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Letter to the Editor

The RAC2-PI3K axis regulates human NK cell maturation and function

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To The Editor

Recently, germ-line activating mutations in Rac2, a Rho guanosine triphosphatase (GTPase) with exclusive expression in hematopoietic cells, resulting in increased PI3K activity, were reported in 7 patients causing a combined immunodeficiency with lymphopenia, hypogammaglobulinemia, T and B cell alterations and viral infections [1–3]. Prior studies suggest a role for RAC proteins in natural killer cell biology [4–6]. Thus, patients' susceptibility to viral infections raises the possibility of defective NK cell maturation or function. Data regarding NK cells from patients with RAC2 activating mutations are lacking. Here, we report the first phenotypic and functional description of NK cells from patients with activating mutations in *RAC2*, revealing both developmental and functional alterations. We demonstrate that the latter can be modified *in vitro* by selective inhibition of p110delta.

The clinical description of the patients, as previously reported in detail [1], was notable among other features, for viral infections, in particular HZV and HPV [1]. The patients had constantly reduced numbers of NK cells during follow-up (Fig. 1A). Analysis of the maturational status of patients' peripheral NK cells, evaluated by means of CD57 (on $CD56^{dim}$ NK cells subset) and CD62L expression [7] (Fig. 1B), revealed a reduced expression of both receptors suggesting that RAC2 is implicated in human NK cell maturation. These cells showed increased expression of the activating receptor CD69 [8] (Fig. 1C, left upper panel), compatible with an activated status. Of note, patients' NK cells displayed reduced levels of the natural cytotoxicity receptors (NCRs) NKp46, NKp30 and of the activating receptor CD16 (Fig. 1C, lower panel) suggesting that RAC2 hyperactivation negatively modulates their expression. The expression levels of the inhibitory receptor NKG2A and of its corresponding activating receptor NKG2C (Fig. 1C, mid and right upper panel) on patients' NK cells resulted increased. Chemokine receptors CXCR1, CXCR3 and CCR7 expression levels resulted decreased on patients' NK cells, suggesting a potential involvement of RAC2 in human NK cell migration ability (Fig. 1D). Evaluation of additional NK cell markers (Pan-KIRs, NKG2D and Perforin content) did not show significant alterations (Supplementary Fig. S1A).

To assess the patients' NK cell function, the surface expression of CD107a, which reflects degranulation, a hallmark of $CD56^{dim}$ NK cells, was measured on resting NK cells from the patients and controls against the human erythroleukemia cell line K562: patients' NK cells demonstrated significantly increased CD107a expression ($p < 0.01$) (Fig. 1E). Instead, upon IL-2 stimulation, patients' NK cell degranulation against the K562 cell line was impaired when compared to healthy controls in a statistically significant manner ($p < 0.01$) (Fig. 1E), which is in line with the reduced expression of NKp46 and NKp30 (activating NK cell receptors directly involved in killing of K562 target cell line [8]) on patients' NK cells (Fig. 1C, left and mid lower panel). In order to further investigate the specific effect of Rac2 mutation in the degranulation ability of single NK cell receptors, redirected killing of IL-2 stimulated NK cells derived from patients against the mouse mastocytoma cell line P815 was performed. Cross-linking of NKp46 and NKp30 resulted in reduced degranulation against the target cell line when compared to NK cells from healthy controls (Fig. 1F), while CD16 cross-linking resulted equally efficient between patients' and healthy controls (Fig. 1F) in line with its normal expression in terms of percentages (data not shown). Overall, these data suggest a direct regulation of Rac2 on expression and function of NKp46 and NKp30. Production of IFN-γ, a hallmark of $CD56^{bright}$ NK cells, in Rac2 hyper-activated NK cells upon *in vitro* stimulation with IL-12 plus IL-18 did not result significantly impaired (Supplementary Fig. S1B). These results collectively suggest that Rac2 plays a critical, previously unrecognized, role in NK cell cytotoxicity upon *in vitro* NK cell stimulation.

RAC2 activating mutations have been shown to induce PI3K activation both in primary immunodeficiencies (germline mutations) [1–3] and in human tumors (somatic mutations) [9]. PI3K has been shown to play important roles in various aspects of NK cell biology, such as development/maturation, homing, and function [10]. Considering the NK cell alterations here described, we decided to evaluate pS6 levels on total patients' $CD56^{+}$ NK cells as well as on $CD56^{dim}$ and $CD56^{bright}$ NK cells. As shown in Fig. 1G (left panel), pS6 levels in patients' total NK cells were significantly higher at steady state when compared to healthy controls. Evaluation of pS6 levels in $CD56^{dim}$ and $CD56^{bright}$ NK cells revealed that the $CD56^{dim}$ population is the main subset accounting for the increased pS6 levels (Fig. 1G mid panel), while the $CD56^{bright}$ ones do not show major differences when compared to healthy controls (Fig. 1G right panel). Of note, *in vitro* inhibition with CAL-101, a specific p110delta inhibitor, restored normal pS6 levels in patients' NK cells, both total and $CD56^{dim}$ ones (Fig. 1G left and mid panel), while

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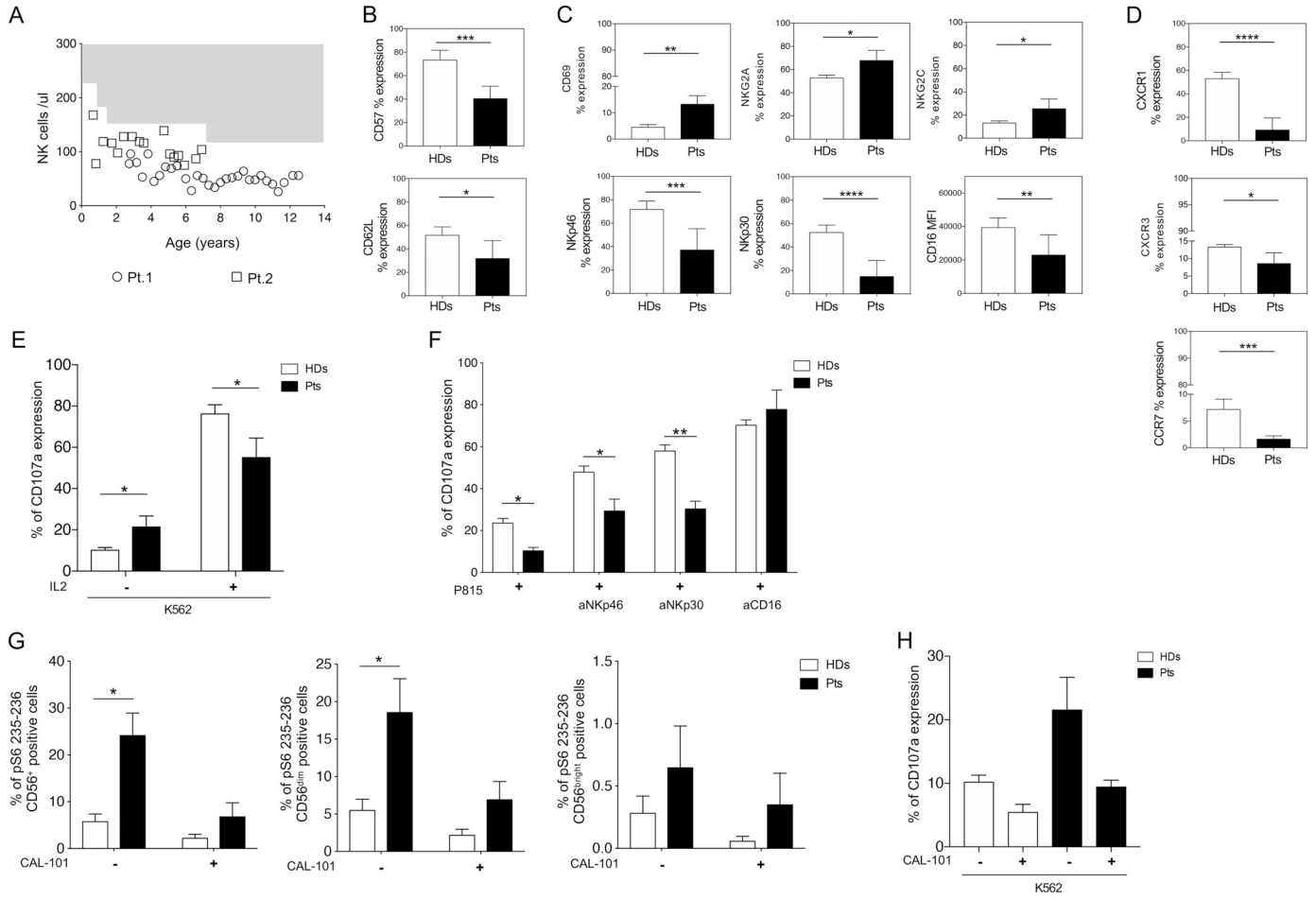

Fig. 1. Characterization and functional evaluation of NK cells from patients with activating mutation in Rac2. A. Patients' absolute NK cell counts over time. The grey area depicts normal NK cell counts for age. B. Expression of the NK cell maturation markers CD57 (on $CD56^{dim}$ NK cells) and CD62L on patients' NK cells compared to healthy controls. C. Expression of activating receptors CD69, the inhibitory receptor NKG2A and its corresponding activating receptor NKG2C, NKp46, NKp30 and CD16 on patients' NK cells compared to healthy controls. D. Expression of the NK cell chemokine receptors CXCR1, CXCR3 and CCR7 on patients' NK cells compared to healthy controls. E. Degranulation assay measured as CD107a expression against the K562 human erythroleukemia cell line without or with IL-2 stimulation. F. Redirected killing assay of IL-2 stimulated patients' NK cells against the mouse mastocytoma cell line P815 upon cross-linking of NKp46, NKp30 and CD16 activating receptors. G. Expression of pS6 in total patients' NK cells and in $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets with or without CAL-101 (p110delta specific inhibitor). H. Degranulation assay measured as CD107a expression against the K562 human erythroleukemia cell line with or without CAL-101, the p110delta specific inhibitor. Data in Fig. 1, B-H are pooled from 4 independent experiments performed on both patients and eight healthy controls. Statistical analysis was performed using the Student's *t*-Test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

the $CD56^{bright}$ ones remained almost unaffected (Fig. 1A right panel). Accordingly, *in vitro* inhibition of p110delta with CAL-101 reduced spontaneous degranulation in patients' NK cells against the K562 cell line to levels similar to those of resting NK cells from healthy controls (Fig. 1H) highlighting that Rac2 hyperactivation modulates PI3K activity in human $CD56^{dim}$ NK cells and affects their degranulation, a finding that has not been reported yet.

Our findings offer novel information on the biological role of Rac2 in human NK cell maturation and function. Since the clinical spectrum of affected patients includes recurrent viral infection and, as previously reported, patients' $CD8^+$ T cells present a senescent phenotype [1], the here described NK cell alterations may contribute to the viral infectious history of Rac2 mutated patients. Rac2 hyperactivation leading to increased PI3K activity appears to functionally affect mainly the $CD56^{dim}$ NK cell subset which can be pharmacologically modified *in vitro* with commercially available drugs with possible implications in the management of conditions where Rac2 activating mutations are germ-line (primary immunodeficiencies) [1–3] or somatic (tumors) [9].

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.108257.

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Disclosure of Competing Interest

The authors declare no conflict of interest.

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