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published in
Clinical Immunology
2020

DOI (link to publisher)
10.1016/j.clim.2019.108248

document version
Publisher's PDF, also known as Version of record

Clinical Immunology 212 (2020) 108248

Contents lists available at ScienceDirect

Clinical Immunology

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

Letter to the Editor

A dominant activating *RAC2* variant associated with immunodeficiency and pulmonary disease

To the Editor

Combined immunodeficiencies (CID) include combined defects of lymphoid cell development and function. We identified a previously reported heterozygous c.184G > A (pE62K) variant in the *RAC2* gene, in a family with three CID affected members [1].

RAC2 is part of the small Rho GTPase superfamily and is specifically expressed in hematopoietic cells. RAC2 is involved in ROS production through NADPH activation and f-actin remodeling, and is activated upon GTP binding [2]. The GTP/GDP exchange is catalyzed respectively by guanine exchange factors (GEFs) for GTP binding and GTPase activating proteins (GAPs) for GTP hydrolysis [2]. Normal activity of RAC2 and other small Rho GTPases were shown to be crucial for neutrophil and macrophage motility in larval zebrafish and for normal lymphocyte development in mice [3,4]. In humans, earlier loss of function mutations have been described (c.169G > A (p.D57N), c.167G > A (p.W56X) that cause reduced chemotaxis and ROS production in neutrophils and sometimes abnormal lymphocyte numbers [5,6].

Recently, activating RAC2 variants were described such as the c.184G > A (pE62K) variant, previously reported by Hsu et al. in three unrelated CID patients. Here, decreased GTP hydrolysis relayed an increase in active RAC2 leading to severe lymphopenia and neutrophil dysfunction [1]. They replicated these findings in cell culture-based experiments and mouse models and showed upregulated signaling that resulted in increased ROS production and polymerized actin [1]. We report on three related patients with a similar phenotype that carry this RAC2-E62K variant, and elaborate on the molecular changes.

The affected members were a 1-year old (P1), his father (P2) and grandfather (P3). P1 had suffered from respiratory tract infections (RTIs) requiring treatment with oral antibiotics. P2 and P3 had suffered from similar recurrent RTIs causing pulmonary damage leading to end-stage pulmonary failure for which they both required lung transplantation; P3 passed away during this procedure. Lymphocyte phenotyping of P1 and P2 showed T-cell and B-cell lymphopenia with high relative percentages of effector/memory T-cells (Supplementary Table S1), without bone marrow abnormalities. This differed from previously encountered *RAC2* mutations, which did not present with an absolute CID nor with end-stage pulmonary failure [5,6].

NGS-based immunodeficiency panel analysis showed a heterozygous variant c. 184G > A in *RAC2* causing a glutamic acid to lysine substitution at position 62 (p.E62K). Given the progressive disease course of P2 and P3, we performed hematopoietic cell transplantation (HCT) with unrelated cord blood after myelo-ablative conditioning in P1. Neutrophils reconstituted > $1{,}8 * 10^9$/mL after 23 days and CD4 T-cells reconstituted > $100 * 10^6$/mL after six months. Absolute CD4, CD8 and B-cell numbers normalized one year after transplant, during which P1 suffered from CMV and HHV6 reactivation and acute graft versus host (GvHD) of the gut and acute chronic GvHD of the skin which was accompanied by increased numbers of CD4 and CD8 effector/memory cells. He is being treated with prednisone, tacrolimus and mycophenolate which sufficiently suppresses his skin GvHD and his T-cell subsets are normalizing. He currently suffers from frequent infections and his pulmonary function test results are suboptimal. P2 is clinically well after pulmonary transplantation with normalization of his pulmonary function test and a reduction of his RTIs. He is still severely immunocompromised with CD4 counts < $100 * 10^6$/mL and lymphocyte counts < $0.5 * 10^9$/mL.

We used RAC2 crystal structures (PDB IDs 2W2V, 2W2T) and HADDOCK to structurally investigate variant E62K. We found no direct effect on GTP binding with minimal atom-atom distances > 1 nm and hence analyzed Rac2/RacGAP1 interaction [7]. We observed two salt-bridges in the highest-ranked docked RAC2-RacGAP1 structure that disappear upon introduction of the mutation (Fig. 1A). This could hamper GAP binding to RAC2, which would reduce GTP hydrolysis.

To confirm this, we measured relative GTP-bound RAC2 levels in patient neutrophils [5]. We immunoprecipitated GTP-bound RAC2 with PAK-loaded beads and found that only after fMLF stimulation there was a 2-fold increase of GTP-bound RAC2 in patient neutrophils (Fig. 1B). Data shown is from normalized data, obtained after merging three controlled experiments that each included healthy donor and patient data; examples of individual blots are shown.

To assess neutrophil function, we cultured neutrophils in 3D fibrin matrices [8]. Migratory speed was reduced by 50% (Fig. 1C) and when cultured with GFP-expressing *S. aureus* we found reduced bacterial killing, triggering outgrowth of *S. aureus* at a concentration of 5×10^6/mL and a multiplicity of infection of one (Fig. 1D).

Finally, we investigated the suppressed T-cell numbers in RAC2-E62K patients, which could be caused by decreased thymic emigration, because the patients expressed no bone marrow abnormalities, and normal thymic populations and outgrowth in RAC2-E62K mice were found [1]. The number of CD4 T-cells expressing recent thymic emigrant (RTE) markers were severely reduced in both patients, but partially restored in P1 after HCT (Fig. 1E). These results could be biased by relative abundance of effector memory T-cells that were previously shown in RAC2 patients and in P2, however, P1 had relatively normal naive and effector/memory numbers before transplantation and only showed an expansion of memory T-cells after transplant. Thus, the lymphopenia in RAC2-E62K patients could involve disturbances in RAC2 signaling-mediated chemotactic function of (precursor) T-cells.

This additional data supports that deficient GAP binding is the underlying cause for the clinical phenotype of the recently reported RAC2-E62K variant [1]. It corroborates that internal non-catalyzed GTP hydrolysis is sufficient to regulate RAC2 activation under unstimulated conditions. When patient neutrophils become activated, however, GAPs cannot bind efficiently and consequently GTP-bound RAC2 increases. The same mechanism might be relevant for other *RAC2* variants at the interface with GAP binding, supported by the fact that patients with a

https://doi.org/10.1016/j.clim.2019.108248
Received 16 June 2019; Received in revised form 1 August 2019; Accepted 1 August 2019
Available online 02 August 2019

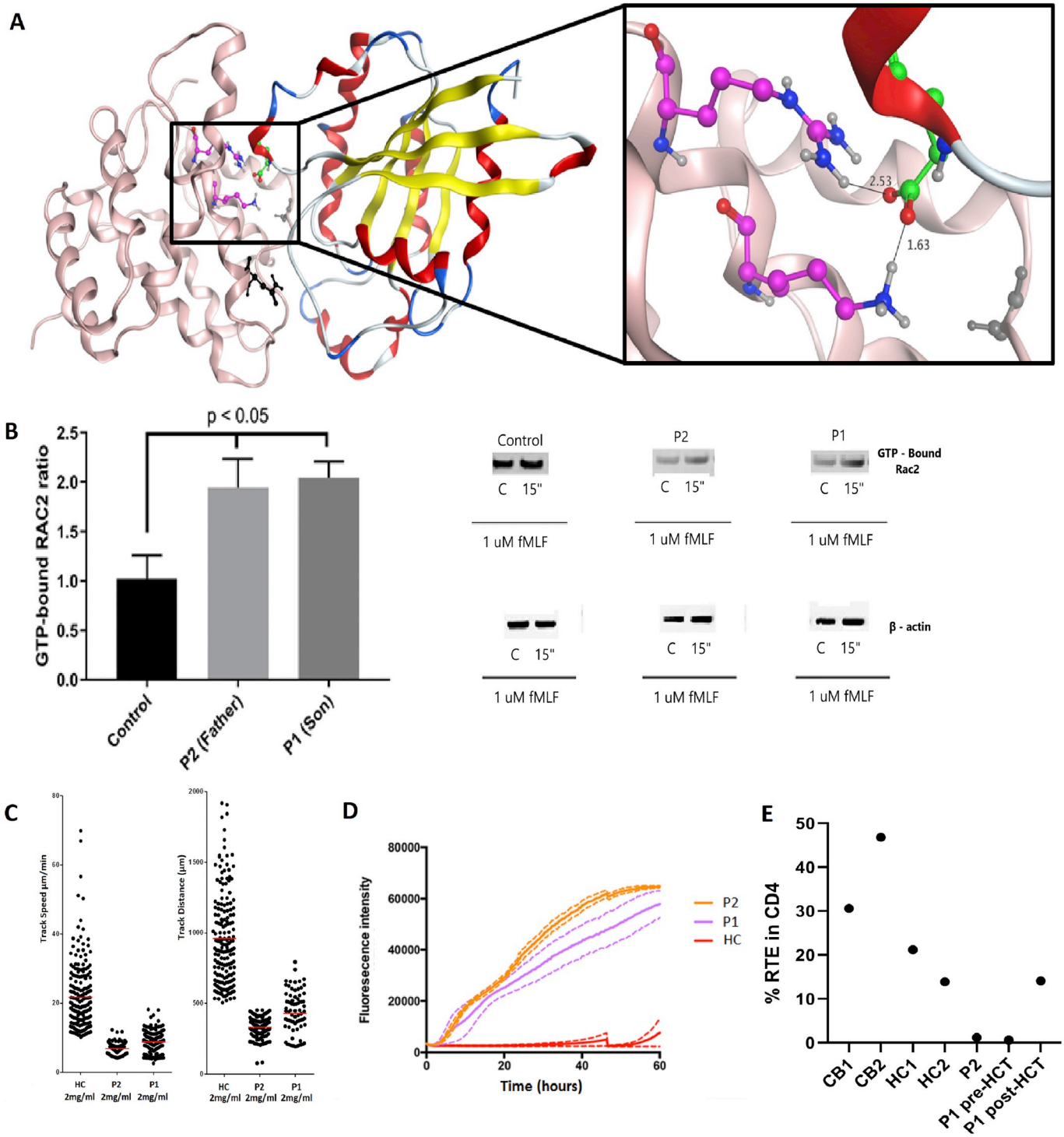

Fig. 1. A. Docked model of the RAC2-RacGAP1 complex; the right panel zooms in on the interface region that includes residue E62 of RAC2 (highlighted in ball-and-stick representation, carbon atoms in green). Within the complex, RAC2 is the protein on the right-hand side, RacGAP1 is given in pink. Two candidates for salt-bridge formation with E62 (K423 and R427 of RacGAP1) are shown in ball-and-stick representation with carbons in magenta; atomic distances are indicated in Å. Other key residues for RacGAP1 binding (N495, black; G388, grey) are also highlighted.

B. GTP-bound RAC2 ratio, normalized for GTP-bound RAC2 at t = 0, both father and son have significantly more GTP-bound RAC2 after 15 seconds of stimulation with fMLF (1µM, one-way ANOVA, $p < 0,05$) when compared to neutrophils of one healthy donor. This effect was replicated twice after which the average GTP-bound ratios were compared.

C. Chemotaxis of affected neutrophils compared to a healthy control. Both the track speed (left) and distance (right) of patient neutrophils is diminished when compared to healthy controls when stimulated with fMLF in varying concentrations of fibrin gel (concentration under y axis).

D. Fluorescence after incubation of GFP-expressing S. aureus with healthy and patient neutrophils. Patient neutrophils are incapable of killing S. aureus resulting in immediate colonization at an S. aureus concentration of 5 x 10^6 (S4A), at a concentration of 10 x 10^6 the healthy neutrophils can also not prevent S. aureus from growing.

E. Number of recent thymic emigrants in father (P2) and son (P1) compared to adult healthy controls (HC) and cord blood of two new-borns (CB) in the CD4 T-cell population gated as CD3 +, CD4 +, CD8-, CD45RA +, CD27 +, CD25 +, CD62L-, CD31-, and CD21 +.

P34H mutation show a similar phenotype [7,9].

The increase in GTP-bound RAC2 might impede physiological f-actin polymerization, which could cause neutrophilic dysfunction, and possibly, the underlying lymphopenia by hampering thymic emigration. This is reminiscent of phenotypes of WASp and DOCK8 variant cells, which are both effectors of the small GTPase Cdc42 [10].

In conclusion, we report additional clinical and molecular findings on the recently discovered dominant activating RAC2-E62K variant, which we found in three related patients. Our data support that the RAC2-E62K variant leads to more GTP-bound, active RAC2 by hampered GAP binding.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.108248.

Authorship contributions

BS, CK, MD, DG, SN, MB, MG, LK, AB, HW and MT conceived and designed experiments.

BS, FV, CK, MD, PL, LC and MT performed the experiments.

BS, CK, MD, DG, MB, and LK analyzed the data.

JM, SN, HL, JJB, CL, AB and LC provided clinical care.

SN, MB, MG, LK, AB and HW provided reagents, materials and analysis tools.

BS, MB, HRW and LK wrote and edited the manuscript.

All authors read and approved the final manuscript.

Declaration of Competing Interest

None to disclose.

Acknowledgements

We thank Ninke Nieuwenhuis and Willemien Zuilhof for discussions on and reviewing of previous studies on RAC2-GAP interactions. We also would like to thank our patients for providing us with the information and samples needed to perform this study.

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