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# Mutations in PI3K110δ cause impaired NK cell function partially rescued by rapamycin treatment

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## Abstract

**Background—**Heterozygous gain-of-function mutations in PI3K110 lead to lymphadenopathy, lymphoid hyperplasia, Epstein-Barr virus (EBV) and cytomegalovirus (CMV) viremia, and sinopulmonary infections.

**Objective—**The known role of NK cell function in the control of EBV and CMV prompted us to investigate the functional and phenotypic effect of PI3K110δ mutations on NK cell subsets and cytotoxic function.

**Methods—**Patient mutations were identified by whole exome or targeted sequencing. We performed NK cell phenotyping and functional analysis of patient cells by flow cytometry, standard $Cr^{51}$ cytotoxicity assays, and quantitative confocal microscopy.

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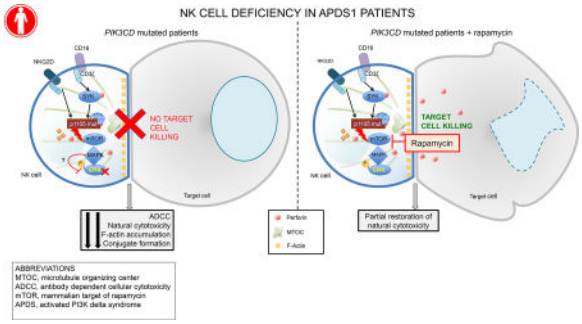
**Competing interests:** The remaining authors declare that they have no competing interests.

**Results—**PI3K110δ mutations led to an altered NK cell developmental phenotype and cytotoxic dysfunction. Impaired NK cell cytotoxicity was due to decreased conjugate formation with susceptible target cells and abrogated activation of cell machinery required for target cell killing. These defects were partially restored following the initiation of treatment with rapamycin in three patients.

**Conclusion—**We describe novel NK cell functional deficiency due to PI3K110δ mutation, which is a likely contributor to the severe viremia observed in these patients. Rapamycin treatment partially restores NK cell function, providing further rationale for its use in this disease.

## Capsule Summary




Gain-of-function mutations in PI3K110δ lead to impaired NK cell cytolytic function that can be partially restored with rapamycin treatment.

### Keywords

NK cell deficiency; combined immunodeficiency; cytotoxicity; APDS; PI3K signaling

## Introduction

NK cells play an important role in host defense, and NK cell deficiency (NKD) leads to severe and often fatal viral infection and malignancy[1–4]. While primary immunodeficiencies (PID) leading to isolated NKD are relatively rare, currently greater than 40 congenital deficiencies affect NK cell function in a broader immunological context[5]. Human NK cells play a critical role in the control of viral infections through the secretion of cytokines and the direct, contact dependent killing of virally infected cells. As such, PID that include an effect on NK cell function are frequently caused by mutations in genes that affect the cytolytic machinery of NK cells. These conditions include the familial hemophagocytic lymphohistiocytosis diseases, mutations affecting the cytoskeleton such as DOCK8[6, 7] and Wiskott-Aldrich syndrome[8, 9], defects in the NFκB pathway[10, 11], and others[5].

NK cell killing of virally infected targets is mediated by the directed secretion of lytic granules following the formation of a lytic immunological synapse (IS). NK cell IS formation and function includes multiple tightly regulated steps, and the perturbation of these checkpoints leads to a blunting of the cytotoxic response. NK cell cytotoxicity is controlled through a balance of activating and inhibitory receptor signaling. Overcoming a critical threshold of activating receptor signaling leads to commitment to formation of an IS,

which includes F-actin remodeling and *de novo* polymerization, polarization of the microtubule organizing center (MTOC) and lytic granules to the IS, and exocytosis of perforin- and granzyme-containing lytic granules. An early priming step in IS formation is the convergence of lytic granules to the MTOC, which occurs in response to diverse activating signals, including cytokine stimulation, integrin ligation and activating receptor ligation[12–14]. This step, which is independent of actin remodeling, precedes integrin-mediated firm adhesion and *de novo* F-actin polymerization, which occurs downstream of activating receptor signaling. Convergence is followed by polarization of the MTOC, which delivers lytic granules to the IS. Lytic granules then traverse the F-actin network at the IS and undergo exocytosis, leading to target cell apoptosis.

The phosphoinositide 3-kinase (PI3K) pathway is a key axis for NK cell cytotoxicity. Signaling downstream from NK cell activating receptors, such as CD16 and NKG2D, leads to PI3K recruitment and activation through adaptors including DAP10 and CD3ζ[15–20]. Downstream signalling to cytotoxicity is mediated by Rac1, p21 activated kinase-1 (PAK1), mitogen-activated protein kinase kinase (MEK) and extracellular regulated kinases (ERK) 1/2[21]. The requirement for PI3K in NK cell cytotoxic function has been shown using pharmacologic inhibitors, with varying effects dependent upon the target and killing not being entirely abrogated in all cases[16, 19]. Further insight has been gained using isoform-specific mouse models to dissect the role of individual subunits in the cytotoxic process. Class 1A PI3K in lymphocytes are comprised of a p110 catalytic subunit, encoded by the *PIK3CA, PIK3CB* or *PIK3CD* gene, paired with a p85, p55 or p50 regulatory subunit. Catalytic inactivation specifically of the PI3K110δ subunit in mice leads to reduced NK cell number, impaired maturation, and decreased cytotoxic function[22]. The effect of gain-of-function *Pik3cd* mutations has not been directly tested in mice, however their effect can be predicted based on loss-of-function mutations in *Pten*, which have a similar effect of elevating PtdIns(3,4,5)P3 levels.

Mutations in PI3K isoforms lead to distinct PID; loss of p85 in both mice and humans leads to loss of B cell function and subsets, whereas gain-of-function mutations in p110δ patients lead to “p110δ-activating mutation causing senescent T cells, lymphadenopathy and immunodeficiency” (PASLI) disease[23] or “activating PI3Kδ syndrome, class 1” (APDS1)[24], characterized by EBV and CMV viremia in conjunction with T cell lymphoproliferation and $CD8^+$ T cell metabolism defects, lymphopenia, increased IgM and circulating transitional B cells, and recurrent respiratory infections[23–27]. In these patients, hyperactive PI3K pathway signalling through protein kinase B (AKT) drives premature senescence of $CD8^+$ T cells and subsequent loss of $CD8^+$ T cell mediated IL-2 secretion[23], as reviewed in Lucas et al.[28]. The hyperactivation of the PI3K pathway leads to increased mammalian target of rapamycin (mTOR) activity and glycolytic function. These effects are partially counteracted by treatment with the mTOR inhibitor rapamycin. Rapamycin treatment of APDS1 patients results in decreased $CD8^+$ cellularity and increased IL-2 secretion, which is accompanied by observed clinical improvement of the disease[23].

In this study, we identified a family of three siblings with E525K mutations in the *PIK3CD* gene[29, 30]. The 3 affected brothers had clinical and immunologic features consistent with *PIK3CD* gain-of-function disease, including persistently elevated EBV PCR titers, diffuse

lymphadenopathy, hepatosplenomegaly, markedly elevated percentages of transitional B cells, and poor responses to polysaccharide antigens. The identification of this mutation in these patients afforded us the opportunity to evaluate NK cell function and phenotype prior to and following the initiation of rapamycin treatment. Further, we expanded our cohort to describe NK cell deficiency in 5 previously described patients[23] and 2 additional previously unreported patients. We show that both E1021K and E525K gain-of-function mutations in PI3K110δ lead to functional NK cell deficiency because of decreased frequency of conjugate formation with susceptible target cells and impaired signalling leading to the execution of cytotoxicity. Further, skewing of the NK cell phenotype occurs, notably with decreased expression of the Fc receptor (CD16) and IL-2 receptor β (CD122) on patient cells. Following stabilization while on rapamycin therapy a significant improvement in NK cell function was observed, reflected by partially corrected immune synapse formation. Therefore, we define gain-of-function mutations in PI3K p110δ as a novel cause of NK cell functional and developmental impairment and a likely contributor to disease in these patients.

## Materials and Methods

### Cell isolation and cell lines

All human samples were obtained using written informed donor consent and were used with the approval of the National Institutes of Health (NIH) and Baylor College of Medicine Institutional Review Boards for the Protection of Human Subjects. All samples were obtained in compliance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation over Ficoll-Paque according to manufacturer's instructions. For $Cr^{51}$ release assays, Raji and K562 cell lines were used as target cells for NK cell cytotoxicity and were maintained in supplemented RPMI media with 10% fetal calf serum. Target cells were routinely tested and confirmed to be mycoplasma free.

### Genetic studies

Whole exome sequencing for research at Baylor College of Medicine was performed as previously described[31]. Clinical sequencing was performed by Baylor Genetics[32, 33]. Variants were confirmed by Sanger sequencing by the Baylor DNA Sequencing Core Facility. Patients from the NIH were identified genetically by targeted sequencing or whole exome sequencing as previously described[23]. For deep sequencing, the *PIK3CD* c.G1573A variant was validated by amplicon deep sequencing on MiSeq sequencer (Illumina). Briefly, an amplicon of 234bp defined by forward primer of 5'-ACCGAGGAGGAGGTGAGTG-3' and reverse primer of 5'-ACTTGGTGACCAGCAGCAG-3' was generated through polymerase chain reaction (PCR) from 6 genomic DNA samples of the proband and family members using PrimeSTAR GXL DNA Polymerase (Takara). PCR products were purified with AMPure beads (Beckman Coulter) and further end-repaired and ligated with TruSeq indexed adaptors. Equal amounts of ligated amplicons were pooled and sequenced by MiSeq Nano Kit v2 300 cycles with PhiX spike-in. The raw sequencing data were demultiplexed and converted to Fastq files. The reads were then aligned to human genome assembly 37/

hg19 reference sequence by BWA-MEM. Variants were called and number of variants read were counted from each de-multiplexed sample.

## $Cr^{51}$ release assays

Chromium release assays for the analysis of NK cell activity were performed as previously described[34]. Freshly isolated PBMC were incubated at varying ratios (50:1, 25:1, 12.5:1, 6.25:1, 3.13:1) with $10^4$ K562 or Raji target cells that had been labeled with 50 μCi of $Cr^{51}$ then washed. Where indicated, assays were performed in the presence of 1000 U/ml IL-2 (Roche) or, as a stimulus for antibody-mediated cytotoxicity, 20 μg/ml Rituximab. Effector:target conjugates were incubated in 200 μl in round-bottomed 96 well plates (Corning). Following 4 hours of incubation, positive controls for maximal lysis were produced by lysing labeled target cells with 1% octylphenoxypolyethoxyethanol. Supernatant was harvested and transferred to LumaPlates (PerkinElmer). The supernatant was dried and plates were read on a TopCount gamma counter (PerkinElmer). Percent specific lysis was calculated as [(Experimental release – spontaneous release)/maximum release*100]. Lytic units were calculated as the number of effectors required to lyse 10% of K562 target cells using the slope of the curves generated by cytotoxicity assay data over the range of effector to target ratios[8].

## Flow cytometry

Multi-parametric flow cytometry was done using the antibodies described in Supplemental Table 1. For cell surface receptors, all incubations were performed for 30 minutes at 4°C. For intracellular staining of cytok ines and effector molecules, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience) followed by incubation with antibodies of interest. For activating and functional assays, cells were pre-incubated with phorbol myristate acetate (PMA) and ionomycin (Sigma Aldrich) or vehicle control for 6 hours. Brefeldin A (10 μg/mL-Sigma Aldrich) was added 4 hours before antibody staining. Flow cytometric data were acquired on a BD Fortessa with 18-color configuration. Data were exported as FCS files. SPADE analysis was performed with Cytobank Premium[35] following gating on lymphocytes then $CD56^+CD3^-$ cells. Clustering analysis was based upon the following parameters: CD94, CD117, CD57, CD62L, CD16, CD27 and CD127. Pairwise comparison of single markers and routine flow cytometric analyses were performed using FlowJo X (TreeStar Inc.).

For detection of downstream signaling, NK cells were activated for 0, 2, 4, 8 or 16 minutes with PMA and ionomycin and then harvested, fixed, permeabilized and stained for intracellular phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr2014, D13.14.4E, Cell Signaling Technology). Samples were acquired and analyzed as above.

For flow cytometric analysis of conjugation frequency, PBMC were pre-incubated with anti-CD56 (clone HCD56, Biolegend) and anti-CD3 (clone SK7, Biolegend) to identify NK cells as $CD56^+CD3^-$. Susceptible K562 target cells labeled with vital dye PKH26 were mixed with PBMC and incubated for 0, 20, 40 or 60 minutes prior to fixation. Data were acquired on a BD Fortessa and analyzed using FlowJo X. NK cells were gated and the frequency of cells found in conjugates was calculated as $[PKH26^+CD56^+CD3^-]/[CD56^+CD3^-]$.

### Confocal microscopy

Confocal microscopy was performed as previously described[36]. Briefly, patient PBMC were incubated with susceptible K562 target cells for 45 minutes to facilitate conjugate formation. Following incubation, cells were fixed and permeabilized, then stained intracellularly with anti-tubulin biotin (Life Technologies) followed by streptavidin AlexaFluor 488 (Life Technologies). These steps were followed by staining with anti-perforin AlexaFluor 647 (clone δG9; Biolegend) and phalloidin AlexaFluor 568 (Life Technologies). Slides were mounted with ProLong Gold anti-fade media (Life Technologies). Images were acquired on a Zeiss Axio Observer Z1 microscope with Yokogowa CSU-10 spinning disk, Hamamatsu ORCA-AG camera, and Zeiss 63X 1.43 NA oil immersion lens. Excitation lasers (405 nm, 488 nm, 568 nm) were managed through an LMM5 laser merge unit (Spectral Research). Images were taken in a single Z-plane. Images were acquired and analyzed with Volocity 6.0 software (PerkinElmer). Image analysis was performed as described for F-actin accumulation, lytic granule convergence and MTOC polarization[36–38]. Briefly, F-actin accumulation at the synapse was calculated with Volocity software (PerkinElmer) by measuring the intensity and area of F-actin above a uniform threshold. Cortical actin from both effector and target cells was subtracted from F-actin at the synapse so that only specific F-actin accumulation was measured[37]. For measurement of granule convergence, centroids of lytic granules were detected by perforin intensity and the MTOC was detected by intensity of tubulin immunostaining. Distance of granules to the MTOC was measured for each granule using Volocity software; each data point represents the mean of all granules within a given cell[38].

### Graphing and statistical analysis

Pairwise comparisons were made by Student's two-tailed t test. For cytotoxicity assays, conditions were compared by ordinary one-way ANOVA with post hoc analysis by Tukey's multiple comparisons test. For conjugation assays, statistical outliers were identified using ROUT analysis (1%) and excluded from analysis. All graphing and statistical analyses was performed with Prism 6.0 (GraphPad Software).

## Results

### Clinical history and genetic confirmation of PI3K110δ mutation

The index family consists of 3 brothers from Qatar who presented at 9 years, 5 years, and 1 year of age, respectively, due to diffuse lymphadenopathy, hepatosplenomegaly, and persistently elevated EBV PCR titers. X-linked lymphoproliferative disease, autoimmune lymphoproliferative syndrome, and hemophagocytic lymphohistiocytosis were excluded, and the family returned to Qatar without a formal diagnosis. They returned 4 years later for further evaluation. The older brother had developed bronchiectasis and mild pulmonary fibrosis in the interim due to recurrent pneumonias. They otherwise denied any other recurrent infections. The parents denied consanguinity, and the 3 siblings had 2 other older twin brothers who were healthy. Immunologically, the 3 affected boys had markedly elevated transitional B cell percentages and poor antibody responses to polysaccharide antigens. Whole exome sequencing was performed and revealed the presence of a heterozygous missense *PIK3CD* E525K mutation in the 3 affected brothers; the 2 twin

siblings carried wild-type *PIK3CD* sequences (see Supp. Table 2 for other variants found at low frequency within the EXaC database). The father was noted to have 6 variant reads out of 38 total reads, suggestive of mosaicism[29, 30] or heterozygosity. As the resolution of Sanger sequencing did not permit us to determine whether the father was truly heterozygous (Supp. Fig 1A), we performed deep sequencing of the region of interest, generating greater than 200,000 reads (Supp. Fig 1B). This analysis confirmed the presence of the heterozygous mutation in the 3 affected siblings and homozygosity of the wild-type allele in the unaffected brothers. The father was also found to have full heterozygosity at this locus, with 49% of reads having the mutation present in the affected children. Unfortunately, the father was not available for further immunologic evaluation. It is regrettable that further clinical information about the father is not available as this would additionally demonstrate the pathogenicity of the mutation shared by the father and the patients, however the previous reports of the *PIK3CD* E525K mutation as disease-causing with high penetrance (23) underscore the likelihood that there is clinical impact of this mutation on affected family members.

We identified further individuals with *PIK3CD* mutations. First, we were introduced to a 5-year-old asymptomatic boy who had clinical whole exome sequencing performed due to mild developmental delay. The results incidentally revealed the presence of a single heterozygous E1021K mutation in *PIK3CD*, which was confirmed by clinical Sanger sequencing. This subject was also noted to have an elevated transitional B cell percentage (over 20% of $CD19^+$ B cells) and lack of response to polysaccharide antigens. Serologic and PCR testing showed that he had never been previously exposed to EBV. Next, we obtained samples from 5 previously reported subjects and one unreported individual who had *PIK3CD* mutations identified by whole exome or targeted sequencing[23]. Three of the 6 subjects carried the E525K mutation, 2 had the E1021K mutation, and 1 patient had the N334K mutation (summarized in Table 1). As previously described[23, 24], the E1021K mutation is located within the kinase domain of PI3K110δ and the E525K mutation is found within the helical domain (Supp. Fig 1C).

## NK cells from PI3K110δ patients have decreased natural and antibody-mediated cytotoxicity

NK cells from PI3K110δ mutant mice have decreased natural cytotoxicity[22, 39] and PASLI/APDS1 patients show susceptibility to viral infection. As NK cells participate in antiviral defense, we tested the function of patient NK cells using $Cr^{51}$ release assays against susceptible K562 target cells in the presence or absence of IL-2. Patients showed decreased cytotoxic function, although interestingly stratification based on mutation was observed for both unstimulated and IL-2 stimulated cells (Fig 1A). Patients with E525K and N334K mutations had a greater reduction in NK cell lytic function than those with E1021K mutation relative to grouped healthy controls. While response to IL-2 was varied, a relative increase in cytolytic function in response to IL-2 was noted (Fig 1A, B). We further evaluated the cytolytic capacity of patient NK cells by calculating lytic units required to lyse 10% of K562 target cells following adjustment for the frequency of NK cells within the PBMC population (Fig. 1B). With the exception of Patient 5, patient NK cells had reduced lytic capacity when directly compared to the healthy donor control used for their respective assays.

To determine the effect of PI3K110δ mutation on antibody-mediated cellular cytotoxicity (ADCC), Raji target cells were coated with rituximab, and cytotoxicity assays were performed as above. While Raji cells in the absence of antibody were not lysed (not shown), the presence of antibody led to effective killing of targets by controls. However, NK cells from patients with E525K and E1021K mutations had significantly decreased function (Fig. 1C). Taken together, these results show impaired NK cell function in patients with gain-of-function PI3K110δ mutations, producing significantly reduced cytolytic function towards both susceptible tumor cells and antibody-coated targets.

### PI3K110δ mutation leads to skewed NK cell developmental phenotypes in human NK cells

Patients with PI3K110δ gain-of-function mutations have some variability in NK cell numbers and altered T cell subsets due to premature senescence[25,23]. In addition, alteration of PI3K function in mice is known to lead to reduced NK cell numbers with abnormal NK cell developmental subsets[22, 39]. To fully explore the NK cell phenotype in these patients, we performed quantitative flow cytometry analysis with 5 panels assessing 40 parameters including NK cell surface receptors, intracellular cytokines, and markers of NK cell activation (Supplemental Table 1). To visually compare the distribution of phenotypic subsets between healthy donor and patient samples and to identify novel populations, we performed visual spanning tree progression of density normalized events (SPADE)[40]. Analysis was performed on flow cytometry data of 8 key NK cell maturation markers following gating for $CD56^{+}CD3^{-}$ NK cells from 2 patients with E525K mutations and 5 healthy donors from 3 independent experiments. Consistently, Patients 1 and 3 (E525K mutation) had unique subsets preserved between the two patients that were not seen in healthy donors (Fig 2). Healthy donor populations from 5 individuals were remarkably similar when visualized by SPADE given the known intra-individual heterogeneity in NK cell subsets (Fig 2, not shown)[41, 42].

Guided in part by our SPADE analysis that identified visually distinct subsets within patient NK cell populations, we defined markers from our panels that were differentially expressed on patient cells compared to healthy controls. To quantitatively compare these, we performed pairwise analyses of frequency of receptor expression (percent positive) on NK cells. Since $CD56^{bright}$ and $CD56^{dim}$ NK cells represent phenotypic and functional subsets, and $CD56^{bright}$ NK cells are thought to be the developmental precursors of $CD56^{dim}$ NK cells, we quantified expression on these subsets individually. As previously described, we saw significant reduction in NK cell numbers in many PI3K110δ patients[23] (Fig 3A). While the frequency of patient $CD56^{bright}$ cells was normal (Fig 3B), skewing of the NK cell phenotype towards a generally immature one was present, with a decrease in CD16 and increase in NKG2A expression on $CD56^{dim}$ NK cells (Fig 3C). Further, CD62L and CD127 expression were decreased on $CD56^{bright}$ NK cells, and CD122 expression was diminished on both subsets (Fig 3C). In contrast to the phenotype observed in the mouse model, no detectable difference in CD27 expression was observed on patient NK cells (not shown)[22]. Granzyme B and CD57 were also unchanged between patient and healthy donor cells, in contrast to the phenotype of effector T cells in these patients[23]. Intracellular IFNγ expression in response to PMA/ionomycin stimulation was lower in patient NK cells, although not significantly. While TNFα expression was also not affected, a significant

decrease in GM-CSF expression by patient NK cells was observed (Supp. Fig. 2). Taken together, these results indicate an alteration in NK cell phenotype or activation state. These effects are independent of global NK cell development when taken in light of the normal distribution of $CD56^{bright}$ and $CD56^{dim}$ subsets. Furthermore, they are distinct from effects on both murine NK cells and human $CD8^{+}$ effector cells, which have unique markers affected by PI3K110δ mutation. Collectively, however, these significant alterations in NK cell phenotype provide interesting insight into the loss of cytotoxic function in patient NK cells.

## Impaired conjugate formation, ERK phosphorylation and lytic machinery polarization in PI3K110δ NK cells

To further probe the mechanism of cytotoxic dysfunction, we sought to identify the affected stage(s) of PI3K110δ patient NK cell-mediated killing of target cells. The initial stage of NK cell cytotoxicity is recognition and conjugate formation leading to firm adhesion (reviewed in[43]). Given the requirement for F-actin rearrangement in conjugate formation and the potential impact of deregulated PI3K activity on this pathway, we analyzed the frequency of NK- target cell conjugate formation by FACS. Differentially labeled NK cells and targets were mixed and allowed to form conjugates for 0, 10, 20, 30 and 60 minutes. Despite comparable expression of integrins and activating receptors between healthy donors and patient cells (not shown), NK cells from PI3K110δ patients showed a trend towards lower conjugate formation that was significant in patients with E1021K mutations at later time points (60 minutes: HD 7.9±1.9%; E525K 4.4±0.7%; E1021K 1.9±0.4%) ($p<0.05$ by one-way ANOVA, Fig 4A).

PI3K110δ catalytically inactive mutant mice show impaired signaling in the MAPK pathways, specifically phosphorylation of JNK1/2[22], and *Pik3cd*$^{-/-}$ *Pik3cg*$^{-/-}$ mice have defective ERK1/2 signaling[39]. To test this pathway in patient cells, we performed intracellular FACS for phosphorylated ERK1/2, JNK1/2 and p38 following NK cell activation by PMA and ionomycin. While phosphorylation of JNK1/2 and p38 were intact in patient cells, the percentage of NK cells with ERK1/2 phosphorylation was significantly reduced in patients with both E525K and E1021K mutations 15 minutes following activation (HD 43.3±9.2%; E525K 7.2±2.2%; E1021K 9.2±1.4%) ($p<0.05$ by one-way ANOVA, Fig 4B).

Upon conjugate formation and activating signaling, NK cells rapidly converge lytic granules to the MTOC, which is followed by the effector stage of cytotoxicity, marked by accumulation of F-actin and polarization of lytic granules and the MTOC to the immunological synapse[38, 43]. To further investigate the impact of impaired PI3K110δ signaling on NK cell lytic function, we performed quantitative analysis of the polarization of lytic machinery in NK cells conjugated to susceptible target cells and imaged by confocal microscopy (Fig. 4C). While healthy donor NK cells had accumulation of Factin following conjugation to target cells, aggregation of F-actin at the immunological synapse was significantly decreased in all patient cells tested, regardless of mutation (Fig 4D). Furthermore, significant defects in the convergence of lytic granules to the MTOC and

MTOC polarization were present in the E525K cohort (Fig 4D). Interestingly, these parameters were not significantly affected in the E1021K patients.

### Partial increase in NK cell function following initiation of treatment with rapamycin

Rapamycin treatment has proven relevant for the amelioration of symptoms in PI3K110δ patients[23]. To determine the specific effect of treatment on NK cell function, we evaluated NK cell cytotoxicity in Patients 1–3 both prior to initiation of rapamycin and following the establishment of treatment. While NK cell number increased in one patient only (Fig 5A) and cell surface receptor phenotype was not significantly changed (not shown), improvement in the specific lysis of K562 target cells was seen in all three patients (Fig 5B). When normalized to healthy donor controls, the mean specific lysis at 50:1 effector to target for all three patients was raised from 0.14±0.01 to 0.49±0.1 ($p<0.01$, Fig 5B). Finally, to ascertain if this increase in cytotoxic function correlated with a restoration of previously impaired effector mechanism, we re-evaluated NK-target conjugates by confocal microscopy. We found that following the establishment of rapamycin therapy in the patients, who had all failed to accumulate F-actin at the synapse previously, we now observed that F-actin rearrangement was comparable to that of healthy donor controls (Fig 5C). Therefore, the increase in NK cell function following mTOR inhibition in PI3K110δ patients occurs in concert with restoration of cytolytic machinery.

## Discussion

The PI3K signaling axis is an important one in NK cell biology, as demonstrated by studies in both mouse[22, 39] and humans[23, 24, 44]. In particular, the PI3K signaling pathway is required for the development of NK cells in mouse[22, 39] and the polarization of lytic machinery during human NK cell-mediated killing of target cells[45]. The recent description of patients with gain-of-function mutations in *PIK3CD* highlights the importance of balanced signaling through this pathway in human health, as these patients suffer severe lymphoproliferative disease and immunodeficiency. The prevalence of EBV disease, a hallmark of NK cell dysfunction, and the documented role for this signaling pathway in NK cell function led us to investigate further the functional and developmental phenotype of NK cells in these patients. Here we show that in addition to the reduced NK cell number seen in some PI3K110δ patients[23], significant impairments in NK cell phenotype and function exist in patients with *PIK3CD* gain-of-function mutations.

Previous studies of murine models have revealed both developmental and functional abnormalities in NK cells following disruption of PI3K110δ. Loss of PI3K110δ function leads to reduced NK cell number in the periphery and an immature phenotype when combined with loss of the PI3K110γ subunit[39]. Catalytically inactive PI3K110δ results in a similar phenotype, with a block in NK cell development leading to decreased NK cell numbers and specific decrease in the $CD27^{hi}$ mature population[22]. In both previous studies, developmental defects were accompanied by specific loss of NK cell function, as impairment in JNK1/2 and ERK1/2 activation leads to defective *in vitro* and *in vivo* tumor clearance[22, 39]. We have now demonstrated that hyperactive PI3K110δ function in humans leads to NK cell derangements.

The careful examination of the NK cell phenotype in PI3K110δ patients shows select developmental defects. Interesting conserved defects were observed between the gain-of function mutations in our patients and murine loss-of-function models. This semblance includes the immature NK cell phenotype seen in patient cells, although this defect is not as absolute as the halt in NK cell development seen in the mouse model. The presence of CD27 on patient cells at comparable levels to healthy donors is a notable difference between gain- and loss-of-function mutations, although the significant divergence between mouse and man in terms of NK cell development may also account for this discrepancy. Whereas human NK cells are defined largely phenotypically and functionally by CD56 density, with $CD56^{bright}$ being the cytokine producing subset and $CD56^{dim}$ the predominantly cytotoxic subset, murine NK cells do not express a CD56 homologue. As human NK cells are thought to pass first through the $CD56^{bright}$ stage and then mature to the $CD56^{dim}$ stage, it has been difficult to completely correlate these stages in the mouse. However, a surrogate scheme based on CD27 and CD11b (Mac-1) density has been designed in terms of function[46], and thus the lack of $CD27^{hi}$ NK cells in the mouse model is reflective of a block in maturation. That said, the relative frequency of $CD56^{bright}$ and $CD56^{dim}$ cells was not directly affected as a result of gain-of-function mutations in the patients we examined. Instead, other very consistent differences were observed that indicated a less mature NK cell functional phenotype as a result of gain-of-function mutations in patient NK cells.

These phenotypic changes were initially illuminated by SPADE analysis that showed unique NK cell subsets in patient cells relative to healthy donors. Further analysis showed a generally immature $CD56^{dim}$ population, with decreased CD16 and increased NKG2A expression. Interestingly, both $CD56^{bright}$ and $CD56^{dim}$ subsets had decreased CD122 expression, which could indicate that at least some of the functional hyporesponsiveness shown by patient NK cells is due to decreased IL-2 mediated activation. While $CD56^{dim}$ NK cells appeared less mature, the $CD56^{bright}$ NK cells in our patients had decreased CD62L and CD127 receptors, and it would be of interest to determine whether patient T cells were similarly affected and whether this decreased expression contributes to the T cell phenotype seen in these patients[23, 24, 44]. Of note, $CD56^{bright}$ NK cells appeared to have normal cytokine production, including IFNγ, upon stimulation, and levels of granzyme B were not affected unlike in cytotoxic T lymphocytes[23].

The significance of the differences between the mutations is not entirely clear. While both E525K and E1021K result in impaired NK cell cytotoxic function and seemingly similarly affect NK cell number and phenotype, the E1021K mutation had a significant effect on conjugate formation, whereas the E525K mutation had a greater effect on polarization of lytic machinery following the formation of conjugates. The E1021K mutation, located in the kinase domain, has been shown to increase membrane association and kinase activity[24, 47]. In contrast, both N334K and E525K mutations interrupt inhibitory interactions between the regulatory p85α subunit and p110δ catalytic subunit, leading to a different mechanism of PI3K hyperactivation. While both mutations have the effect of promoting PtdIns(4,5)$P_2$ phosphorylation, subtle differences may exist as a result of the mislocalization of protein due to E1021K mutations that have yet to bet determined.

Despite hyperphosphorylation of AKT as a result of both E525K and E1021K mutations[23, 24], the effect of gain-of-function mutations on both T cells and NK cells appears to be one of cellular hyporesponsiveness. This conclusion is supported by our results obtained on a single cell level by confocal microscopy image analysis. It is interesting that rarer loss-of-function mutations in PI3K also lead to immunodeficiency and impaired immune responses[48, 49], however the cellular phenotype of NK cells was not specifically studied in these instances. Gain-of-function mutations in PTEN, which would be predicted to have an effect counter to that of PI3K gain-of-function, also lead to a very similar NK cell phenotype. Human NK cells overexpressing PTEN have impaired F-actin accumulation, granule convergence and MTOC polarization as seen in APDS1 patients[50]. Interestingly, PI3K inhibitors also impair conjugate formation, cytotoxic function and MTOC polarization[51]. This effect underscores the importance of carefully regulated PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ signaling in NK cell cytotoxic function and suggests that deregulation of this signalling globally leads to hyporesponsiveness. In T cells, this hyporesponsiveness is likely due to premature senescence of the effector population. However, as NK cell senescence is poorly defined it is difficult to draw a parallel. The similarities between loss- and gain-of-function mutations in terms of NK cell phenotype suggests that overactivation of this signaling pathway leads to an inability to tune its responsiveness and may be as detrimental as inactivation through loss of function. This signaling can be partially restored through dampening hyperactive signaling, and inhibition of mTOR signaling is effective in partially restoring NK cell function in these patients. Treatment with rapamycin in our cohort led to increased cytotoxic function with increased F-actin accumulation at the NK cell synapse, a critical step in NK cell cytotoxicity. Interestingly, this was independent of reversal of the phenotypic changes that we detected in patient NK cells, suggesting that it may represent a partial restoration of intracellular activation that has been rendered hyporesponsive in APDS patient NK cells. While the exact mechanism of action is not known, the known importance of the PI3K signaling pathway through AKT and mTOR in NK cells suggests that re-tuning responsiveness to cytokine or activating receptor signaling may enable restoration of function in patient cells. mTOR plays a role in both the generation and activation of NK cells, and primary human cells from healthy donors treated with rapamycin have impaired cytotoxic function, suggesting a direct role for mTOR signaling in the regulation of lytic function[52].

Taken together, our data confirm speculation[28] that extreme susceptibility to herpesviral infection in APDS patients is due in part to impaired NK cell function and provides additional rationale for the use of rapamycin in patients, particularly those with viral illness.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **NKD** | NK cell deficiency |
| **PID** | primary immunodeficiency |
| **MTOC** | microtubule organizing center |
| **EBV** | Epstein-Barr virus |
| **CMV** | cytomegalovirus |
| **PASLI disease** | p110δ-activating mutation causing senescent T cells, lymphadenopathy and immunodeficiency |
| **APDS** | activated PI3K delta syndrome |
| **PI3K** | phosphoinositide 3 OH-kinase |

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**Key Messages**

- Patients with *PIK3CD* gain-of-function mutations have impaired NK cell cytotoxic function due to decreased conjugate formation and NK cell polarization
- Patients with *PIK3CD* gain-of-function mutations have decreased NK cell frequency which is accompanied by decreased expression of CD16, CD122 and CD127 and increased expression of NKG2A
- Functional, but not phenotypic, defects are partially restored by rapamycin treatment

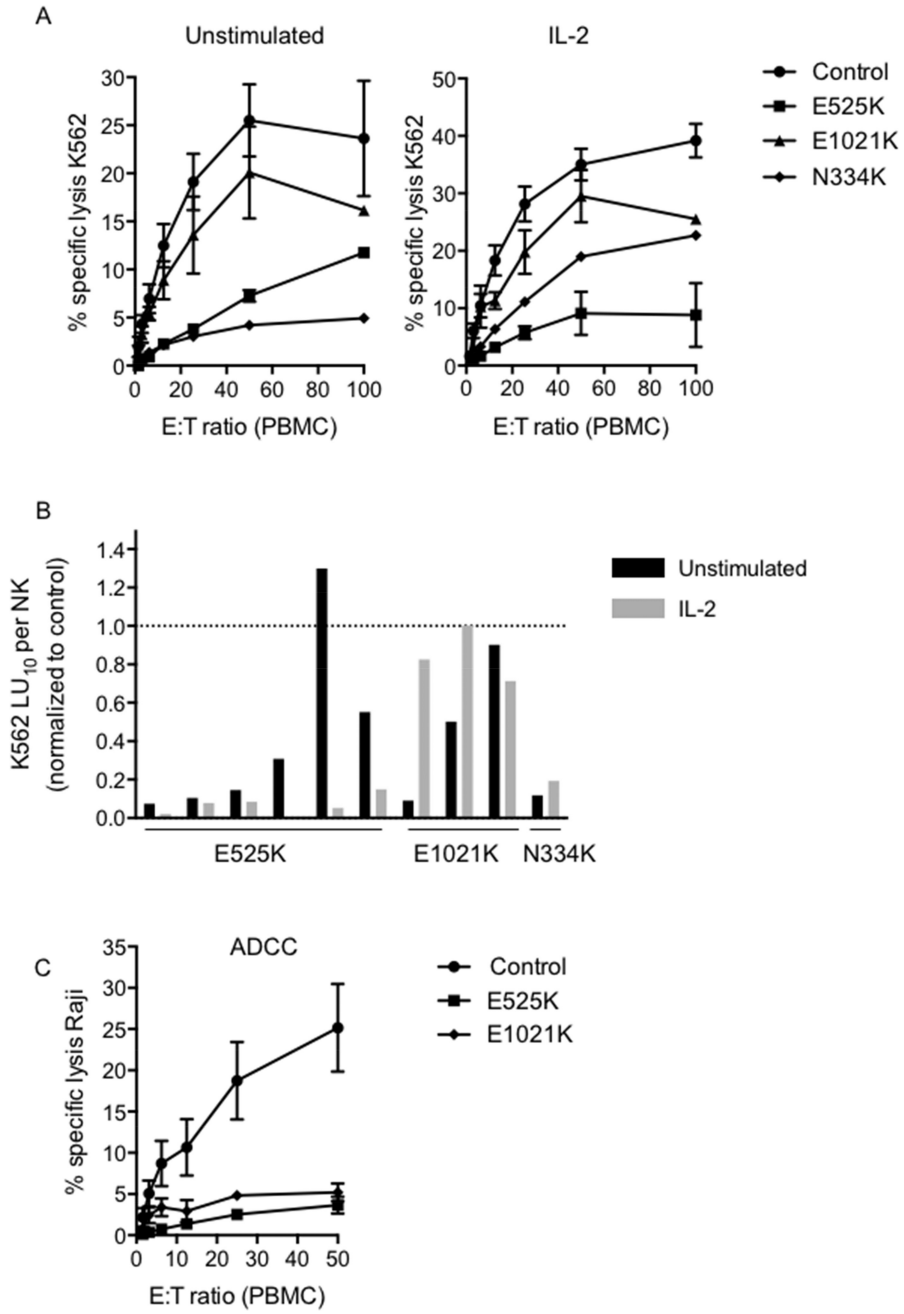


**Figure 1. PI3K110δ mutation results in impaired NK cell cytotoxic function**

PBMC isolated from healthy donors (circles, n=18), PI3K110δ E525K patients (squares, n=6), PI3K110δ E1021K patients (triangles, n=3) or PI3K110δ N334K (diamonds, n=1) were incubated with A) susceptible K562 target cells in the absence (left) or presence of 1000 U/ml IL-2 (right). Cytotoxicity was measured by $Cr^{51}$ release assay. B) Results from (A) are shown as the lytic units ($LU_{10}$) required to lyse 10% of target cells when adjusted for the frequency of NK cells within PBMC. Patient $LU_{10}$ is normalized to the healthy donor control for each assay. C) PBMC isolated from healthy donors (circles, n=10), PI3K110δ E525K patients (squares, n=4) or PI3K110δ E1021K patients (triangles, n=2) were

incubated with Patient Raji B cells in the presence of 20 μg/ml Rituximab. Cytotoxicity was measured by $Cr^{51}$ release assay.

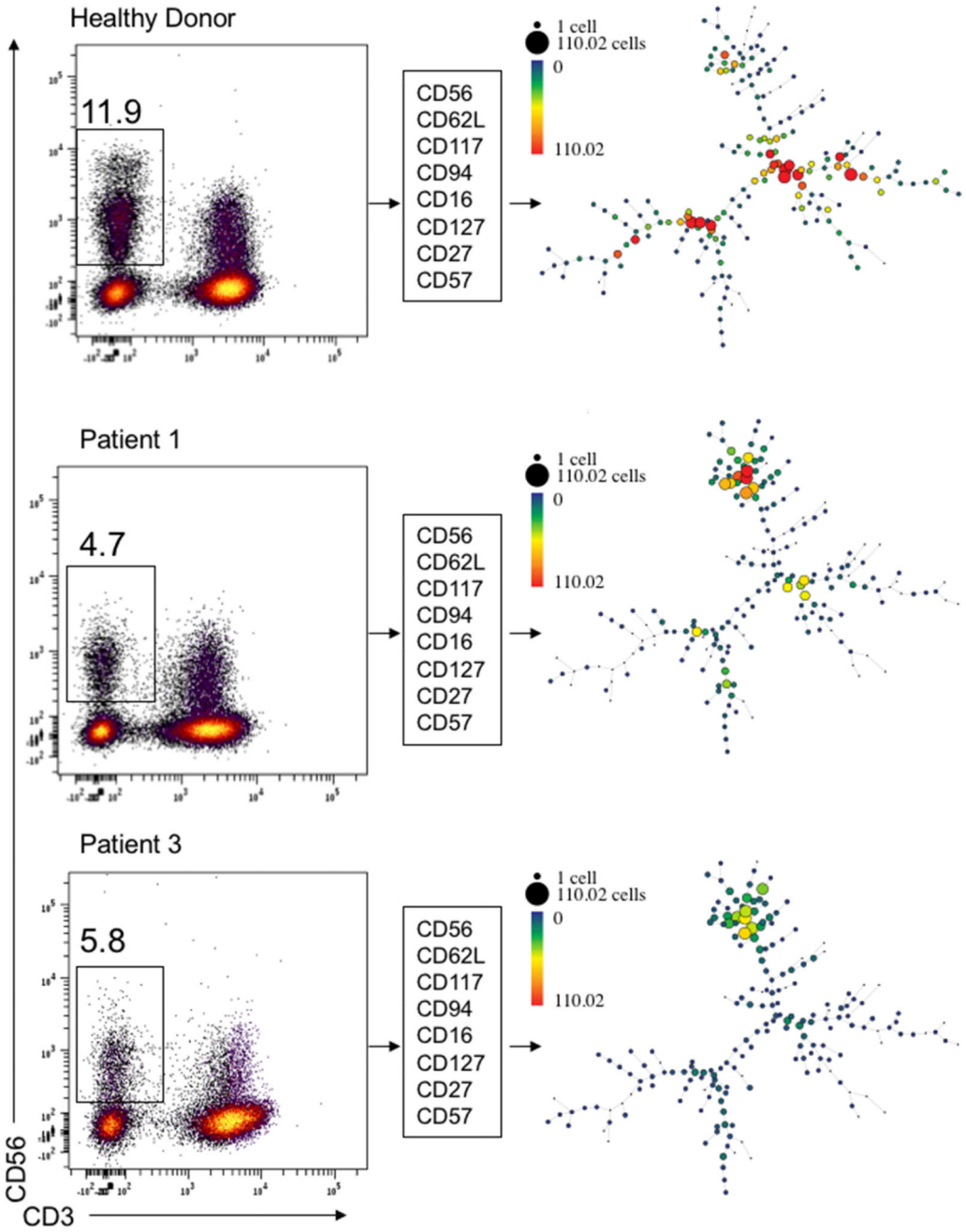


**Figure 2. PI3K110δ mutation leads to altered NK cell subset distribution**

Flow cytometry data from 8-color analysis of NK cell surface receptors associated with developmental subsets on healthy donor (top) and two E525K PI3K110δ patients (bottom) and was visualized by spanning analysis of density events (SPADE). Gating strategy for each patient or control is shown on the left with the frequency of NK cells within the lymphocyte gate. Clustering parameters are shown in the center. SPADE plot node sizes and intensities are based upon cell counts as shown in the heat map legend.

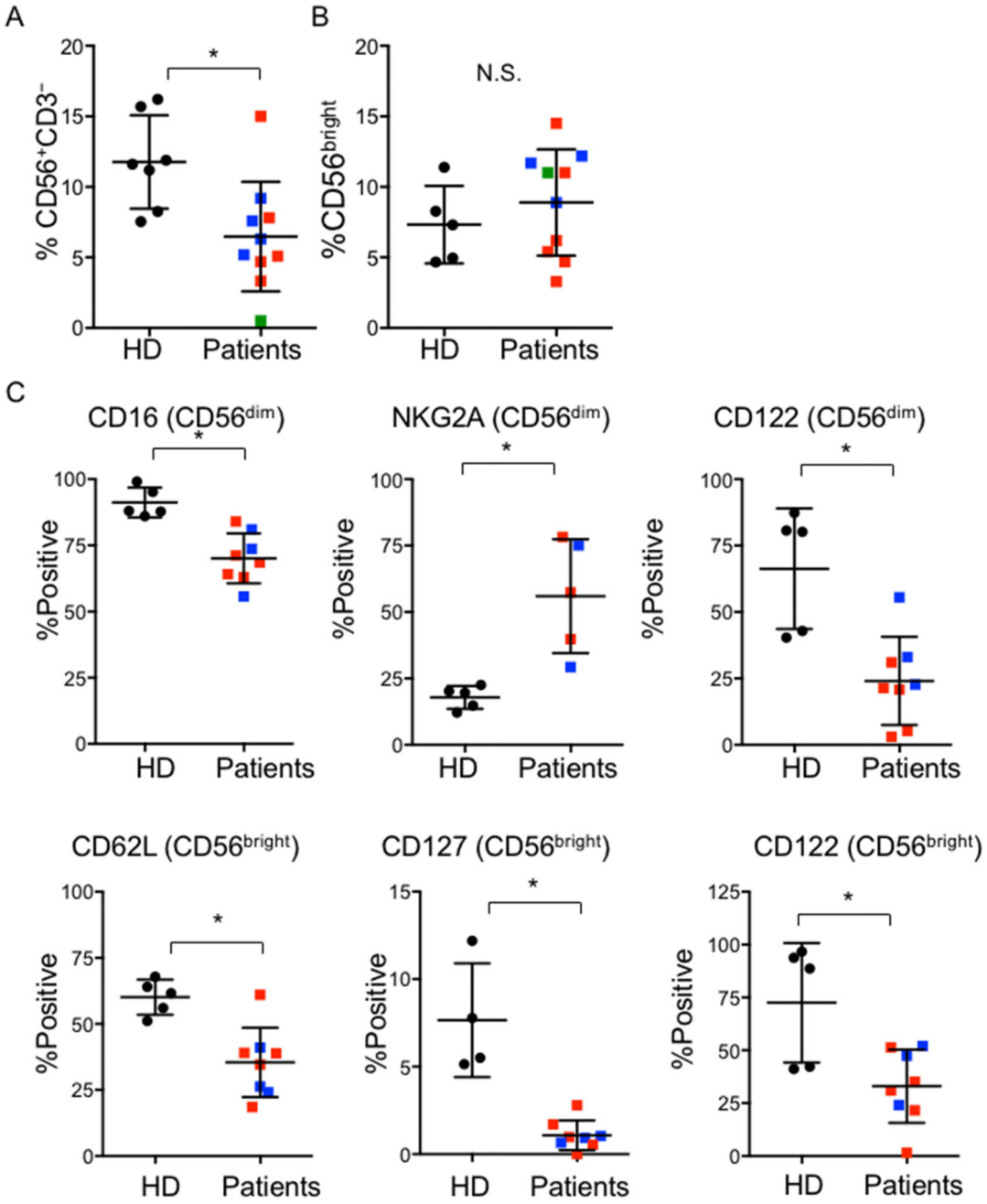


**Figure 3. Pairwise analysis of differentially expressed receptors on NK cells from patients and healthy donors**

PBMC from 5 healthy donors (black circles) and PI3K110δ patients with E525K (red), E1021K (blue) or N334K (green) mutations were analyzed by flow cytometry as in Figure 2. A) NK cell frequency and B) $CD56^{bright}$ subset distribution. C) NK cell receptor expression on $CD56^{bright}$ and $CD56^{dim}$ subsets as indicated. *$p$<0.05 by Student's two-tailed T test.

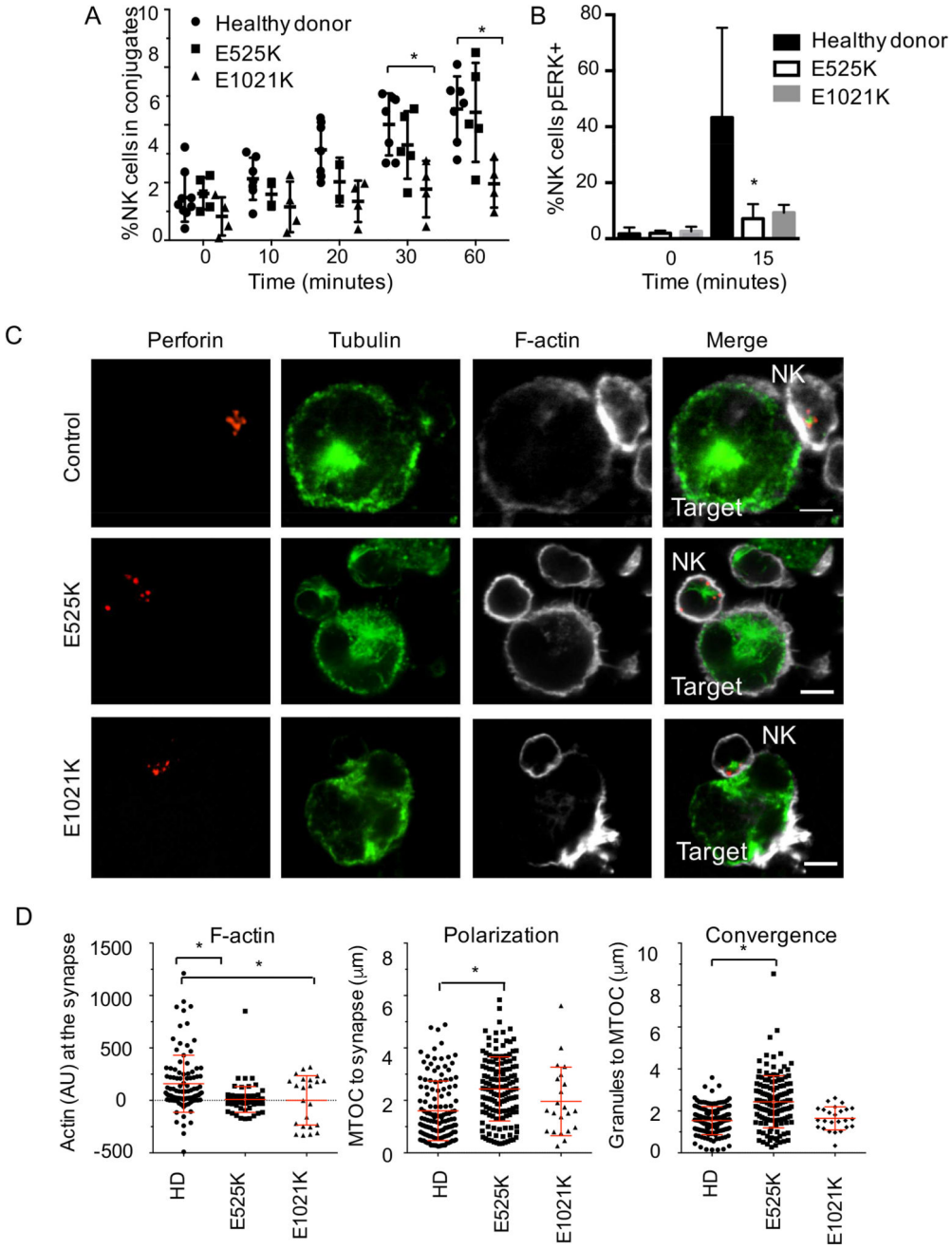


**Figure 4. Impaired conjugate formation, ERK phosphorylation and effector cell polarization in NK cells from PI3K110δ patients**

A) Differentially labeled NK cells within PBMC and K562 targets were incubated together for times indicated, then fixed, and conjugate frequency was determined by flow cytometry. Healthy donor, n=7; E525K, n=5; E1021K, n=4. B) Intracellular phospho-ERK was analyzed by flow cytometry following activation by plate bound anti-NKG2D. *$p$<0.05 by ordinary one-way ANOVA with post hoc comparison, n=5. C) NK cells were conjugated to K562 targets then fixed, permeabilized and stained with anti-perforin, anti-α-tubulin and phalloidin. Scale bar=5 μm. D) F-actin accumulation, lytic granule convergence and MTOC polarization were measured for >25 conjugates. *$p$<0.05 by Student's two-tailed T test.

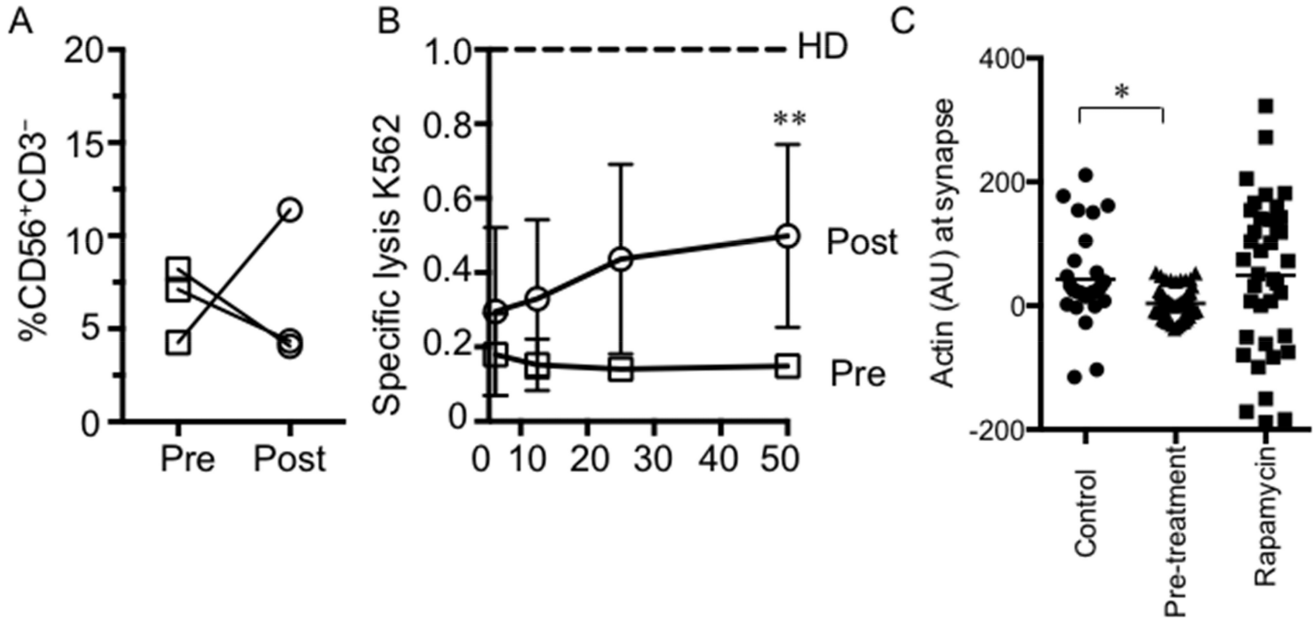


**Figure 5. Partial restoration of NK cell function following initiation of rapamycin treatment in PI3K110δ patients**

A) NK cell number in three patients prior to (open squares) and following initiation of (open circles) rapamycin treatment. B) Evaluation of NK cell cytotoxic function without IL-2 stimulation in the same three patients relative to normal controls (dashed line). C) NK cell-target conjugates were analyzed as in Figure 4. ** $p<0.01$ by Student's two-tailed T test at 50:1 ratio. * $p<0.05$ by ordinary one-way ANOVA with post hoc comparison.

**TABLE I**

Clinical and immunologic phenotype of PI3K110δ patients

| Patient | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Family * | NA | NA | NA | F.II.1 * | E.1 * | D.I.1 * | B.III.1 * | NA | NA | G.1 * |
| Mutation | E525K | E525K | E525K | E525K | E525K | E525K | E1021K | E1021K | E1021K | N334K |
| Clinical/immunologic phenotype | | | | | | | | | | |
| Transitional B cells | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ND | ↑ | NL |
| Specific antibody response | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ND | ↓ | NL |
| IgA | NL | NL | NL | ↓ | NL | NL | NL | ND | NL | ↓ |
| IgM | ↑ | NL | NL | NL | NL | NL | ↑ | ND | NL | ↑ |
| IgG | ↑ | ↑ | ↑ | NL | NL | ↑ | NL | ND | NL | ↓ |
| Switched B memory | ↑ | ↑ | ↑ | ↓ | ↓ | NL | ↓ | ND | Low/NL | ↓ |
| $CD4^+$ | NL | Low/NL | NL | NL | ↓ | ↓ | ↓ | ND | ↓ | ↓ |
| $CD8^+$ effector | ND | ND | ND | ↑ | ↑ | ↑ | ↑ | ND | ND | ↑ |
| $CD8^+$ central memory | ND | ND | ND | ↑ | NL | NL | NL | ND | ND | NL |
| EBV | Yes | Yes | Yes | Yes | Yes | Yes | Yes | ND | No | Yes |
| Lymphadenopathy | Yes | Yes | Yes | Yes | Yes | No | Yes | ND | No | Yes |
| Hepatosplenomegaly | Yes | Yes | Yes | No | No | No | No | ND | No | ND |
| Other | | | | $EBV^+$ lymphoma | CMV | CMV | | | | Sinopulmonary bacterial infections |

*CMV*, Cytomegalovirus; *NA*, not applicable; *ND*, not determined; *NL*, normal.

*Refers to Lucas et al.[24]

*J Allergy Clin Immunol*. Author manuscript; available in PMC 2019 August 01.