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ORIGINAL ARTICLE

The Patterns of MHC Association in Aplastic and Non-aplastic Paroxysmal Nocturnal Hemoglobinuria

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Abstract The deficiency of glycosyl-phosphatidylinositol (GPI)-anchored proteins in plasma membranes of *PIG-A* gene mutated hematopoietic stem cells (HSCs) is so far insufficient to explain the domination of paroxysmal nocturnal hemoglobinuria (PNH) clone over the normal HSC. We attempted to elucidate possible link between MHC and initial severe aplastic anemia (ISAA/PNH) type and non-aplastic (n/PNH) outcome of PNH. In 50 PNH patients assigned as ISAA/PNH ($n = 13$), n/PNH ($n = 33$) or nonassigned ($n = 4$) and 200 ethnically matched controls we analyzed MHC associations. Our data confirmed strong associations of *DRB1*15:01* (RR = 3.51, $p = 0.0011$) and *DQB1*06:02* (RR = 7.09, $p = 0.000026$) alleles, especially with n/PNH subtype. *B*18:01* allele was associated with increased risk of ISAA/PNH subtype (RR = 5.25, $p = 0.0028$). We conclude that both class II and class I MHC alleles are associated with different subsets of PNH. Clonal selection of *PIG-A* mutated cells with cognate metabolic block is associated with MHC class II alleles *DRB1*15:01* and *DQB1*06:02* independent from initial severe AA clone selection. MHC class I molecule B*18:01 can additionally influence the domination of PNH clone in PNH subjects with initial severe aplastic anemia.

Keywords Paroxysmal nocturnal hemoglobinuria · Major histocompatibility complex · Association study · Bone marrow aplasia

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Abbreviations

HSCs	Hematopoietic stem cells
ISAA/PNH	Initial severe aplastic anemia domination PNH
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
FITC	Fluorescein isothiocyanate
GPI	Glycosyl-phosphatidylinositol
HLA	Human leukocyte antigens
IBGRL	International Blood Group Reference Laboratory
LDH	Lactate dehydrogenase
MHC	Major histocompatibility complex
NADH	Nicotinamide adenine dinucleotide, reduced
n/PNH	Non-aplastic anemia type PNH
p_{corr}	Corrected p
PCR	Polymerase chain reaction
PIG-A	Phosphatidylinositol glycan complementation group A gene
PLD	Phosphatidylinositol-glycan-specific phospholipase D
PNH	Paroxysmal nocturnal hemoglobinuria
RBCs	Red blood cells
SSP	Sequence specific primers

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal nonmalignant disease associated with the deficiency of proteins that are normally anchored in plasma membranes through glycosyl-phosphatidylinositol (GPI) (Crosby and Dameshek 1950). The direct cause of the GPI-deficiency in PNH is the hematopoietic progenitor cell somatic mutation of phosphatidylinositol glycan complementation group A (*PIG-A*) gene located in Xp22.1 chromosome (Takeda et al. 1993). Total or partial lack of the GPI leads to the absence or deficiency of such GPI-anchored proteins, as decay accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) at plasma membranes of all descent cells of the mutated hematopoietic progenitor cell (Bessler and Fehr 1991; Boccuni et al. 2000; Holguin et al. 1989). Complement-mediated lysis of the mature red blood cells (RBCs) is highly promoted by the lack of these proteins at the cell membrane. The pathogenesis of simultaneously observed thrombosis and the bone marrow insufficiency or myelodysplasia is less understood. Although, in the course of PNH the bone marrow aplasia, dysplasia, hemolysis or thrombosis may dominate in different phases of the disease, the domination of PNH clone over normal hematopoietic clones is universal phenomenon throughout the course of a clinically apparent disease. In fact, some healthy individuals have small *PIG-A* mutated polyclonal cell subsets that do not dominate normal hematopoiesis (Hu et al. 2005; Żupańska et al. 2005). This makes the pathogenesis even more difficult to be justified. In many patients with PNH, the *PIG-A* mutant clone size does not change or change slowly and dynamic balance between normal and PNH clones is achieved, which in clinically apparent PNH commonly riches the level of 20–95% (Hillmen et al. 1995).

The immune background of PNH is supported by the finding of MHC association. In PNH patients an increased frequency of HLA-DR2 phenotype, *DRB1*15:01* genotype and *DRB1*15:01-DQA1*01:02-DQB1*06:02* haplotype have been revealed (Lombardi et al. 2008; Maciejewski et al. 2001; Shichishima et al. 2002). In Italian and North American white PNH patients an increased frequency of the haplotype *B*14:02-Cw*08:02* has been additionally suggested (Lombardi et al. 2008; Maciejewski et al. 2001).

Various mechanisms have been proposed to be responsible for MHC associations with diseases. Antiviral and anti-pathogen repertoires of the MHC molecules have mostly been exploited in this regard (Lombardi et al. 2008; Nowak et al. 2009). In fact, the peptide presentation repertoires stand for main functional differences between MHC molecules. The association study is prerequisite for the assessment of possible mechanisms of the association.

Materials and Methods

Patients

Fifty PNH patients of European Polish ancestry were investigated in regard with MHC alleles and compared with 200 perfectly ethnically matched healthy controls (Nowak et al. 2008). Patients were not treated with eculizumab prior to and during the study. The female to male patient ratio (Table 1) was 27–23 (54–46%), with mean age of 34 (18–73) in whole group of 50 PNH patients. The research protocol was approved by the institutional ethics committee and all human participants gave written informed consent prior to enrolment in the study.

Diagnosis of the PNH

Blood samples from 50 patients were examined by flow cytometric analysis of RBCs and granulocytes separated by

Table 1 Initial characteristics of PNH patients with non-aplastic PNH or initial severe aplastic anemia PNH type

Characteristics	n/PNH N = 33		ISAA/PNH N = 13		*p*
	Value	Range	Value	Range	
Age (median, years)	37	18–73	27	18–38	0.022
Haptoglobin (g/L)	0.03	0.00–0.48	0.15	0.00–0.94	0.024
LDH (U/L)	2,109	239–5,780	1,270	450–2,480	0.16
Platelets ($\times 10^9$/L)	127	13–347	52	6–126	0.0023
Leukocytes ($\times 10^9$/L)	4.5	1.9–17.8	3.0	0.1–4.2	0.058
Reticulocytes (‰)	63	14–185	29	2–94	0.0062
Erythrocytes ($\times 10^{12}$/L)	2.72	1.77–3.51	2.37	1.50–2.94	0.027
Hemoglobin (g/dL)	8.7	4.1–12.8	7.7	5.6–10.3	0.075
E/CD59 (%)[b]	38[a]	4–89	29[a]	1–65	0.12
E/CD55 (%)[b]	34[a]	2–89	22[a]	1–52	0.067
G/CD59 (%)[b]	73[a]	8–99	49[a]	1–98	0.012
G/CD55 (%)[b]	75[a]	7–99	38[a]	1–75	0.0028
G/CD66b (%)[b]	73	8–100	53	1–96	0.035
	F (%)	M (%)	F (%)	M (%)	
Sex	21 (64)	12 (36)	4 (31)	9 (69)	0.051

n/PNH non-aplastic PNH patients, *ISAA/PNH* initial severe aplastic anemia PNH patients, *p* statistical significance of the difference, *LDH* lactate dehydrogenase, *E* erythrocyte fraction, *G* granulocyte fraction, *CD* cluster of differentiation, *F* female, *M* male patients

[a] Percent of negative cells

[b] The percentages of CD59 and CD55 deficient granulocytes were significantly higher than percentages of CD59 and CD55 deficient erythrocytes both in ISAA/PNH, $p = 0.0014$ and $p = 0.022$, respectively and n/PNH groups, $p = 7.8 \times 10^{-8}$ and $p = 1.7 \times 10^{-7}$, respectively

dextran sedimentation and subsequent hemolysis of RBCs (Bogdanik et al. 1998). Following monoclonal antibodies against GPI-anchored proteins were used: anti-CD59 FITC-conjugated (clone BRIC 229, IBGRL Research Products), anti-CD55 FITC-conjugated (clone BRIC 216, IBGRL Research Products) and anti-CD66b FITC-conjugated (clone G10F5, Becton–Dickinson). PNH was diagnosed by the evidence of GPI-deficient granulocyte and erythrocyte subsets higher than 1% (Bogdanik et al. 1998). Together with GPI-deficient clone minimal requirement for PNH diagnosis was clinical evidence of hemolysis (reticulocytosis, abnormally high level of serum lactate dehydrogenase (LDH) and abnormally low concentration of serum haptoglobin without evidence of another defined bone marrow abnormality, according to Parker et al. (2005).

Bone Marrow Biopsy

Forty-six out of 50 PNH patients were available for conclusive bone marrow biopsy. The patients were subdivided into initial severe aplastic anemia domination PNH (ISAA/PNH) and non-aplastic anemia type PNH (n/PNH) groups according to presence or absence of initial severe bone marrow aplasia confirmation in bone marrow biopsy before or within 2 months from PNH diagnosis. The subgrouping of the patients was based on a decrease in bone marrow cellularity with the absence or depletion (<25% of normal cellularity according to the age group) of all hematopoietic cells or normal cellularity due to focal erythroid hyperplasia with depletion of granulopoietic cells and megakaryocytes. These patients fulfilled severe aplastic anemia (sAA) criteria by Camitta et al. (1976). For the purpose of this study, patients with higher bone marrow cellularity, including patients with hypocellular bone marrow or moderate aplastic anemia according to Camitta et al. (1976) were included in n/PNH group. Patients with pancytopenia due to cytostatic therapy or irradiation and patients with significant fibrosis or neoplastic infiltration were not classified. For 4 out of 50 PNH patients the biopsy data were not conclusive and they were not included into ISAA/PNH or n/PNH group. Bone marrow specimens were reviewed by experienced pathologist and experienced hematologist.

Laboratory Characteristics

In PNH patients peripheral blood morphology tests were performed including RBC, leukocyte and platelet counts, total hemoglobin and reticulocytes. As markers of hemolysis, haptoglobins were evaluated using Nor-partigen radial immunodiffusion kits, according to manufacturer's protocol and LDH activities were tested with the method based on the NADH-dependent reduction of the tetrazolium salt.

HLA Typing

HLA-A, -B, -C, -DRB1 and -DQB1 alleles were typed according to manufacturers' protocols, using polymerase chain reaction sequence specific primer (PCR-SSP) amplification based commercial kits (Olerup SSP AB, Saltsjöbaden, Sweden or One Lambda, Canoga Park, CA, USA). In some cases (when quantity of DNA material was smaller) PCR-sequence specific oligonucleotide probe tests based on reverse hybridization of amplified DNA fragments were used (Innogenetics N·V., Ghent, Belgium). In total, the DNA material was sufficient to type HLA of 36 patients in locus A, 49 in B, 46 in Cw, 50 in DRB1 and 39 in DQB1.

Statistical Analysis

The associations of HLA with PNH were statistically tested using the method described by Tiwari and Terasaki (1985) including relative ratios (RR) that corresponded to odds ratio calculations and p estimated using χ^2 test and 1 degree of freedom. Briefly, $RR = ((a + 1)(d + 1))/((c + 1)(b + 1))$; where a—patients positive for the allele, b—patients negative for the allele, c—controls positive for the allele, d—controls negative for the allele and $\chi^2 = wy^2$; where $w = 1/V$, $V = 1/a + 1/b + 1/c + 1/d$ and $y = \ln RR$. Alleles with the incidence lower than 10 in the control group have been excluded from the association analysis. To define the possible influence of multiple comparisons p values were corrected using Bonferroni inequality method for number of comparisons for each HLA locus, according to the formula: $p_{corr} = 1 - ((1 - p)\text{^}X)$; where p_{corr}—corrected p, X—the number of comparisons, was the number of considered alleles (those with the incidence higher or equal to 10 in the control group) of each locus of $N = 200$ individuals, i.e. 10 alleles in A locus, 13 in B, 10 in C, 12 in DRB1 and 10 in DQB1. To keep comparable the scaling of comparisons of strength of associations the uncorrected p were compared. The allele incidence was estimated by direct counting method.

Results

Thirteen (26%) patients were classified to ISAA/PNH group according to sAA traits that dominated in bone marrow biopsy before the PNH diagnosis or at the onset of the PNH. Four (8%) patients were not eligible for conclusive bone marrow biopsy and remaining 33 (66%) patients were classified to n/PNH since absence of full

Table 2 Association analysis of HLA-A, B, C, DRB1 and DQB1 alleles with total PNH and n/PNH and ISAA/PNH subsets

Allele	Controls	PNH				n/PNH				ISAA/PNH			
	(%)	Patients (%)[a]	RR	p	p_{corr}	Patients (%)	RR	p	p_{corr}	Patients (%)	RR	p	p_{corr}
*A*24:02*	50 (25)	3 (8)	0.27	0.038	0.32	3 (15)	0.53	0.33	0.98	0	0.12	0.043	0.36
*A*25:01*	25 (13)	11 (31)	3.08	0.0074	0.072	5 (25)	2.33	0.15	0.80	5 (42)	5.00	0.010	0.10
B*18:01	28 (14)	13 (27)	2.24	0.030	0.33	6 (19)	1.42	0.48	1.00	6 (46)	**5.25**	**0.0028***	**0.036**
*DRB1*04:01*	10 (5)	7 (14)	3.09	0.030	0.31	5 (15)	3.39	0.036	0.36	2 (15)	3.45	0.14	0.84
DRB1*15:01	51 (26)	26 (52)	**3.17**	**0.00041***	**0.0049**	18 (55)	**3.51**	**0.0011***	**0.013**	5 (38)	1.83	0.31	0.99
DQB1*06:02	44 (22)	24 (63)	**6.08**	**0.0000017***	**0.000017**	16 (67)	**7.09**	**0.000026***	**0.00026**	5 (45)	2.95	0.085	0.59

Only significantly ($p < 0.05$) associated alleles are presented

RR relative ratio, p level of significance in χ^2 test, p_{corr} p value corrected according to Bonferroni inequality method, for the number of comparisons for each HLA locus, see "Statistical analysis"

* Significant associations that withstand the Bonferroni corrections (for contrast assigned bold)

[a] The percentage of carrier patients is related to the total number of patients HLA typed in specific locus, i.e. 36 in locus A, 49 in B, 46 in C, 50 in DRB1 and 39 in DQB1

aplasia has been found in the bone marrow at the PNH diagnosis or earlier, irrespective the level of peripheral blood measures.

ISAA/PNH patients were significantly younger than n/PNH patients (27 vs. 37 years of mean age, respectively; $p = 0.022$). In the ISAA/PNH group male patients (9 of 13) and in n/PNH group female patients were more frequent (21 of 33, $p = 0.051$). The platelet and reticulocyte counts were significantly lower in ISAA/PNH than in n/PNH (52 vs. 127 $\times$ 10^9/L, $p = 0.0023$ and 29 vs. 63‰, $p = 0.0062$, respectively). Hemolysis as measured by the decay of haptoglobin was more strongly pronounced in n/PNH group (0.03 vs. 0.15 g/L, $p = 0.024$) whereas LDH activity, hemoglobin levels and leukocyte counts did not differ significantly between groups.

As measured at PNH diagnosis, the percentages of CD59 and CD55 deficient granulocytes were significantly higher than those of deficient erythrocytes ($p = 1.2 \times 10^{-10}$ and $p = 2.8 \times 10^{-8}$). The percentages of CD66b, CD59 and CD55 deficient granulocytes significantly differed between n/PNH and ISAA/PNH, being higher in the former subgroup (73 vs. 53%, $p = 0.035$, 73 vs. 49%, $p = 0.012$ and 75 vs. 38%, $p = 0.0028$, respectively). The CD59- and CD55-deficient clones have shown generally lower values for erythrocytes and did not differ significantly between n/PNH and ISAA/PNH groups (38 vs. 29%, $p = 0.12$ and 34 vs. 22%, $p = 0.067$, respectively).

In association analysis (Table 2) six MHC alleles associated significantly with PNH. For five of them (*A*25:01, B*18:01, DRB1*04:01, DRB1*15:01* and *DQB1*06:02*) increased risk of PNH has been revealed and for *A*24:02* allele the protective role in PNH has been suggested.

The stratification of the patients into n/PNH and ISAA/PNH groups demonstrated significant difference in MHC association patterns. The associations of class II alleles were preferentially focused in n/PNH group whereas class I alleles associated mainly with ISAA/PNH (Table 2). Only one class I allele (*B*18:01*, 46 vs. 14%; RR = 5.25, $p = 0.0028$) has shown strong association with ISAA/PNH and withstand the Bonferroni correction ($p_{corr} = 0.036$). Two PNH associated class II alleles (*DRB1*15:01* and *DQB1*06:02*) associated strongly with n/PNH subtype (55 vs. 26%; RR = 3.51, $p = 0.0011$ and 67 vs. 22%; RR = 7.09, $p = 0.000026$, respectively) and withstand the Bonferroni corrections ($p_{corr} = 0.013$ and $p_{corr} = 0.00026$, respectively). For three remaining weakly PNH associated alleles (*A*24:02, A*25:01 and DRB1*04:01*) their associations with PNH subtypes did not withstand the corrections.

Discussion

Our total association results confirmed earlier findings of strong *DRB1*15:01* and *DQB1*06:02* allele associations with PNH (Lombardi et al. 2008; Maciejewski et al. 2001; Shichishima et al. 2002).

In studies by Maciejewski et al. (2001) and Saunthararajah et al. (2002), patients with both moderate and sAA were incorporated into AA/PNH and pure AA cohorts, in respective studies. In current analysis of moderate and severe AA/PNH patients combined in one group (excluding the PNH group with normocellular bone marrow) the proportions of *DRB1*15:01* carriers in patients and controls (47 vs. 26%; RR = 2.56, $p = 0.019$, not published) were very close to the previously published data (42 vs. 21% and 56 vs. 28% for Maciejewski et al. 2001 and Saunthararajah et al. 2002 cohorts, respectively). This comparison suggests our global results on strong

association of *DRB1*15:01* allele with PNH and AA/PNH are confirmatory to the previous findings indicating involvement of *DRB1*15:01* both in PNH and AA pathogenesis.

To separate, as far as possible, the pathogenetic role of AA clone as a source of PNH clone selection we used different highly restrictive criteria for ISAA/PNH group selection, where only those patients with pre-PNH bone marrow severe aplasia were incorporated. All PNH patients with hypoplastic bone marrow and those with normal cellularity were in our study incorporated in n/PNH group. Because of more restrictive criteria of ISAA/PNH patients' assignment, in our n/PNH patients the pathogenetic role of AA clone as a source of PNH clone selection was less likely than in previous study (Maciejewski et al. 2001). As a result, in our detailed association analysis of stratified ISAA/PNH and n/PNH groups unexpected patterns of MHC associations were revealed. Despite the limits of small sample size, we found not earlier described significant association of *B*18:01* allele with ISAA/PNH. Simultaneously, *DRB1*15:01* and *DQB1*06:02* alleles were clustered preferentially within n/PNH, and much less in ISAA/PNH group. Similar but weaker trend of decreased frequency of HLA-DR2 (and its main allele *DRB1*15:01*) within AA with GPI-anchored protein-deficient clone present as compared to PNH hemolytic syndrome was observed previously (Maciejewski et al. 2001). When compared the frequency of HLA-DR2 in AA patients with GPI-anchored protein-deficient cells to those in whom these cells were absent, HLA-DR2 was stronger associated with the presence of an expanded clone (Maciejewski et al. 2001). Current data strongly support this previously suggested link of the expansion of the PNH clone itself to the *DRB1*15:01* allele, rather than to the aplastic anemia in which PNH occurs. Our findings of more significant association of *DRB1*15:01* with n/PNH rather than ISAA/PNH can be explained by different criteria applied for patient grouping in previous studies and by us. Moreover, only small part of AA patients in Maciejewski et al. (2001) cohort were PNH patients and only 35 PNH patients (16 with primary PNH and 19 with AA/PNH) have been finally analyzed. Indeed, because of the rarity of the disease and its subsets the small sampling sizes of patient groups in previous and our studies can adversely influence the power of statistical inferences. In our study the precision of the association coefficients like RR and p were improved by the use of perfectly ethnically and geographically matched controls and patients (European, Polish ancestry) that was not the case in previous study. Historical control group data from NMDP Registry and blood center donors of not precisely described ethnical origin have been used previously and patient groups were not uniform with 32 and 40% of patients of non-North American white origin in Maciejewski et al. (2001) and Saunthararajah et al. (2002) cohorts, respectively. These differences may account for more sharp results in our association study both in MHC class I and II.

Diseases with overlapping physiopathology, like AA, PNH and MDS (Hillmen et al. 1995; Marsh and Elebute 2003), may have different etiology and the sub-grouping of patients can be a rewarding strategy (Spurkland and Sollid 2006). Setting ISAA/PNH population apart from the normo- and hypoplastic PNH patients was expected to uniform some physiopathological features in subgroups. Fitness of the patients' dissection made by our model was confirmed by demography characteristics and those outcome measures that differentiated our ISAA/PNH patients from n/PNH patients (Table 1). Significantly younger age, higher prevalence in males, significantly lower hemolysis and platelet and reticulocyte counts may suggest different pathogenetic mechanisms to be involved in ISAA/PNH than n/PNH. Yet, partial overlap in the clinicopathological features of the two groups has been confirmed by the data, with no significant differences observed in LDH, hemoglobin concentrations and leukocyte counts. In accordance with earlier findings (Żupańska et al. 2002), the PNH clone sizes of GPI-deficient erythrocytes were lower than for granulocytes. In current study the GPI-deficient granulocyte clone sizes were major discriminative parameter between ISAA/PNH and n/PNH groups. Although these features clearly indicated complex and partly overlapped physiopathology they strongly suggest involvement of independent pathogenetic pathways in the two PNH presentations.

HLA-DR2 and its main allele *DRB1*1501* have been described as closely associated with multiple sclerosis (Olerup and Hillert 1991), autoimmune glomerular nephritis (Fisher et al. 1997), narcolepsy (Matsuki et al. 1992) and prolonged survival of non-Hodgkin lymphoma patients (Juszczynski et al. 2002). HLA linkage to autoimmune diseases and immune surveillance-dependent diseases is believed to arise from HLA-molecule dependent ability to present pathogenic peptides derived from self, foreign or HLA-derived themselves peptide antigens. PNH, like AA, was reported to be strongly associated with DRB1*15:01 molecule (Lombardi et al. 2008; Maciejewski et al. 2001; Shichishima et al. 2002) and abnormal T-cell repertoires were observed in PNH patients. The identification of expanded T-cell clones by CDR3 size analysis gave the evidence of hematopoietic stem cell (HSC)-specific T cells effect on normal and PNH HSCs (Karadimitris et al. 2000). In this context, the more conceivable hypothesis of MHC class II association with PNH points to the involvement of specific (self-) peptides likely able to bind the associated HLA allele product in the immune-mediated mechanisms underlying emergence of GPI-

defective clones. Young (1992) proposed that within the hypocellular marrow environment, cytotoxic T lymphocytes were involved in the positive selection of the PNH clone. According to the model proposed by Luzzatto et al. (1997) normal HSCs are eliminated via hypothetical molecule which, upon binding to a GPI-linked protein, can cause damage to the stem cell. In the model promoted by Marsh and Elebute (2003) the *PIG-A* gene mutation is followed by immunological attack to PIG-normal stem cells with limited clonal expansion caused by immunoselection. A secondary mutation in *PIG-A* mutated cell is necessary step in this model. Despite the extensive research of immune cell repertoires, no uniform peptide, source protein or systematic additional mutation have been found in PNH patients that could explain MHC associations (Luzzatto and Bessler 1996; Luzzatto et al. 1997; Marsh and Elebute 2003; Tiu and Maciejewski 2006; Young 1992). In this context the mechanisms that may deregulate the balance between normal and pathological hematopoiesis in PNH remain poorly recognized.

HLA class I molecules are profoundly involved in immune response including initiation and regulation of T-cell cytotoxicity and antigen-presenting cell licensing. Our discovery of moderate association of *B*18:01* with ISAA/PNH and lack of the association with n/PNH can support the hypothesis of independent immune processes to be involved in selection of aplastic and non-aplastic GPI-deficient clones. The peptide presentation repertoire specific for B*18:01 molecule might be responsible for more intense suppression of HSC in *B*18:01* allele carriers with PNH (Papadopoulos et al. 1996).

Other Possible Explanations

Although, the expression of immune recognition elements on the cell surface is changed in PNH clone (Bessler and Fehr 1991; Maciejewski et al. 1996; Rosse and Dacie 1966), the MHC expression is not deficient because of transmembranal anchoring of MHC molecules. What's more, the overexpression of MHC is promoted by the increased levels of interferon γ in PNH (Zeng et al. 2006). It has been confirmed in EL4 cell lines the 21% higher MHC class II expression on GPI^- than GPI^+ cells (Murakami et al. 2002). Conversely, the expression of MHC class II molecules on activated human T cells seems to be lower in PNH clones than in normal T cells (Richards et al. 1999). The disproportional expression of MHC vs. GPI-anchored proteins can shape the cell membrane characteristics of the PNH clone and influence interactions with cytotoxic T cells (Murakami et al. 2002).

In contrast to other HLA alleles, the HLA-*DRB1*15:01* is restrictive element for the number of peptides endogenously derived from DR15β (the molecule encoded by its own restrictive allele), for the peptides derived from DQB1*0602 molecule and for the number of peptides derived from other class II molecules (Chicz et al. 1993; Vogt et al. 1994). The contribution of HLA-DR triggering in suppressed hematopoiesis has been confirmed both in animal (Yamaguchi et al. 1999a) and human models (Lee et al. 1997; Yamaguchi et al. 1999b). If the HSC proliferation is regulated by the expression of MHC class II, we postulate that the high expression of MHC (Zeng et al. 2006) and the strong self-protective signal assembled by the broad presentation of self class II peptides in *DRB1*15:01* carrier PNH patients can disrupt T cytotoxic cell autoregulative attack directed at the level of MHC class II expressing HSC and redirect this attack to normal HSC.

This model fits well to our observation of higher association of *DQB1*06:02* than *DRB1*15:01* with PNH. The *DQB1*06:02* allele in the tested population remains in complete linkage disequilibrium with *DRB1*15:01*, but the last allele is associated with several different *DQB1* alleles (Nowak et al. 2008). In *DRB1*15:01* carriers broad MHC self-peptide presentation by DR15 molecule can be broader and disruption of autoimmune attack can be more efficient in the presence of *DQB1*06:02* allele than in *DRB1*15:01* individuals with alternative *DQB1* allele not presented in the context of DR15. Taken together, class II association data suggest the level of self peptide presentation by the MHC class II expressing HSC can upregulate the relative PNH clone proliferation at the expense of normal hematopoietic clone.

Our previous analysis of HLA haplotypes in PNH suggested independent roles of class I and II molecules in PNH pathogenesis (Nowak et al. 2010) and justified the assumptions on different mechanisms of HLA class I and II association with PNH subtypes. It has been shown the *B*18:01* allele to be specific restrictive element for immunodominating octapeptide GEDGRVYV, a fragment 753–760 of phosphatidylinositol-activated phospholipase D (PLD) (Papadopoulos et al. 1996; Protein Information Resource Georgetown University Medical Center 2010). Investigations focused on PLD have shown important anti-apoptotic and cell protective regulatory effect of this intracellular enzyme in hematopoietic cells (Nozawa 2002; Park et al. 2002). The PLD can be activated stronger in PNH than in normal hematopoietic cells because PNH clone emergency is in its nature associated with the metabolic block at the level of phosphatidylinositol, the strong activator of PLD (Hillmen et al. 1993). Considering coincidence of B*18:01 molecule presentation ability and PNH pathologies, the metabolic pathway of apoptosis inhibition via PLD can be a target for further investigation upon PNH pathogenesis.

In conclusion, the detailed sequence of events that ultimately result in MHC association with PNH clone domination remains to be further elucidated. In our study

PNH associated strongly with *DRB1*15:01* allele and the association was strongest in the presence of *DQB1*06:02*. This association remains in agreement with hypothesis of autoimmune surveillance upon PNH and possibly normal hematopoiesis. The stratification analysis indicated the stronger association of *DRB1*15:01* with n/PNH than with ISAA/PNH, that indicates close coincidence of this allele with the occurrence of PNH clone and probable independent pathogenetic role to autoimmune mechanism of aplastic anemia. Simultaneously, HLA-*B*18:01* associated strongly with ISAA/PNH, that needs further investigations and may suggest deregulated balance in the machinery of apoptosis. It has been widely confirmed that increased apoptosis in $CD34^+$ bone marrow cells is a major feature in the pathophysiology of AA (Ismail et al. 2001; Killick et al. 2000a; Killick et al. 2000b; Philpott et al. 1995). The idea that *PIG-A* mutation and not necessarily GPI-anchored protein deficiency can be responsible for relative clonal expansion of PNH cells under proapoptotic stress was recently strongly supported by Savage et al. (2009).

We conclude that clonal selection of *PIG-A* mutated cells with cognate metabolic block is associated with MHC class II alleles *DRB1*15:01* and *DQB1*06:02* independent from initial severe AA clone selection. PNH associated MHC class I molecule B*18:01 can additionally influence the domination of PNH clone in initial sAA type PNH subjects. Future study of clonal selection and expansion in association with MHC selfpresentation are warranted to elucidate stem cell biology in PNH.

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