

Letter to the Editor

p85 α is an intrinsic regulator of human natural killer cell effector functions

To the Editor:

The inositol phospholipid signaling plays an important role in the biology of natural killer (NK) cells.¹ A major role in this signaling cascade has been attributed to phosphoinositide-3 kinases (PI3K). Class I PI3Ks are divided into class IA (p110 α , p110 β , p110 δ) and class IB (p110 γ) kinases, which interact with the regulatory subunits p85 α , p50 α , p55 α , p85 β and p55 γ

(class IA) and p101 and p84 (class IB).¹ Mice lacking only the p85 α regulatory subunit, encoded by the *PIK3R1* gene, did not show any NK-cell defects,² whereas mice lacking both the p85 α and its alternatively spliced variants p50 α /p55 α showed maturational and functional NK-cell defects.³ Recently, the same monoallelic gain-of-function *PIK3R1* mutation was identified in 12 patients affected with a hyper-IgM-like primary immunodeficiency associated with T- and B-cell maturational and functional defects.^{4,6} However, the impact of this p85 α mutant on human NK-cell maturation and function has not been studied yet. We report on defective NK-cell effector functions,

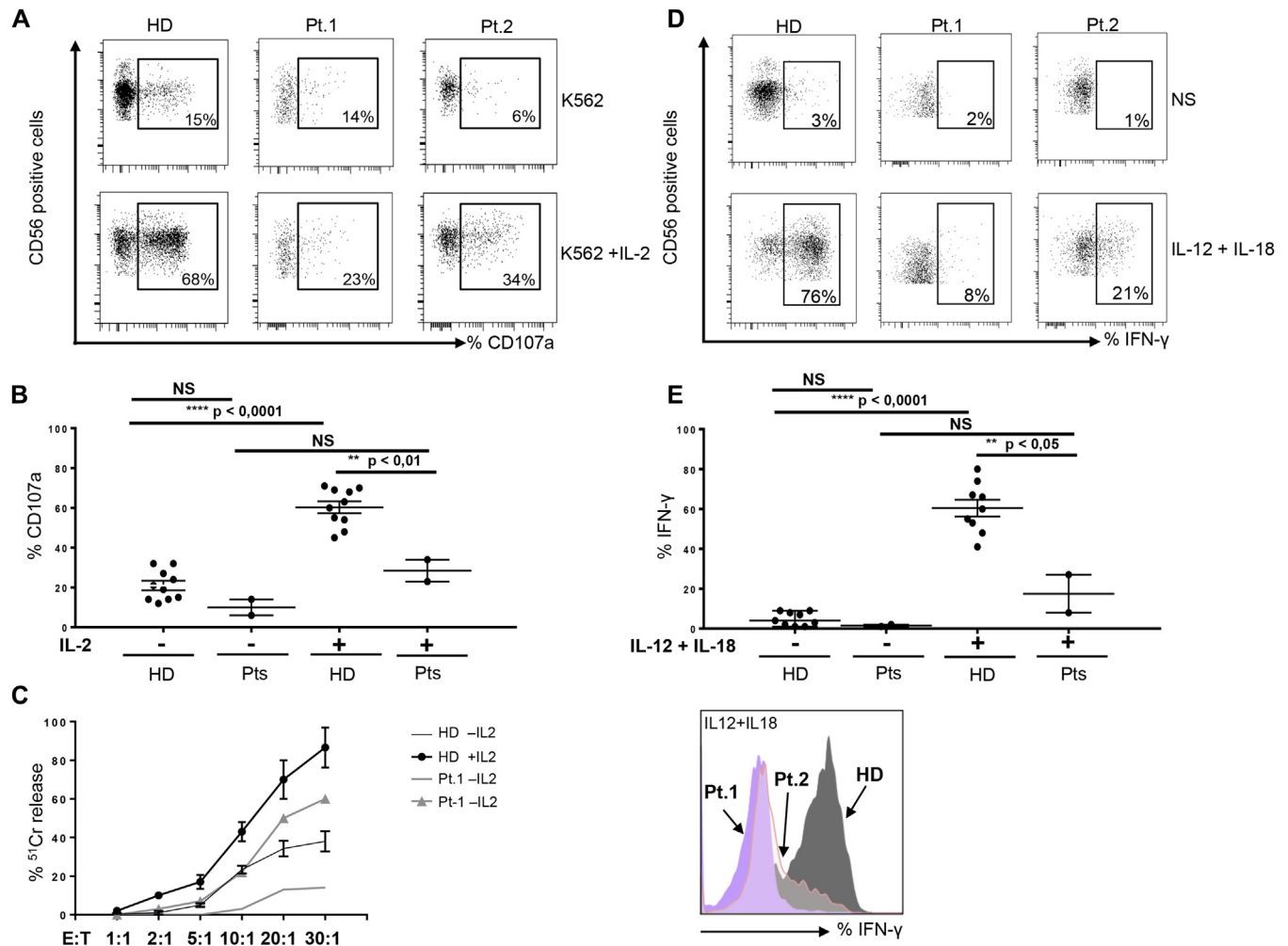


FIG 1. NK-cell cytolytic activity and IFN- γ production in the presence of the p85 α mutation. **A**, Patients' NK cells (patients 1 and 2) fail to upregulate CD107a on IL-2 stimulation against the human K562 erythroleukemia cell line when compared with a representative healthy control (HD). **B**, Summarized data from 3 experiments for CD107a upregulation from 10 healthy controls (HD) and the 2 index patients (patients 1 and 2). **C**, Defective cytotoxic activity of patient's PBMCs (patient 1) against NK-cell-susceptible human K562 cell line either before (gray continuous line) or after overnight incubation in the presence of IL-2 (gray line with triangle) when compared with 5 healthy controls (HD) (black line without or with circle, respectively). **D**, Evaluation of IFN- γ production before (NS, not stimulated; upper panel) and after (lower panel) stimulation with IL-12 and IL-18 in NK cells from the index patients (patients 1 and 2) and a representative healthy donor. **E**, Summarized data for IFN- γ production (shown as percentages of positive cells) from 9 healthy controls (HD) and the 2 index patients (patients 1 and 2) (upper panel). Statistical analysis was performed using the student *t* test (NS, not statistically significant). Histogram showing IFN- γ production from patients 1 and 2 and a healthy control (HD) (lower panel). Pt., Patient.

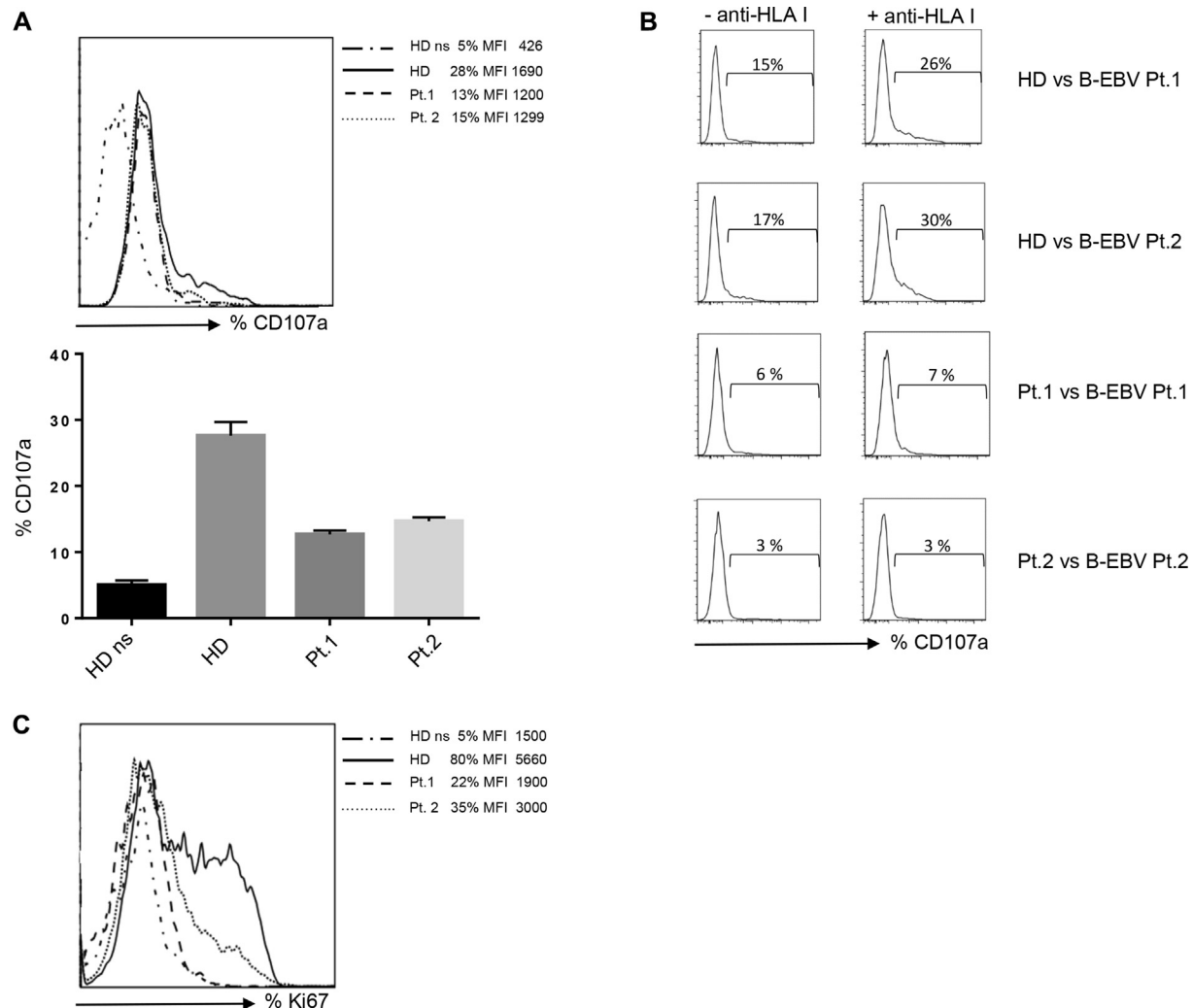


FIG 2. NK-cell cytolytic activity against EBV-infected B cells and IL-2-dependent proliferative capacity from p85 α -mutant patients. **A**, Evaluation of the cytolytic capacity of IL-2-activated NK cells from patients (patients 1 and 2) and representative healthy control (HD) against the NK-cell-susceptible human target cell line HLA class I- LCL 721.221 B-cell line. Representative experiment of 3 independent experiments. **B**, Evaluation of the cytolytic capacity of IL-2-activated NK cells from patients (patients 1 and 2) (lower panels) and healthy controls (HD) (upper panels) against patients' EBV-infected B cells with and without anti-HLA-I-blocking antibody (right and left column, respectively). Representative experiment of 3 independent experiments. **C**, Evaluation of the proliferative capacity of IL-2-activated NK cells from the index patients (patients 1 and 2) and representative healthy control (HD) as measured by Ki-67 intracellular expression. Representative experiment of 3 independent experiments. *Pt.*, Patient.

including degranulation, IFN- γ secretion, and lysis of EBV-infected cells in patients mutated in *PIK3R1* with dominant activation of the PI3K pathway.

NK-cell maturation from *PIK3R1*-mutated patients was normal (see Fig E1, A-C, and Results in this article's Online Repository at www.jacionline.org), which is in contrast with maturational defects described in the animal model.³ Although human NK-cell maturation was not perturbed, we decided, on the basis of animal model data,³ to investigate the functional consequences of the *PIK3R1* mutation in human NK-cell biology. Baseline degranulation against the human erythroleukemia cell line K562, measured as CD107a expression of resting NK cells, was similar between healthy controls and patients: 15% (healthy donor [HD]) versus 14% (patient 1) and 6% (patient 2) (Fig 1, A, upper panel, and B). Upon IL-2 stimulation, patients'

NK-cell degranulation against the human erythroleukemia cell line K562 was significantly reduced as compared with healthy controls: 23% (patient 1) and 34% (patient 2) versus 68% (HD) (Fig 1, A, lower panel, and B). This impairment was also confirmed with the cytotoxic chrome release assay (Fig 1, C), underscoring an important role for p85 α in this process in humans, similar to what was observed in the p85 α knockout mice.³ We then investigated whether IFN- γ production, a prominent biological feature of NK cells, was affected by the presence of the p85 α -dominant activating mutant. We thus evaluated IFN- γ production by patients' and healthy controls' NK cells before and after simultaneous stimulation with IL-12 and IL-18. Although, as expected, nonactivated NK cells from the healthy controls and the patients did not produce IFN- γ (2% patient 1 and 1% patient 2 vs 3% HD [Fig 1, D, upper panel]),

upon combined IL-12 and IL-18 stimulation, IFN- γ production by patients' NK cells was significantly reduced as compared with healthy controls (8% patient 1 and 21% patient 2 vs 76% HD [Fig 1, D, lower panel, and E]). These results were confirmed in 3 independent experiments, suggesting that p85 α plays a critical role in IFN- γ production upon *in vitro* NK-cell simulation with IL-12 and IL-18. Evaluation of IL-2-activated NK-cell populations from affected patients did not show phenotypical alterations or functional impairment in terms of redirected killing experiments (see Fig E2 and Results in this article's Online Repository at www.jacionline.org). An important effector function of NK cells is recognition and lysis of EBV-infected B cells.⁷ We decided therefore to investigate whether the p85 α mutation influenced this NK-cell function in affected patients. NK cells are known to express inhibitory receptors, recognizing autologous HLA class I molecules; therefore, the lack of HLA class I-LCL 721.221 renders this human cell line an optimal target for the evaluation of human NK-cell cytotoxic capacity.⁸ Thus, IL-2-activated NK cells from the mutated patients (patients 1 and 2) and healthy controls (HDs) were tested against the NK-cell-susceptible human target cell line HLA class I-LCL 721.221 and against patients' EBV-infected B cells. Degranulation was measured by CD107a upregulation. At difference with healthy controls (Fig 2, A, upper panel), IL-2-activated NK cells from patients 1 and 2 displayed reduced degranulation against the LCL 721.221 B-cell line (Fig 2, A) and failed to upregulate CD107a on encounter with the autologous EBV-infected B cells (Fig 2, B, far upper and mid upper panels), even after masking of HLA class I molecules (Fig 2, B, mid lower and far lower panels), suggesting an essential role for p85 α in human NK-cell lysis of EBV-infected B cells. The defect of p85 α mutant NK cells in recognizing EBV-infected B cells may underline an increased susceptibility to EBV-related infections and/or lymphoproliferative disorders for affected patients. To date, only 1 of the 12 reported p85 α -mutated patients⁴⁻⁶ presented silent EBV-viremia without mention of fulminant mononucleosis or lymphoma development; nonetheless, patients mutated in p110 δ , a catalytic unit regulated by p85 α and other subunits,¹ have been reported to present an elevated incidence of B-cell lymphomas, many of which are EBV-related.⁹ These findings therefore may be of particular interest for clinicians following these patients in terms of lymphoproliferation monitoring and may be of particular relevance because pharmacologic manipulation of the PI3K cascade has been recently introduced in the treatment of lymphoid malignancies.¹⁰

In a further set of experiments we evaluated the intracellular expression of Ki-67 as an index of proliferative capacity of IL-2-activated NK cells from patients and healthy controls. Our results showed that the proliferative responses on IL-2 activation of p85 α -mutated NK cells, expressing normal surface levels of IL2R (data not shown), were impaired as compared with healthy controls (Fig 2, C), suggesting an intrinsic role for p85 α in the IL-2-dependent human NK-cell proliferative response.

Taken together, our findings define a novel intrinsic role for p85 α in human NK-cell effector functions, broaden the spectrum of primary immunodeficiencies with NK-cell defects, and underscore that only longer follow-up and more numerous patients' cohorts will allow to better define the clinical implications of human NK-cell impairment in the presence of the p85 α mutation.

We thank Fondazione Camillo Golgi, Comunità Bresciana, Brescia, Italy, and Consorzio Interuniversitario Biotecnologie Trieste, Italy. We also thank Alessandro Moretta for useful comments on the manuscript and for providing monoclonal antibodies anti-NK-cell receptors, produced in his Laboratory of Department of Experimental Medicine, University of Genoa, Italy. We also thank the patients, the patients' families, and the nurses for all their efforts.

Vassilios Lougaris, MD^a
Ornella Patrizi, PhD^a
Manuela Baronio, PhD^a
Giovanna Tabellini, PhD^b
Giacomo Tampella, PhD^a
Gaetana Lanzi, PhD^{b,c,d}
Filippo Salvini, MD, PhD^e
Antonino Trizzino, MD^f
Silvia Parolini, PhD^b
Alessandro Plebani, MD, PhD^a

From ^athe Department of Clinical and Experimental Sciences, Pediatrics Clinic and Institute for Molecular Medicine A. Nocivelli, ^bthe Department of Molecular and Translational Medicine, ^cthe Institute for Molecular Medicine A. Nocivelli, and ^dthe Department of Pathology, Laboratory of Genetic Disorders of Childhood, University of Brescia, Spedali Civili di Brescia, Italy; ^ethe Pediatric Department, San Paolo Hospital, University of Milan, Milan, Italy; and ^fthe Department of Pediatric Oncology and Hematology, ARNAS CIVICO, Palermo, Italy. E-mail: vlougaris@yahoo.com.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no. 201549 (EURO-PADnet HEALTH-F2-2008-201549) and from the Italian Ministerial grant (grant no. GR-2010-2315762). The research leading to these results also received funding from the "Fondazione C. Golgi," Brescia, Italy.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

- Kerr WG, Colucci F. Inositol phospholipid signaling and the biology of natural killer cells. *J Innate Immun* 2011;3:249-57.
- Suzuki H, Terauchi Y, Fujiwara M, Aizawa S, Yazaki Y, Kadowaki T, et al. Xid-like immunodeficiency in mice with disruption of the p85 α subunit of phosphoinositide 3-kinase. *Science* 1999;283:390-2.
- Awasthi A, Samarakoon A, Dai X, Wen R, Wang D, Malarkannan S. Deletion of PI3K-p85 α gene impairs lineage commitment, terminal maturation, cytokine generation and cytotoxicity of NK cells. *Genes Immun* 2008;9:522-35.
- Deau MC, Heurtier L, Frange P, Suarez F, Bole-Feysot C, Nitschke P, et al. A human immunodeficiency caused by mutations in the PIK3R1 gene. *J Clin Invest* 2014;124:3923-8.
- Lucas CL, Zhang Y, Venida A, Wang Y, Hughes J, McElwee J, et al. Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K. *J Exp Med* 2014;211:2537-47.
- Lougaris V, Faletra F, Lanzi G, Vozzi D, Marcuzzi A, Valencic E, et al. Altered germinal center reaction and abnormal B cell peripheral maturation in PI3KR1-mutated patients presenting with HIGM-like phenotype. *Clin Immunol* 2015;159:33-6.
- Chijioke O, Müller A, Feederle R, Barros MH, Krieg C, Emmel V, et al. Human natural killer cells prevent infectious mononucleosis features by targeting lytic Epstein-Barr virus infection. *Cell Rep* 2013;5:1489-98.
- Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell types. *J Exp Med* 1993;178:1321-36.
- Kracker S, Curtis J, Ibrahim MA, Sediva A, Salisbury J, Campr V, et al. Occurrence of B-cell lymphomas in patients with activated phosphoinositide 3-kinase δ syndrome. *J Allergy Clin Immunol* 2014;134:233-6.
- Curran E, Smith SM. Phosphoinositide 3-kinase inhibitors in lymphoma. *Curr Opin Oncol* 2014;26:469-75.

<http://dx.doi.org/10.1016/j.jaci.2016.01.026>

RESULTS

Normal human NK-cell maturation in the presence of dominant activating p85 α mutations

To determine whether activating mutations in *PIK3R1* affect NK-cell maturation, PBMCs from 2 index patients (patients 1 and 2) were evaluated by multicolor flow cytometry. Both patients presented normal percentages of circulating CD56⁺ NK cells (Fig E1, A).^{E1} The evaluation of the expression pattern of activating and inhibitory NK-cell receptors, including CD16, natural cytotoxicity receptors (NKp46 and NKp30), KIRs (KIR2D and KIR3D), NKG2A (inhibitory receptor), NKG2C (activating receptor), CD62L, CD57, and chemokine receptors (CXCR1 and CCR7) on human NK cells (both CD56^{bright} and CD56^{dim} subsets) allowed us to discriminate NK-cell subsets in terms of maturational and functional status.^{E2} We did not observe abnormalities in the expression profile of activating and inhibitory NK-cell receptors in human NK cells from p85 α -mutant patients. In addition, the expression of the chemokine receptors CXCR1 (responsible for the preferential migration of CD56^{dim} CD16⁺ into inflamed tissues) and CCR7 (responsible for the preferential migration of CD56^{bright} CD16⁺ NK cells into lymphoid organs)^{E3} on patients' NK cells was unaltered as compared with healthy controls (Fig E1, A). These results, together with the normal expression of the maturation marker CD57 on CD56^{dim} NK cells (Fig E1, A), a classical NK maturational marker,^{E4} are in contrast to the maturational defects described in the animal model.^{E5} Moreover, CD62L expression on patients' NK cells was similar to that observed on healthy controls' NK cells (Fig E1, A), suggesting that the sequential steps of regular NK-cell maturation CD56^{bright}, CD56^{dim}CD62L⁺, and CD56^{dim}CD62L⁻^{E6} are conserved in the presence of the dominant p85 α mutation. The expression of the activation marker CD69 on NK cells was evaluated in patient 1 and did not show differences as compared with the healthy control (data not shown). The histograms of percentage expression and mean fluorescence intensity of surface NK-cell receptor profile (Fig E1, B and C, respectively) endorsed the normal immunophenotype observed in the patients as compared with HDs. On the whole, these data provide evidence that the p85 α -dominant activating mutation does not impair human NK-cell maturation.

IL-2-activated p85 α -mutated human NK cells do not display phenotypical or functional alterations

Because human NK cells showed impaired *in vitro* degranulation and IFN- γ production in the presence of the p85 α mutation, we decided to also evaluate whether IL-2-activated NK-cell populations from affected patients showed phenotypical or functional alterations. Evaluation of the expression of activating NK-cell receptor NKG2D and coreceptor CD244 (2B4), besides the ones already tested on resting patients' NK cells (Fig E1, A), on patients' and healthy controls' IL-2-activated NK cells did not show any significant differences (Fig E2, A). Cross-linking of activating molecules such as CD16, NKp46, 2B4 (CD244), and NKG2D (Fig E2, B) on patients' and healthy controls' IL-2-activated NK cells showed similar degranulation capacity against the murine P815 cell line (Fc γ R⁺), in redirected killing experiments,^{E7} as evaluated by CD107a expression. These data suggest, therefore, that the activating function of human NK-cell receptors is unaffected in the presence of the p85 α mutation. Finally, patient's NK cells showed normal perforin expression, comparable with the healthy control (Fig E2, C).

REFERENCES

- E1. Lougaris V, Faletra F, Lanzi G, Vozzi D, Marcuzzi A, Valencic E, et al. Altered germinal center reaction and abnormal B cell peripheral maturation in PI3K δ -mutated patients presenting with HIGM-like phenotype. *Clin Immunol* 2015;159:33-6.
- E2. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001;22:633-40.
- E3. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat Immunol* 2001;2:123-8.
- E4. Moretta L. Dissecting CD56dim human NK cells. *Blood* 2010;116:3689-91.
- E5. Awasthi A, Samarakoon A, Dai X, Wen R, Wang D, Malarkannan S. Deletion of PI3K-p85 α gene impairs lineage commitment, terminal maturation, cytokine generation and cytotoxicity of NK cells. *Genes Immun* 2008;9:522-35.
- E6. Juelke K, Killig M, Luetke-Eversloh M, Parente E, Gruen J, Morandi B, et al. CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells. *Blood* 2010;116:1299-307.
- E7. Moretta A, Bottino C, Pende D, Tripodi G, Tambussi G, Viale O, et al. Identification of four subsets of human CD3-CD16⁺ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J Exp Med* 1990;172:1589-98.

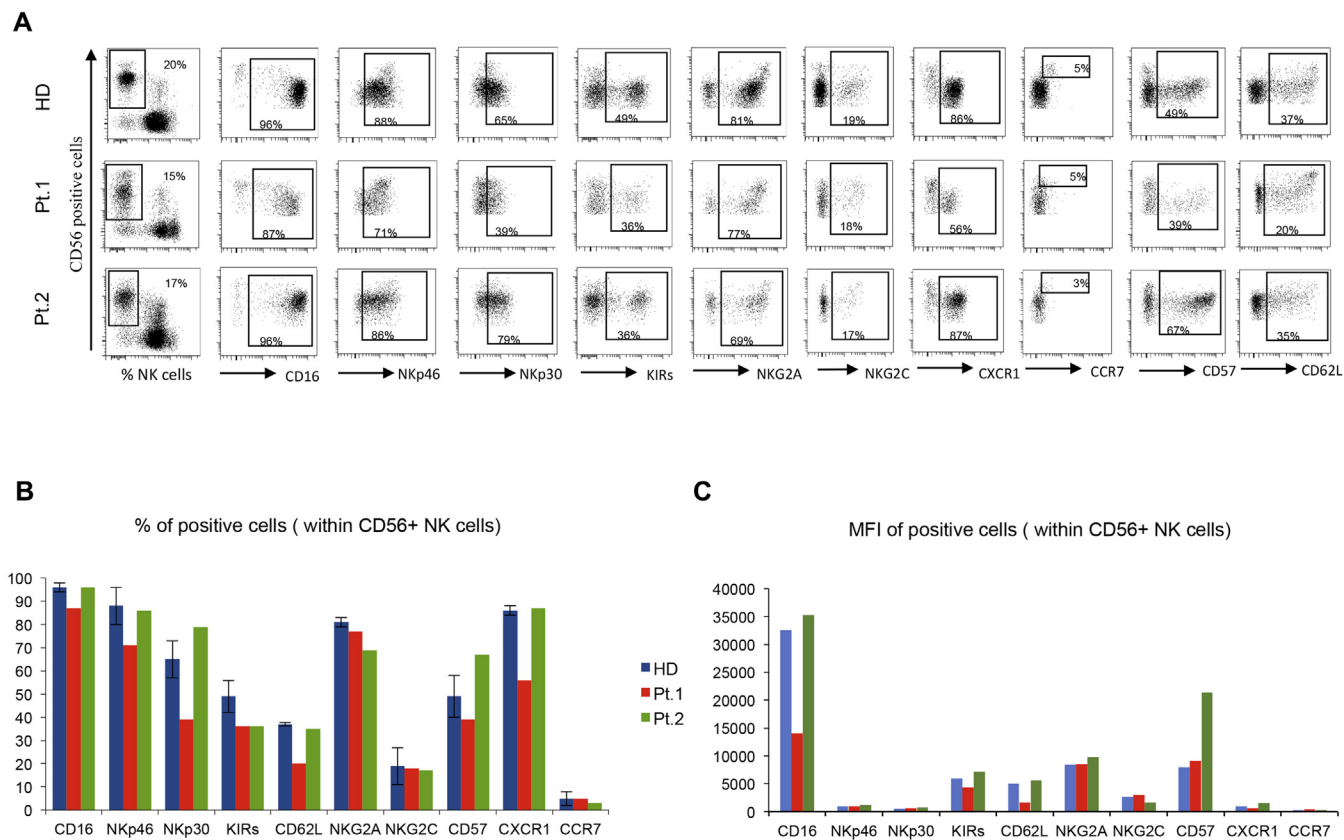


FIG E1. NK-cell phenotype in p85 α -mutated patients. **A**, Flow cytometric evaluation of human NK-cell receptor repertoire positivity on patients' (patients 1 and 2) and a representative HD CD56+CD3 $^{-}$ gated NK cells. Values are expressed as percentages (%). **B**, Comparison of percentages of expression of the NK-cell markers used for NK evaluation in this experiment. SD is shown only for values derived from 10 HDs. **C**, Comparison of MFI of different NK-cell receptors on CD56-positive NK cells from the patients and a representative healthy control. *MFI*, Mean fluorescence intensity; *Pt.*, patient.

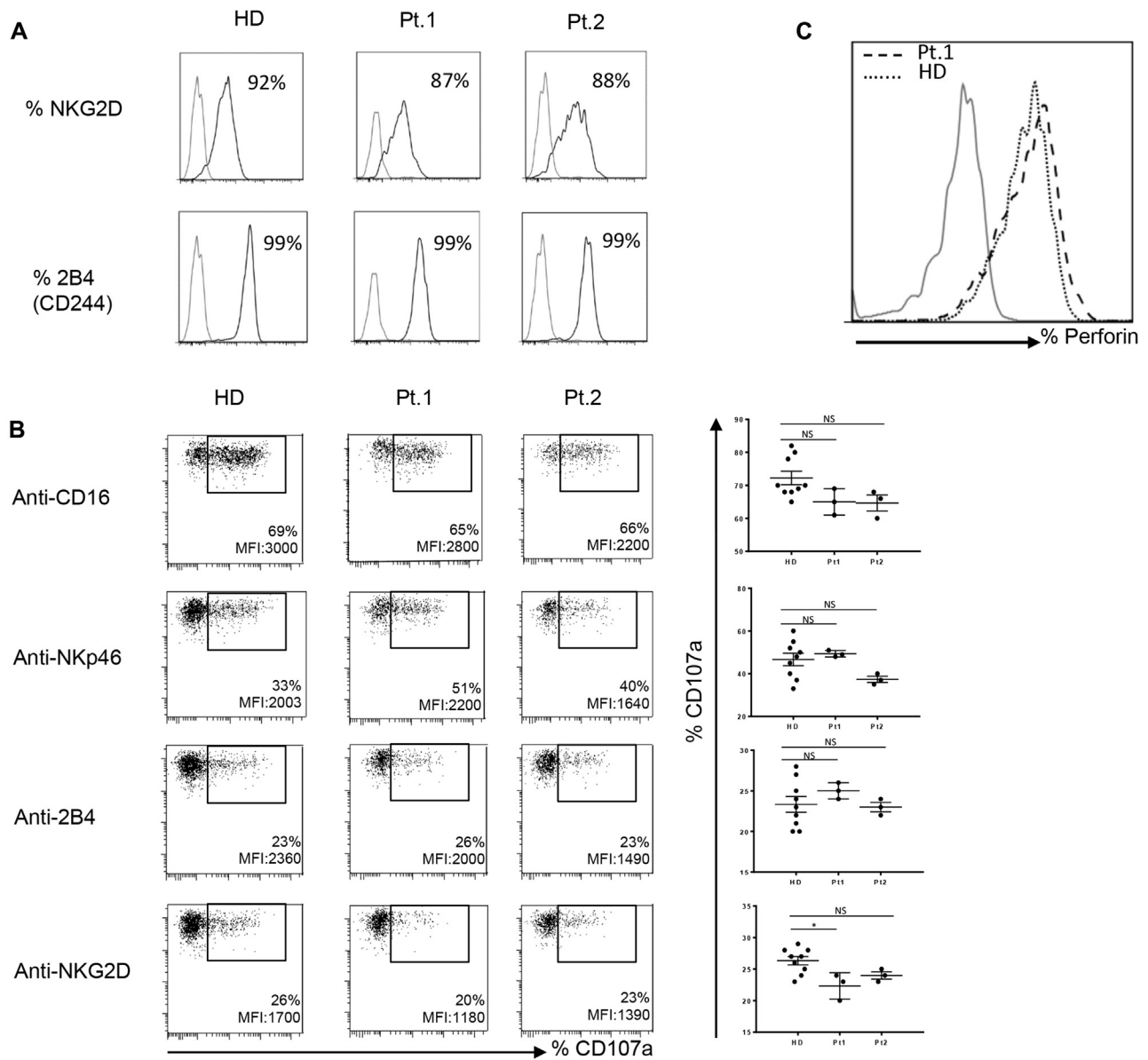


FIG E2. Evaluation of IL-2-activated human NK cells from p85 α mutant patients. **A**, Flow cytometric evaluation of the expression of activating NK-cell receptor NKG2D and coreceptor CD244 (2B4) on IL-2-activated NK cells from patients (patients 1 and 2) (*mid and right column*) and a representative healthy control (HD) (*left column*). Representative experiment of 3 independent experiments. **B**, Evaluation of cytotoxic activity of human IL-2-activated NK cells from the patients (patients 1 and 2) (*mid left and mid right column*) and a representative healthy control (HD) (*far left column*) upon cross-linking of CD16, NKp46, 2B4 (CD244), and NKG2D against the murine P815 cell line (Fc γ R⁺) measured as upregulation of CD107a expression. Summarized data from 3 independent experiments are shown in the *far right column*. **C**, Perforin content in gated NK cells CD56⁺CD3⁻ derived from patient (patient 1) and from a representative HD. *Pt.*, Patient. *Statistical significance was defined by using the unpaired Student *t* test ($P < .05$).