Functional evaluation of Natural Killer cell cytotoxic activity in NFKB2-mutated patients

Letter to editor

Functional evaluation of Natural Killer cell cytotoxic activity in NFKB2-mutated patients

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Capsule Summary: defects of NK-cell cytotoxic activity have been recently reported in subjects with *NFKB2* mutations. Herein we present an extensive functional evaluation of NK cells, indicating that NK function is normal at least in some *NFKB2*-mutated patients.

Key words: immunodeficiency; NK cells; NFKB2

To the Editor:

Germline mutations affecting the C-terminus of NFKB2 are associated with early-onset hypogammaglobulinemia and anterior pituitary hormone deficiency. Autoimmunity and defects in peripheral T and B cells have been also described in these patients [1–4].

Recently, Lougaris et al. reported a male patient with immunodeficiency, central adrenal insufficiency, *alopecia totalis*, and trachyonychia, who carried a heteroyzgous truncating mutation (c.2557C>T, p.Arg853*) in *NFKB2*. The patient displayed a defective NK-cell cytotoxic activity *in vitro*, which could not be restored by the addition of human recombinant interleukin-2 (hr-IL2). Of note, NK-cell subsets showed regular expression of different NK receptors, and normal intracellular levels of perforin in terminally differentiated NK cell population. CD69 expression in mature NK cells was increased, suggesting an activated steady state [5]. Herein we report the results of an *in vitro* investigation of NK-cell function in three novel patients from two unrelated families, where we identified the same reported heterozygous mutation.

Patient 1 (II-1, family 1) is a woman born from unrelated Italian parents. At the age of six years, we diagnosed hypogammaglobulinemia with normal B cell count, following recurrent infections of upper and lower airways. At 12 years, she presented a severe hypoglycemic episode due to hypothalamic adrenocorticotropic hormone (ACTH) deficiency, and she was treated with hydrocortisone substitution therapy. Simultaneously, we observed a severe B-cells reduction ($CD19^+$ cells <1%). During follow-up, the patient presented occasional infections of the upper respiratory tract and developed *lichen planus pigmentosus* at the age of 25 years.

Patient 2 (III-1, family 1) was the second child of patient 1. Due to familial history, serum immunoglobulin and endocrinological assessment had been monitored since birth. At 12 months, immunoglobulin replacement was started, because the persistence of profound hypogammaglobulinemia, even in the absence of severe and recurrent infections. At 6

years, ACTH deficiency was diagnosed, and he was placed on treatment with hydrocortisone. He is now 8 years old, and does not present other signs or symptoms of anterior pituitary deficiency or other autoimmune features. B-cell count is still in the normal range.

Patient 3 (II-1, family 2) is a boy born from unrelated, healthy Italian parents. At 6 years, laboratory investigation was performed because of recurrent infections of upper airways and documented hypogammaglobulinemia and slightly reduced peripheral B cell population ($CD19^+$ cells 3.7%). Immunoglobulin replacement treatment was started. At 10 years, he presented hypotensive shock and prolonged hypoglycemia during an episode of enteric infection. Endocrinological evaluation revealed ACTH deficiency, and substitutive therapy was started. Over the subsequent follow-up, he developed also hypothyroidism and growth hormone deficiency requiring hormone replacement therapies. B cell count is now severely reduced ($CD19^+$ cells <1%).

In the context of a genetic screening program focused on immunological defects, whole exome sequencing was performed on the three patients, allowing to identify the nonsense c.2557C>T (p.Arg853*) change in *NFKB2* as the causative mutation in both families (Figure 1B). Sanger sequencing validated the identified change in probands, and genotyping confirmed co-segregation of the mutation with disease in family 1 and its *de novo* origin in family 2 (Figure 1A). The c.2557C>T (p.Arg853*) mutation had previously been reported in three unrelated cases with similar clinical features [1,6].

Extensive immunological evaluation of our three patients revealed anomalies in both T- and B-cell lineages (Table E1, Online Repository). B cells showed a slow decline over time, reaching <1% levels. We also disclosed a B-cell maturation defect, with a low switched-memory population (CD27+IgM-). Interestingly, follicular T helper cells were virtually absent in all patients, even before the disappearance of B cells.

Herpes viral susceptibility, reduced NK-cell levels, and defective NK-cell cytotoxic activity have been reported in *NFKB2*-mutated patients [1,2,5]. Therefore we performed an extensive functional evaluation of NK cells in order to confirm previous results and better characterize NK cells in NFKB2-deficient patients.

In all our patients NK cells count was normal. Surface expression of NKp46 and NKp30 (Natural Cytotoxic Receptors, NCR) - the major NK activating receptors - was similar to what observed in healthy control (Figure 2A). Intracellular expression of SLAM-Associated Protein (SAP) and perforin were also normal in NK cells (not shown).

We tested resting and activated NK-cell degranulation upon K562 stimulation, after overnight incubation of PBMC, basally or following IL2 stimulation, and measuring surface CD107a expression on $CD3^{-}CD56^{+}$ cells by cytofluorimetric analysis. In all cases, we observed normal degranulation compared with healthy control (Figure 2B).

To evaluate proliferation and expansion of polyclonal NK-cell populations, NK cells from patients and healthy controls were purified using the RosetteSep method (StemCell Technologies, Vancouver, BC, Canada), and cultured on irradiated feeder cells in the presence of phytohemagglutinin and r-IL2. The assay revealed a lower proliferation rate of *NFKB2*-mutated NK cells compared to healthy control.

To test the function of various NK cell triggering receptors, polyclonal activated NK-cell populations were tested in reverse antibody-dependent cellular cytotoxicity (R-ADCC) assays against ^{51}Cr-labelled P815 ($Fc\gamma Rc^{+}$) murine targets, in the presence of appropriate monoclonal antibodies. All the investigated pathways demonstrated a fully activation when compared with healthy control (see Figure 2C).

In summary, we confirm c.2557C>T (p.Arg853*) is a recurrent mutation in *NFKB2*-mutated patients. We documented proper physiologic maturation and cytotoxic activity, and regular recruitment of the activation pathways in NK cells from three subjects carrying this

heterozygous mutation. A slightly reduced stimulus-dependent proliferation was the most relevant feature in these cells.

Overall, our data indicate that NK function is not significantly impaired at least in some subjects with *NFKB2* mutation, which is in contrast to what previously reported [5]. Considering the fact that our patients share the same mutation of previously reported one, it could be hypothesized that some modifying genes are needed in determining functional impairment in *NFKB2*-mutated NK cells.

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LEGEND TO TABLE AND FIGURES

Figure 1. Families and sequence data. **A**, pedigrees of the families studied are depicted. Filled symbols indicate affected subjects; a short line above the symbol indicates available genomic DNA; “+/mut“ indicates the heterozygous state for the truncating (p.Arg853*) mutation. **B,** Sanger electropherograms showing the heterozygous state for the c.2557C>T change (mut). The corresponding wild-type sequence (wt) is also eported, together with the mutated codon and nucleotide substitution.

Figure 2. NK cell phenotype and function. **A**, NK cells ($CD3^-CD56^+$ PBMC) of the three affected subjects and a healthy control (H1) were analyzed by flow-cytometry; percentage of positive cells are indicated. **B,** resting and activated NK cell degranulation assay; results are percentage of ΔCD107+ cells (K562 stimulated – un-stimulated). **C,** polyclonal activated NK cells were tested against ^{51}Cr-labeled P815 target cells (E:T ratio 4:1), either in the absence (CTR) or in the presence of mAbs to the indicated molecules.

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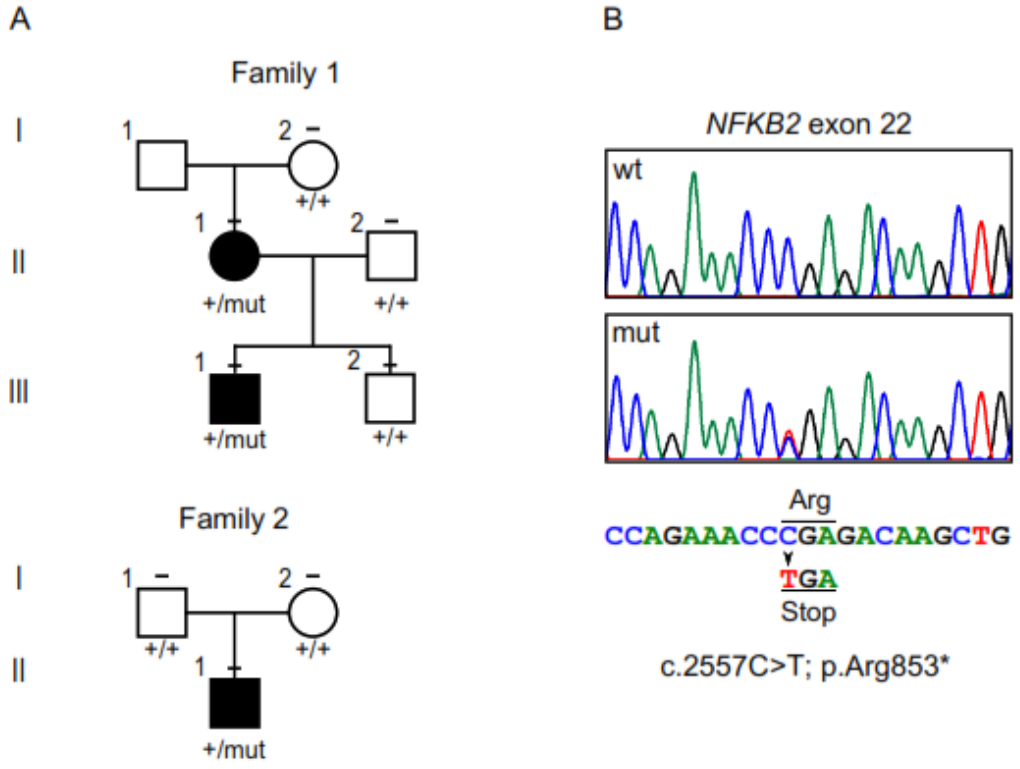
Figure 1


A
Family 1
I
II
III
1
2
+/+
+/mut
Family 2
B
NFKB2 exon 22
wt
mut
Arg
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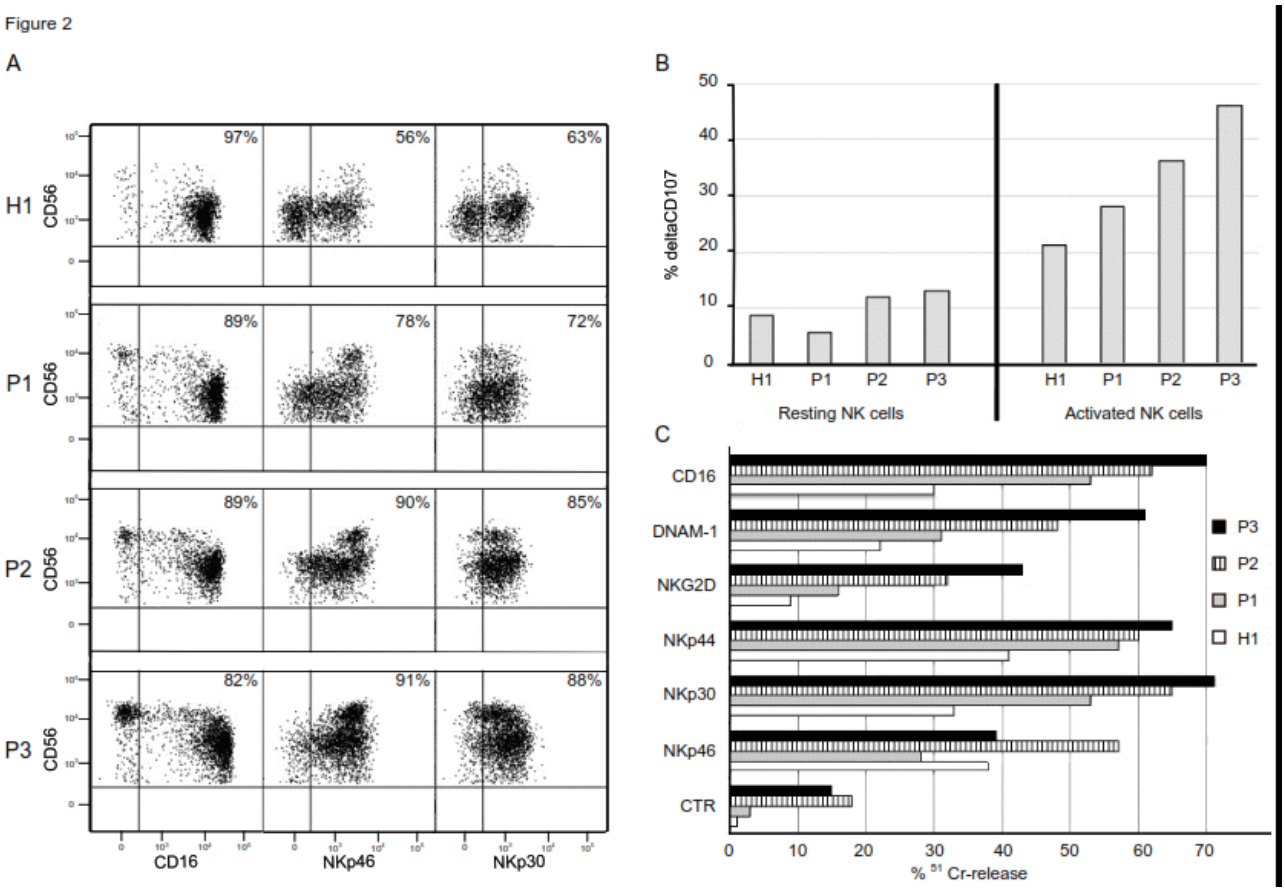
TGA
Stop
c.2557C>T; p.Arg853*

Figure 2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H1
P1
P2
P3
CD56
97%
56%
63%
89%
78%
72%
89%
90%
85%
82%
91%
88%
CD16
NKp46
NKp30
B
% deltaCD107
50
40
30
20
10
0
H1
P1
P2
P3
Resting NK cells
Activated NK cells
C
CD16
DNAM-1
NKG2D
NKp44
NKp30
NKp46
CTR
P3
P2
P1
H1
0
10
20
30
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60
70
% 51 Cr-release