson BH. Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-2. *J Immunol* 2001;166:1723-9.
32. Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, et al. Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J* 1999;18:1367-77.
33. Magné S, Caron S, Charon M, Rouyez MC, Dusanther-Fourt I. STAT5 and Oct-1 form a stable complex that modulates cyclin D1 expression. *Mol Cell Biol* 2003;23:8934-45.
34. Lavoie JN, L'Allemain G, Brunet A, Müller R, Pouysségur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* 1996;271:20608-16.
35. Newbern J, Zhong J, Wickramasinghe RS, Li X, Wu Y, Samuels I, et al. Mouse and human phenotypes indicate a critical conserved role for ERK2 signaling in neural crest development. *Proc Natl Acad Sci U S A* 2008;105:17115-20.
36. Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* 1988;54:541-52.
37. Hu E, Mueller E, Oliviero S, Papaioannou VE, Johnson R, Spiegelman BM. Targeted disruption of the c-fos gene demonstrates c-fos-dependent and -independent pathways for gene expression stimulated by growth factors or oncogenes. *EMBO J* 1994;13:3094-103.
38. Pandey MK, Liu G, Cooper TK, Mulder KM. Knockdown of c-Fos suppresses the growth of human colon carcinoma cells in athymic mice. *Int J Cancer* 2012;130:213-22.
39. Fu L, Dong Q, Xie C, Wang Y, Li Q. CRKL protein overexpression enhances cell proliferation and invasion in pancreatic cancer. *Tumour Biol* 2015;36:1015-22.
40. Zheng P, Noroski LM, Hanson IC, Chen Y, Lee ME, Huang Y, et al. Molecular mechanisms of functional natural killer deficiency in patients with partial DiGeorge syndrome. *J Allergy Clin Immunol* 2015;135:1293-302.
41. Lin F, Chengyao X, Qingchang L, Qianze D, Enhua W, Yan W. CRKL promotes lung cancer cell invasion through ERK-MMP9 pathway. *Mol Carcinog* 2015;54(suppl 1):E35-44.
42. Nosaka Y, Arai A, Miyasaka N, Miura O. CrkL mediates Ras-dependent activation of the Raf/ERK pathway through the guanine nucleotide exchange factor C3G in hematopoietic cells stimulated with erythropoietin or interleukin-3. *J Biol Chem* 1999;274:30154-62.
43. Lord JD, McIntosh BC, Greenberg PD, Nelson BH. The IL-2 receptor promotes proliferation, bcl-2 and bcl-x induction, but not cell viability through the adapter molecule Shc. *J Immunol* 1998;161:4627-33.
44. Ravichandran KS, Burakoff SJ. The adapter protein Shc interacts with the interleukin-2 (IL-2) receptor upon IL-2 stimulation. *J Biol Chem* 1994;269:1599-602.
45. Gu H, Pratt JC, Burakoff SJ, Neel BG. Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Mol Cell Biol* 2000;20:7109-20.
46. Gu H, Maeda H, Moon JJ, Lord JD, Yoakim M, Nelson BH, et al. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol Cell Biol* 2000;20:7109-20.
47. Gadina M, Sudarshan C, O'Shea JJ. IL-2, but not IL-4 and other cytokines, induce phosphorylation of a 98-kDa protein associated with SHP-2, phosphatidylinositol 3'-kinase, and Grb2. *J Immunol* 1999;162:2081-6.
48. Hou J, Schindler U, Henzel WJ, Wong SC, McKnight SL. Identification and purification of human Stat proteins activated in response to interleukin-2. *Immunity* 1995;2:321-9.
49. Gilmour KC, Pine R, Reich NC. Interleukin 2 activates STAT5 transcription factor (mammary gland factor) and specific gene expression in T lymphocytes. *Proc Natl Acad Sci U S A* 1995;92:10772-6.
50. Wang LH, Kirken RA, Erwin RA, Yu CR, Farrar WL. JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrosine kinase AG-490 regulation of IL-2-mediated T cell response. *J Immunol* 1999;162:3897-904.
51. Gelkop S, Babichev Y, Isakov N. T cell activation induces direct binding of the Crk adapter protein to the regulatory subunit of phosphatidylinositol 3-kinase (p85) via a complex mechanism involving the Cbl protein. *J Biol Chem* 2001;276:36174-82.
52. Gelkop S, Isakov N. T cell activation stimulates the association of enzymatically active tyrosine-phosphorylated ZAP-70 with the Crk adapter proteins. *J Biol Chem* 1999;274:21519-27.
53. Nolz JC, Nacusi LP, Segovis CM, Medeiros RB, Mitchell JS, Shimizu Y, et al. The WAVE2 complex regulates T cell receptor signaling to integrins via Abl and CrkL-C3G-mediated activation of Rap1. *J Cell Biol* 2008;182:1231-44.
54. Rapoport MJ, Lazarus AH, Jaramillo A, Speck E, Delovitch TL. Thymic T cell anergy in autoimmune nonobese diabetic mice is mediated by deficient T cell receptor regulation of the pathway of p21ras activation. *J Exp Med* 1993;177:1221-6.
55. Zipris D, Lazarus AH, Crow AR, Hadzija M, Delovitch TL. Defective thymic T cell activation by concanavalin A and anti-CD3 in autoimmune nonobese diabetic mice. Evidence for thymic T cell anergy that correlates with the onset of insulinitis. *J Immunol* 1991;146:3763-71.
56. Rapoport MJ, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, et al. Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J Exp Med* 1993;178:87-99.
57. Kar B, Reichman CT, Singh S, O'Connor JP, Birge RB. Proapoptotic function of the nuclear Crk II adaptor protein. *Biochemistry* 2007;46:10828-40.
58. Colucci F, Bergman ML, Penha-Gonçalves C, Cilio CM, Holmberg D. Apoptosis resistance of nonobese diabetic peripheral lymphocytes linked to the Idd5 diabetes susceptibility region. *Proc Natl Acad Sci U S A* 1997;94:8670-4.
59. Cheung HW, Du J, Boehm JS, He F, Weir BA, Wang X, et al. Amplification of CRKL induces transformation and epidermal growth factor receptor inhibitor resistance in human non-small cell lung cancers. *Cancer Discov* 2011;1:608-25.
60. Pierdominici M, Marziani M, Giovannetti A, Oliva A, Rosso R, Marino B, et al. T cell receptor repertoire and function in patients with DiGeorge syndrome and velocardiofacial syndrome. *Clin Exp Immunol* 2000;121:127-32.
61. Zemble R, Luning Prak E, McDonald K, McDonald-McGinn D, Zackai E, Sullivan K. Secondary immunologic consequences in chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome). *Clin Immunol* 2010;136:409-18.

METHODS

Cell culture

Whole blood from all subjects was collected in BD Vacutainer Plus plastic tubes (BD Bioscience, San Jose, Calif).

PBMCs were obtained by using Ficoll-Hypaque Lympholyte density gradient centrifugation (Cederlane, Burlington, Ontario, Canada). PHA-activated T cells were generated by stimulating PBMCs with 5 μ g/mL PHA (Sigma-Aldrich, St Louis, Mo) and IL-2 at 600 U/mL (PeproTech, Rocky Hill, NJ). Afterward, PBMCs were seeded at a density of 5×10^5 cell/mL in 96-well round-bottom plates and maintained in culture with RPMI supplemented with 60 mg/L penicillin, 12.5 mg/L streptomycin, 2 mmol/L L-glutamine, and 10% heat-inactivated FBS (EuroClone, Sizzano, Italy) with 1200 U/mL IL-2. These cells were used 3 weeks after culture and expansion for different experiments. PHA-activated T lymphocytes have been cultured for 12 hours with medium alone and then stimulated as indicated. Jurkat T cells were maintained in fresh culture medium (RPMI supplemented as above) and used 48 hours after transfection with *CRKL*-specific siRNA.

Copy number variation analysis

CRKL haploinsufficiency was evaluated by means of copy number variation analysis with a *CRKL*-specific probe. Twenty nanograms of DNA were extracted with the DNA Blood Mini Kit (Qiagen, Hilden, Germany) from peripheral blood and incubated in quadruplicate by using a TaqMan *CRKL*-specific probe with TaqMan Genotyping Master mix (Life Technologies, Grand Island, NY). The PCR amplification reaction was carried out with the 7500 Fast Real Time System (Life Technologies), and data were analyzed with CopyCaller 2.0 (Life Technologies).

Flow cytometry

Proliferation assay. CFSE (Sigma-Aldrich)-labeled PBMCs were seeded in a 24-well plate at a density of 5×10^5 cells/mL in the presence of PHA at 5 μ g/mL (Sigma-Aldrich) or 5 μ g/mL α CD3 (BD Biosciences) pre-coated to the wells and 750 ng/mL α CD28 (BD Biosciences). After 72 hours, cells were collected, washed in PBS twice, and acquired with a FACSCalibur cytometer (BD Biosciences); data were analyzed with FlowJo 7.5 software (TreeStar, Ashland, Ore). Analysis of division, division index, proliferation index, and cell percentage at each generation were calculated according to the manufacturer's instructions.

Briefly, the division index expresses the average number of divisions a cell has undergone in the original population. It is an average value and also includes cells that have never divided. It was calculated as the number of cell divisions performed by a selected cell population.

The proliferation index is the total number of divisions divided by the number of cells that underwent division. The proliferation index includes only the cells that divided at least once. It was calculated as the total number of cell replications divided by the number of cells that underwent division, and thus only the cells that respond to stimulation are reflected in the proliferation index.

In selected experiments we have investigated lymphocyte proliferation after addition of increasing concentrations (0.5, 5, and 10 μ g/mL) of IL-2 neutralizing antibody (Anti-IL2 mAb, clone 5334; Sigma-Aldrich).

Apoptosis assay. PBMCs (5×10^5 cells/mL) were seeded in 24-well plates in culture medium with LPS at 200 ng/mL (PeproTech), in medium alone, or in medium without serum. After 96 hours, cells were collected and stained with Annexin V and propidium iodide, according to the manufacturer's protocol (Dead Cell Apoptosis Kit; Invitrogen, Eugene, Ore), and acquired.

Cell-surface staining. Whole blood (100 μ L) was stained for immunophenotypic analysis. Lymphocyte subset analyses were performed with the following combination of mAbs (Becton Dickinson): (1) anti-CD14/fluorescein isothiocyanate (FITC), CD56/phycoerythrin (PE), CD16/peridinin-chlorophyll-protein complex (PerCP)-Cy5.5, CD3/PE-Cy7, CD19/allophycocyanin (APC), CD8/APC-H7, CD4/Bv421, and CD45/V500 and (2) anti-CD45RA/FITC, CD31/PE, CD8/PerCP-Cy5.5, CD3/PE-Cy7, CD4/APC, and CCR7/Bv421. After staining, samples were lysed, fixed, and acquired on FACSCanto II cytometer.

For analysis of activation markers, 100 μ L of whole blood was stimulated with IL-2 (100 U/mL), α CD3 (5 μ g/mL), and α CD28 (750 ng/mL) for 2, 4, or 24 hours. Cells were washed in PBS and stained with α CD4/PerCP-Cy5, α CD25/PE, and α CD69/FITC (BD Bioscience); washed in PBS; and acquired.

STAT5 phosphorylation. Whole blood (100 μ L) was stimulated with an increasing concentration of IL-2 (6, 60, 600, and 3000 U/mL) at 37°C for 10 minutes. Samples were fixed with Lyse/Fix Buffer at 37°C for 10 minutes and then washed twice with PBS, permeabilized with Perm Buffer III on ice for 30 minutes, stained with anti-phosphorylated (p)-STAT5/PE and α CD3/PerCP, washed in PBS, and acquired.

IL-2 secretion analysis. IL-2 protein levels were evaluated in supernatants (25 μ L) isolated from activated PBMCs by using the Flow-Cytomix Human Kit (eBioscience, San Diego, Calif), according to the manufacturer's instructions. Samples were acquired with BD FACSCalibur cytometer and analyzed by using FlowCytomix Pro 2.0 software.

Western blotting

PHA-activated T cells were used to evaluate protein expression levels. For CRKL, p-CRKL, p-ERK1/2, and p-STAT5, 2×10^6 cells were stimulated with SDF-1 α (300 ng/mL, PeproTech) for 5 minutes or IL-2 (200 U/mL) for 10 minutes. For cyclins D1, D2, and D3 and c-Fos, cells were stimulated for 24 hours with α CD3 (5 μ g/mL) and α CD28 (750 ng/mL) or with IL-2 (200 U/mL). Reactions were stopped by using ice-cold PBS, and cells were lysed in RIPA lysis buffer (EuroClone) supplemented with 0.5 mol/L EDTA and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, Mass).

After protein quantification with the Bradford method, 30 μ g of protein extract was loaded onto a NuPage 10% Bis-Tris Gel (Invitrogen, Carlsbad, Calif) and run for 2 hours at 80 V in NuPage MES SDS Running Buffer (Invitrogen). Then the proteins were transferred on Immobilon-P Transfer Membrane PVDF (Millipore, Temecula, Calif) for 1 hour at 350 mA in NuPage Transfer Buffer (Invitrogen). The membranes were blocked in 5% no-fat milk in Tris-buffered saline (TBS)-Tween buffer for 30 minutes at 37°C and further incubated overnight with primary antibodies diluted in TBS-Tween buffer. In these experiments antibodies against the following proteins were used: CRKL, c-Fos, cyclin D1, cyclin D2, cyclin D3, p-CRKL (pY207), p-STAT5 (pY694), and p-ERK1/2 (pT202/Y204; Cell Signaling, Danvers, Mass), or α -tubulin (Sigma-Aldrich) as a housekeeping control protein. Membranes were incubated with secondary antibodies diluted in TBS-Tween buffer (ie, goat anti-rabbit IgG-horseradish peroxidase or goat anti-mouse IgG-horseradish peroxidase; Santa Cruz Biotechnology, Dallas, Tex) for 1 hour, developed with LiteAbloT Extend ECL (EuroClone), and acquired by using the Biospectrum AC Imaging System (UVP, Upland, Calif). The computational analysis of Western blot images was done with Gel-Pro Analyzer 4.0 software. p-CRKL levels were normalized against CRKL. Levels of CRKL, cyclin D1, cyclin D2, cyclin D3, c-Fos, p-ERK1/2, and p-STAT5 were normalized against tubulin levels. Protein expression was evaluated by means of immunoblotting and quantitative densitometric analysis of the image. The extent of expression was calculated as the percentage of protein expressed by unstimulated cells (100% of expression levels).

Chromatin Immunoprecipitation assays

PHA-activated T cells (10×10^6) were stimulated with IL-2 at 200 U/mL (PeproTech) or α CD3 (5 μ g/mL) and α CD28 (750 ng/mL) for 45 minutes, and chromatin immunoprecipitation experiments were performed, as described elsewhere,^{E1} with minor modifications. In brief, nuclear extracts were prepared from 10×10^6 cells and then sonicated and immunoprecipitated with 5 μ g of anti-STAT5 rabbit polyclonal antibodies (sc-835, Santa Cruz Biotechnology). The coimmunoprecipitated material was then subjected to quantitative PCR analysis with the following promoter-specific primers (purchased from Life Technologies): *IL2RA*, forward TTCCACAGTTCTGA-GAAAGGTG and reverse CATGGCAAGGGTTTATGAG; prolactin (*PRL*), forward AGGGAAACGAATGCCTGATT and reverse GCAGGAAACA-CACTTACCA; and *BCL2*, forward GCTGCCTGCTTCTCTCTTAC and reverse GGAGGGGGAATCTGTGTTC. STAT5 recruitment to the *PRL*

promoter was quantified and used as a control for the specificity of the assay because it is completely silent in lymphoid and myeloid cells.

Real-time RT-PCR

CRKL mRNA expression was evaluated on Jurkat cells transfected with CRKL-specific siRNA after 48 hours of culture. Total RNA was extracted with the RNA Mini Kit (Qiagen) and retrotranscribed with the High Capacity Retro Transcription Kit (Applied Biosystems, Foster City, Calif). CRKL, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and actin (Applied Biosystems) mRNA expression was evaluated by using real-time PCR analysis with the CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, Calif).

siRNA transfection

Jurkat cells were transfected with CRKL siRNA or nontarget siRNA (Applied Biosystems) by using Amaxa 4D Nucleofector Systems (Lonza, Cologne, Germany), according to the manufacturer's instructions. In brief, 1×10^6 cells were transfected with CRKL siRNA (150 or 300 nmol/L) or nontarget siRNA (300 nmol/L) by Cell Line-Nucleofector Kit SE 4D (Lonza, Basel, Switzerland). The transfection efficiency, as evaluated after transfection with a green fluorescent protein-labeled control plasmid (pmax GFP control vector), was about 70% for each experiment.

Cells (1×10^6 cells/per sample) were harvested in a 15-mL falcon tube and resuspended in 10 mL of complete RPMI supplemented with 10 mmol/L HEPES. Then cells were centrifuged at 1200 rpm for 6 minutes and washed before adding 100 μ L of the Nucleofector solution. Cell suspensions were transferred into the Nucleocuvette and processed with the Jurkat Nucleofector protocol (CL120 program), according to the manufacturer's instructions. Transfected cells were then collected and mixed with RPMI supplemented with HEPES and transferred to 12-well plates. Real-time, immunoblot, or proliferation assays were then performed at 48 hours after transfection. CRKL siRNA sequences were as follows: 5'-GGUGAGAUCCUAGUGAUAAtt-3' (siRNA sense) and 5'-UUAUCACUAGGAUCUCACCct-3' (siRNA antisense).

Statistical analysis

All experiments were done in triplicate, and statistical analysis was performed by using nonparametric tests, including the Kruskal-Wallis test. A *P* value of less than .05 was considered statistically significant.

REFERENCE

- E1. Zimmermann M, Aguilera FB, Castellucci M, Rossato M, Costa S, Lunardi C, et al. Chromatin remodelling and autocrine TNF α are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun* 2015; 6:6061.

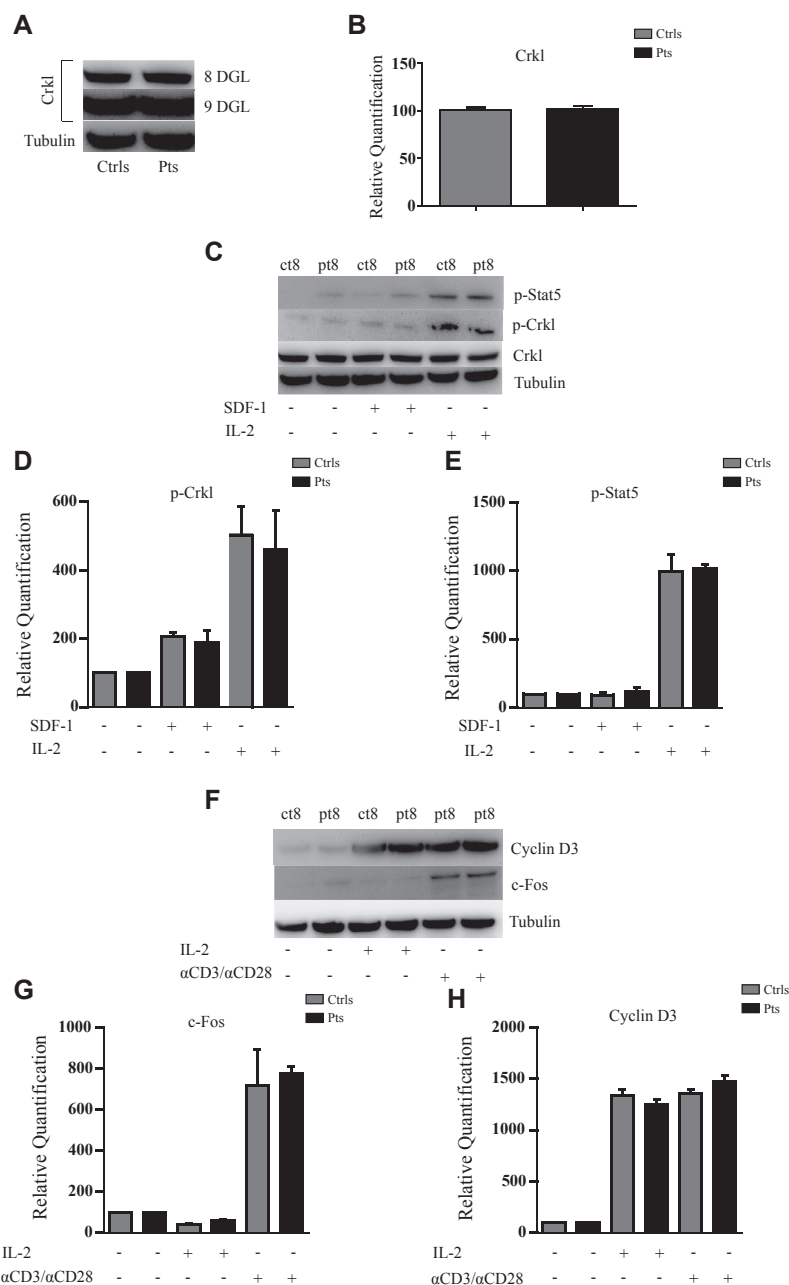


FIG E1. Expression of CRKL and p-CRKL in patients with DGL syndrome. **A**, Immunoblot analysis of CRKL levels in 2 patients with DGL syndrome and 2 control subjects. **B**, CRKL expression levels in patients with DGL syndrome and healthy control subjects. **C**, CRKL and STAT5 phosphorylation in response to IL-2 or SDF-1 α in a representative patient with DGL syndrome (pt8) and a healthy control subject (ct8). **D** and **E**, Relative quantification of p-CRKL (Fig E1, **D**) and p-STAT5 (Fig E1, **E**) in 2 patients with DGL syndrome and 2 healthy subjects. **F**, Cyclin D3 and c-Fos protein expression determined by immunoblot analysis in PHA-activated T cells from a representative patient with DGL (pt8) and a healthy control subject (ct8). **G** and **H**, Relative quantitation of c-Fos and cyclin D3 protein expression in 2 patients and 2 healthy control subjects. Protein expression was measured by using immunoblot analysis followed by quantitative densitometry and normalized with tubulin levels.