# Phenotypic and functional characterization of a mouse model of targeted *Pig-a* deletion in hematopoietic cells

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## Supplementary methods.

### Animals

Inbred B6 and congenic C.B10-H2$^b$/LilMcdJ (C.B10) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). *Pig-a*$^{flox}$ mice were provided by Dr. T. Kinoshita (Osaka University, Japan) and the Fes-Cre transgenic mice were a generous gift from Dr. P. Pandolfi (Memorial Sloan-Kettering Cancer Center, NY, USA). *Pig-a*$^{-/-}$ mice were produced by cross-breeding *Pig-a*$^{flox}$ and Fes-Cre mice to avoid embryonic lethality and to enable *Pig-a* deletion specifically in hematopoietic cells. We first mated *Pig-a*$^{-/-}$ females with Fes-Cre males (*Pig-a*$^{+/0}$) in order to produce *Pig-a*$^{+/-}$ females and *Pig-a*$^{-/0}$ males, which were mated to produce *Pig-a*$^{+/-}$ and *Pig-a*$^{-/-}$ females and *Pig-a*$^{+/0}$ and *Pig-a*$^{-/0}$ males. We also mated *Pig-a*$^{+/-}$ females with normal Fes-Cre males (*Pig-a*$^{+/0}$) to produce *Pig-a*$^{+/+}$ and *Pig-a*$^{+/-}$ females and *Pig-a*$^{+/0}$ and *Pig-a*$^{-/0}$ males. All animals used in this study were confirmed to have the *Pig-a* gene deletion and the presence of Fes-Cre transgene by polymerase chain reaction (PCR). We also phenotyped animals by analyzing the presence of GPI$^-$ cells in red blood cells using CD24 and in B and T cells using CD48 as the GPI-anchored protein markers.

Mice were bred and maintained at the National Institutes of Health animal facility under standard conditions for care and nutrition, and they were used at 2-10 months of age. Experiments were performed using female *Pig-a*$^{-/-}$ and male *Pig-a*$^{-/0}$, mice and comparing them with female heterozygous (*Pig-a*$^{+/-}$) and wild type (WT, *Pig-a*$^{+/+}$) animals. All animal study protocols were approved by the National Heart, Lung, and Blood Institute's Animal Care and Use Committee.

### Genotyping

The genotype of the *Pig-a*$^{-/-}$ mice was determined by PCR using three sets of primers, as previously described. After an initial denaturation at 93 °C for 3 min, 28 cycles of PCR were performed at 93 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min. Fes-Cre transgene was also detected by PCR using two primer sets as described earlier with the same PCR conditions except that the annealing temperature was 56 °C.

### Cell analysis and cell sorting

Peripheral blood was obtained by orbital sinus bleeding. Bone marrow cells were extracted from femora and tibiae of *Pig-a*$^{-/-}$, *Pig-a*$^{-/0}$, *Pig-a*$^{+/-}$, WT *Pig-a*$^{+/+}$ or normal B6 mice into Iscove's modified Dulbecco's medium (IMDM, Gibco/Life Technologies, Carlsbad, CA, USA), and were filtered through a 90 µM nylon mesh to remove debris. Nucleated cells were counted by a ViCell counter (Beckman Coulter, Fullerton, CA, USA).

Antibodies for murine CD3 (145-2C11), CD4 (RM4-5), CD8 (5.3-6.7), CD11a (2D7), CD11b (M1/70), CD24 (M1/69), CD44 (IM7), CD45R (RA3-6B2), CD48 (HM48-1), Gr1/Ly6-G (RB6-8C5), Ter 119 (TER-119), interferon-γ (XMG1), and the T-cell receptor Vβ screening panel were obtained from BD Biosciences (San Diego, CA, USA), and they were conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanine 5 (PE-Cy5), or allophycocyanin (APC). Anti-FoxP3 (FJK-16s) was obtained from e-Biosciences (San Diego, CA, USA).

For the staining of cell surface antigens, bone marrow and peripheral blood cells were first incubated with Gey's solution (130.68 mM NH$_4$Cl, 4.96 mM KCl, 0.82 mM Na$_2$HPO$_4$, 0.16 mM KH$_2$PO$_4$, 5.55 mM dextrose, 1.03 mM MgCl$_2$, 0.28 mM MgSO$_4$, 1.53 mM CaCl$_2$ and 13.39 mM NaHCO$_3$) on ice for 10 min to lyse red blood cells and then stained with different antibody mixtures on ice for 30 min. For the intracellular staining of FoxP3 and IFN-γ, cells were pre-stained with cell-surface markers, fixed and permeabilized, and then incubated on ice for 30 min with the specific antibody for intracellular staining. Interferon-γ expression was detected on peripheral blood leukocytes after stimulation with phorbol 13-myristate 12-acetate (PMA) (50 ng/mL) and ionomycin (1 µg/mL) at 37°C 5% CO$_2$ for 5 h in combination with monensin (2 µM final concentration). Stained cells were analyzed by LSR II flow cytometry with FACSDiva software (Becton Dickinson, San Jose, CA, USA).

For cell sorting, bone marrow cells from *Pig-a*$^{-/-}$ or littermate WT mice, or from recipients of GPI$^-$ bone marrow cells or normal B6 bone marrow cells, were stained with a FITC-conjugated antibody mixture containing CD24, CD48 and Gr1, and were sorted into [CD24/CD48/Gr1]$^+$ (GPI$^+$) and [CD24/CD48/Gr1]$^-$ (GPI$^-$) cell fractions using a FACSVantage cell sorter (Becton Dickson, San Jose, CA, USA). For some analyses, CD4 and CD8 antibodies were added to sort out GPI$^+$ T cells (GPI$^+$CD4$^+$ and GPI$^+$CD8$^+$) and GPI$^-$ T cells (GPI$^-$CD4$^+$ and GPI$^-$CD8$^+$).

### Cell transplantation

Bone marrow cells from female *Pig-a*$^{-/-}$ donors were incu-

bated with 10 nM aerolysin (Protox Biotech, Victoria, BC, Canada) at 37 °C for 30 min to lyse GPI+ cells. The residual GPI- bone marrow cells were then transplanted into lethally-irradiated (9 Gy total body irradiation from a Shepherd Mark 1 [137]cesium gamma source, J. L. Shepherd & Associates, Glendale, CA, USA) B6 mice at $10^6$ cells/recipient (TR-GPI-). Five lethally-irradiated B6 mice were transplanted with $10^6$ normal bone marrow cells (TR-GPI+) from female Pig-a+/+ donors as controls.

### RNA extraction and microarray

Total RNA was extracted from sorted GPI+ and GPI- bone marrow cells of Pig-a-/- animals, or from sorted TR-GPI+ and TR-GPI- bone marrow cells obtained from the transplantation experiment and WT-GPI+ cells derived from B6 mice, using RNeasy mini kits (Qiagen, Valencia, CA, USA). We combined cells from three to six female Pig-a-/- and male Pig-a-/0 animals to make GPI+ and GPI- cell pools and, in total, we had three GPI+ and three GPI- cell pools for RNA extraction. RNA was extracted from three TR-GPI+, four TR-GPI- and four WT-GPI+ animals. The quantity of RNA was measured using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) while the quality and integrity of the RNA was assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The complementary RNA (cRNA) labeling and hybridizations were performed according to protocols from Affymetrix Inc. (Santa Clara, CA, USA). Briefly, 500 ng of total RNA derived from GPI- and GPI+ cell pools and 1 μg derived from TR-GPI-, TR-GPI+ and WT-GPI+ cells were converted to double-stranded cDNA with a T7-(dT)24 primer. The cDNA was transcribed to biotinylated cRNA in vitro by incorporating biotin-CTP and biotin-UTP using an Affymetrix IVT labeling kit. Twenty micrograms of biotin-labeled RNA were broken down into approximately 200 bp fragments by incubation in fragmentation buffer containing 200 mM Tris-acetate pH 8.2, 500 mM potassium acetate and 500 mM magnesium acetate for 35 min at 94°C prior to hybridization. Fragmented RNA was assessed for relative length on an Agilent 2100 bioanalyzer and hybridized to Affymetrix mouse genome 430 2.0 arrays for 16 h, washed, stained on an Affymetrix fluidics station and scanned using an Affymetrix genechip scanner. The primary CEL files were deposited in the public database Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) with accession number GSE14561.

### CDR3 size distribution analysis (CDR3 skewing)

Sorted GPI-CD8+ and GPI+CD8+ cells from splenocytes of male Pig-a-/0 and female Pig-a+/- mice were collected in a lysis buffer containing guanidinium thiocyanate. Total RNA was extracted using an RNAqueous-Micro kit (Ambion, Austin, TX, USA) following the manufacturer's directions. Genomic DNA was removed by DNAse treatment and 100 ng of eluted RNA were reverse transcribed into complementary RNA (cDNA) by a SuperScript II RT kit (Invitrogen). cDNA was amplified by PCR with Vβ 5.1 and Vβ 5.2 specific primers together with a hex-labeled constant region primer. PCR products were separated on 1.5% agarose gels, excised from the gels, purified using a

QIAquick Gel Extraction Kit (Qiagen) and subjected to CDR3 size distribution analysis. Spectratyping was performed as described previously using a 3100 DNA sequencer (Applied Biosystem, Foster City, CA, USA) and the analysis was carried out using Gene Mapper Software version 4.0 (Applied Biosystem). Samples were judged to be oligoclonally skewed when the CDR3 patterns differed from the usual Gaussian distribution and the region was dominated by one prominent peak.

### Immunoblotting

GPI+ T cells and GPI- T cells were separated from the splenocytes of female Pig-a-/- mice while normal CD4 and CD8 T cells were obtained from B6 mice. Sorted cells were lysed in 1% NP-40 buffer (Pierce, Rockford, IL, USA) containing 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA, USA) and complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA) for 25 min on ice. After removal of nuclear debris by centrifugation, the supernatants were analyzed by immunoblotting with the following antibodies: rabbit antiserum to TCR-associated ζ, rabbit antiserum to TCR-ZAP-70, rabbit antibody to extracellular

**Online Supplementary Table S1.** Shared gene expression change between GPI- and TR-GPI- bone marrow cells.

| Gene names | Fold change GPI- vs. GPI+ | Fold change TR-GPI- vs. TR-GPI+ |
|---|---|---|
| CD8 antigen, β chain | 23.6 | 4.8 |
| CD8 antigen, α chain | 12.1 | 3.7 |
| CD3 antigen, γ polypeptide | 15.4 | 4.7 |
| CD3 antigen, δ polypeptide | 12.2 | 4.9 |
| CD7 antigen | 10.3 | 3.7 |
| CD28 antigen | 7.9 | 2.3 |
| CD247 antigen | 7.4 | 2.2 |
| Chemokines (C-X-C motif) receptor 3 | 8.2 | 3.4 |
| Chemokines (C-C motif) receptor 9 | 7.4 | 3.9 |
| Chemokines (C-C motif) receptor 5 | 3.5 | 2.2 |
| Chemokines (C-X-C motif) receptor 6 | 6.2 | 2.1 |
| Chemokines (C-C motif) ligand 5 | 22.9 | 7.5 |
| T-cell receptor β variable 13 | 40.1 | 8.3 |
| T-cell receptor α chain 19 | 6 | 4.9 |
| T-cell receptor γ chain | 11.4 | 2.8 |
| Transcription factor 7, T-cell specific | 15.7 | 3.1 |
| Lymphocyte protein tyrosine kinase | 16.6 | 2.7 |
| Linker for activation of T cells | 8.0 | 2.4 |
| Fyn proto-oncogene | 6.1 | 2.3 |
| Complement component 1β subcomponent | 0.5 | 0.5 |
| Vascular cell adhesion molecule 1 | 0.4 | 0.4 |
| Complement component 1α subcomponent | 0.3 | 0.4 |
| Elastase 2, neutrophil | 0.3 | 0.5 |
| Phosphatidylinsositol glycan anchor | 0.2 | 0.1 |
| biosynthesis, class A Ficolin B | 0.2 | 0.3 |

*Shared genes with drastically different expression between GPI- and GPI+ cells and between TR-GPI- and TR-GPI- bone marrow cells.*

signal-regulated kinase (ERK)-2 (C-14) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody to Lck (3A5) (Santa Cruz Biotechnology), and peroxidase-linked goat antibodies to mouse and rabbit Ig (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were visualized after staining with the enhanced chemiluminescence staining kit (Pierce).

### In vitro *lymphocyte cytotoxicity assay*

Sorted GPI+ T cells and GPI- T cells from bone marrow and splenocytes of female *Pig-a*−/− mice were used as effectors, and fresh bone marrow cells from a female C.B10 mouse were used as targets in an *in vitro* cytotoxicity assay using the CyToxiLux PLUS kit (OncoImmunin, Inc., Gaithersburg, MD, USA). We first labeled target cells with an APC-conjugated fluorescent dye (TLF4) at 37°C for 90 min, dispensed cells into 96-well culture plates at 750 target cells per well, and then added to each well $1.5 \times 10^4$ sorted GPI+ T cells or GPI- T cells as effectors (E:T ratio = 20:1). Effector and target cells were first co-incubated at 37°C 5% $CO_2$ for 60 min and were then incubated with the FITC-conjugated caspase substrate at 37°C 5% $CO_2$ for an additional 30 min. Target cell apoptosis was measured by caspase activity through fluorescent emission detected by the LRS-II flow cytometer. Wells with targets or effectors only or with targets plus caspase substrate only were used as controls.

### Data analysis

Microarray analysis was performed using Affymetrix mouse 430 2.0 chips. Image processing and expression analysis were achieved using Affymetrix GCOS version 1.2 to calculate the signal intensity and the percent present calls on the hybridized Affymetrix chip. The signal intensity values obtained for probe sets in the microarrays were transformed using an adaptive variance-stabilizing, quantile-normalizing transformation (Munson, P.J., GeneLogic Workshop of Low Level Analysis of Affymetrix GeneChip Data, 2001, software available at *http://abs.cit.nih.gov/gene-expression.html*). Transformed data from all the chips were subjected to principal component analysis to detect outliers. One-way analysis of variance (ANOVA) and post-hoc t-tests were performed to evaluate each probe set. Probesets/genes were selected with thresholds of 2.0 fold change, false discovery rate less than 10% and percentage of present calls using the MAS5 algorithm. Two-way hierarchical clustering was used to bring together sets of samples and genes with similar expression pattern. The hierarchical cluster was run from the JMP6 software (SAS Institute Inc., Cary, NC, USA) using the ward method. JMP6 software was also used to analyze all the flow cytometry data.