

Letter to the Editor

Human phagocyte defect caused by a *Rac2* mutation detected by means of neonatal screening for T-cell lymphopenia

To the Editor:

T-cell receptor excision circles (TRECs) are byproducts of T-cell receptor DNA rearrangement and can be quantified as a surrogate marker for thymic output of naive T cells. Infants with severe combined immunodeficiency have low or absent TRECs in peripheral blood samples.¹ In 2008, Wisconsin added the TREC assay to its newborn screening program and demonstrated that this assay is able to detect a variety of causes of T-cell lymphopenia.² During the first year of screening, we encountered the surprising finding of a primary neutrophil migration defect caused by a mutation in *Rac2* in an infant with low TREC numbers, which we will present here.

The patient was a full-term male born to nonconsanguineous parents. Newborn screening detected low TRECs with adequate β -actin controls (Table I). He was evaluated at 2 weeks of age and appeared healthy. Confirmatory laboratory testing showed leukocytosis (white blood cell count, 29,800/ μ L) and neutrophilia (absolute neutrophil count, 23,800/ μ L). Flow cytometry demonstrated modest CD4⁺ T-cell lymphopenia with an increased percentage of memory CD4⁺ T cells (CD45 RO⁺ cells, Table I). Although the patient's lymphocyte subsets were low, they were not decreased to the levels typically seen in patients with severe combined immunodeficiency.

On day of life 26, he presented with fever and periumbilical erythema. He subsequently underwent surgical debridement for omphalitis with appropriate healing of the surgical site. Further immunologic evaluation did not disclose other abnormalities at that time (Table I). On day of life 56, he was evaluated and found to be pale and lethargic with severe macrocytic anemia (hemoglobin, 6.8 g/dL), continued leukocytosis, and neutrophilia (white blood cell count, 26,300/ μ L; absolute neutrophil count, 18,300/ μ L). A computed tomographic scan revealed a paratracheal abscess that was drained, and cultures grew *Stenotrophomonas* and *Prevotella* species. Biopsy of the abscess wall showed minimal inflammation with few neutrophils. This prompted further investigation of the patient's neutrophil function. Results of flow cytometry with antibodies to CD18 and CD15 was normal, excluding leukocyte adhesion deficiency types I and II, respectively (Table I). Neutrophil chemotaxis, however, was severely decreased when compared to neutrophils from control subjects (Fig 1, A). Because of the severity of the infection and a presumed neutrophil migration defect, the patient received granulocyte transfusions over 3 weeks and then underwent a 5 of 6 matched unrelated umbilical cord blood transplantation at 3.5 months of life. He is currently 25 months old, fully engrafted from his transplant, and doing well. A detailed report of the pretransplantation conditioning of this and 4 other patients detected by the TREC assay will be discussed in a forthcoming article (manuscript in preparation).

Further studies examining neutrophil function included a normal oxidative burst to phorbol 12-myristate 13-acetate (PMA), demonstrating a normal nicotinamide adenine dinucleotide phosphate oxidase complex (Fig 1, B; the methods are described in the Methods section of this article's Online

TABLE I. Laboratory results

Laboratory test	Day of life			
	14	26	30	>56
Lymphocyte enumeration				
Absolute lymphocyte count/mm ³ (normal, 3400-7600)	4158	2795	4986	3077
Absolute CD3/mm ³ (normal, 2500-5500)	2869	1425	3041	1231
Absolute CD3 ⁺ CD4 ⁺ /mm ³ (normal, 1600-4000)	1497	783	1795	769
Absolute CD3 ⁺ CD8 ⁺ /mm ³ (normal, 560-1700)	1372	643	1247	462
CD4 ⁺ CD45RO (%) (normal, 2% to 22%)	49	37	51	32
CD56 ⁺ /mm ³ (normal, 170-1100)	144	615	1196	677
Absolute CD19 ⁺ /mm ³ (normal, 300-2000)	166	252	698	800
Neutrophil markers				
CD18 ⁺		100%		100%
CD15 ⁺		98%		100%
CD11b ⁺		92%		99%
ADA enzyme activity (nmol · h ⁻¹ · mg ⁻¹ ; normal, 63 ± 41.4)			55.8	
PNP enzyme activity (nmol · h ⁻¹ · mg ⁻¹ ; normal, 1.336 ± 443)			1185	
IgG (139-725 mg/dL)			413	411
IgA (1-40 mg/dL)			<7	<6
IgM (11-68 mg/dL)			<4	7
TRECs (repeated assays)	0, 4, 0	5, 4	5, 13	20, 33

PNP, Purine nucleoside phosphorylase; ADA, adenosine deaminase.

Repository at www.jacionline.org). However, our patient's neutrophils failed to respond to N-formyl-methionyl-leucyl-phenylalanine (fMLP), a physiologic activator of neutrophils (Fig 1, B).³ The combination of defective oxidative burst in response to fMLP but not PMA and defective neutrophil chemotaxis suggested a signaling defect. Our patient did not exhibit a bleeding diathesis typical of leukocyte adhesion deficiency type III (eg, mutations in *KINDLIN3*).⁴ A patient with a similar clinical phenotype, defective neutrophil chemotaxis, and defective oxidative burst to fMLP has been described, with these effects caused by a mutation of *Rac2*.^{5,6} Sequence analysis of our patient's PBMCs demonstrated a guanine to adenine substitution at codon 57 (GAC → AAC) that resulted in the substitution of asparagine for aspartic acid (data not shown). Neither parent carried this mutation (data not shown). We next tested the adhesive properties of this patient's neutrophils to fibronectin upon stimulation with TNF- α or fMLP. The patient's neutrophils exhibited abnormally enhanced adhesion at baseline that increased after activation (Fig 1, C). We also investigated actin polymerization by using phalloidin staining of migrating neutrophils and demonstrated that control neutrophils activated with fMLP showed actin capping and cortical actin polarization, whereas the patient's neutrophils showed evidence of disorganized actin formation without polarization (Fig 1, D).

Rac2 is a member of the Rho family of guanosine triphosphatases, a group of signaling molecules that bind downstream

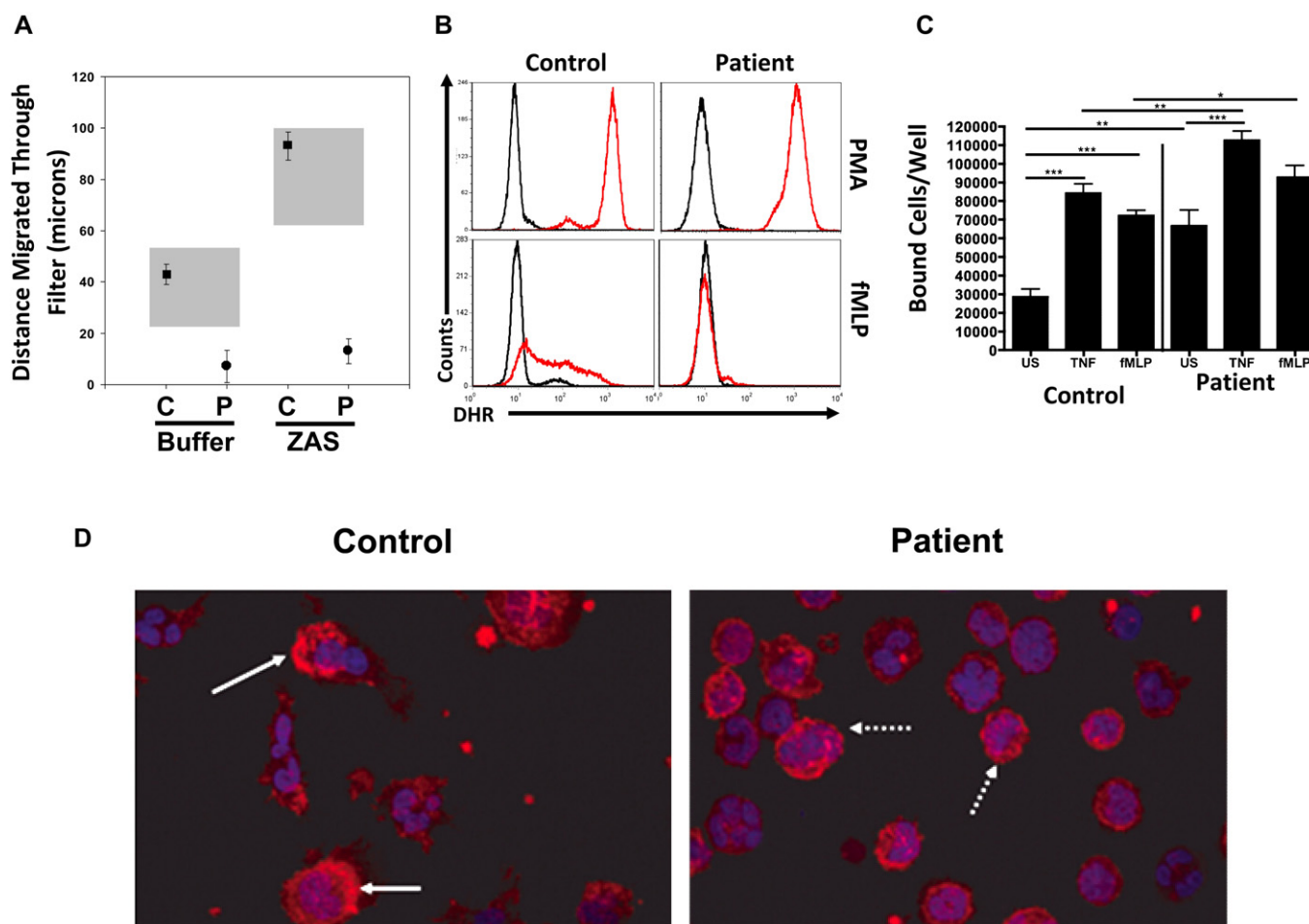


FIG 1. **A**, Neutrophil migration of control (C) or patient (P) neutrophils in response to buffer or zymosan-activated serum (ZAS) using a Boyden Chamber. The shaded areas show the range of normal. **B**, Oxidative burst of control and patient neutrophils in response to PMA or fMLP. DHR, dihydrorhodamine. **C**, Neutrophil adhesion to fibronectin following TNF- α or fMLP activation. *** $P < .001$, ** $P < .01$, * $P < .05$. **D**, Actin staining of neutrophils following stimulation with fMLP. Notice the polarization and actin cap formation in control neutrophils (solid arrows), and disorganized pattern of actin without evidence of polarization in the patient's neutrophils (dashed arrows).

effector molecules and activate a variety of cellular signaling pathways, including actin polymerization and formation of the reduced nicotinamide adenine dinucleotide phosphate oxidase complex.⁷ The mutation in Rac2 occurs at a highly conserved amino acid involved in binding guanosine triphosphate.^{5,6} The mutant protein fails to bind guanosine triphosphate and appears to prevent downstream signaling of not only Rac2 but also Rac1.⁸ How this mutation affects Rac1 is unclear, but sequestration of guanine nucleotide exchange factors has been suggested.⁸ Rac proteins and other members of the Rho family work together for normal actin-filament polymerization and turnover during cell migration.⁹ Defects in any component can lead to dysfunctional cell migration and abnormal neutrophil adhesion. Rac2-deficient mice have defects in neutrophil migration that are similar to those seen in our patient.¹⁰ However, our patient's neutrophils also demonstrated enhanced adhesion to fibronectin, which is not apparent in Rac2-deficient mice¹⁰ and is different from the adhesion defect previously reported in a human patient.⁶ The reason for this discrepancy is unclear.

The most surprising aspect of these results is that the patient was detected by means of screening for TRECs. To understand the role of this mutation in lymphocyte function, we assessed lymphocyte intracellular calcium mobilization in response to activation with CD3 and CD28 antibodies and proliferation in response to mitogens. The patient's lymphocytes demonstrated normal intracellular calcium mobilization and proliferation (see this article's Fig E1, A and B in the Online Repository at www.jacionline.org). Control PBMCs, however, exhibited a characteristic aggregation of lymphocytes 24 hours after PHA stimulation that was not apparent in the patient's PBMCs (Fig 2, A). The aggregation of normal lymphocytes after PHA stimulation can be blocked with CD18 antibodies, indicating an integrin-dependent event (Fig 2, B). Thus although the patient's lymphocytes mobilized calcium and proliferated normally, they demonstrated abnormalities in integrin-dependent aggregation.

Studies with Rac1 and Rac2 knockout mice have shown defects in T-cell development, proliferation, survival, adhesion, and migration.¹¹ However, our patient did not demonstrate defects

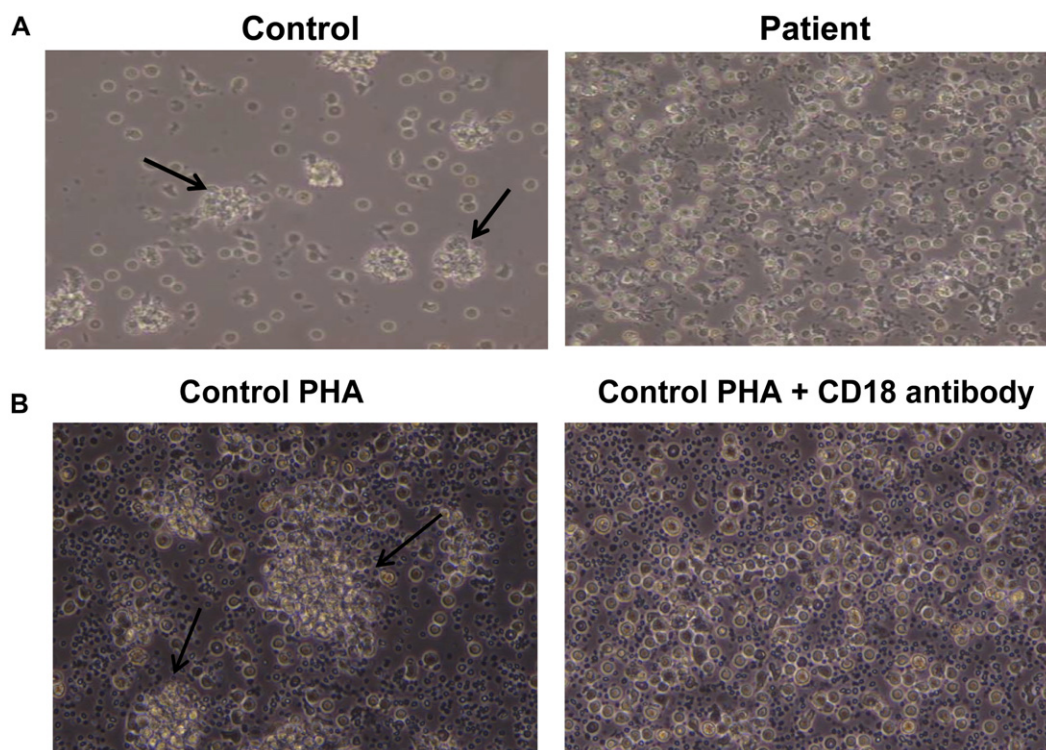


FIG 2. **A,** Phase contrast photography of patient or control PBMCs activated with PHA for 24 hours (*top*). **B,** Control PBMCs were PHA activated without (*left*) or with (*right*) CD18 blocking antibodies for 24 hours. Arrows indicated integrin dependent aggregation of lymphocytes (*dark arrows*).

in T-cell proliferation or activation *in vitro*. Interestingly, mice that express a dominant-negative Rac1 mutation have abnormal T-cell development, despite nearly normal T-cell activation.¹² Thus the phenotype of mice expressing the dominant-negative Rac1 mutation is similar to the phenotype found in our patient with a dominant-negative Rac2 mutation.

Collectively, these observations suggest that abnormal T-cell development in our patient was a result of the cumulative effects of abolishing Rac1 and Rac2 function. We hypothesize that abnormal integrin-dependent functions of T cells lead to defective T-cell development in the thymus due to defects in migration, adhesion, and proper T-cell activation and selection. Furthermore, this report demonstrates that newborn screening by means of TREC analysis is a powerful tool that may detect rare mutations that affect the function of leukocytes apart from T cells.

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METHODS

TREC analysis

TREC analysis was performed as previously described.^{E1} Briefly, dried blood spots from newborn screening (Guthrie) cards were obtained, a 3.2-mm disk was punched from the dried blood spot, DNA was extracted, and real-time quantitative PCR of TRECs or β -actin was performed.

Flow cytometric evaluation

One to 2 mL of whole blood collected in sodium heparin tubes was stained with antibodies to CD3–fluorescein isothiocyanate (SK7), CD4–phycoerythrin (PE; SK3), CD8–PE (SK1), CD19–PE (SJ25C1), CD45–PE(2D1) (BD Immunocytometry Systems, San Jose, Calif), CD56–allophycocyanin (N901; Beckman Coulter, Fullerton, Calif), and CD45RO–peridinin-chlorophyll-protein complex (2D1; Invitrogen, Carlsbad, Calif) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Measurement of the neutrophil respiratory burst

The ability of neutrophils to generate a normal oxidative burst was measured by using the dihydrorhodamine 123 (DHR) assay. Briefly, whole blood was labeled with DHR (50 μ g/mL, Invitrogen) for 10 minutes. PMA (10 ng/mL; Sigma, St Louis, Mo) or fMLP (2.2 μ g/mL, Sigma-Aldrich, St Louis, Mo) was added to the DHR-labeled neutrophils and incubated for 15 minutes. The oxidation of DHR in activated neutrophils results in an increase in fluorescence, as detected by using flow cytometry.

Neutrophil adhesion and actin polymerization

Peripheral blood neutrophils were purified from human blood by using Polymorphprep, according to the manufacturer's recommendations (Nycomed, Sheldon, United Kingdom). For immunofluorescent staining, neutrophils (1×10^6) were plated on glass cover slips coated with 10 μ g/mL fibronectin (Sigma-Aldrich) in the presence of 0.25 μ g/mL recombinant human TNF- α (BioSource, Camarillo, Calif) or 10 nmol/L fMLP for 30 minutes. Cells were then fixed with 6.6% paraformaldehyde, 0.05% glutaraldehyde, and 0.25 mg/mL saponin in PBS with Ca^{2+} / Mg^{2+} for 15 minutes, as described previously.^{E2} This reaction was quenched with 0.15 mol/L glycine, blocked in PBS containing 10% heat-inactivated FBS and saponin, and then stained with rhodamine/phalloidin (Molecular Probes, Junction City, Ore) at a 1:40 dilution and 4'-6-diamidino-2-phenylindole dihydrochloride at 1:10,000 in PBS. Cover slips were imaged with a 60 $\times$ /1.4 oil objective on a laser scanning confocal microscope (CI; Nikon, Tokyo, Japan).

For the neutrophil adhesion assay, neutrophils were incubated with 0.48 μ mol/L of the cell-permeant fluorescent indicator calcein-

acetoxymethyl ester (Molecular Probes) for 15 minutes at 37°C. Cells were then washed, suspended at 1×10^6 cells/mL in PBS, and then treated with either 0.25 μ g/mL TNF- α or 1 μ mol/L fMLP. Neutrophils (1×10^5) were allowed to adhere for 30 minutes on a 2.5 μ g/mL fibrinogen (Sigma Chemical Co)–coated, black-sided, black-bottomed 96-well plate (Greiner, Kremsmuenster, Austria). Unbound cells were then removed by means of washing and vigorous shaking, and fluorescence excitation/emission at 485/535 nm was determined with a Multilabel HTS counter (Victor V; PerkinElmer, Waltham, Mass). Samples were run in quadruplicates. A standard curve with control neutrophils was included on the plate.

T-cell proliferation

PBMCs were isolated from heparinized blood by using Ficoll-Hypaque (Amersham, Piscataway, NJ) and resuspended at 1×10^6 cells/mL in PBS. An equal volume of 500 nmol/L 5,6-carboxyfluorescein diacetate succinimidyl ester in PBS was added to the PBMCs and incubated at 37°C. Carboxyfluorescein diacetate succinimidyl ester labeling was quenched by adding media (RPMI-1640 supplemented with 2 mmol/L L-glutamine [Gibco, Grand Island, NY], 50 μ mol/L 2-mercaptoethanol, 100 U/mL penicillin [Gibco], 100 μ g/mL streptomycin [Gibco], 10 U/mL IL-2, and 10% FCS [HyClone, Logan, Utah]). Cells were washed, then resuspended at 2×10^6 cells/mL in media, and activated with concanavalin A, PHA, PMA plus ionomycin, and anti-CD3. After 4 days of incubation at 37°C, cells were harvested, washed with PBS plus 0.1% azide, and stained with anti-CD8 peridinin-chlorophyll-protein complex–Cy5.5 and anti-CD4 allophycocyanin at room temperature for 15 minutes. Stained cells were washed, and flow cytometric data were acquired on a BD FACSCalibur (BD Biosciences). In some experiments cells were analyzed by means of phase-contrast microscopy after 24 hours of activation.

Intracellular calcium mobilization

PBMCs were incubated with 5 μ mol/L Fluo-4 in PBS (Molecular Probes). Cells were then resuspended in 2 mL of RPMI media, labeled with antibodies to CD3 (2.5 μ g/mL) and CD28 (2.5 μ g/mL), and then activated with goat anti-mouse IgG (Sigma). Cells were then analyzed on a BD FACSCalibur over time to measure Fluo-4 fluorescence.

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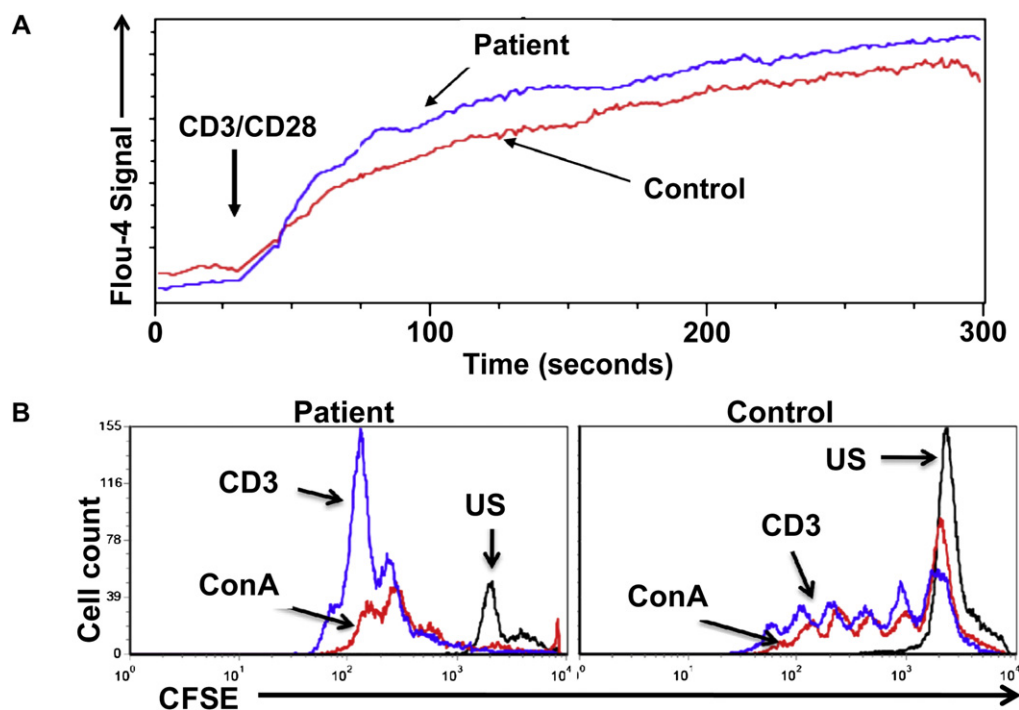


FIG E1. A, Intracellular calcium levels of patient and control lymphocytes following CD3 and CD28 activation. **B,** Lymphocyte proliferation in response to CD3 antibodies or Concanavalin A (Con A) by carboxy-fluorescein succinimidyl ester (CFSE) dilution.