The small population of *PIG-A* mutant cells in myelodysplastic syndromes do not arise from multipotent hematopoietic stem cells

Jeffrey J. Pu, Rong Hu, Galina L. Mukhina, Hetty E. Carraway, Michael A. McDevitt, and Robert A. Brodsky

Division of Hematology, Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, MD, USA

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Online Supplementary Design and Methods

Selection of proaerolysin-resistant CFCs

Bone marrow CD34+ cells ($1x10^5$ - $3.5x10^5$) were mixed with 1 mL of Methcult complete medium with cytokines (StemCell Technologies, Vancouver, BC, Canada) and 1 nM of proaerolysin. The mixture was plated in a tissue culture dish (35 x 10 mm). In each experiment, 10 or more dishes were plated. Three thousand CD34+ cells mixed with 1 mL of Methcult complete medium with cytokines, but without proaerolysin, were plated in a culture dish (35 x 10 mm) as a control with total of 4 dishes per experiment. After incubation in an incubator containing 5% CO2 with humidified air at 37°C for 14-16 days, the culture dishes were scored for CFCs under an inverted phase contrast microscope (Leitz, Wetzlar, Germany). The *PIG-A* mutational frequency (mf) were calculated using the following formula:

total number of proaerolysin-resistant CFC formation/efficiency of control CFC formation x total number of CD34+ cells plated in proaerolysin-contained medium

Cell surface GPI-anchor deficiency of these CFCs was confirmed by flow cytometry (BD FACSCalibur) after labeling with phycoerythrin (PE)-conjugated mouse anti-human glycophorin A monoclonal antibody (Immunotech, Marseille, France) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD59 monoclonal antibody (Immunotech, Marseille, France) for BFU-Es, and PE-conjugated mouse anti-human CD15 monoclonal antibody (Immunotech, Marseille, France) and fluorescent proaerolysin variant (FLAER) (Protox Biotech, Victoria, BC, Canada) for CFU-GMs. Positive control cells, negative control cells, and mouse isotype labeled cells were used to set up the gates, and to monitor autofluorescence and non-specific antibody binding.

Flow cytometry analysis of peripheral blood PNH cells

We diluted 3 uL of peripheral blood in 4 mL of PBS. This was washed with cold PBS and labeled with PE-conjugated mouse anti-human glycophorin A and FITC-conjugated mouse anti-human CD59 for flow cytometry identification of PNH erythrocytes. Then 100 μL of peripheral blood mixed with 4 mL of red blood cell lysis buffer was incubated in a 37°C water bath for 20 min, washed with cold PBS, and stained with PE-conjugated mouse anti-human CD15 or PE-conjugated mouse anti-human CD3 and FLAER to measure the percentage of PNH granulocytes or PNH T lymphocytes. The sources of antibodies and FLAER reagent, and the gate set-up were the same as before.

PNH T-lymphocyte enrichment culture

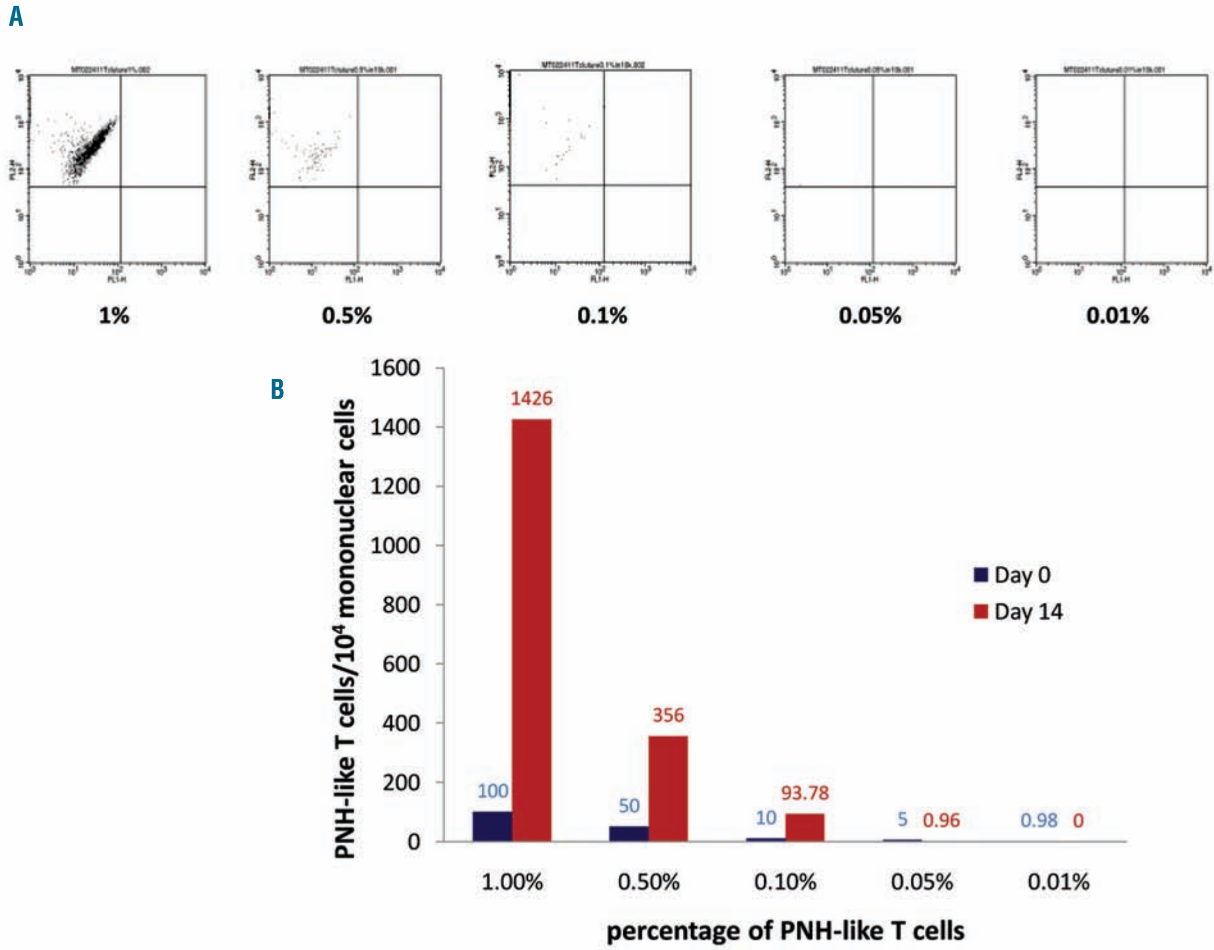
Mononuclear cells ($2.5\text{-}5 x10^6$) isolated from 5-10 mL peripheral blood sample were suspended in 1 mL of RPMI1640 medium (Cellgro; Mediatech, Manassas, VA, USA), containing 10% human serum, 25 μL of antibody (CD2, CD3, and CD28)-coated microparticles (Miltenyi Biotec, Auburn, CA, USA), 1.2% non-essential amino acid (Cellgro, Manassas, VA, USA), 1.2% sodium pyruvate (Cellgro, Manassas, VA, USA), and 1.2% L-glutamine (Cellgro; Manassas, VA, USA) and were cultured in an incubator containing 5% CO_2 with humidified air at 37°C for 14 days. The culture medium was supplemented with 20 units of rIL-2 (Miltenyi Biotec, Auburn, CA, USA) per mL every three days starting from Day 3 of incubation. The culture was treated with proaerolysin (1nM) for 48 h on Day 0 and then replaced with fresh medium cocktail as mentioned above. Clonal proaerolysin-resistant T lymphocytopoiesis processed around a single microparticle and formed a colony cell mass after 14 days of culture. These colonies were picked, assayed by standard flow cytometry for GPI anchor expression as described above, and genomic DNA was extracted for *PIG-A* gene mutation analysis as described below. As a control for our T-lymphocyte enrichment assay, T lymphocytes were also grown in T-lymphocyte growth medium without proaerolysin selection. The viability of the enriched T lymphocytes was measured by trypan blue staining.

PIG-A gene sequencing assay

Proaerolysin-resistant CFCs from semisolid Methcult complete medium and T-lymphocyte clones from liquid T-lymphocyte enrichment culture medium were individually picked-up and genomic DNAs were extracted using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The genomic DNAs were further purified using PCR Purification Kit (Qiagen, Valencia, CA, USA). Next, all 6 exons of the *PIG-A* gene were PCR amplified and sequenced as previously described.[1] The retrieved sequences were aligned with human PIG-A gene sequence via the online Blast nucleotide database comparison program (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) to search for sites of mutation. Each mutation site was confirmed by sequencing the complementary strand and identifying the same mutation using a reverse primer.

References

1. Hu R, Mukhina GL, Piantadosi S, Barber JP, Jones RJ, Brodsky RA. PIG-A mutations in normal hematopoiesis. Blood. 2005;105 (10):3848-54.



Online Supplementary Figure S1. Proaerolysin-selected T-cell enrichment assay can detect GPI-anchor deficient T-cell population as low as 0.05% and significantly enriches GPI-anchor deficient T-cell population at 0.1%. (A) A serial dilution panel of GPI-anchor deficient T cells (from 1% to 0.001%) was used to perform proaerolysin-selected T-cell enrichment assay. After culturing for 14 days, the GPI-anchor deficient T cells were collected and counted. GPI-anchor deficient T cells (as shown in panel A) were confirmed by flow cytometry analyses using PE-conjugated mouse anti-human CD3 and FLAER, (B) The enrichment power of this assay in various sizes of GPI-anchor deficient T lymphocytes by comparing the counts of GPI-anchor deficient T cells before and after enrichment culture.