

## Glycosylphosphatidylinositol-anchored protein deficiency confers resistance to apoptosis in PNH

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**Objective.** Investigate the contribution of *PIG-A* mutations to clonal expansion in paroxysmal nocturnal hemoglobinuria (PNH).

**Materials and Methods.** Primary CD34<sup>+</sup> hematopoietic progenitors from PNH patients were assayed for annexin-V positivity by flow cytometry in a cell-mediated killing assay using autologous effectors from PNH patients or allogeneic effectors from healthy controls. To specifically assess the role of the *PIG-A* mutation in the development of clonal dominance and address confounders of secondary mutation and differential immune attack that can confound experiments using primary cells, we established an inducible *PIG-A* CD34<sup>+</sup> myeloid cell line, TF-1. Apoptosis resistance was assessed after exposure to allogeneic effectors, NK92 cells (an interleukin-2 – dependent cell line with the phenotype and function of activated natural killer [NK] cells), tumor necrosis factor (TNF) –  $\alpha$ , and  $\gamma$ -irradiation. Apoptosis was measured by annexin-V staining and caspase 3/7 activity.

**Results.** In PNH patients, CD34<sup>+</sup> hematopoietic progenitors lacking glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-AP<sup>–</sup>) were less susceptible than GPI-AP<sup>+</sup> CD34<sup>+</sup> precursors to autologous (8% vs 49%;  $p < 0.05$ ) and allogeneic (28% vs 58%;  $p < 0.05$ ) cell-mediated killing from the same patients. In the inducible *PIG-A* model, GPI-AP<sup>–</sup> TF-1 cells exhibited less apoptosis than induced, GPI-AP<sup>+</sup> TF-1 cells in response to allogeneic cell-mediated killing, NK92-mediated killing, TNF- $\alpha$ , and  $\gamma$ -irradiation. GPI-AP<sup>–</sup> TF-1 cells maintained resistance to apoptosis when effectors were raised against GPI-AP<sup>–</sup> cells, arguing against a GPI-AP being the target of immune attack in PNH. NK92-mediated killing was partially inhibited with blockade by specific antibodies to the stress-inducible GPI-AP ULBP1 and ULBP2 that activate immune effectors. Clonal competition experiments demonstrate that the mutant clone expands over time under proapoptotic conditions with TNF- $\alpha$ .

**Conclusion.** *PIG-A* mutations contribute to clonal expansion in PNH by conferring a survival advantage to hematopoietic progenitors under proapoptotic stresses. © 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem cell disorder manifested by complement-mediated intravascular hemolysis, thrombosis,

smooth muscle dystonias, and bone marrow failure [1]. Disease results from the expansion of an abnormal clone that harbors a somatic mutation in the X-linked gene, *PIG-A* [2–4]. The *PIG-A* gene product is required for the initial step in the biosynthesis of glycosylphosphatidylinositol (GPI), a lipid moiety that attaches various proteins to the cell surface. The hallmark of PNH blood cells is a clonal population that is deficient in cell-surface GPI-anchored

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proteins (GPI-AP<sup>−</sup>) [5]. The mechanisms of clonal dominance in PNH are not entirely understood. One hypothesis to explain the close relationship between PNH and aplastic anemia, as well as the mechanisms whereby the PNH clone achieves dominance, involves a two-step model. This model first proposes that early hematopoietic stem cells randomly and spontaneously acquire *PIG-A* mutations. Indeed, *PIG-A* mutations can be found at low frequency in mature blood cells from most healthy controls; however, in contrast to *PIG-A* mutations in PNH, most of these mutations do not occur in hematopoietic stem cells [6–8]. Step two in this model proposes that the immunologic attack that targets hematopoietic stem cells, as in acquired aplastic anemia, spares PNH cells (i.e., immune escape), possibly because of the absence of one or more GPI-AP [9,10]. There is evidence for such an autoimmune attack in the classical form of PNH that does not arise from aplastic anemia [11,12]. Although the one feature common to all cases of PNH is a mutant *PIG-A* gene, it remains unclear to what degree the mutation contributes to the clonal expansion of GPI-AP<sup>−</sup> cells. It has been proposed that expansion of PNH cells may involve a second somatic mutation in the *PIG-A* mutant stem cell [13]. Inoue et al. [14] recently reported two PNH patients who possessed a chromosome 12 rearrangement that resulted in ectopic expression of the transcription factor *HMGA2*. While the chromosome 12 rearrangement may have contributed to the clonal expansion in these two patients, most PNH patients have normal karyotypes.

Using hematopoietic progenitors from PNH patients and an inducible *PIG-A* myeloid cell line, we studied the mechanism of clonal dominance in PNH. We find that there is an ongoing immune attack against hematopoietic progenitors in both de novo PNH and that arising from aplastic anemia, and this attack spares GPI-AP<sup>−</sup> cells. Moreover, the *PIG-A* mutations appear to contribute to the resistance of GPI-AP<sup>−</sup> cells to the immune attack by conferring a global cellular resistance to proapoptotic stresses.

## Materials and methods

### Patients

Peripheral blood and/or bone marrow samples from three normal controls and six patients with clinically evident PNH were collected after obtaining informed consent according to requirements of the Institutional Review Board of the Johns Hopkins School of Medicine. The diagnosis of severe aplastic anemia was based on the criteria of Camitta et al. [15]. Diagnosis of PNH was confirmed by flow cytometry as described previously [16,17].

### TF-1-inducible cell line

TF-1 is a granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent, human CD34<sup>+</sup> hematopoietic progenitor cell line (CRL-2003; ATCC, Manassas, VA, USA) that expresses GPI-AP and possesses a wild-type *PIG-A* gene [6]. TF-1 cells

were selected in growth medium containing 1 nM proaerolysin [18] (Protox Biotech, Victoria, Canada). Proaerolysin binds the glycan core of the GPI anchor and lyses cells expressing GPI-AP. Thus, proaerolysin does not directly induce DNA damage; rather, it selects for spontaneous *PIG* gene family mutants that lead to failed expression of GPI anchored proteins. A proaerolysin-resistant clone was expanded and shown to be GPI-AP-deficient. TF-1 proaerolysin-resistant cells had a seven-nucleotide deletion at position 291–297 (TTGTCAC) in exon 2 of *PIG-A*, which results in a frameshift mutation (data not shown). Inducible TF-1 cells were prepared as described previously [19]. Briefly, the *PIG-A*–inducible TF-1 cells were transduced by the two lentiviral vectors comprising the tetracycline (tet)-SUPER inducible transgene expression system. In this system, the first vector expresses a transcriptional suppressor domain fused to the tet repressor DNA-binding domain. The chimeric transcription suppressor (tTS) binds to its cognate DNA site, tetO, and inhibits transcription from nearby promoters. The second lentiviral vector expresses the human *PIG-A* cDNA controlled by the human EF1 $\alpha$  promoter adjacent to the tetO DNA site. The binding of tTS to the tetO site suppresses the EF1 $\alpha$  promoter-mediated transcription of the *PIG-A* transgene. Addition of tetracycline or its synthetic analogue doxycycline (Dox) will abolish the binding of the tTS, and thereafter releases its suppression of *PIG-A* transgene expression. Thus, we established an isogenic system for induced *PIG-A* expression: *PIG-A* expression is absent when Dox is absent; conversely, once Dox is added, *PIG-A* expression is turned on, and maximal GPI-AP expression is reached after 2 days. Cells were maintained in RPMI-1640 medium supplemented with 2 ng/mL GM-CSF (Biosource, Camarillo, CA, USA), 2 mM L-glutamine, penicillin/streptomycin, and 10% fetal calf serum under BL2 lab containment. Phenotyping of TF-1 cells was performed with the following reagents: fluorescent aerolysin (FLAER; Protox Biotech, Victoria, Canada; mouse monoclonal antibodies to human ULBP1 and ULBP2 (R&D Systems, Minneapolis, MN, USA) with secondary goat anti-mouse IgG2a fluorescein-isothiocyanate-conjugated (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations.

### Induction of apoptosis in inducible TF-1 cells

Inducible TF-1 cells were split into two equal volumes during log phase growth and 10 ng/mL Dox was added to one flask for at least 2 days to induce GPI anchor expression. For tumor necrosis factor (TNF)- $\alpha$ –induced apoptosis, at least 10<sup>6</sup> cells in log phase growth were subjected to TNF- $\alpha$  (Promega, Madison, WI, USA) for 2 hours at 37°C and then washed in cold phosphate-buffered saline. For irradiation experiments, at least 10<sup>6</sup> cells from each induced and uninduced sample were washed 1 $\times$  in RPMI-1640 to remove serum prior to radiation and assayed 6 hours after irradiation exposure at 2 or 4.5 Gy using a cesium GammaCell irradiator (GammaCell, Ontario, Canada).

Caspase 3/7 activity was measured in cell lysates using a Caspase colorimetric assay (Promega). All steps after incubation were performed at 4°C. Caspase activity was normalized to the total protein content of each lysate, as measured by the Bradford method. Time-course experiments were done to determine the optimal sampling time for caspase activity (2 hours for TNF- $\alpha$  and 6 hours for irradiation).

### Flow cytometric cell-mediated lysis assay

Effector peripheral blood mononuclear cells (PBMC) isolated by Ficoll/Hypaque density gradient ( $\text{sg} = 1.077$ ) were cocultured at 37°C, 5%CO<sub>2</sub>, and 100% humidity for 5 to 7 days with normal, irradiated allogeneic PBMC (12 Gy), or with original or *PIG-A* mutant TF-1 cells (40 Gy) as stimulators at a concentration of  $1 \times 10^6/\text{mL}$  (each cell type) in RPMI-1640 medium supplemented with 20% fetal bovine serum, 50  $\mu\text{M}$  2-mercaptoethanol, and 2 mM L-glutamine. CD34<sup>+</sup> bone marrow mononuclear cells were isolated by iron-colloid conjugated anti-CD34 antibody and double passage through a magnetically activated column (Miltenyi Biotech, Auburn, CA, USA). CD34<sup>+</sup> targets were then labeled with 4  $\mu\text{M}$  PKH-26 (Sigma, St. Louis, MO, USA); induced or uninduced TF-1 cells were labeled with 10  $\mu\text{M}$  carboxyfluorescein diacetate succinimidyl ester (CFSE). Stimulated effectors were mixed with the PKH-26<sup>+</sup> or CFSE<sup>+</sup> targets at a 20:1 effector-to-target ratio, lightly pelleted (100g for 3 minutes) in round-bottom 12  $\times$  75-mm tubes, and cocultured for 4 to 7 hours at 37°C. Primary CD34<sup>+</sup> cells were then stained with FLAER (Protox Biotech), propidium iodide (PI), and annexin-V–biotin/streptavidin–peridinin-chlorophyll-protein complex (Pharmingen, San Diego, CA, USA). TF-1 targets were stained with PI and annexin-V-APC. Data was acquired on a FACScan or FACSCalibur instrument (Becton Dickinson, Mansfield, MA, USA). Apoptosis was measured as the sum of all PI-positive and annexin-only positive PKH-26<sup>+</sup> or CFSE<sup>+</sup> target cells. Results for TF-1 experiments represent apoptosis of the treated cells minus the background apoptosis untreated induced and uninduced controls.

### NK92 cell-mediated lysis assay with the inducible TF-1 cell line

The natural killer (NK) cell line NK92 (ATCC) was incubated in  $\alpha$ -minimum essential medium without ribonucleosides and deoxyribonucleosides, supplemented with 12.5% horse serum, 12.5% fetal bovine serum, 2 mM L-glutamine, 0.2 mM inositol, 0.02 mM folic acid, and 0.1 mM 2-mercaptoethanol. NK92 cells were incubated with CFSE-labeled induced or uninduced TF-1 cells at 37°C and 5% CO<sub>2</sub> after 3-minute centrifugation at 100g in 12  $\times$  75-mm round bottom tubes. Cells were stained after incubation with annexin-V-APC and PI. CFSE<sup>+</sup>-gated events were analyzed for apoptosis (annexin and/or PI-positive events) and reported as the percent apoptosis minus the background apoptosis in untreated, CFSE-labeled controls. Time-course and dosing experiments showed that a 2-hour incubation with an effector:target ratio of 1:1 yielded the most consistent apoptosis in the range of

30% to 70% by annexin-V and PI staining. For NK92 inhibition experiments, target cells were incubated with mouse monoclonal antibodies to ULBP1 and ULBP2 (25  $\mu\text{g}/\text{mL}$  each) for 15 minutes at room temperature prior to incubation with NK92 cells.

### Clonal competition assay

Original and *PIG-A* mutant cells were maintained at similar cell concentrations, counted, and resuspended in RPMI-1640 media (serum-free) at 1:1 or 19:1 ratios of original to mutant cells. Cell mixtures were treated with nothing or TNF- $\alpha$  (1 or 10 ng/mL). Cell clone sizes were measured as above by flow cytometry with FLAER and PI exclusion at 1 and 2 days after exposure. Percent GPI-negative populations were compared to the untreated control mixtures sampled at the same time points.

### Statistics

Groups were compared using a two-tailed paired *t*-test using Microsoft Excel software.

## Results

### Sensitivity of CD34<sup>+</sup> cells from PNH patients to immune cell-mediated killing

In order to assess differences in apoptosis resistance in primary PNH cells, the sensitivity of GPI-AP<sup>−</sup> and GPI-AP<sup>+</sup> CD34<sup>+</sup> targets from PNH patients to both autologous effectors from PNH patients and allogeneic effectors from healthy controls was analyzed using a flow cytometry–based cell-mediated killing assay. Patient characteristics and the results of cell-mediated killing from the six PNH patients are listed in Table 1. The diagnosis of PNH was preceded by aplastic anemia in patients 1 to 3; all of these patients received immunosuppressive therapy (anti-thymocyte globulin/ cyclosporin) for their aplastic anemia before being diagnosed with PNH. In these patients, autologous effectors killed a mean of 55% CD34<sup>+</sup>GPI<sup>+</sup> cells compared to only 5% of the CD34<sup>+</sup>GPI<sup>−</sup> cells. CD34<sup>+</sup> cells from patients with classical PNH (patients 4–6) were also studied. Similar to the patients whose PNH evolved from aplastic anemia, autologous effectors killed 42% CD34<sup>+</sup>GPI<sup>+</sup> cells compared to only 11% of the CD34<sup>+</sup>GPI<sup>−</sup> cells. Overall (patients 1–6), the CD34<sup>+</sup>GPI<sup>−</sup> cells were more resistant

**Table 1.** Autologous and allogeneic cell-mediated killing of wild-type and paroxysmal nocturnal hemoglobinuria (PNH) CD34<sup>+</sup> hematopoietic progenitors

Patient	Age (y)/gender	Disease	Diagnosis	duration (y)	% GPI <sup>−</sup> CD34 <sup>+</sup> target cells	% Apoptosis			
						Autologous effectors vs CD34 <sup>+</sup> GPI <sup>−</sup>	Autologous effectors vs CD34 <sup>+</sup> GPI <sup>+</sup>	Allogeneic effectors vs CD34 <sup>+</sup> GPI <sup>−</sup>	Allogeneic effectors vs CD34 <sup>+</sup> GPI <sup>+</sup>
1	31/F	AA/PNH		1	81	3	34	ND	ND
2	13/F	AA/PNH		1	65	8	50	18	52
3	52/M	AA/PNH		20	65	3	83	53	93
4	19/F	PNH		1	75	5	21	6	42
5	34/M	PNH		5	70	18	52	24	67
6	61/F	PNH		0.2	65	10	54	27	53
Mean	35			4.8	70	8 $\pm$ 2	49 $\pm$ 8	28 $\pm$ 8	58 $\pm$ 10

AA = aplastic anemia; F = female; M = male.

than the CD34<sup>+</sup>GPI<sup>+</sup> cells to autologous cell-mediated killing (8% vs 49%;  $p < 0.05$ ). Allogeneic effectors from normal controls kill based on MHC disparities rather than specific GPI-AP expression [20–23]. When allogeneic effectors were incubated with CD34<sup>+</sup> cells from PNH patients, the CD34<sup>+</sup>GPI<sup>−</sup> cells again were more resistant than the CD34<sup>+</sup>GPI<sup>+</sup> cells (28% vs 58%;  $p < 0.05$ ). Differences in apoptosis are unlikely due to FLAER staining because the channel forming portion of proaerolysin has been modified and previously shown not to induce apoptosis at this concentration [24], and control experiments show no differences in apoptosis between FLAER stained and unstained cells (Suppl. Fig. 1).

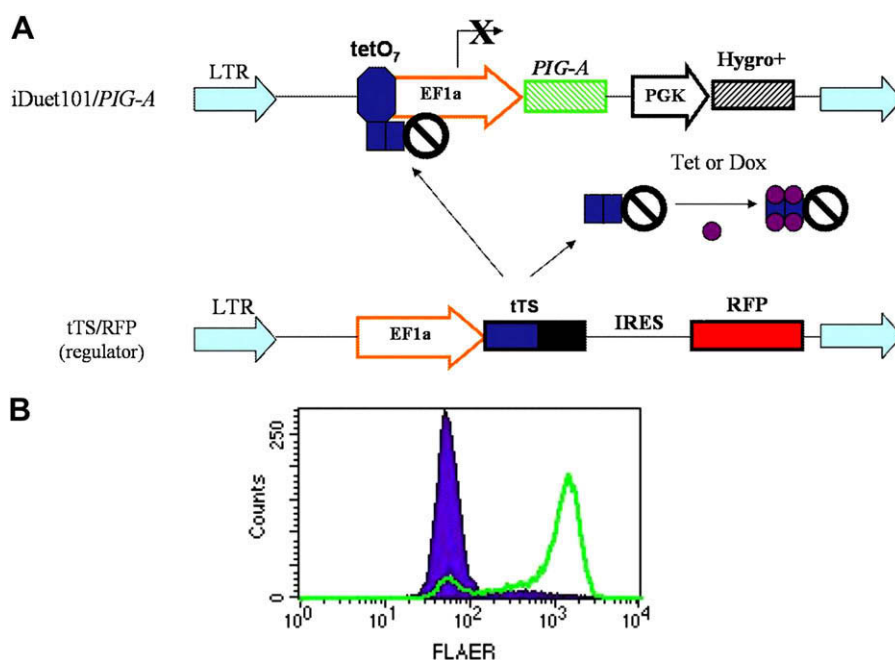
#### Effects of *PIG-A* mutations on immune cell-mediated killing of TF-1 cells

The relative resistance of PNH progenitors to allogeneic killing suggests that a GPI-anchored epitope is not the target of immune attack, because such killing is mediated by major histocompatibility differences. However, it is possible that the increased in vitro T-cell killing [22] of normal progenitors from PNH patients is the result of injury the cells had suffered during the in vivo autoimmune attack [25]. To control for genetic heterogeneity and mitigate differences in background apoptosis, a *PIG-A* mutant myeloid cell line was established by proaerolysin selection of the CD34<sup>+</sup>, GM-CSF-dependent TF-1 cell line. The *PIG-A* mutant TF-1 cells were then transduced with *PIG-A* cDNA using a lentiviral vector under the control of

a Dox-inducible promoter to generate inducible *PIG-A* TF-1 cells (Fig. 1A). The inducible TF-1 cell line becomes >90% GPI-AP-positive after *PIG-A* induction with Dox (Fig. 1B). PNH and wild-type cells in this system are isogenic and are not subject to ongoing immune selection.

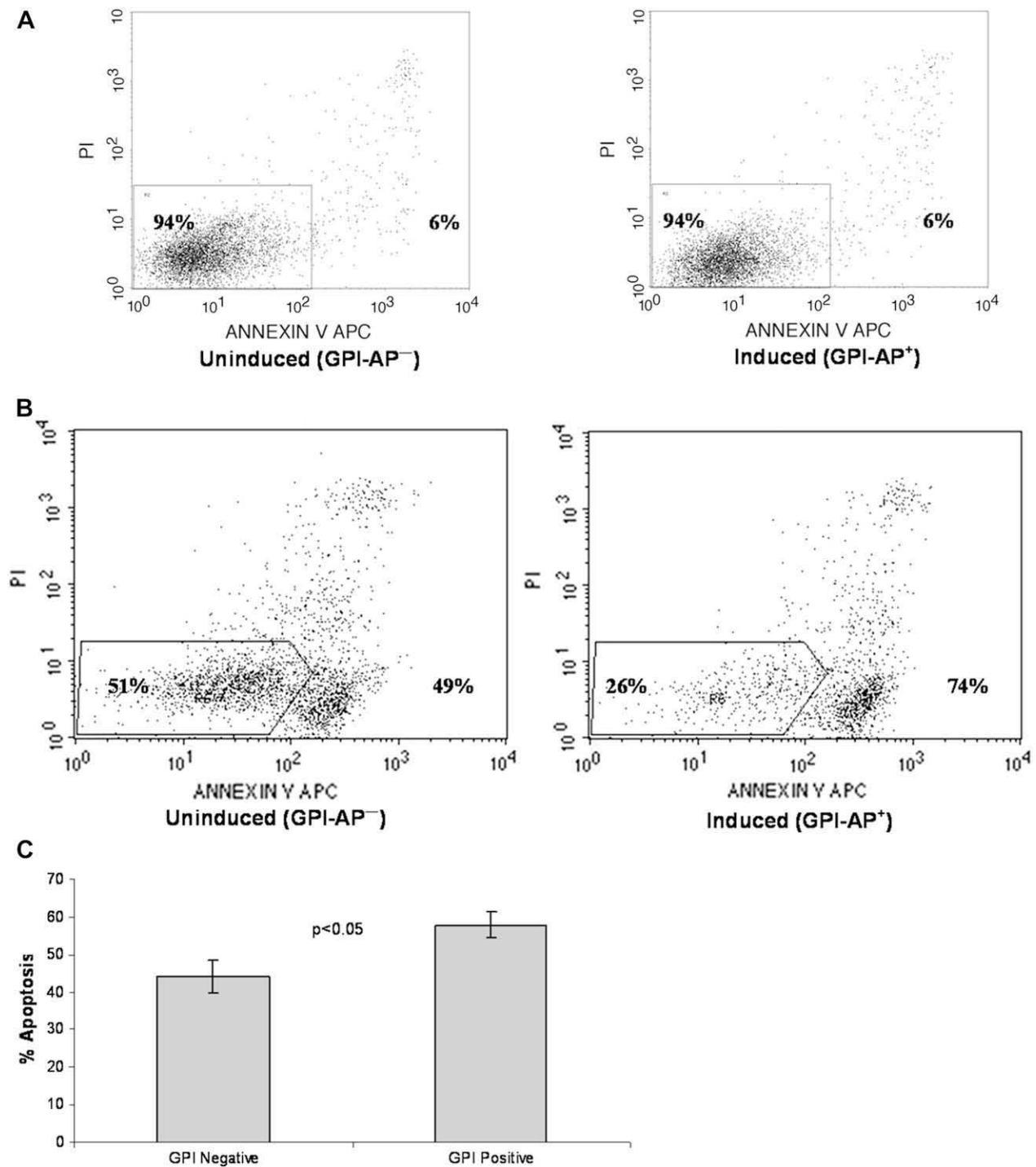
The effects of both NK92 cells and primed effector PBMCs on inducible *PIG-A* TF-1 cells were studied. NK92 is an IL-2-dependent NK cell line with the phenotype and function of activated NK cells; like NK cells, NK92 cells kill targets in an MHC-independent manner by either granule exocytosis or binding TNF family receptors [26]. Compared to the induced, GPI-AP<sup>+</sup> TF-1 cells, the uninduced, GPI-AP<sup>−</sup> cells were relatively resistant to NK92-mediated killing (44% vs 58%;  $p < 0.05$ , Fig. 2), in agreement with prior results [27]. The induced and uninduced cells exhibited similar amounts of background apoptosis, demonstrating that Dox was not responsible for the differences in apoptosis (Fig. 2A).

Allogeneic PBMCs from normal controls were primed against either GPI-AP-replete or -deficient TF-1 cells and used to test resistance to allogeneic cell-mediated killing. Using GPI-AP<sup>−</sup> TF-1 cells to stimulate effectors yielded a mean 18% apoptosis in uninduced TF-1 targets and 28% apoptosis in induced TF-1 targets. Using GPI-AP-replete TF-1 cells to stimulate effectors yielded a mean 16% apoptosis in uninduced TF-1 targets and 28% apoptosis in induced TF-1 targets. Induced (GPI-AP<sup>+</sup>) TF-1 targets consistently underwent more apoptosis than uninduced (GPI-AP<sup>−</sup>) targets, regardless of whether



**Figure 1.** *PIG-A* inducible TF-1 cell line. (A) The tetracycline (tet)-SUPER *PIG-A* inducible lentiviral system. A tet transcriptional suppressor (tTS), located on a regulator vector, blocks EF1a-driven expression of *PIG-A*, until tet or its analogue, doxycycline, is added. (B) Inducible TF-1 cells are <5% glycosylphosphatidylinositol (GPI)-anchor-positive, as identified by fluorescent aerolysin (FLAER, solid). After 2 days induction with 10 ng/mL doxycycline, cells become >90% GPI-anchor-positive (outline).

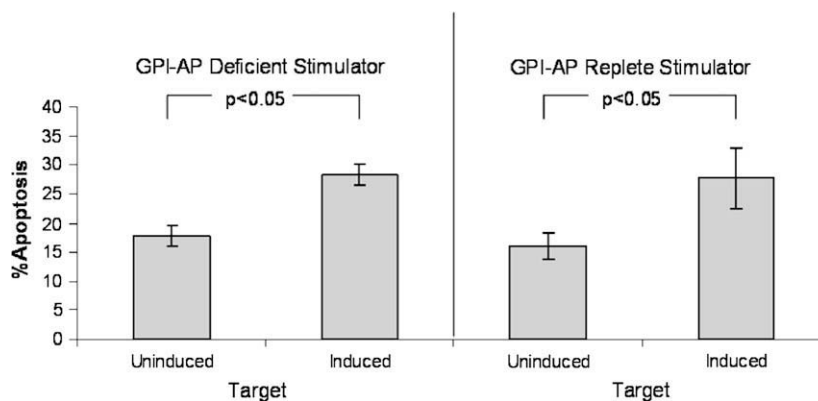




**Figure 2.** Glycosylphosphatidylinositol-negative (GPI<sup>-</sup>) TF-1 cells are more resistant to NK92-mediated killing. (A) An example of Annexin-V-APC and propidium iodide (PI) staining of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled, control uninduced (GPI<sup>-</sup>, left) and induced (GPI<sup>+</sup>, right) TF1 cells, showing similar background apoptosis. Numbers on the left inside the gate represent percent viable cells, and numbers on the right represent percent apoptotic cells. (B) An example of NK92-mediated cell lysis. NK92 cells were mixed 1:1 with CFSE-labeled uninduced (GPI<sup>-</sup>) or induced (GPI<sup>+</sup>) TF-1 cells for 2 hours. Apoptosis is measured as percent annexin<sup>+</sup> and/or PI<sup>+</sup> of CFSE<sup>+</sup> events. (C) Results of five experiments  $\pm$  standard error of mean. Results represent percent apoptosis minus background apoptosis of untreated, CFSE-labeled controls.

wild-type or PNH TF-1 cells were used to stimulate effectors (Fig. 3). Because the allogeneic cell-mediated lysis results were independent of whether GPI-AP<sup>-</sup> or GPI-AP<sup>+</sup>

cells were used to stimulate the effectors, the difference in apoptosis does not appear to be a function of the immunogenicity of specific GPI-AP epitopes.



**Figure 3.** Glycosylphosphatidylinositol-negative (GPI<sup>-</sup>) TF-1 cells are more resistant to allogeneic cell-mediated killing. Allogeneic effectors (peripheral blood mononuclear cells) were raised against either original (WT) or *PIG-A* mutant TF-1 cells as stimulators. Effectors were then incubated with either uninduced (GPI-deficient) or induced (GPI-replete) carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled TF-1 cells. Bars represent apoptosis (Annexin<sup>+</sup> and/or propidium iodide-positive [PI<sup>+</sup>]) caused by cell-mediated killing minus background apoptosis of CFSE-labeled, untreated controls. GPI<sup>+</sup> TF-1 cells had more apoptosis regardless of whether GPI-deficient or GPI-replete cells were used to stimulate effectors. Results represent the mean ± standard error of the mean of five experiments.

#### Effects of *PIG-A* mutations on TNF- $\alpha$ and radiation-mediated apoptosis in TF-1 cells

Resistance to cell-mediated apoptosis in PNH could result from the absence of a GPI-AP required for immune recognition or killing, or alternatively from an intrinsic resistance to apoptosis caused by *PIG-A* mutations. To investigate the effects of *PIG-A* mutations on resistance to alternate mechanisms of apoptosis induction, caspase 3/7 activity was analyzed in the inducible TF-1 cells treated with either TNF- $\alpha$  or  $\gamma$ -irradiation (Fig. 4). Induced cells (GPI-AP<sup>+</sup>) showed dose-dependent, increased caspase activity as compared to the uninduced (GPI-AP<sup>-</sup>) cells to both irradiation (Fig. 4A) and TNF- $\alpha$  (Fig. 4B).

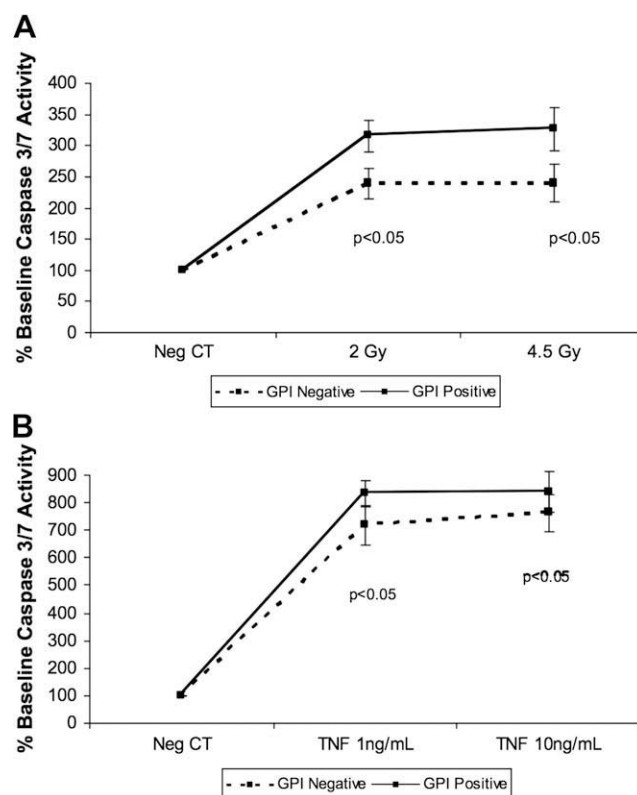
#### ULBP1 and ULBP2 in TF-1 apoptosis resistance

ULBP1 and ULBP2 are stress-inducible GPI-AP that activate immune effectors. Hanaoka et al. [28] demonstrated that *PIG-A* mutant K562 cells are resistant to NK-mediated killing because of the lack of expression of these proteins. Similar to K562 cells, *PIG-A* induced TF-1 cells express ULBP1 and ULBP2, whereas uninduced TF-1 cells do not (Fig. 5A). In response to  $\gamma$ -irradiation and TNF- $\alpha$  no increase in expression is noted (Fig. 5B, C). When induced TF-1 cells have ULBP1 and ULBP2 blocked with antibody and are incubated with NK92 cells, a 3.4% decrease in apoptosis is observed ( $p < 0.05$ ) when assayed with annexin-V and PI positivity. This decrease is less than the 14% decrease observed with total absence of GPI-AP.

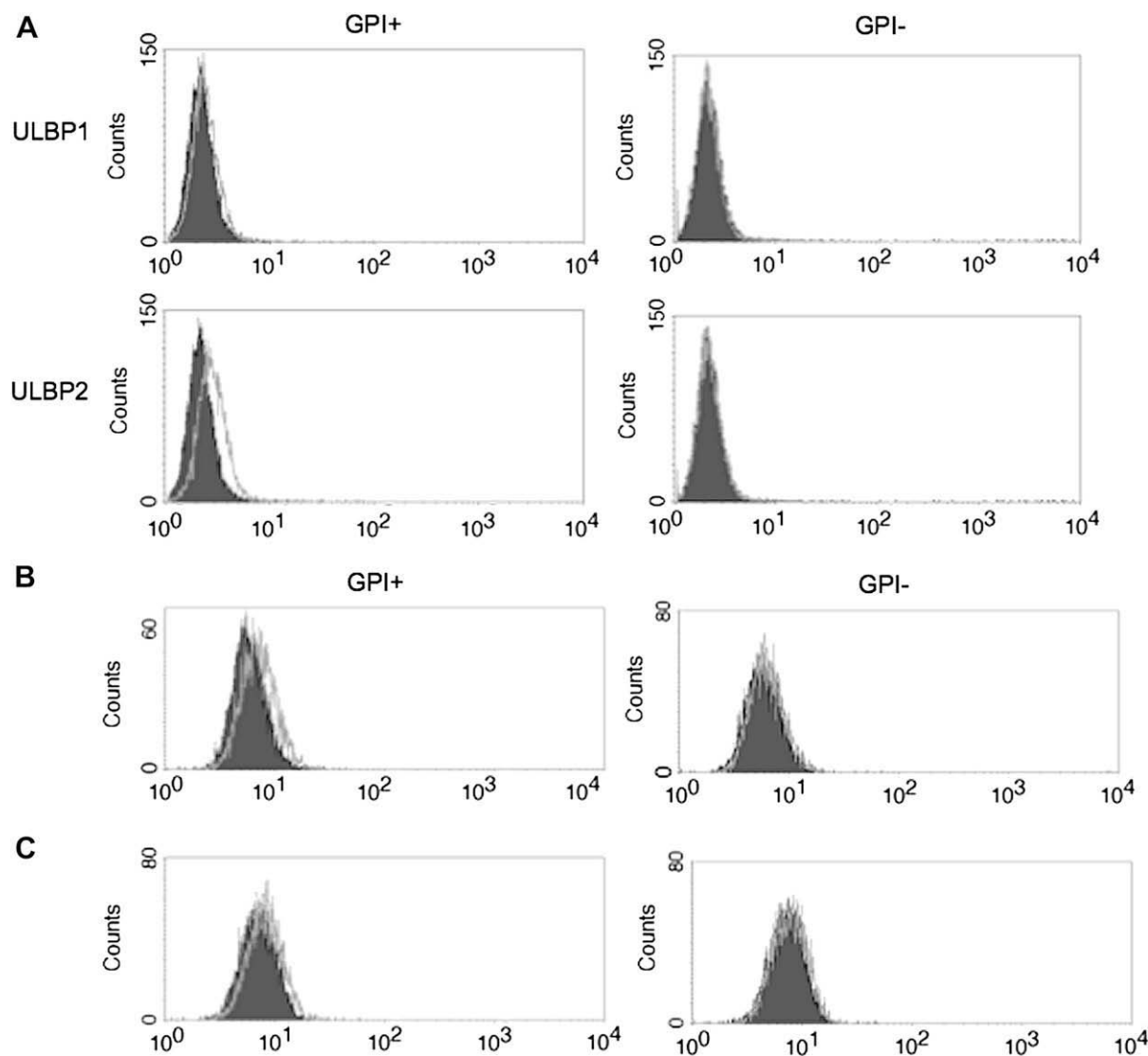
#### Apoptosis resistance and clonal selection

*PIG-A*-inducible TF-1 cells showed differences in apoptosis under a variety of stressors, but it is important to demonstrate that apoptosis resistance translates into clonal expansion, as is often the case in the natural history of PNH. Because *PIG-A*-inducible TF-1 cells need constant

induction with Dox to maintain *PIG-A* expression, it is not feasible to mix induced and uninduced TF-1 cells in clonal competition experiments. Therefore, we mixed



**Figure 4.** TF-1 cells lacking *PIG-A* expression are resistant to apoptosis. (A) Relative induction of caspase 3/7 activity 6 hours after radiation exposure. (B) Relative induction of caspase 3/7 activity 2 hours after tumor necrosis factor- $\alpha$  exposure. Time-course experiments were done to determine the optimal sampling time for caspase activity. Results represent the mean ± standard error of the mean of six experiments.



**Figure 5.** ULBP 1 and 2 expression on *PIG-A* inducible TF-1 cells. (A) *PIG-A* inducible TF-1 cells were treated with (glycosylphosphatidylinositol-positive [GPI<sup>+</sup>]) or without (GPI<sup>-</sup>) doxycycline for 2 days. Expression of ULBP1 and ULBP2 was determined by comparison to isotype control staining (solid) by flow cytometry. (B) ULBP2 expression is not increased after 16-hour exposure to tumor necrosis factor- $\alpha$  (outline) or (C) 4.5-Gy radiation (outline). Isotype controls are displayed as solid histograms.

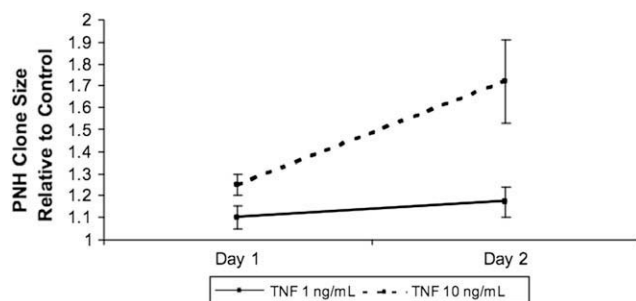
original and *PIG-A* mutant TF-1 cells and exposed them to TNF- $\alpha$  as a surrogate for autoimmune attack. After exposure to TNF- $\alpha$ , mutant TF-1 cells outgrew wild-type cells in a time and dose-dependent relationship at a 19:1 original to mutant TF-1 ratio (Fig. 6). Similar results were observed using a 1:1 original to mutant ratio (data not shown).

## Discussion

The mechanisms underlying clonal expansion of PNH *in vivo* remain unclear. There are three leading hypotheses to explain such clonal expansion: PNH cells evade immune attack possibly because a cell surface GPI-AP is the target of the immune attack [10,11], the *PIG-A* mutation itself confers an intrinsic resistance to apoptosis [29], and second

antiapoptotic mutation occurs in the PNH clone [14]. There are data that support each of these potential mechanisms for clonal expansion, as well data that conflict. For example, it would seem unlikely that a specific GPI-AP is the target of an autoimmune attack: GPI-AP are synthesized and degraded intracellularly in the setting of a *PIG-A* mutation [30]; therefore, protein epitopes should be displayed extracellularly by histocompatibility antigens and be immunogenic even if the intact protein is not on the cell surface.

Whether PNH cells are inherently less susceptible to apoptosis also remains controversial. No survival advantage has been found under a variety of conditions in the mouse model of PNH [31]. Moreover, several studies using human lymphoid cell lines found that the *PIG-A* mutant lymphocytes had equivalent apoptosis to wild-type cells [32–35].



**Figure 6.** Clonal competition of wild-type and *PIG-A* mutant TF-1 cells. Original and *PIG-A* mutant TF-1 cells were mixed 19:1, respectively, and exposed daily to nothing, 1 ng/mL or 10 ng/mL tumor necrosis factor- $\alpha$ . The percentage of glycosylphosphatidylinositol-negative (GPI<sup>-</sup>) cells in treated and untreated controls were measured on days 1 and 2. Data are reported as the ratio of GPI<sup>-</sup> cells in treated groups to percent GPI<sup>-</sup> cells in untreated control cell mixtures. Both treatment groups showed statistically significant expansion over controls ( $p < 0.05$ ).

In contrast, reports consistently have found that primary GPI-AP<sup>-</sup> myeloid cells from PNH patients were relatively resistant to apoptosis [12,29,32,36–38], even when the GPI-AP<sup>-</sup> lymphocytes were not [32]. However, two of these studies concluded that because the degree of resistance was not proportional to PNH clone size, the resistance could be independent of the *PIG-A* mutation [32,36]. Because primary GPI-AP<sup>-</sup> CD34<sup>+</sup> progenitors exhibited similar proliferative and survival rates to CD34<sup>+</sup> progenitors from healthy controls, other studies concluded that the survival advantage of the primary GPI-AP<sup>-</sup> cells resulted from diminished survival of GPI-AP<sup>+</sup> progenitors in a “hostile” PNH environment [12,37,38]. However, it is possible that primary GPI-AP<sup>-</sup> CD34<sup>+</sup> progenitors may also be damaged by the same hostile environment present in the PNH patients, and an intrinsic survival advantage in PNH cells may be obscured when PNH progenitors are compared to normal control progenitors which had not been exposed to such an environment. Thus, we designed experiments to address the potential methodological issues associated with studying lymphocytes, which are rarely affected in PNH, as well with comparing normal and PNH progenitors that had experienced different in vivo environments.

PNH CD34<sup>+</sup> progenitors displayed less apoptosis than their wild-type counterparts when incubated with both autologous and allogeneic effectors (Table 1). This finding suggested to us that the resistance to apoptosis of the PNH cells was not the result of the absence of a particular GPI-AP. Allogeneic effectors kill based on human leukocyte antigen incompatibility recognition, not GPI-AP expression. Thus, we would expect allogeneic effectors to kill GPI-AP<sup>-</sup> and GPI-AP<sup>+</sup> CD34<sup>+</sup> progenitors to the same degree. However, the GPI-AP<sup>-</sup> cells have less apoptosis, suggesting that the improved survival is not due to a specific GPI-AP.

Experiments with primary cells cannot control for additional mutations that could contribute to a survival

advantage or an in vivo immune attack that targets both the GPI-AP<sup>-</sup> as well as the GPI-AP<sup>+</sup> cells in PNH patients. To specifically test the role of mutant *PIG-A* in the apoptosis resistance of myeloid cells, we developed an inducible *PIG-A* myeloid cell line, TF-1. PNH and wild-type TF-1 cells in this system are isogenic as there are no other genetic differences between cells in which *PIG-A* is induced or suppressed. Additionally, the background levels of apoptosis are similar in both the *PIG-A* induced and uninduced cells, and the similar apoptosis controls for confounding effects of an autoimmune attack targeting the PNH cells. Thus, any differences in susceptibility to apoptosis could only be attributed to effects of the *PIG-A* gene. We found that PNH TF-1 cells consistently displayed less apoptosis than their isogenic, wild-type counterparts when subjected to radiation, TNF- $\alpha$ , NK92, and allogeneic T-cell-mediated apoptosis. This resistance to apoptosis translated into clonal expansion of *PIG-A* mutant TF-1 cells under immune selection by TNF- $\alpha$ .

Resistance to apoptosis was observed regardless of whether *PIG-A* mutant or original TF-1 cells were used to stimulate the allogeneic effectors, providing further evidence that the resistance to apoptosis of the PNH cells is not due to the absence of a particular GPI-AP. It is possible that stimulating effectors with the original TF-1 cell line could generate effector clones that target a specific GPI-AP. By using mutant TF-1 cells, which do not express GPI-AP, to stimulate effectors, we exclude the possibility that effectors are primed against a GPI-AP. The observed differences in apoptosis attributable to a *PIG-A* mutation were small but consistent across experiments and statistically significant. This is not surprising, given that clonal expansion in PNH often occurs over many years.

Collectively, these data support an immune escape model in which an autoimmune attack provides a selective pressure on all hematopoietic progenitors, and under this stress the PNH clone expands because of an intrinsic survival advantage. These results further indicate that a second hit is not required to confer apoptotic resistance to PNH cells, although a second genetic alteration could certainly further augment survival and proliferation in a subset of PNH, as has been described [14].

A potential mechanism by which *PIG-A* mutations may produce resistance to apoptosis is through the disruption of lipid rafts by GPI anchor depletion. Lipid rafts are cell surface microdomains consisting primarily of sphingolipid, cholesterol, and GPI-AP [39]. It has been observed that cell signaling for Fas and TNF receptors is localized to lipid rafts [40,41], and that disruption of lipid rafts by depletion of sphingolipid or cholesterol can lead to apoptosis resistance [42–43]. Disruption of GPI anchors may destabilize lipid rafts in the same way, as has been described with the mutation of the *PIG-A* homologue in yeast [45]. Dispersion of lipid rafts may also explain the reported decreased Fas receptor expression on PNH cells [12].



The absence of ULBP1 and ULBP2 on GPI-AP<sup>+</sup> TF-1 cells explains at least some of this resistance to apoptosis, as well. In the PIG-A inducible TF-1 model system, blocking ULBP1 and ULBP2 did not completely overcome the change in apoptosis; nor was a significant change in ULBP1 or ULBP2 expression observed in apoptosis resistance in response to radiation and TNF- $\alpha$ . Nevertheless, it is feasible that the absence of ULBPs may play a larger role in apoptosis resistance in vivo.

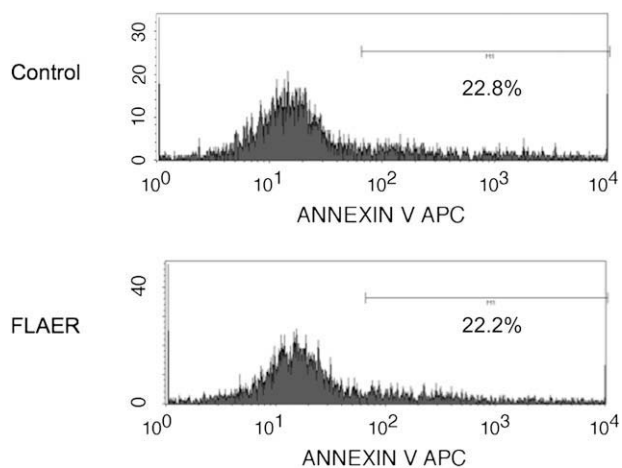
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**Supplemental Figure 1. Fluorescent aerolysin (FLAER) staining and apoptosis of leukocytes.** Peripheral blood mononuclear cells were stained with nothing or 1 nM (FLAER) and incubated at 37°C, 5% CO<sub>2</sub> in RPMI-1640 + 10% fetal calf serum. Apoptosis was measured by Annexin-V staining among CD45<sup>+</sup> cells at 24 hours. Trypan blue exclusion demonstrated 94.2% viability of control cells and 95.0% viability of FLAER stained cells at 24 hours.