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Natural killer (NK) cell function in paroxysmal nocturnal hemoglobinuria; a deficiency of NK cells, but not an NK cell deficiency.

To the editor;

Treatment of the glycosylphosphatidylinositol (GPI) anchor-deficiency, paroxysmal nocturnal hemoglobinuria (PNH), has been revolutionized by use of the anti-C5 antibody eculizumab, which blocks complement-mediated hemolysis and the associated pathology.[1,2] In addition to complement susceptibility, GPI-anchor deficiency alters cellular function with the potential to further contribute to disease. For example, defective natural killer (NK) cell activity in PNH was first described more than three decades ago.[3,4] NK cell deficiencies are associated with susceptibility to infection[5,6], suggesting that NK function in PNH should be analyzed in more detail. Here we show that functional defects in NK cell activity in PNH result from reduced NK cell numbers rather than cell intrinsic defects.

Early PNH studies showed that impaired NK cell activity was associated with reduced large granular lymphocyte (LGL) counts.[3,4] However, these reports preceded the definition of NK cells and our understanding of the molecular basis of PNH, prompting reassessment. Mosaicism in PNH[1] allows side-by-side functional comparisons of GPI+ and GPI^{neg} NK cells within individual patients, enabling the assessment of NK cell activity on a per cell basis (Figure 1 and Supplemental Figure 1). Despite reports of impaired activity[3,4], the GPI-deficient NK cells were proficient at target cell induced granule exocytosis (Figure 1A-B and Supplemental Figure 1). Thus, early findings associating reduced NK cell activity with reduced LGL numbers rather than intrinsic cellular activity are correct.[4] The absolute number of NK cells (and more variably, other lymphocytes) are indeed reduced in PNH[7]; in our cohort of thirty-nine patients, two thirds had NK cell counts below the reference range (Figure 1C) and NK cell numbers were not significantly correlated with neutrophil, monocyte or platelet counts (Supplemental Figure 2). The basis for reduced NK cell numbers in PNH is unclear, although this might be related to impaired chemotactic or homeostatic mechanisms, as we recently reported.[8] Whilst the activity of GPI-deficient NK cells is unimpaired, a reduction in absolute numbers of NK cells will reduce NK cell activity in the blood as a whole.

Clearly, PNH should not be classified as a functional NK cell deficiency (NKD). Classical NKD is characterized by approximately one tenth the normal number of NK cells and counts in most of our PNH patients exceeded this (Figure 1C). Furthermore, the term NKD is reserved for where "the impact upon NK cells need represent the major immunological abnormality in the patient".[6] In PNH, all hematopoietic lineages are affected due to the presence of PIGA mutations in hematopoietic stem cells.[1] More compelling is the clinical phenotype; the defining feature of NKD is the heightened susceptibility to viruses[5,6] which has not been observed in PNH.[7,9,10] Instead, infection in PNH is bacterial in origin[10] and likely to be associated with neutropenia secondary to underlying bone marrow failure or associated with use of eculizumab which increases the risk of infection with encapsulated bacteria normally eliminated by terminal complement components.[1] In summary, the low numbers of NK cells in PNH affect overall cytotoxicity, but this defect is not severe enough to manifest as heightened susceptibility to viral infection as seen in NKD.

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Authorship

Contribution: R.J.K., A.H., and P.H. manage the PNH clinic and recruited the patient cohort; Y.M.E.-S. performed the experimental work; G.P.C. and Y.M.E.-S. designed the study; Y.M.E.-S., G.M.D., R.J.K and G.P.C. analyzed the data; G.P.C. wrote the article with input from all authors.

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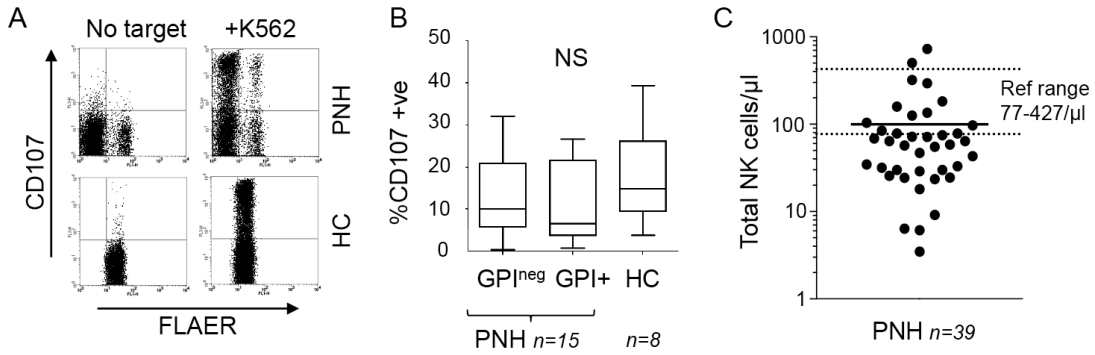


Figure 1

A) NK cell granule exocytosis in PNH. NK cells were purified from blood samples from healthy controls (HC) or PNH patients using indirect selection reagents from Miltenyi. NK cell degranulation was assayed using a modification of a standard method to allow detection of GPI+ and GPI-deficient NK cells using fluorescent aerolysin (FLAER; Ref 8 and Supplemental information). Briefly, purified NK cells were co-cultured with K562 target cells (for four hours). Co-cultures were then stained with anti-CD56 antibody (to identify NK cells), FLAER to distinguish GPI-deficient and GPI+ cells and anti-CD107 to identify degranulated NK cells. This analysis is from one PNH patient and one healthy control (gating on the purified CD56+ NK cells) with the data from the cohort shown in B). We also compared the mean fluorescence intensity of CD107 staining in paired GPI-deficient and GPI+ cells within each patient. This demonstrated a significant increase in CD107 display on the GPI-deficient NK cells (Supplemental Figure 1).

B) Summary of NK cell degranulation activity from 15 PNH patients and 8 healthy controls (HC). The box plot shows the percentage of GPI-deficient (GPI^{neg}) and GPI+ NK cells that have degranulated in response to target cells. The range (whiskers), median (horizontal line) and interquartile range (box) are shown. The percentage of CD107+ NK cells was not statistically significant (NS) between any two groups according to the Mann Whitney test. Comparison of the percentage of CD107+ NK cells within individual patients showed no significant differences between matched GPI+ and GPI^{neg} NK cells (Supplemental Figure 1).

C) Number of total NK cells in 39 PNH patients (cells per microliter). Patient values ranged from 3-725 cells per microliter (mean; 100 cells/µl). A European reference

range (77-427 cells/μl) is shown by the dotted lines. The patients with NK cell counts within the normal range were unremarkable in terms of gender, age or treatment (ciclosporin or eculizumab). Furthermore, there was no correlation between the NK cell counts and the absolute numbers of other cell types (Supplemental Figure 2). Peripheral blood samples used in this work were collected after informed consent in accordance with the Declaration of Helsinki.

Supplemental Information

i) Supplemental Methods

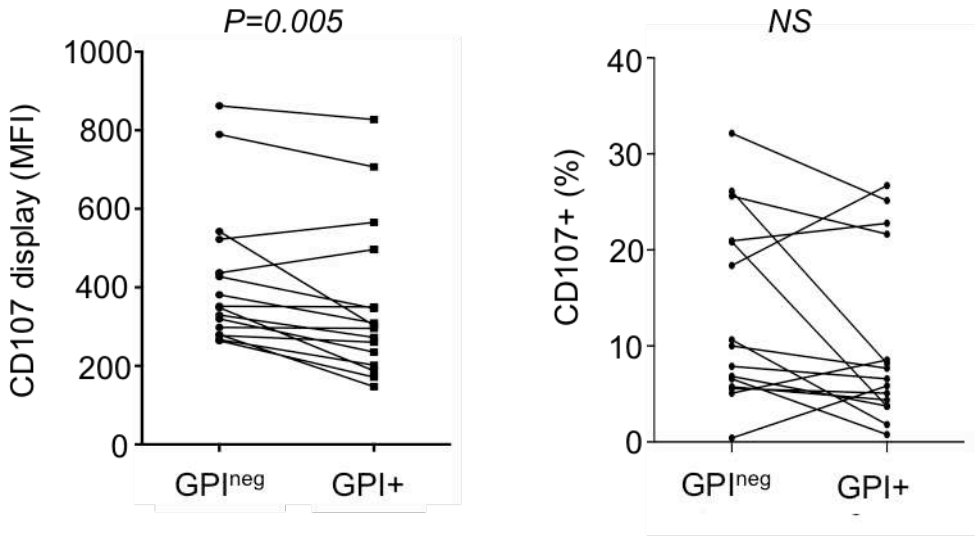
A modified degranulation assay to analyse responses of GPI-deficient and GPI+ NK cells.

We modified a lymphocyte cytotoxic granule exocytosis assay (Reference S1) to include staining with Alexa-488 conjugated aerolysin (Fluorescent aerolysin or FLAER from Pinewood Scientific Services, Canada). FLAER binds to intact GPI anchors thereby allowing identification of the GPI+ and GPI^{neg} NK cells (Reference S2 and S3). NK cells were purified from blood samples from healthy controls (HC) or PNH patients using indirect selection reagents from Miltenyi Biotec. This procedure typically yields NK cells at >95% purity. Purified NK cells were co-cultured with K562 target cells at an effector:target ratio of 2:1 for four hours (in the presence of Golgi Stop from BD Biosciences). Co-cultures were then stained with anti-CD56 antibody (to identify NK cells), FLAER to distinguish GPI^{neg} and GPI+ cells and anti-CD107 to identify degranulated NK cells.

Stained cells were analyzed by flow cytometry (using a BD Biosciences LSRII). The co-culture contains both NK cells and K562 cells; we gated on the NK cells on the basis of their reduced forward and side scatter profile and on the basis of CD56 expression. Gated NK cells were then analysed for CD107 display on the $FLAER^{neg}$ (GPI^{neg}) and FLAER+ (GPI+) populations. We analyzed the percentage of GPI+ and GPI^{neg} NK cells displaying CD107 on their surface (shown in Figure 1A and B of the main manuscript) and the mean fluorescence intensity of the CD107 staining on the degranulating GPI+ and GPI^{neg} NK cells (Supplemental Figure 1).

ii) Supplemental Figure 1.

GPI^{neg} NK cells exhibit unimpaired degranulation against K562 target cells

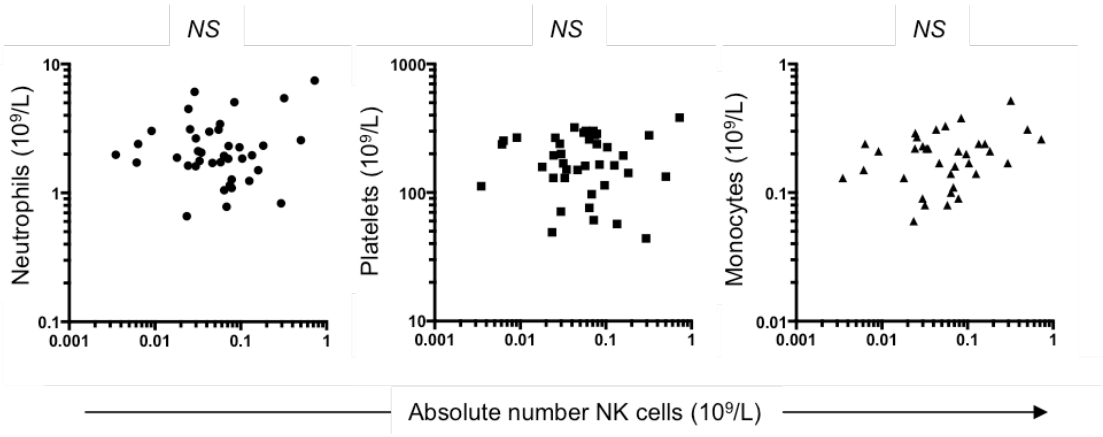


The left hand plot shows the mean fluorescence intensity (MFI) of CD107 staining on the degranulating GPI+ and GPI^{neg} NK cells following co-culture with K562 target cells. The data was analyzed in a pairwise manner, comparing CD107 display in GPI+ and GPI^{neg} NK cells within the same patient (joined by lines). Analysis using the Wilcoxon matched-pairs signed rank test indicated a significant difference between the paired samples, with greater CD107 display shown on the GPI^{neg} population. This demonstrates that the GPI-deficient NK cells exhibit enhanced degranulation on a per cell level (for example, releasing more granules per cell).

The right hand plot shows paired samples analyzed according to the percentage of CD107+ cells in the GPI+ and GPI^{neg} NK cell populations. This analysis did not reveal statistically significant differences (in agreement with the data shown in Figure 1B of the main manuscript).

iii) Supplemental Figure 2.

A lack of significant correlation between absolute numbers of NK cells and other blood cell types in the PNH patient cohort.



The plots show the absolute numbers of total NK cells (encompassing both GPI+ and GPI^{neg} populations) on the x-axis, compared to total neutrophils (left hand plot), total platelets (center plot) and total monocytes (right hand plot) on the y-axis. Each point represents one PNH patient; for neutrophils and platelets, n=39 and for monocytes, n=35. Correlation was analyzed using Spearman's Rank test, no significant correlations were observed.

iv) Supplemental references

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