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Recovery of NK cell cytotoxicity in a p.A91V perforin homozygous patient following severe HLH

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The pore-forming protein perforin in essential for the cytotoxicity of natural killer (NK) cells and CD8+ T cells. Perforin and granzyme proteases are stored in secretory vesicles that migrate to the immune synapse formed with a target cell, fuse with the plasma membrane and release both toxins into the synaptic cleft. Perforin then forms transmembrane pores on the target cell membrane, enabling granzymes to diffuse into the cytosol to initiate apoptosis (Voskoboinik et al., 2015). A complete failure to release perforin due to mutations in the granule exocytosis machinery or bi-allelic loss-of-function (null) mutations in perforin itself cause an autosomal recessive immune dysregulation syndrome in infants, familial haemophagocytic lymphohistiocytosis (FHLH) which, if untreated, results in a fatal cytokine storm (Janka, 2012, Stepp et al., 1999).

The clinical presentation of fulminant FHLH is distinctive, but many other cases have more subtle, delayed or atypical onsets because many mutations still permit partial perforin function (Clementi et al., 2005). The disorder can present in adolescence or even adulthood with life-threatening organ failure or neurological manifestations; or cryptically in children with growth retardation (Gray et al., 2017) or lymphoma (Lopez et al., 2018). Most early-onset FHLH-associated mutations are exceedingly rare, but the *PRF1* polymorphism c.272C>T (encoding the amino acid substitution p.A91V), is very common, being present in ~8% of Caucasian subjects. Several independent studies have found p.A91V homozygosity (or even heterozygosity) to be associated with FHLH (Mancebo et al., 2006, Zhang et al., 2014), chronic inflammatory or auto-immune conditions (Cappellano et al., 2008, Vastert et al., 2010), or haematological cancers (Clementi et al., 2005, Chia et al., 2009, Mehta et al., 2006).

p.A91V causes severe loss of perforin function *in vitro*, as it causes abnormal perforin folding, trafficking and cytotoxic activity (Trambas et al., 2005, Voskoboinik et al., 2007, House et al., 2015). Poor *in vitro* function and the reported associations with serious illness led us recently to investigate whether p.A91V homozygotes (predicted to be ~ 1/700 of all Caucasians) become depleted in the general population as they age. However, in a population study of over 13,000 individuals aged 75 or older (Voskoboinik et al., 2020), we found no reduction in the number of p.A91V homozygotes, nor did the 24 individuals we identified have a preponderance of prior

immune-mediated disease or cancer. Surprisingly then, the lifetime health risk of p.A91V homozygosity is actually very low (Voskoboinik et al., 2020).

Despite our findings at the population level, NK cell cytotoxicity of p.A91V homozygous patients tested during acute FHLH has frequently been found to be markedly reduced, posing a dilemma: if such patients have such poor cytotoxic function, how are they able to survive to old age? Here, we report a unique instance where it has been possible to measure the NK cell cytotoxicity of a p.A91V homozygous adult with FHLH, both at the onset of disease and after he recovered.

A 21-year-old male from non-consanguineous parents was admitted to hospital with severe Epstein-Barr virus (EBV) infection (420,000 virus DNA copies/mL of plasma), fever (38.6^{o}C), pericardial effusion, diffuse lymphoproliferation, hepatitis (AST 305U/L), pulmonary infiltrates and constitutional symptoms. Blood examination revealed bicytopaenia (haemoglobin 76g/L, platelets 92x10^{9}/L), hyperferritinaemia (17,846μg/L), elevated soluble CD25 (1,593IU/mL) and hypofibrinogenaemia (1.39g/L). CT-imaging demonstrated generalised lymphadenopathy, mild hepatomegaly (21.4cm at the midclavicular line) and splenomegaly (18.0cm in craniocaudal plane) with numerous small hypodense lesions and multiple pulmonary nodules. Tonsillar and axillary lymph node biopsies were consistent with severe, active EBV infection. Bone marrow biopsy showed a mildly hypercellular marrow with scattered EBV-encoded small nuclear RNA-positive cells and occasional haemophagocytosis. Initial empiric therapy consisted of intravenous methylprednisolone to address tonsillar enlargement sufficient to cause airway obstruction and a worsening inflammatory pericardial effusion. Intragam® P (1g/kg/day for two days) was administered for immune-mediated cytopenia, along with rituximab (375mg/m^2 once weekly for four weeks) for acute EBV infection. Retrospectively, the patient satisfied seven of eight HLH-2004 diagnostic criteria and the probability score (HScore) for a diagnosis of HLH was 293, consistent with a diagnostic probability of >99% (Table 1). He achieved remission of HLH without the use of etoposide. Four weeks later, his ferritin had fallen to 2,187ug/L along with resolution of his constitutional symptoms, hepatitis and cytopenias. There was also a significant reduction in his adenopathy and pericardial effusion. He recovered fully within two months, and remained well 12 months after discharge.

One of the key diagnostic criteria of FHLH is reduced NK cell function. Initial tests conducted two weeks after disease onset revealed low NK cytotoxicity, severely reduced intracellular perforin staining by FACS and normal NK cell degranulation (Figure 1A-C), suggesting perforin deficiency-associated FHLH. Genetic analysis revealed the patient to be homozygous p.A91V. Both parents were confirmed to be heterozygous carriers of the same allele. Sequencing of the patient's *UNC13D*, *STX11*, *STXBP2*, *XIAP*, *SH2D1A*, *FAS*, *RAB27A* and *LYST* genes did not identify any pathogenic sequence variants. Given we have shown that NK cytotoxicity may be reduced transiently during severe acute illness

(Gray et al., 2017), we re-assessed the patient's NK cytotoxicity one and two months after resolution of symptoms and cessation of therapy. Surprisingly, NK cell cytotoxicity had recovered and was now comparable to control samples (Figure 1E). Despite this, intracellular perforin staining remained decreased (Figures 1b,e), and Western immunoblotting under reducing and non-reducing conditions confirmed p.A91V misfolding, abnormal processing and reduced antigenicity (Trambas et al., 2005, Voskoboinik et al., 2007, House et al., 2015) (Supplementary Figure 1).

Collectively, our findings reported recently (Voskoboinik et al., 2020) and here suggest that markedly reduced NK cytotoxicity assessed during severe acute illness in adolescents and adults is not a reliable predictor of the long-term sequelae of inheriting hypomorphic mutations such as p.A91V, or mutations of unknown significance. Rather, as demonstrated here, *in vitro* NK cytotoxicity may be severely compromised during active disease, but be rapidly restored upon clinical recovery. By contrast, NK function remains suppressed in the long-term in patients who inherit genuinely null or severely abnormal bi-allelic mutations (Gray et al., 2017, Lopez et al., 2018, Sullivan et al., 1998). Complete or near-complete restoration of NK cell function with resolution of a patient's acute illness may thus be a useful predictor of future morbidity associated with missense perforin variants that are hypomorphic, but not null. While the current report focuses on a single p.A91V homozygous individual, other patients with a clinical course similar to the one reported here need to be investigated long-term, to determine whether our observation can be generalised for other FHLH-associated mutations.

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Conflict of interest.

The authors declare no conflict of interest.

Author contribution

JHS, FSL and KI diagnosed and treated the patient; FTWH, TKYT, NT, LP, RM, MJJ and BP conducted experiments and data analysis, TJA and VI conceived the study and co-wrote the paper; all authors contributed to writing the paper.

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in the cytotoxic pathway lead to clinical familial hemophagocytic lymphohistiocytosis. *Blood,* 124**,** 1331-4.

Table 1. Patient's fulfilment of HLH-2004 criteria and HScore at initial presentation.

Parameters	**HLH-2004 Criteria**	**HScore**
Fever (°C)	1 (≥38.5)	33 (38.4-39.4)
Splenomegaly	1 (yes)	
Organomegaly		38 (hepatomegaly and splenomegaly)
Cytopaenia	1 (≥two lineages)[a]	24 (two lineages)[b]
Triglycerides (mmol/L) and/or Fibrinogen (g/L)	1 (≥3 for triglycerides) (≤1.5 for fibrinogen)	64 (>4 for triglycerides)
		30 (≤2.5 for fibrinogen)
Ferritin (μg/L)	1 (≥500)	50 (>6,000)
Haemophagocytosis in bone marrow	1 (yes)	35 (yes)
Low or absent NK-cell activity	1 (yes)	
Soluble CD25 (IU/mL)	0 (≤2,400)	
Asparatate aminotransferase (U/L)		19 (≥30)
Known underlying immunosuppression		0 (no)
Total	7 of 8 (≥5 of 8)	293, >99% probability

[a]Defined as haemoglobin <90g/L, platelets <100x10^9/L, and neutrophils <1.0x10^9/L.

[b]Defined as haemoglobin ≤92g/L, platelets ≤110x10^9/L, and leukocytes ≤5x10^9/L.

Figure Legend.

Figure 1. Assessment of NK cell cytotoxicity and degranulation in a patient who is homozygous for p.A91V.

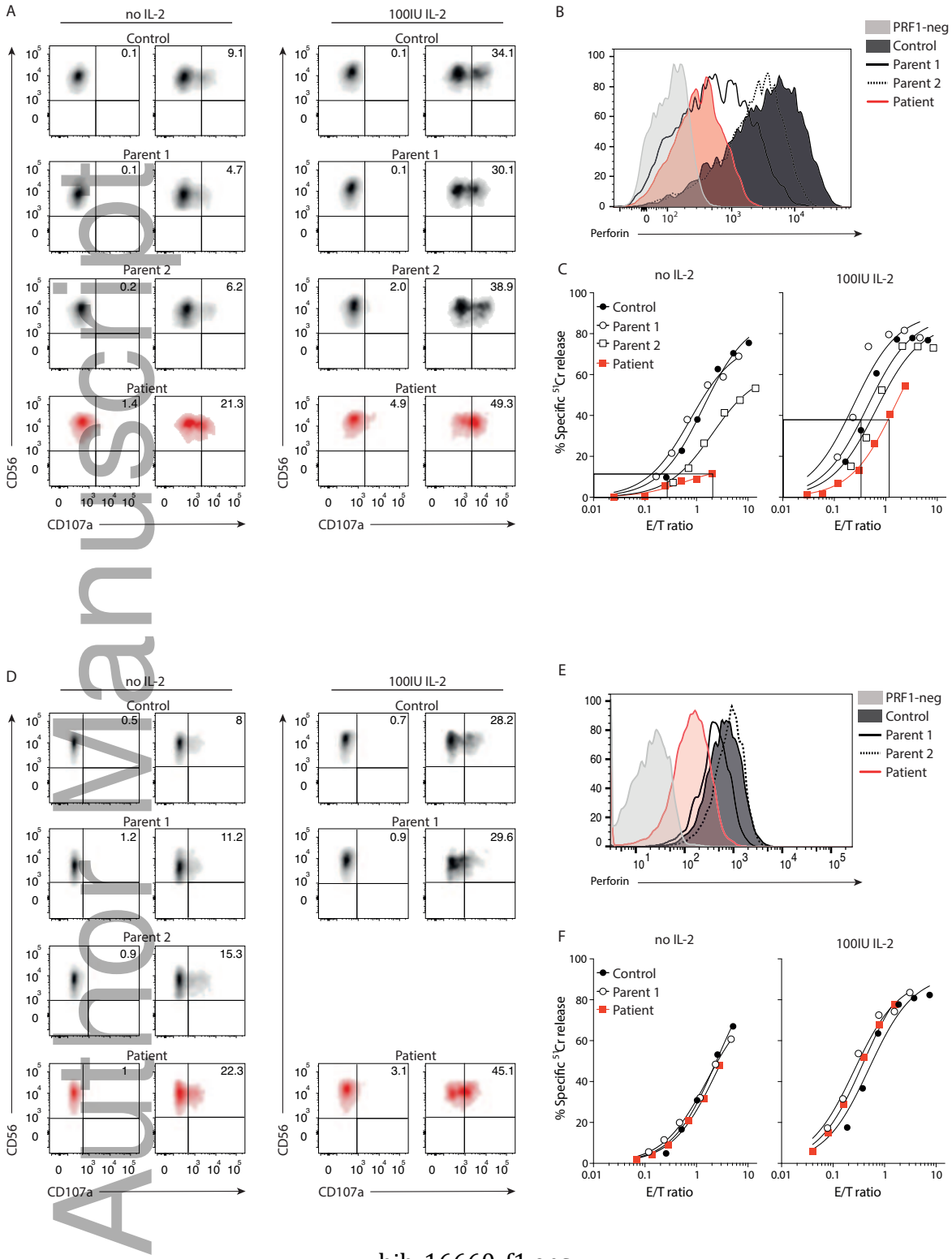
We performed ^{51}Cr release and CD107a externalisation assays as previously described (Gray et al., 2017). **A, D.** CD107a externalisation assay. Peripheral Blood Mononucleated Cells (PBMC) were isolated and cultured in RPMI-1640 culture media supplemented with glutamine, pyruvate, non-essential amino acids, penicillin/streptomycin mixture and 10% Foetal Calf Serum, with or without 100U/mL IL2, for 18 hours. The cells were then incubated without (left column) or with (right column) K562 target cells for 4 hours at 37°C in the presence of anti-CD107a-Alexa488 antibodies. CD107a externalisation was determined in a population of $CD3^-/CD16^+/CD56^{dim}$ NK cells. **B, E.** Intracellular perforin staining (permeabilisation with Perm/Wash buffer 1 (BD Biosciences)) of $CD3^-/CD16^+/CD56^{dim}$ NK cells using δG9-PE anti-perforin antibodies (eBiosciences). "PRF1-neg" –

intracellular perforin staining of non-NK cells from the healthy donor control. **C, F.** A 4-hour ^{51}Cr release assay was conducted using PBMC cultured with or without 100U/mL IL-2 for 18hrs (as described in **A, D**). PBMC and ^{51}Cr-labeled target K562 cells were mixed at various ratios, from 3:1 to 100:1, and incubated for 4 hours. % specific ^{51}Cr release was determined as described in (Gray et al., 2017). The assay was standardised for % NK cells ($CD3^-/CD16^+/CD56^{dim}$) in each sample and plotted as "E/T ratio."

Shown are **A-C** – at discharge, and **D-F** - 2 months after discharge from the hospital.

Surface receptors were detected by staining with the following anti-human antibodies: CD16, CD56 (both from BD Biosciences, San Jose, CA), CD3 and CD107a (Biolegend, San Diego, CA, USA). Cells were analysed on a Fortessa or LSR flow cytometer (BD Biosciences) and data analysed using FlowJo software (version 10, FlowJo LLC, Ashland, OR).

Figure 1



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