No evidence of hypermutability in red cells from patients with paroxysmal nocturnal hemoglobinuria using the *XK* gene

There are two models to account for the large population of GPI-negative cells and the occasional demonstration of oligoclonality in paroxysmal nocturnal hemoglobinuria (PNH): i) immune escape;[1] and ii) hypermutability. In support of immune escape, in PNH, marrow failure is common and is associated with *HLA-DR* alleles and oligoclonal T-cell expansions. Conversely, in support of hypermutability, there is an increased relative risk of leukemia, and cytogenetic abnormalities[2] and secondary mutations[3,4] can occur. Others have reported an increased frequency of *HPRT*-mutants,[5,6] a possible consequence of increased lymphocyte turnover, rather than hypermutability.[7] We previously showed that GPI$^+$ cells from patients with PNH demonstrate no increase in the mutation rate in the *PIG-A* gene itself.[8] However, these prior studies, including our own, utilized lymphoid cells, whereas PNH is a stem cell disorder particularly affecting the myeloid/erythroid lineages.

Here we have applied our analysis[9] for spontaneously arising phenotypic variants using *XK*, the gene mutated in the McLeod syndrome,[10] to explore this question. This assay has several advantages: 1) *XK* is X-linked, as are PIG-A and *HPRT*, and thus a single mutation can produce the mutant phenotype; 2) a broad spectrum of mutations[11] can inactivate the gene; 3) the *XK* mutant (McLeod) phenotype results in a loss of Kell proteins on red blood cells (RBC), which is detected by flow cytometry. Patients and normal donors provided written informed consent. Approximately 10^7 RBC from whole blood were incubated with 50 µL of MIMA91 supernatant (generated as described by Tearina *et al.*[12]) which recognizes a non-polymorphic human Kell antigen. The RBC were washed twice with cold HANKS with 0.1% BSA and incubated with R-phycoerythrin-conjugated F(ab')$_2$ fragment rabbit anti-mouse immunoglobulin (Dako,1:5), washed twice, and incubated with anti-glycophorin-A-FITC (Dako, 1:10). We also analyzed thawed RBC from a patient with the McLeod syndrome and an obligate female carrier. Incubations were performed on ice for 30 min. Prior to each incubation, the cells were resuspended, pelleted, and resuspended. Some experiments used biotinylated anti-CD59 (Serotec,1:20) and streptavidin-PerCP-Cy5.5 (Becton-Dickinson,1:2.5). Cells were analyzed on a BD FACScan using Cellquest and Flow-Jo. Voltage was adjusted so that unstained RBC had a mean fluorescence of 2.5 on FL1 and FL2; RBC were gated by FSC/SSC (log-log scale) and glycophorin-A expression. Cells having less than 20% of the mean FL2 of the overall population were defined as McLeod-like.[9]

First, MIMA91 was validated using RBC derived from a patient with the McLeod syndrome, an obligate carrier, and a normal donor (Figure 1A-C), and the patterns were similar to our previous data using an anti-K14 antibody.[9] In the normal donor, there is a distinct population of RBC with the McLeod-like phenotype at a frequency (*f*) of 31 x 10^{-6}. Pre-treatment of RBC with dithiothreitol greatly attenuated Kell expression, as expected (*data not shown*).

Among 15 normal adult donors, in each, there was a distinct population of McLeod-like RBC; *f* ranged from 13.6 x 10^{-6} to 113 x 10^{-6}, with a median of 48.3 x 10^{-6} (Figure 1D-F). We then tested 17 patients; 13 had "classic" PNH (Table 1). The pattern of Kell and glycophorin-A expression

Table 1.

Frequency of McLeod-like cells in 17 patients with PNH

Pt	M/F	Age	ANC X 10^3/ul	HGB gm/dl	PLT X 10^3/ul	ABS Retics X 10^3/ul	% PNH RBC (type III)	% PNH RBC (type II)	% PNH PMNs	History of Aplastic Anemia	History of thrombosis	On Eculizumab	# McLeod-like Cells	Total # cells Analyzed	Frequency of McLeod-like rbcs ($x10^6$)
1	F	50	2.7	10.8	312	230	40	-	99	N	Y	Y	30	2 160 479	13.9
2	M	20	1.6	11.4	61	89	9	-	40	Y	Y	Y	94	1 903 436	49.4
3	F	32	2	11.5	139	121	60	-	96	Y	N	Y	89	2 164 218	41.1
4	M	52	2.4	8.4	158	146	32	-	75	N	N	N	89	4 421 042	20.1
5	F	24	2.1	10.6	82	37	2.5	1.1	19	Y	N	N	73	2 666 480	27.4
6	F	61	3	10.5	178	131	32	-	77	N	Y	Y	292	3 991 286	73.2
7	F	62	2.9	8.8	149	270	79	-	98	N	N	Y	121	2 602 261	46.5
8	F	46	1.8	10.9	29	56	2	-	10	Y	Y	N	87	3 043 310	28.6
9	M	45	2.7	14.3	120	243	97	-	95	N	N	Y	62	3 408 467	18.2
10	F	63	1.7	11.8	110	58	15	-	39	Y	N	N	150	4 085 902	36.7
11	M	30	3	11.7	138	125	18	10	85	N	N	N	219	4 057 837	54
12	F	46	2.2	8	157	338	75	-	98	Y	N	Y	169	2 625 227	64.4
13	F	37	1.6	9.5	147	155	19	-	77	N	Y	Y	284	4 613 833	61.6
14	F	34	1.2	7.7	117	213	47	9	92	N	N	N	64	3 483 548	18.4
15	F	47	1.9	12.2	97	33	12	-	63	N	Y	Y	171	3 529 347	48.5
16	M	75	12.8	9.2	253	124	20	-	93	N	N	Y	190	2 483 155	76.5
17	F	59	1.6	11.5	100	47	2	2	32	Y	N	N	713	3 489 504	204

Analysis of red cells from 7 additional patients, gated on CD59(-) and CD59(+) populations separately

													CD59-negatives			CD59-positives		
													# McLeod-like cells	Total# cells	Frequency of McLeod-like Cells ($x10^6$)	# McLeod-like cells	Total # cells	Frequency of McLeod-like Cells ($x10^6$)
18	M	46	1.9	9.3	53	506	60	-	88	N	N	Y	1530	4 428 023	346	138	2 951 781	46.8
19	M	51	6.9	11.7	133	112	35	5	83	N	N	N	22	1 885 692	11.7	24	3 167 973	7.6
20	F	38	3.3	7.7	323	266	94	-	99	Y	N	Y	49	5 190 211	9.4	19	431 771	44
21	F	89	1.5	11.8	127	98	37	27	99	N	N	Y	42	3 414 436	12.3	48	1 765 451	27.2
22	F	21	1.5	12.8	117	43	12	-	36	N	N	N	12	675 649	17.8	219	5 535 409	39.6
23	M	45	2.4	10.9	37	75	13	2	95	N	Y	Y	19	574 465	33.1	118	5 576 492	21.2
24	M	31	3.5	10.8	159	94	26	3.8	97	N	N	N	38	1 545 776	24.6	24	3 735 671	6.4

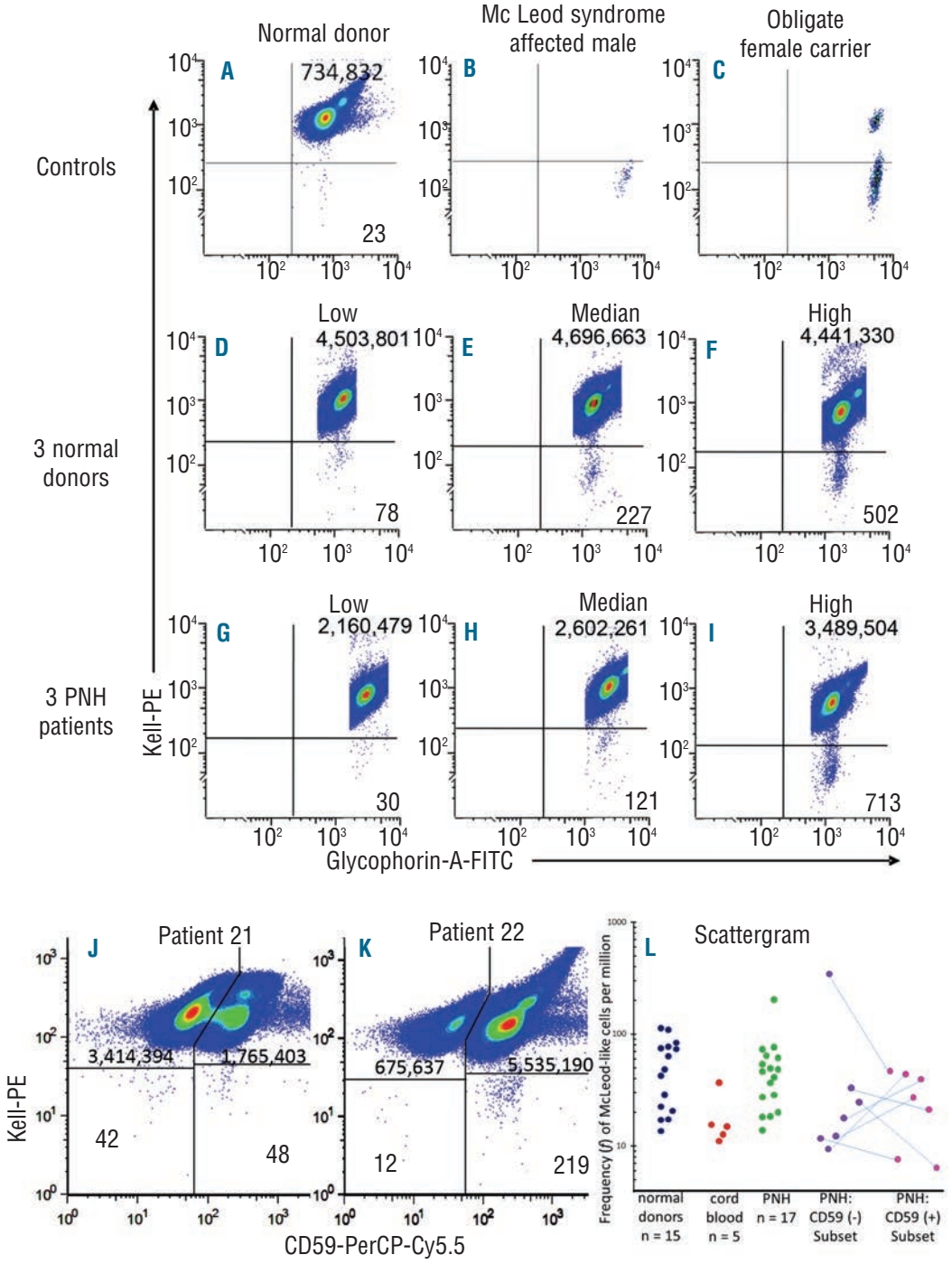

Figure 1. Flow cytometry pseudo-color density plot analyses of RBC from patients and controls are shown, depicting Kell expression *versus* glycophorin A expression. RBC from a normal donor (A) express Kell proteins (measured by FL2 fluorescence, vertical axis) and glycophorin A (FL1, horizontal axis) brightly. The cells from a patient with the McLeod syndrome (B) also express glycophorin A brightly, but express much lower levels of the Kell protein. RBC from an obligate carrier female (C) exhibit two distinct patterns due to random X-chromosome inactivation. Approximately ½ of the events appear similar to the rbcs from the normal donor, and approximately one-half of the events exhibit the McLeod phenotype. When a sufficiently large number of events are collected from the normal donor (A), it can be seen that there is a distinct population of spontaneously arising cells in the lower right quadrant that exhibit a McLeod-like phenotype. In this example, the McLeod-like cells are present at a frequency of $31x10^{-6}$. (D-F) Normal donors, one each representing the low, middle and high range with respect to the frequency of spontaneously arising McLeod-like cells amongst the normal donors. The corresponding frequencies of McLeod-like cells are calculated to be $17.3x10^{-6}$, 48.3×10^{-6}, and $113x10^{-6}$, respectively, in the examples shown. The numbers in the lower right quadrants indicate total number of gated McLeod-like cells, and the numbers at the top of the panels indicate total number of cells analyzed. (G-I) Patients with PNH, one each representing the low, middle and high range for the cohort of 17 patients, with respect to the frequency of spontaneously arising McLeod-like cells. The corresponding frequencies of McLeod-like cells are calculated to be $13.9x10^{-6}$, $46.5x10^{-6}$, and $204x10^{-6}$ respectively in the examples shown. (J-K) RBC from Patients 21 and 22, respectively, showing Kell expression as a function of CD59 expression, after gating on glycophorin-A positive events. The number of events is shown for each quadrant, using non-rectangular gates. (L) Scattergram showing the distribution of *f* values for normal adult donors, cord blood samples, and patients with PNH (on a log scale). For the 7 samples analyzed as per panel (J) and (K), lines connect the results obtained when gating separately on the $CD59^{(-)}$ and $CD59^{(+)}$ subpopulations for each patient.

among patients was the same as for normal donors (Figure 1G-I); for each patient there was a distinct population of McLeod-like RBC, with f ranging from 13.9 to 204 x 10^{-6} (median 46.5 x 10^{-6}). As previously shown,[9] *f* was lower (15 x 10^{-6}, range 11-36.9 x 10^{-6}) among 5 cord blood samples (P<0.006, two-sided non-parametric test) compared to the group of normal donors and patients. We then analyzed an additional 7 patients by gating separately on $CD59^-$ and $CD59^+$ RBC; *f* was 17.8 x 10^{-6} for the $CD59^-$ population (range 9.4-346 x 10^{-6}) and 27.2 x 10^{-6} (range 6.4-46.8 x 10^{-6}) for the $CD59^+$ population (P=NS, signed rank sum test). For Patient 18, *f* was 7.4-fold higher in the $CD59^-$ population than for the $CD59^+$ population. For Patient 20, there was a 4.6-fold difference in the opposite direction. For the others, the difference was less than 4-fold. We did not identify any clinical features that were associated with the fold difference in the *f* values among these 7 patients.

Since hypermutability would have to occur in the stem cell→erythroid differentiation pathway to account for PNH, we believe these data using RBC argue strongly against hypermutability. The frequency of McLeod-like RBC in PNH is similar to that of normal donors, and comparable to other mutation reporter genes.[13-15] Gating specifically on the $CD59^-$ RBC did not overall enrich for McLeod-like cells, arguing against mosaicism for hypermutability. Of note, for both XK and *PIG-A*, a broad spectrum of mutations, including very large deletions, can inactivate the gene, they are of similar length, and have a similar number of sites where a T→G transversion or a demethylation at CpG would produce a stop codon. Therefore, there is no reason to suspect that XK is less susceptible to spontaneous mutagenesis than *PIG-A*.

In a study involving spontaneous loss of *GPA* alleles,[16] it was demonstrated that, among normal donors, the frequency of GPA variants ranged from 0 to 20 x 10^{-6}; among 9 PNH patients, 3 were above this range, and only one was a clear outlier. In our study, Patients 17 and 18 may represent outliers. Of note, we did not specifically study subsets with *JAK2* mutations,[4] complex cytogenetic abnormalities, or multiple PNH clones. However, 8 had separate PNH II and PNH III populations, and most had large PNH populations; if hypermutability were important, we should have seen it in such patients. Another caveat is that we have not yet defined the pathological conditions for which the frequency of McLeod-like cells is definitively elevated, and since RBC are enucleated, it will not be easy to identify the corresponding *XK* mutations. In our work using dividing cells,[8] we could measure the mutation rate, whereas here, with erythrocytes, we must rely upon the mutant frequency as a surrogate. Indeed, it is possible that the kinetics of cell division in stem cells and red cell precursors will be different in patients with PNH due to marrow injury on one hand, and compensation for hemolytic anemia on the other. Despite these caveats, these data support the view that, in most (if not all) patients with PNH, there is another process rather than hypermutability that is the primary cause of expanded *PIG-A*-mutant populations.

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