MEDICAL UNIVERSITY – PLEVEN

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IMMUNOGENETIC ASPECTS OF THE PATHOGENESIS OF JAK2 V617F-POSITIVE MYELOPROLIFERATIVE NEOPLASMS

PhD Thesis Synopsis

Primary field of study: ***7.1 Medicine***

Secondary field of study: 03.01.39 ***Hematology and blood transfusion***

Thesis advisors:

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The PhD thesis report comprises 136 pages and contains 34 figures and 20 tables. The bibliography includes 318 references.

PhD thesis in its final form was presented to an open meeting with external attendees of the Committee of the Department of Nephrology, Haematology and Gatroenterology of the Faculty of Medicine at Medical University – Pleven. It was approved as acceptable for formal review and public defense.

Public PhD thesis defense is scheduled for …… at …… It will take place at …….

All related materials are available to the interested parties in the Library of MU-Pleven.

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ABBREVIATIONS

ALL	Acute lymphoblastic (lymphoid) leukemia
AML	Acute myelogeneous (myeloid) leukemia
APC	Antigen presenting cell
aUPD	Acquired uniparental disomy
BM	Bone marrow
BR	Best rank
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogeneous (myeloid) leukemia
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ES	Enrichment score
ET	Essential thrombocythemia
FWER	Family-wise error rate
GLM	Generalized linear model
GSEA	Gene set enrichment analysis
GWAS	Genome wise association study
HED	HLA evolutionary divergence
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICIs	Immune checkpoint inhibitor
IFN	Interferon
ILCs	Innate lymphoid cells
IPD-IMGT/HLA	Immune polymorphism database – Immunogenetics/HLA
KIR	Killer Immunoglobulin-like receptor
LPC	Lymphoid progenitor cell
LT-HSC	Long term hematopoietic stem cell
MPC	Myeloid progenitor cell
MDS	Molecular dynamics simulations

MHC	Major histocompatibility complex
MPN	Myeloproliferative neoplasm
MPN-SCs	Stem cells for myeloproliferative neoplasms
NGS	Next Generation Sequencing
PB	Peripheral blood
PC	Progenitor cell
PHBR	Patient harmonic mean best rank
PMF	Primary myelofibrosis
PV	Polycythemia vera
RMSD	Root mean squared deviation
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
(sc)RNA Seq	(single cell)RNA sequencing
TCGA	The Cancer Genome Atlas
TILs	Tumor infiltrating lymphocytes
Tregs	Regulatory T cells
WHO	World health organization

I. Introduction

In the third decade of the XXI century neoplastic diseases remain a global medical and social issue. Particularly challenging is the group of rare cancer, which collectively comprises a total of 24% of all cancer cases in Europe, which means that at least 5.1 million Europeans live with the diagnosis of any type of rare cancer (https://www.rarecancerseurope.org/). The definition of a rare disease is based on its prevalence in a given population. However, the definition of a rare cancer is based on its incidence. The cut-off incidence for rare cancer is accepted to be below 6 newly diagnosed cases per 100 000 inhabitants annually. Rare hematological malignancies are included in the general catgory of rare cancers because their cumulative incidence in Europe of 27.73 cases/100 000 persons.

Myeloproliferative neoplasms (MPNs) are classified as rare hematological malignancies with annual incidence for all MPN entities in Europe of 3.31 cases/100 000 persons. The inaugural edition of European roadmap for hematology research issued by the European Hematology Association in 2016 pays special attention to myeloid malignancies and emphasizes the necessity to „characterize cellular and molecular mechanisms involved in disease development, progression and therapy resistance, including germline predisposition, chronic inflammation, microenvironmental and immunological abnormalities, as well as immune escape following stem cell transplantation;“. Obviously, detailed knowledge regarding the mechanisms of immune surveillance and its evasion would allow the rational design of immunotherapeutic approaches.

II. Research hypothesis, goal and tasks

Unsolved questions requiring further investigations

In our opinion the main unresolved questions regarding the pathogenesis of MPM are the following:

Which are the mechanisms driving the transformation of clonal hematopoiesis into clinically significant MPN?

What is the role of inborn genetic factors which predispose to development of clonal hematopoiesis and to the eventual evolution to MPN?

Какво е участието на имунната система като преципитиращ фактор както за инициацииране и поддържането на клоналната хемопоеза и евентуалната еволюция до МПН?

How can contemporary therapeutic approaches influence the evolution of MPNs and the eventually the evasion of immunosurveillance?

Do the mechanisms of evasion of immunosurveillance in MPNs suggest any option for therapeutic intervention so that the truncal mutations (e.g. JAK2 V617F and CALR mutations) can be targeted?

Research hypothesis, goal and tasks

Based on the aforementioned unsolved questions and on our expertise in the fields of immunology, genetics and pathogenesis of MPNs we hypothesized that it would be possible that HLA genotype contributes to the development of JAK2 V617F+ MPNs. This may suggest the existence of HLA–mediated restriction of the development of this subgroup of MPNs and immunosurveillance evasion as part of their pathogenesis through modulation of the expression of key components of

the HLA-I antigen processing and presentation in JAK2 V617F+ transformed MPN-SCs. The hypothesis which this work was based on is summarized in Fig. 1. below.

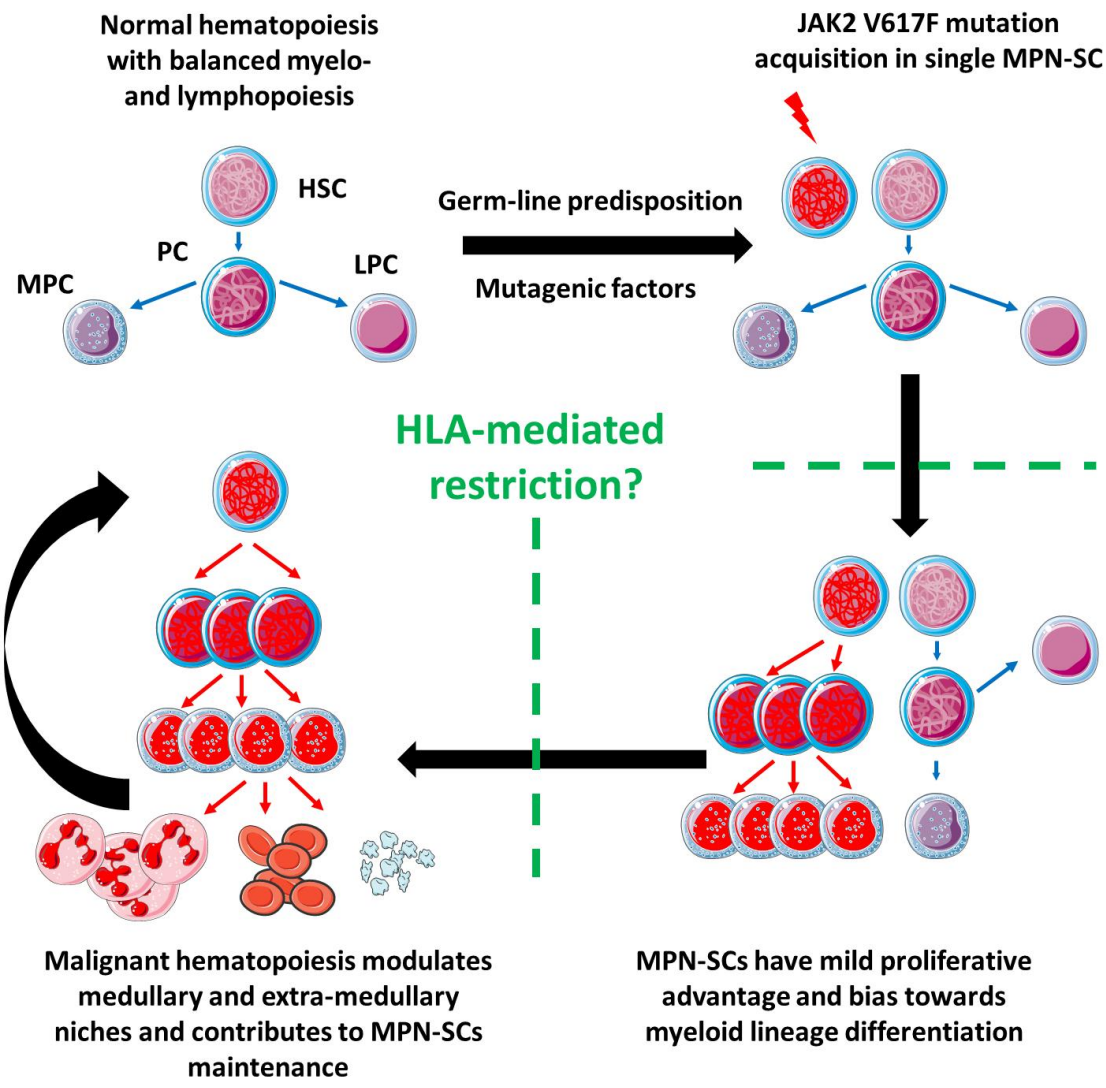

Figure 1. Schematic representation of the development of MPNs in the bone marrow HSCs pool after acquisition of competitive advantage as a consequence of a driver oncogenic mutation such as JAK2 V617F. Our key research question was whether there existed a HLA-mediated restriction at the level of MPN-SCs. Figure was inspired by Mead et al. (2017).

In order to test our hypothesis and to provide any supporting evidence we defined the following main ***research goal:***

1. To investigate the role of the classical immunogenetic factors namely *HLA-I* genotype in the development of JAK2 V67F+ MPN.

In order to achieve our research goal we defined that it would be necessary to complete the following ***investigational tasks:***

1. To curate the data regarding a large cohort of JAK2 V617F+ MPN patients and healthy controls from identical population (Bulgarian) with available data regarding the individual *HLA-I* genotype, determined using contemporary methods for high resolution HLA genotyping;

2. To compare the levels of *HLA-I* loci heterozygosity and the levels of HLA evolutionary divergence in MPN patients and in healthy controls;

3. To determine the theoretical capacity of each individual allele and genotype to present JAK2 V617F-derived neoantigens in MPN patients in comparison to those of healthy controls;

4. To determine individual *HLA-I* alleles frequencies and bi-and tri-loci haplotypes frequencies and to perform association analyses in MPN patients vs. healthy controls;

5. To perform molecular dynamics simulations with potentially protective HLA-I alleles and the theoretical JAK2 V617F-binding alleles;

6. To check the properties of JAK2 V617F-derived neoantigens for effective processing in HLA-I antigen presentation pathway;

7. To investigate the expression profile of the genes from HLA-I antigen processing and presentation pathway in CD34+ cells and granulocytes from patients with JAK2 V617F+ PV, ET and PMF;

8. To investigate the expression profile of the genes from HLA-I antigen processing and presentation pathway in CD34+ cells before and after interferon-alfa and ruxolitinib treatment.

III. Materials and methods

Biological data used

- Data regarding JAK2 V617F mutational status in MPN patients (n=139).
- Data regarding HLA-I genotype in MPN patients (n=139) and healthy controls from Bulgarian population (n=622).
- Publicly available gene expression datasets deposited in Gene Expression Omnibus platforms with accession numbers as follows: GSE53482, GSE103176, GSE54646, GSE47018**,** GSE69827, GSE120363, GSE44961.

Biostatistical and bioinformatics analyses

- Estimation of the HLA evolutionary divergence (HED) for every subject.
- Determination of the binding ranks (BR) for each allele and the patient harmonic binding rank (PHBR) for every subject.
- Association analyses for HLA alleles and haplotypes.
- Neoepitopes evaluation.
- Molecular dynamics simulations.
- Gene expression analyses

All analyses were performed in a blinded fashion of the person who performed them (VS) in strict conformity to the principles of the Helsinki Declaration.

IV. Results

HLA class I genotype does not affect the presence of JAK2 V617F mutation

We hypothesized that if MHC genotype plays any role in shaping the JAK2 V617F pathogenesis one has to expect differences in the distribution of HLA-I alleles and their antigen presentation properties between patients harboring this mutation and healthy controls. Ideally, the patients and control subjects must belong to the same population. We genotyped the three classical HLA-I loci in 139 patients and 622 healthy Bulgarians of Caucasian origin (Table 1). The typing was performed through NGS to achieve 2 fields designation of HLA-I alleles. The patients' and control cohorts were unbalanced in terms of age distribution (Table 1; Fig. 2), which was taken into account in HLA alleles association modeling as described below.

Table 1. Demographic and clinical characteristics of JAK2 V617Fve+ patients and healthy controls. P-values are from t-sided chi-squared test. Abbreviations: "MPN-NOS" – Myeloproliferative neoplasm – not otherwise specified; "NA"-not applicable.

Parameter	***JAK2 V617F+ MPN patients***	***Healthy controls***	***P-value***
Age (n)			<0.0001
≤50 years	13	529	
>50 years	126	93	
Total	139	622	
Gender (n)			0.4647
Males	59	288	
Females	80	334	
Total	139	622	
Diagnosis (n)			NA
ET	51	0	
PV	51	0	
PMF (post-PV/ET MF)	25 (3)	0	
MPN - NOS	12	0	

HLA typing was performed using NGS based technique in order to define HLA-I alleles at 2-field resolution. MPN patients and healthy controls cohorts were unbalanced in regards to age (Table 1 and Fig. 2). This was taken into account in the statistical modeling of HLA allelic and haplotype associations as described below

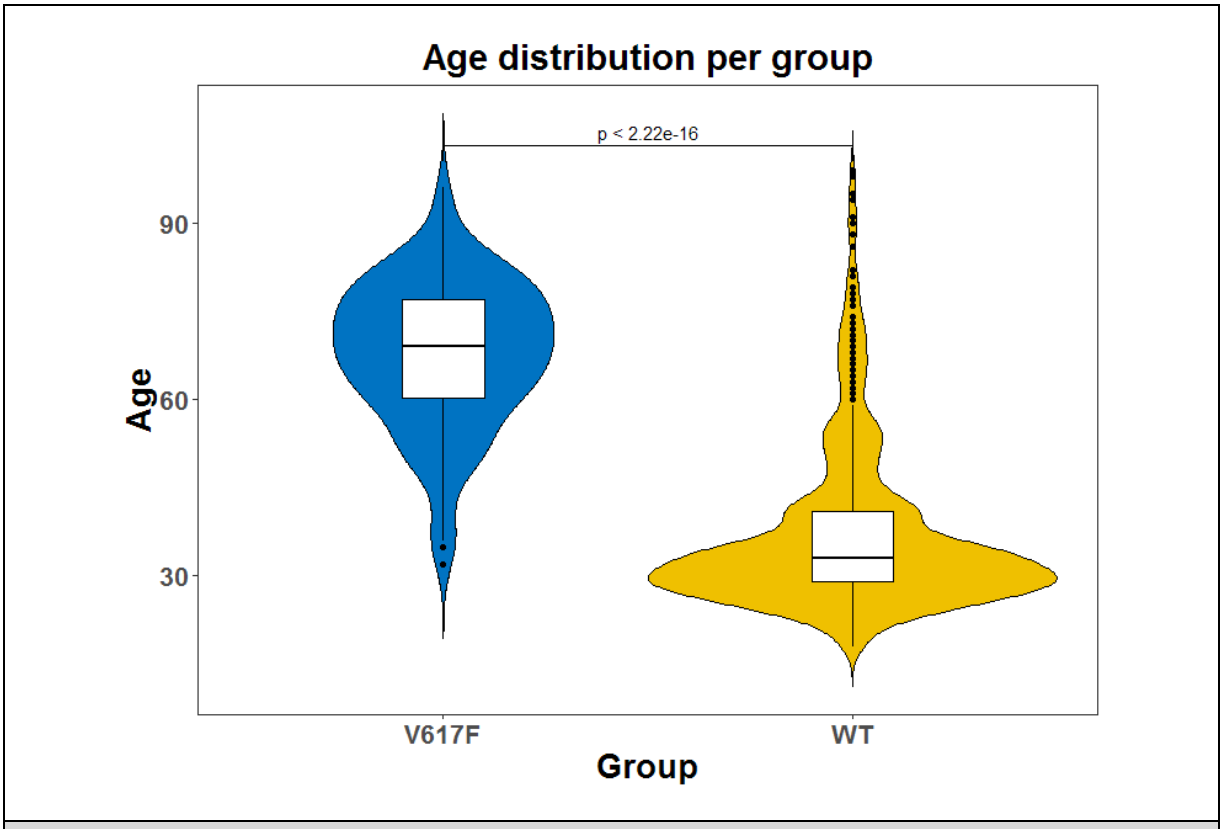

Figure 2. Comparison of age distributions in JAK2 V617F+ MPN patients and healthy controls. P-value is from two-sided Wilcoxon test.

JAK2 V617F mutation has been shown to be a major driver of oncogenesis in MPNs but seems to require the acquisition of additional mutations to sustain clonal overgrowth. Indeed a number of other driver genes have been shown to be mutated in JAK2 V617F positive MPN.

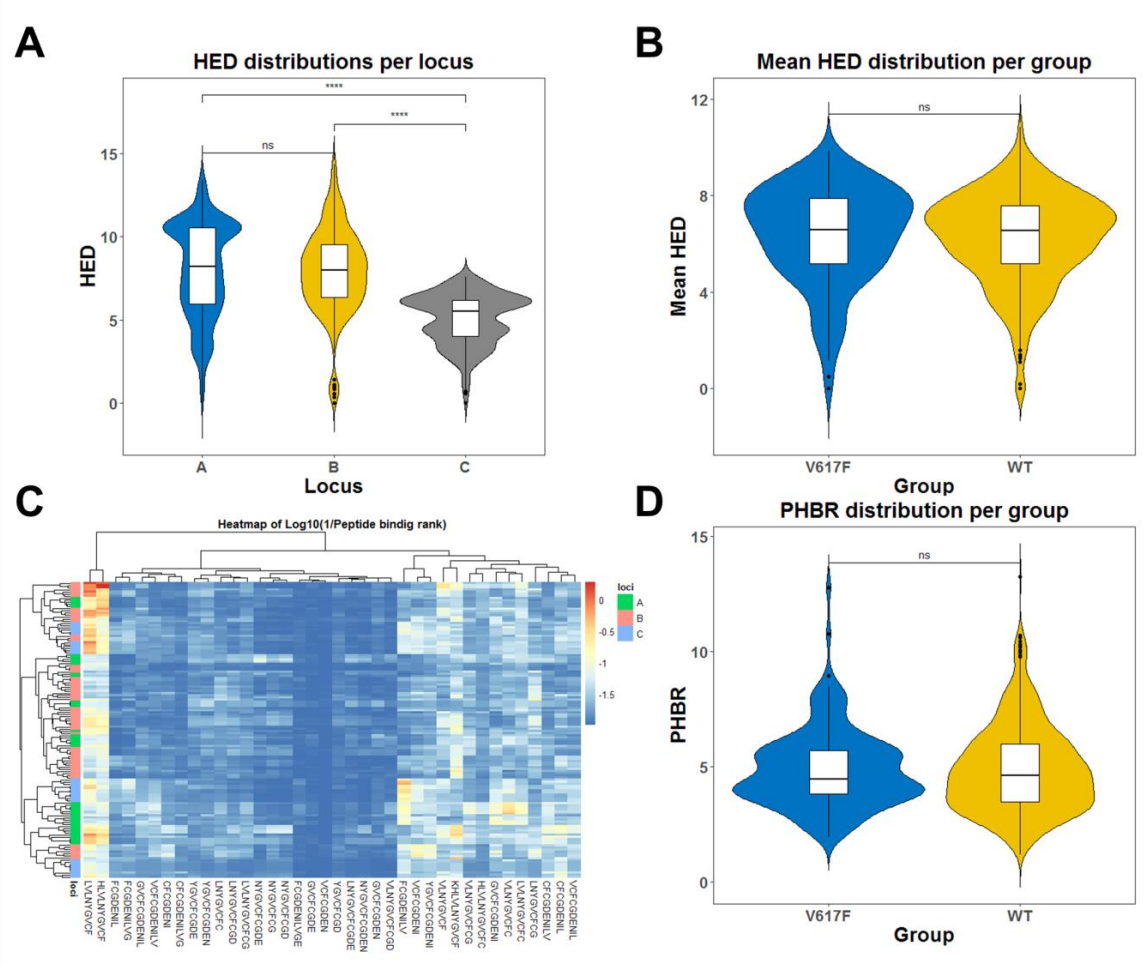

Figure 3. Evaluation of HLA genotype diversity and JAK2 V617F derived neoepitope presentation ability. (A) Distribution of HEDs for all studied subjects per locus. **(B)** Comparison of the distribution of mean HED between JAK2 V617F+ MPN patients and healthy controls. **(C)** Summary of NetMHCpan 4.0 prediction results presented as a heatmap of the log10 of inverted binding ranks for JAK2 V617F derived peptides presentation by every identified HLA-I allele in MPN patients and healthy controls. Each row represents a single HLA-I allele and each column represents one of the 38 tested JAK2 V617F derived peptides. The closer the value of log10 of 1/peptide binding rank is to 0, the better the peptide is binding to the particular HLA-I allele (see legend for color coding). **(D)** Comparison of the PHBR distribution between JAK2 V617F+ MPN patients and healthy controls. P-values are from two-sided Wilcoxon tests. Abbreviations: "HED"- HLA evolutionary divergence; "PHBR" – patient harmonic mean best rank; "WT" – wild type. P-values designation: ns – $p > 0.05$, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$.

As all these mutations can provide neoepitopes which might be subject to immunoediting we first questioned whether the level of global MHC-I diversity is associated with JAK2 V617F mutation. We did not find any association between the level of homozygosity and the presence of mutation (Table 2). As zygosity is assessed based on allele designation heterozygosity at a given locus does not necessarily reflect significant difference in the structure of the peptide binding groove and in the

binding properties of the two HLA alleles in heterozygous state. A global metrics for the evolutionary divergence of two peptides is the Grantham distance, which takes into account the differences in the amino acid composition of two aligned peptide sequences. We estimated the HLA evolutionary divergence (HED) based on the Grantham distance for every locus as well as the mean HED for all three loci for every patient and control subject. Expectedly, *HLA-A* and *–B* loci had significantly higher HED than the *–C* locus. Notably, JAK2 V617F was not associated with any difference in the HED at any locus and mean HED (Figure 3A, B, and Fig. 4).

Table 2. Homozygosity comparison for HLA loci between JAK2 V617F+ patients and healthy controls. P-values are from two-sided chi-squared tests.

Locus	Group	Heterozygous Subjects(n)	Homozygous Subjects(n)	p-value
HLA-A	V617F+	119	20	1
	Controls	530	92	
HLA-B	V617F+	129	8	1
	Controls	584	37	
HLA-C	V617F+	122	11	0.42
	Controls	544	68	
Any	V617F+	100	32	0.724
	Controls	451	160	
Any two	V617F+	136	3	0.27
	Controls	593	20	
All	V617F+	131	1	1
	Controls	605	6	

We further assessed the theoretical ability of the HLA-I molecules to present peptides derived from V617F-mutated JAK2 protein. We followed the procedure developed by Marty et al. (2017) to derive the theoretical best binding rank (BR) for each locus as well as the so called patient best harmonic mean rank (PHBR) for each subject in the study (Figure 3C).

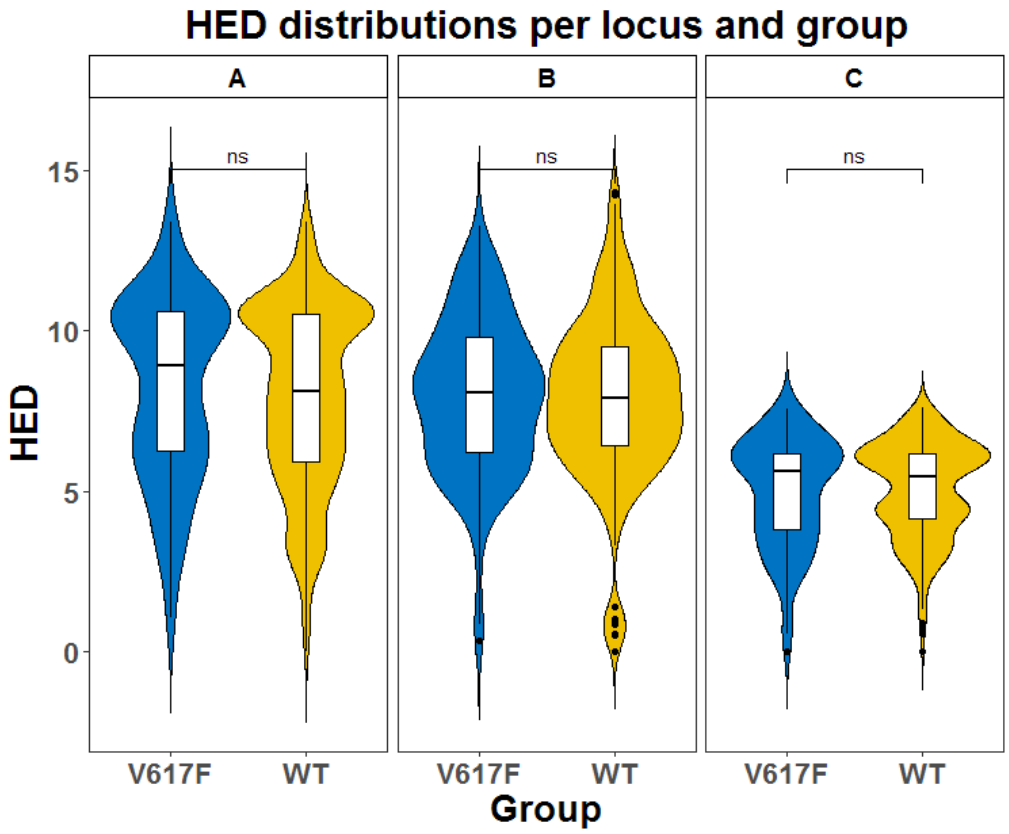

Figure 4. Comparison of HLA evolutionary distances distributions in JAK2 V617F+ MPN patients and healthy controls per HLA locus. P-values are from two-sided Wilcoxon tests. P-values designation: ns – $p > 0.05$, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$.

The higher BR is indicative of a worse binding property of the alleles at a given locus, whereas the higher PHBR points towards a better antigen binding property of the entire HLA-I genotype. It appeared that a number of alleles could theoretically bind a limited set of 9-and 10-mer mutant peptides (Figure 3C). However, the PHBR did not differ between patients and healthy controls (Figure 3D). Mean HED and PHBR did correlate neither for MPNs patients nor for controls (Fig. 5). Collectively, these results suggested that overall HLA-I diversity and the theoretical binding properties of the HLA genotype do not affect the presence of JAK2 V617F mutation. This finding, however, does not exclude the presence of specific HLA alleles that may affect this JAK2 V617F mutation presence.

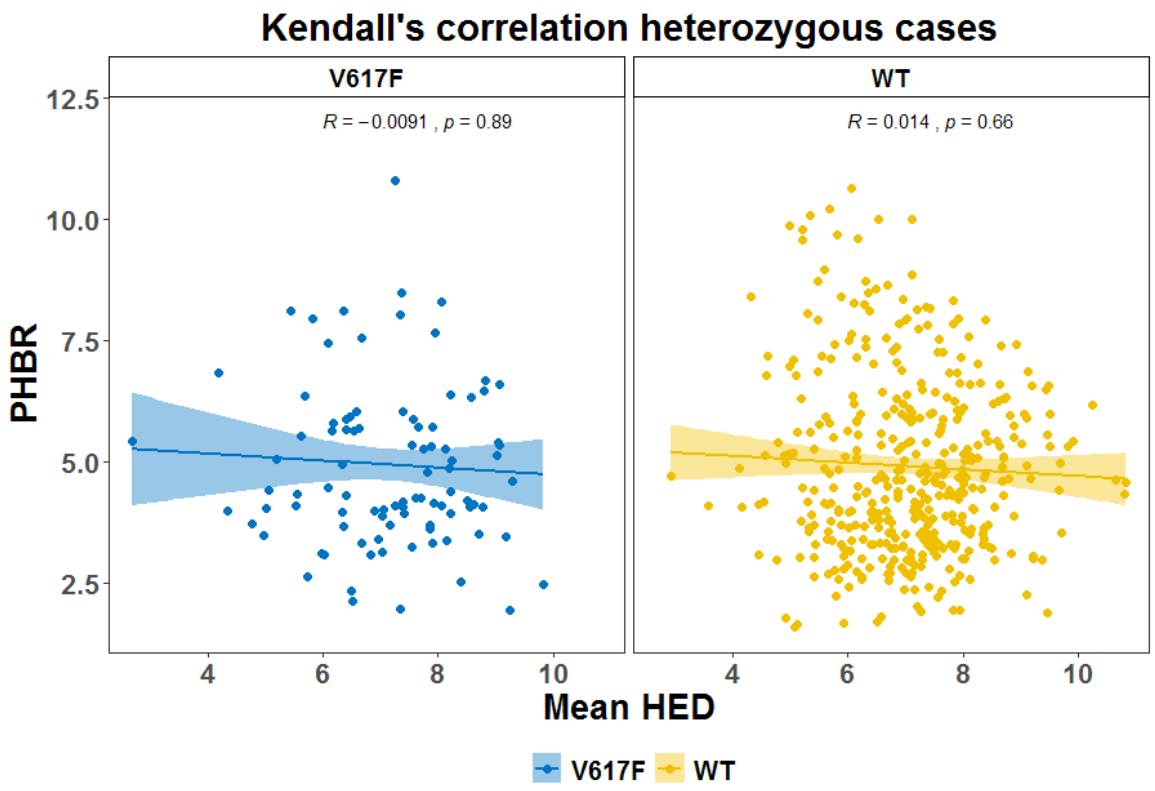

Figure 5. Correlation between mean HLA evolutionary distance (HED) and patient harmonic mean best rank (PHBR) for JAK2 V617F+ MPN patients and healthy controls. Correlation coefficients represent Kendall's tau coefficient. P-values are from two-sided significance test for Kendall's tau different from 0.

Specific HLA class I alleles are associated with decreased risk of JAK2 V617F+ MPN

In order to identify putative HLA-I alleles protective for development of JAK2 V617F positive MPN we performed a comparative allele frequencies analysis for *HLA-A*, *-B*, and *-C* loci. Expectedly, the highest number of unique alleles was observed for the *B* locus, whereas the *C* locus was the least diverse at the allele level (Fig. 6-8).

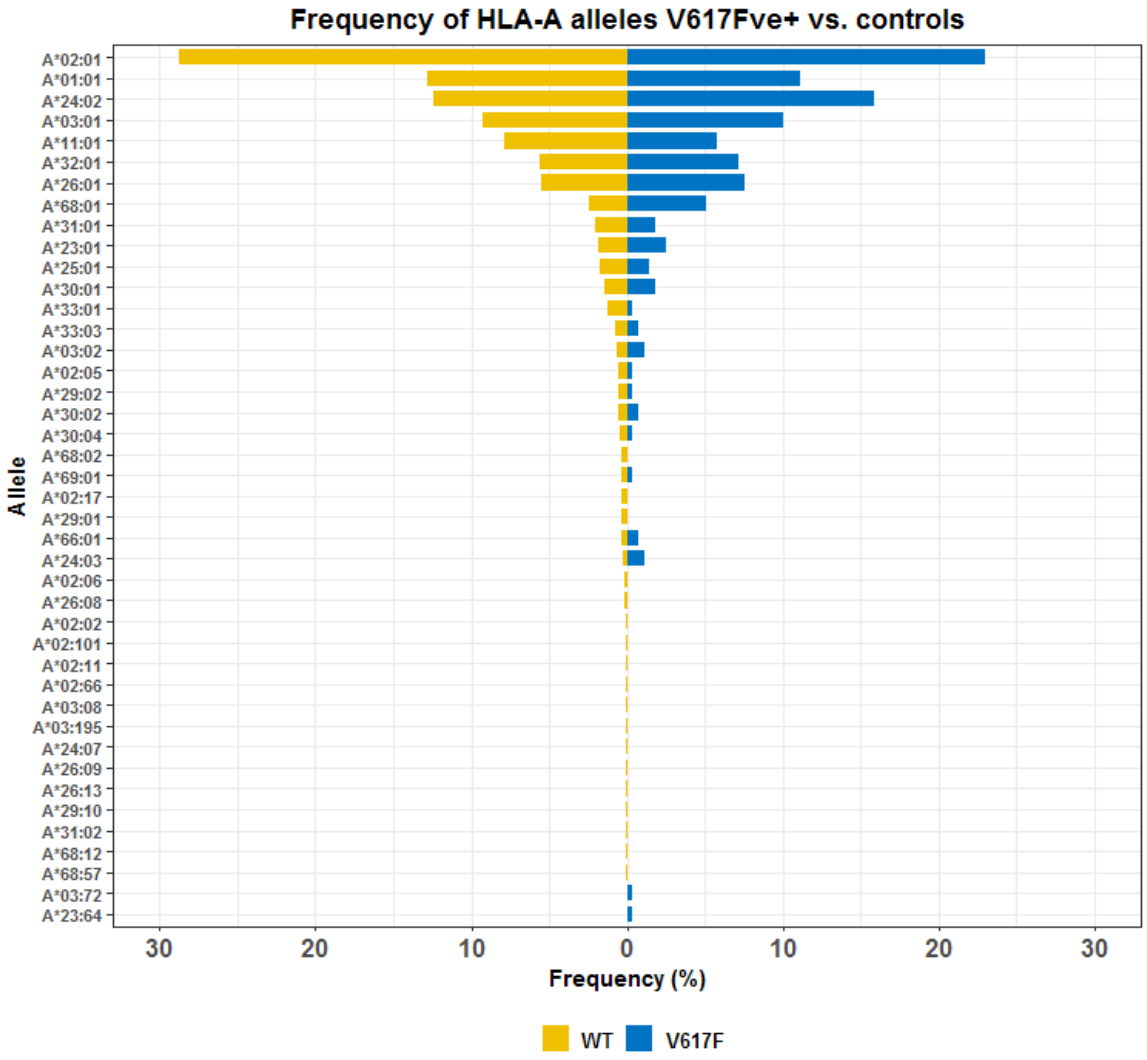

Figure 6. Frequency of identified *HLA-A* alleles at two-field resolution in JAK2 V617F+ MPNs and healthy controls.

To build a rational hypothesis regarding association of specific alleles with JAK2 V617F mutation we fitted multivariate generalized linear models taking gender and age above 50 years as covariates. Three alleles were significantly less frequent in the presence of JAK2 V617F mutation: *HLA-A**02:01 (p=0.036), *HLA-B**35:01 (p=0.017), *HLA-C**15:02 (p=0.033) (Figure 9A). On the other hand, four other alleles were significantly associated with the presence of JAK2 V617F mutation *HLA-B**44:03 (p=0.044), *HLA-B**41:01 (p<0.001), *HLA-C**12:03 (p=0.040) and *HLA-C**17:01 (p<0.0001) (Figure 9A). As these associations might be driven by the relative frequency of the alleles we sought for additional evidence that some alleles are inversely correlated to JAK2 V617F+ disease. Indeed bi-

loci haplotype analysis showed that the haplotype *HLA-A**02:01~*HLA-B**35:01 was the most significantly depleted in JAK2 V617F+ MPNs (p=0.006) (Figure 9B).

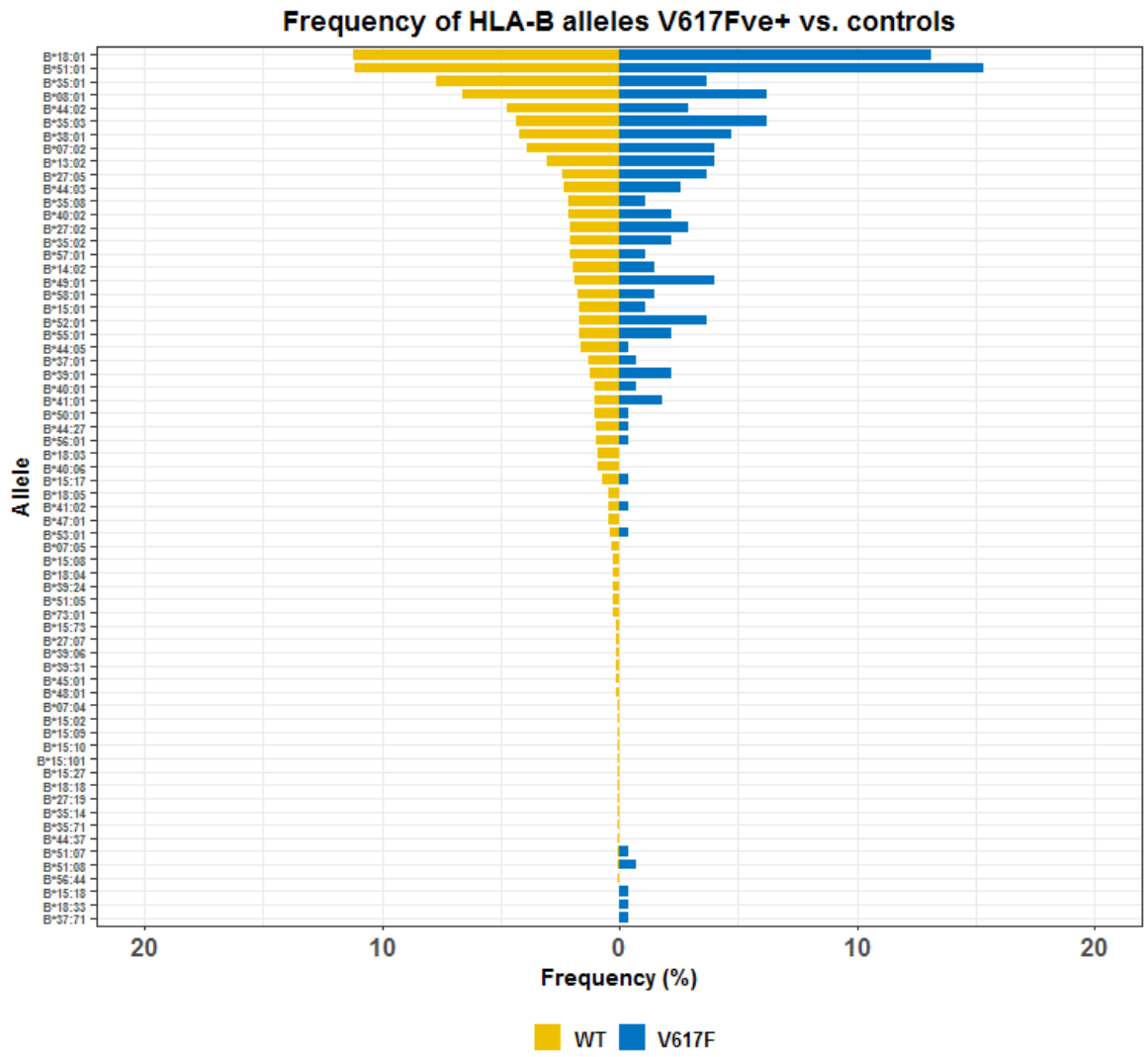

Figure 7. Frequency of identified *HLA-B* alleles at two-field resolution in JAK2 V617F+ MPNs and healthy controls.

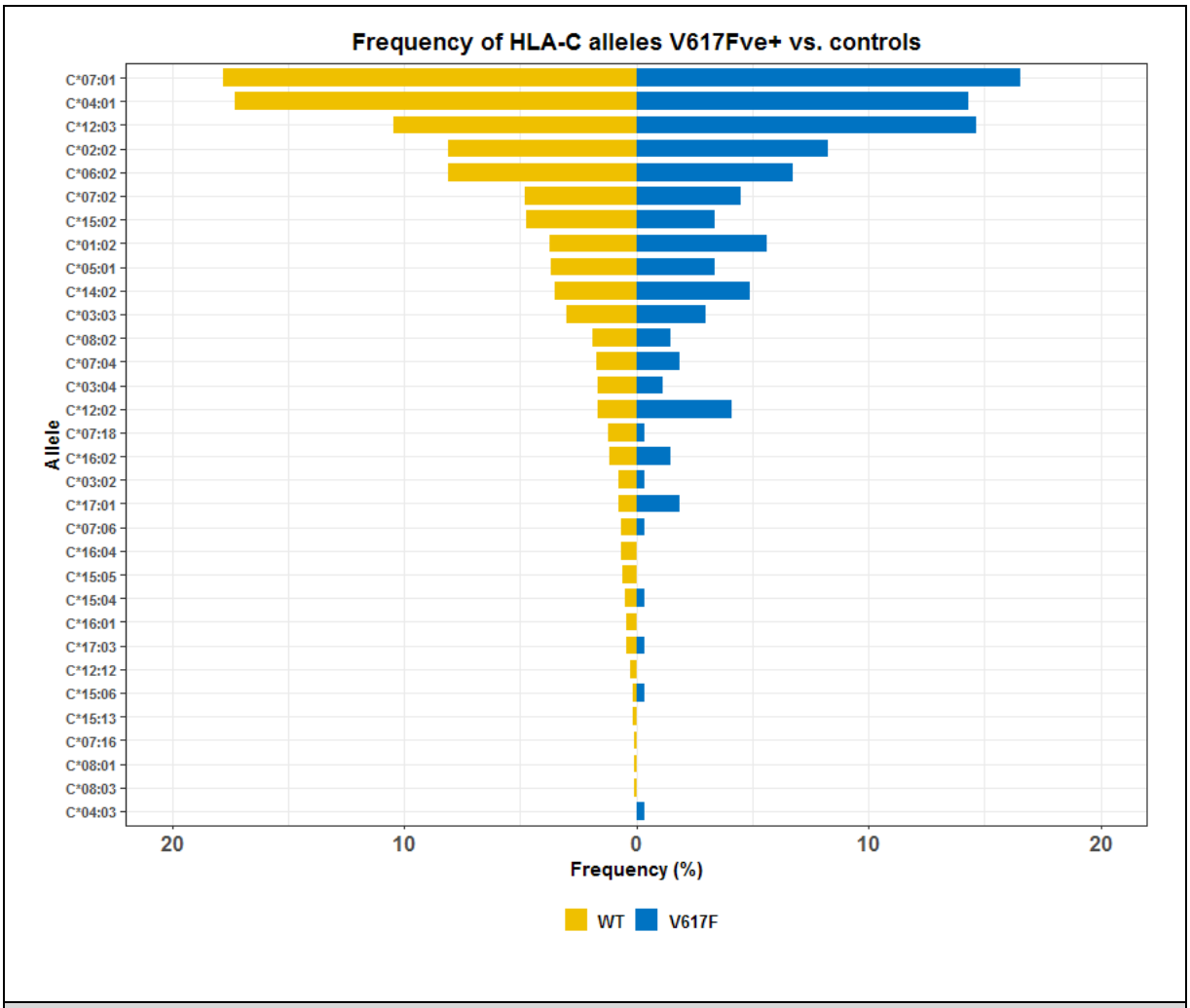

Figure 8. Frequency of identified *HLA-C* alleles at two-field resolution in JAK2 V617F+ MPNs and healthy controls.

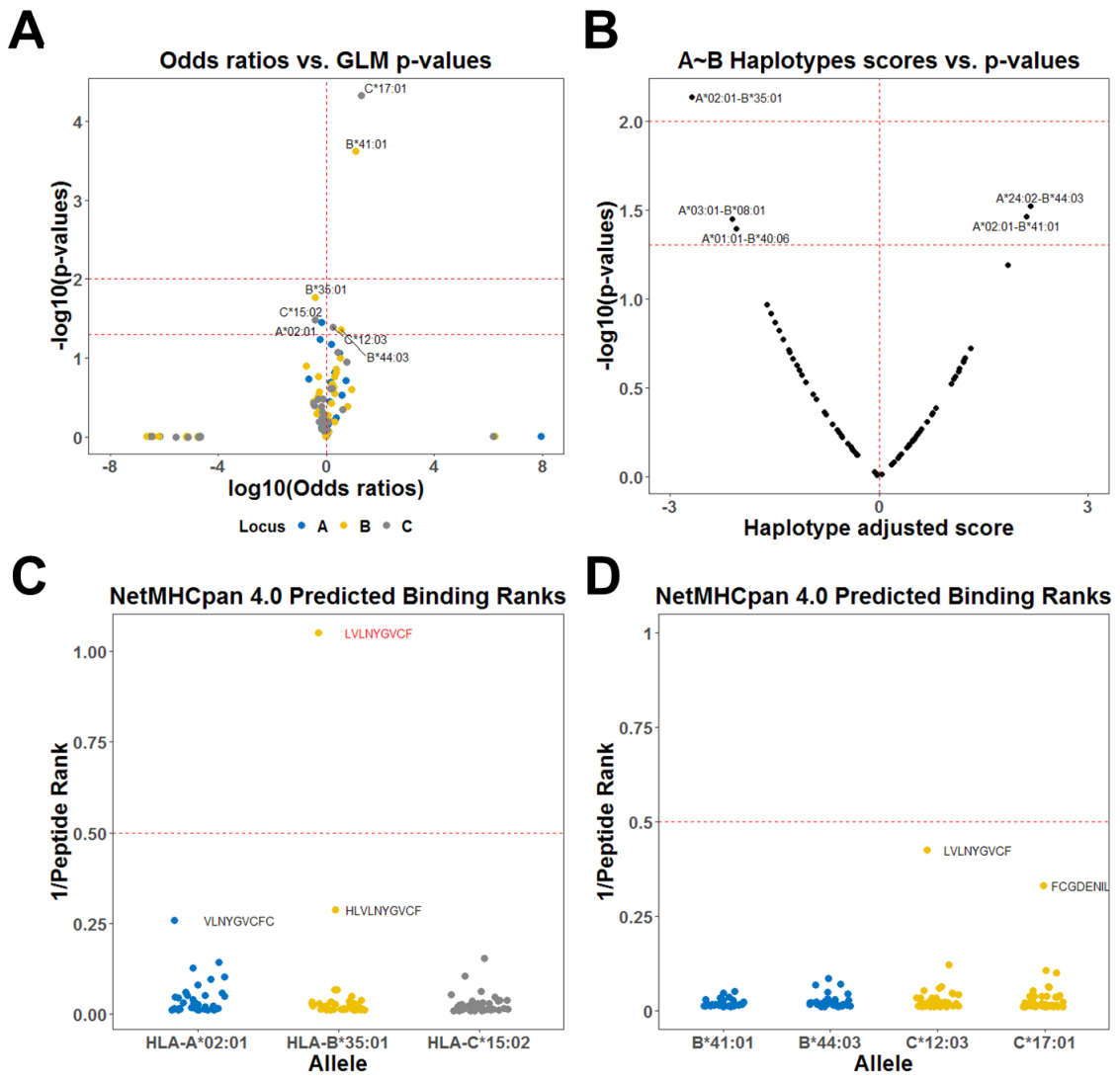

Figure 9. Generalized linear models association test between HLA alleles or haplotypes and presence of JAK2 V617F mutation and predicted peptide binding ranks for negatively or positively associated with JAK2 V617F+ MPNs alleles. (A) Volcano plot for association with HLA class I alleles. **(B)** Volcano plot for association with HLA-A~B biloci haplotypes. **(C)** Inverted values of the predicted binding ranks for JAK2 V617F derived peptides for HLA-A*02:01, HLA-B*35:01 and HLA-C*15:02 alleles. The dashed red line represents the default binder level proposed by the NetMHCpan 4.0 server. The top binding peptide LVLNYGVC**F** is labeled in red. **(D)** Inverted values of the predicted binding ranks for JAK2 V617F derived peptides for HLA-B*41:01, HLA-B*44:03, HLA-C*12:03 and HLA-C*17:01 alleles. The dashed red line represents the default binder level proposed by the NetMHCpan 4.0 server.

The associations with other biloci and triloci haplotypes are presented in Fig. 10-11.

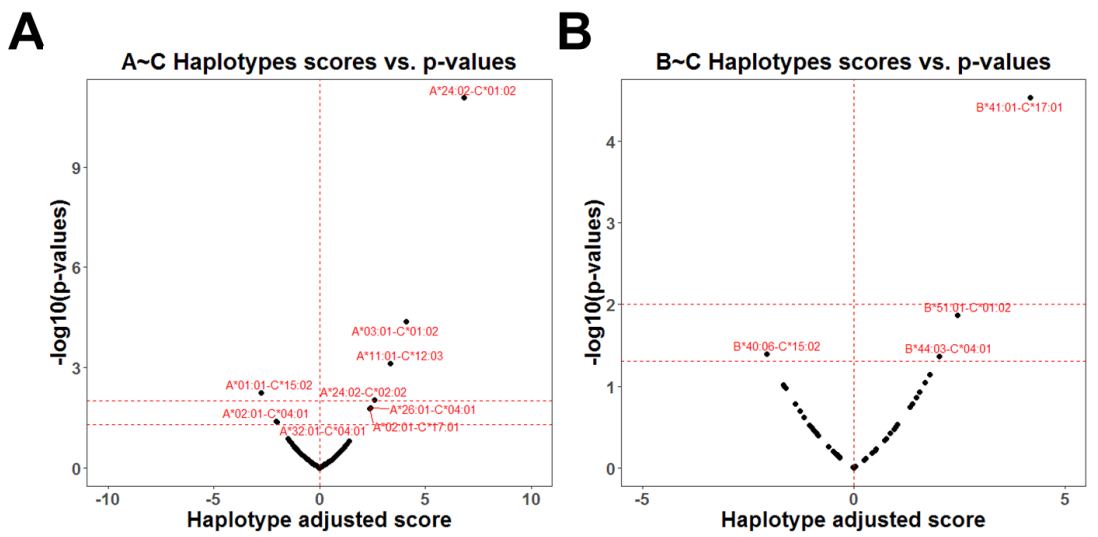

Figure 10. *HLA-I* biloci associations with the presence of JAK2 V617F mutation. (A) *A~C* haplotypes; (B) *B~C* haplotypes.

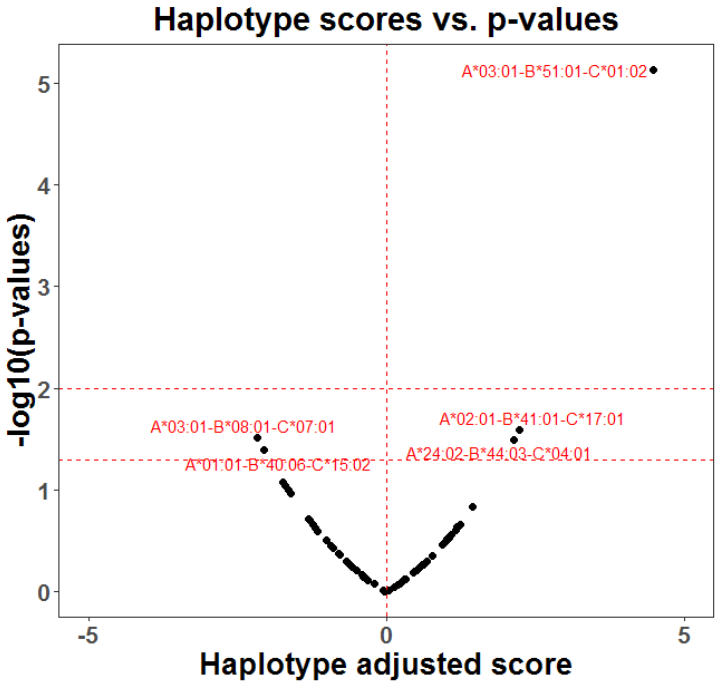

Figure 11. *HLA-I* triloci associations with the presence of JAK2 V617F mutation.

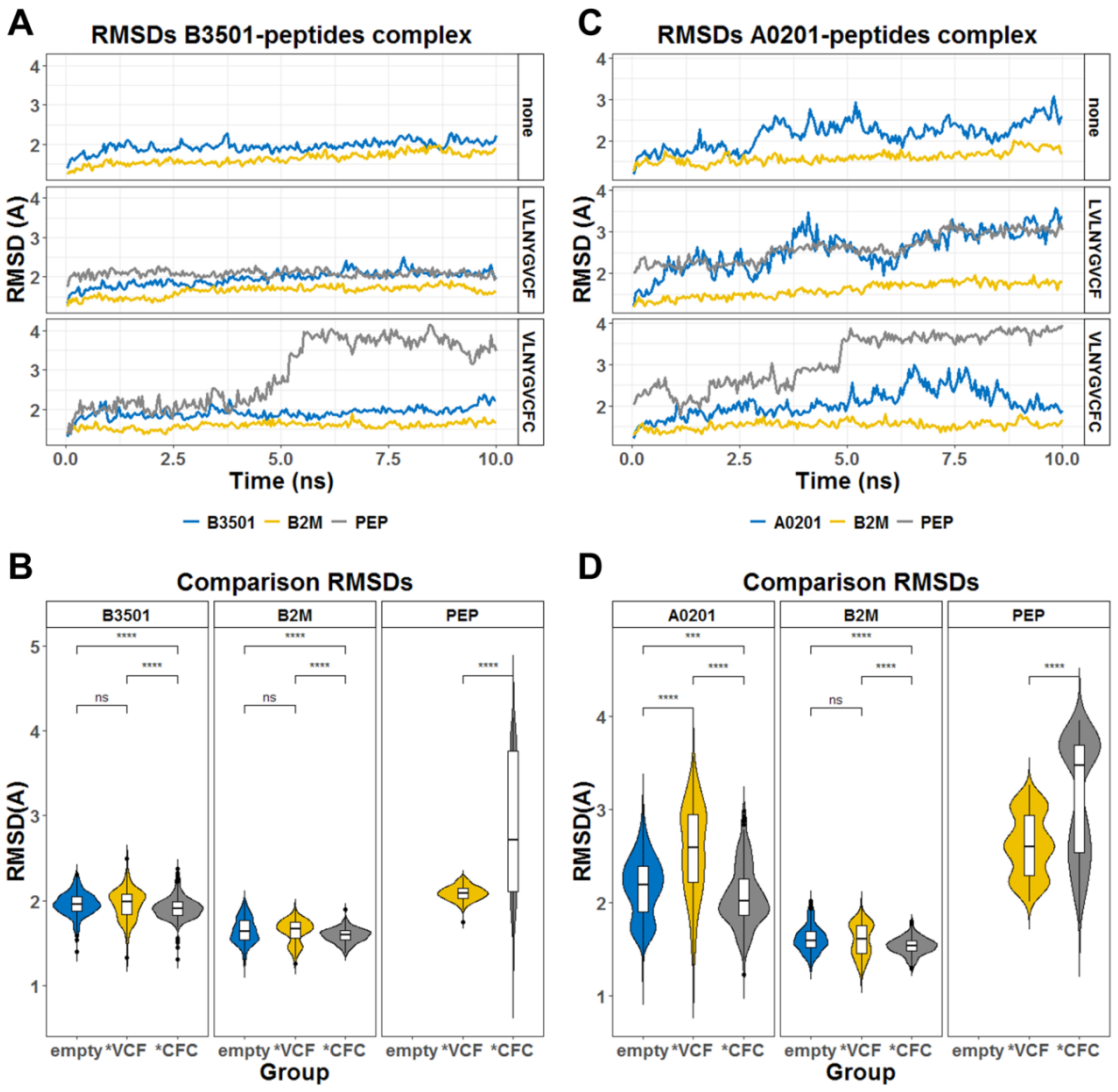

Figure 12. Molecular dynamics simulations of LVLNYGVCF and VLNYGVCFC peptides binding to HLA-B*35:01 and HLA-A*02:01. (A) Dynamics of RMSDs of α carbons over 10 ns of simulation for empty HLA-B*35:01 conformers ("empty") in complex with a predicted binding peptide ("LVLNYGCFC") and nonbinding peptide ("VLNYGVCFC"). **(B)** Comparison of the distribution of RMSD values for each protein in the three simulations in **(A). (C)** Dynamics of RMSD values of α carbons over 10 ns of simulation for empty HLA-A*02:01 conformers ("empty") and in complex with predicted nonbinding peptides ("LVLNYGCFC" and "VLNYGVCFC"). **(D)** Comparison of the distribution of RMSD values for each protein in the three simulations in **(C)**. All *p* values are from two-sided Wilcoxon tests. *PEP*=peptide; *ns*=nonsignificant. $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

*Neoepitope LVLNYGVCF fulfills the criteria for effective presentation by HLA-B*35:01*

To provide mechanistic explanation for the presence of protective HLA-I alleles we rechecked the predicted binding ranks for all 38 8-, 9-, 10-and 11-mer peptides that can be derived from JAK2 V617F mutant protein. Indeed, among those peptides one 9-mer (LVLNYGVC**F**) was predicted to be a weak binder for HLA-B*35:01 according to the predefined criteria of the used NetMHCpan 4.0 server, i.e. binding rank below 2.00% (Figure 3A). Furthermore, NetMHCstab 1.0 Server (http://www.cbs.dtu.dk/services/NetMHCstab/) predicted that among those peptides LVLNYGVC**F** was the most stable binder of HLA-B*35:01 (Table 3).

Table 3. NetMHCstab 1.0 Server (http://www.cbs.dtu.dk/services/NetMHCstab/). 9-mer peptides in complex with HLA-B35:01. The most stably binding peptide is shown in bolded fonts.

Peptide	*Predicted*	*T 1/2 (hours)*	*1-log50k*	*Affinity (nM)*	*Combined*	*Average*
LVLNYGVCF	**0.1764**	**0.7989**	**0.418**	**540.07**	**0.3818**	**0.3818**
VLNYGVCFC	0.0114	0.3097	0.046	30396.07	0.0408	0.0408
LNYGVCFCG	0.0216	0.3615	0.067	24349.41	0.0602	0.0602
NYGVCFCGD	0.0071	0.2799	0.028	36931.66	0.0249	0.0249
YGVCFCGDE	0.111	0.6308	0.068	23957.42	0.0745	0.0745
GVCFCGDEN	0.0134	0.3215	0.041	32259.8	0.0369	0.0369
VCFCGDENI	0.0484	0.4577	0.076	21970.92	0.0719	0.0719
CFCGDENIL	0.0175	0.3425	0.095	17888.25	0.0834	0.0834
FCGDENILV	0.0434	0.442	0.06	25982.58	0.0575	0.0575

The same observation was confirmed by the SYFPEITHI server as shown on Table 4.

Table 4. Ranking of predicted of binding of JAK2 V617F derived nonapeptides to HLA-B*35:01 using SYFPEITHI (www.syfpeithi.de). Peptides of interest are shown in bolded fonts.

Peptide	*Score*
LVLNYGVCF	**11**
CFCGDENIL	11
VCFCGDENI	7
FCGDENILV	4
LNYGVCFCG	1
YGVCFCGDE	1
VLNYGVCFC	**0**
NYGVCFCGD	0

GVCFCGDEN	0

Analogous analyses for 9-mer peptides binding to HLA-A*02:01 showed that VLNYGVCF was the top performing peptide according to NetMHCpan 4.0 (though not reaching the arbitrary weak binder level of 2%) (Fig. 9C), NetMHCstab 1.0 (Table 5) and SYFPEITHI (Table 6). On the other hand, none of the mutant peptides reached the NetMHCpan 4.0 binder threshold for HLA alleles positively associated with JAK2 V617F mutation (Figure 9D).

Table 5. NetMHCstab 1.0 Server (http://www.cbs.dtu.dk/services/NetMHCstab/). 9-mer peptides in complex with HLA-A*02:01. The most stably binding peptide is shown in bolded fonts.

Peptide	*Predicted*	*T 1/2 (hours)*	*1-log50k*	*Affinity (nM)*	*Combined*	*Average*
VLNYGVCFC	**0.5645**	**2.4243**	**0.368**	**932.72**	**0.3975**	**0.3975**
FCGDENILV	0.4358	1.6689	0.252	3272.12	0.2796	0.2796
LVLNYGVCF	0.043	0.4406	0.139	11172.82	0.1246	0.1246
VCFCGDENI	0.0851	0.5625	0.102	16673.41	0.0995	0.0995
CFCGDENIL	0.0078	0.2854	0.054	27725.29	0.0471	0.0471
LNYGVCFCG	0.1034	0.6109	0.052	28485.48	0.0597	0.0597
GVCFCGDEN	0.0077	0.2845	0.034	34610.27	0.03	0.03
NYGVCFCGD	0.0051	0.2626	0.027	37333.42	0.0237	0.0237
YGVCFCGDE	0.0051	0.2629	0.021	39622.53	0.0186	0.0186
FCGDENILV	0.0434	0.442	0.06	25982.58	0.0575	0.0575

Table 6. Ranking of predicted of binding of JAK2 V617F-derived nonapeptides to HLA-A*02:01 using SYFPEITHI (www.syfpeithi.de). Peptides of interest are shown in bolded fonts.

Peptide	*Score*
VLNYGVCFC	**17**
CFCGDENIL	13
VCFCGDENI	12
FCGDENILV	12
LVLNYGVCF	**11**
GVCFCGDEN	6
LNYGVCFCG	5
YGVCFCGDE	3
NYGVCFCGD	1

In vivo presentation of HLA-I antigens requires also efficient proteasomal degradation and efficient TAP mediated transport to endoplasmic reticulum (ER). We used NetMHCchop 3.1 Server to

show that the peptide LVLNYGVC**F** can be produced by proteasomal degradation from JAK2 V617F mutant protein (Table 7). Furthermore, the TAPpred tool showed that LVLNYGVC**F** is the best binder of TAP transport proteins among all possible 9-mer peptides harboring the V617F mutation (Table 8).

Table 7. Prediction of proteasomal cleavage using NetMHC chop 3.1 Server with prediction method 20S 3.0 (http://www.cbs.dtu.dk/services/NetChop/). The submitted 29-mer peptide sequence was: KLSHKHLVLNYGVCFCGDENILVGEFVKF. Assumed cleavage sites are those with score above 0.5 (the default of the server). "S" sign in the Cleavage column denotes predicted cleavage site after the marked amino acid site position. "." sign in the Cleavage column denotes that the score for that amino acid is below 0.5 and therefore is not considered a predicted cleavage site after the marked amino acid site position. The score is result of a neural network predicting algorithm and ranges between 0 and 1. Cleavage sites and spanned amino acids of interest are shown in bolded fonts.

Position	*Amino acid*	*Cleavage*	*Score*
1	K	.	0.052129
2	L	S	0.814683
3	S	.	0.108973
4	H	S	0.963449
5	K	.	0.114662
6	H	**S**	0.578722
7	**L**	S	0.575888
8	**V**	S	0.853866
9	**L**	S	0.948302
10	**N**	S	0.548877
11	**Y**	S	0.920869
12	**G**	.	0.207565
13	**V**	S	0.918516
14	**C**	.	0.289169
15	**F**	**S**	0.970047
16	C	.	0.451041
17	G	.	0.107042
18	D	S	0.932481
19	E	.	0.345346
20	N	.	0.214343
21	I	.	0.291945
22	L	.	0.479267
23	V	.	0.412147
24	G	S	0.834033
25	E	.	0.423634
26	F	.	0.104392
27	V	S	0.907699
28	K	.	0.161427

29	F	S	0.741238

Table 8. Prediction of TAP binding for JAK2 V617F derived 9-mer peptides using the TAPPred server (https://webs.iiitd.edu.in/raghava/tappred/). The submitted protein sequence was the 17-mer peptide: LVLNYGVCFCGDENILV. The best binding peptide is shown in bolded fonts.

Rank	*Start Position*	*Sequence*	*Score*	*Predicted Affinity*
1	**1**	**LVLNYGVCF**	**7.368**	**High**
2	3	LNYGVCFCG	3.476	Intermediate
3	8	CFCGDENIL	2.4	Low or undetectable
4	4	NYGVCFCGD	1.173	Low or undetectable
5	7	VCFCGDENI	0.908	Low or undetectable
6	2	VLNYGVCFC	0.673	Low or undetectable
7	9	FCGDENILV	0.296	Low or undetectable
8	6	GVCFCGDEN	-1.908	Low or undetectable
9	5	YGVCFCGDE	-6.656	Low or undetectable

Finally, to assess the dynamics of the binding of LVLNYGVC**F** to HLA-B*35:01 we performed a 10 ns MDS of the binding using QwikMD (Figure 12B-D). Notably, during simulation RMSD for the alpha carbon atoms of the HLA-B*35:01 molecule, beta-2-microglobulin (B2M) and LVLNYGVC**F** remained stable around the value of the 2 Ångtröms (Å) (Figure 12A). As a comparator simulation we used another V617F derived nonamer VLNYGVC**F**C, which is a predicted non-binder of HLA-B*35:01. In this simulation the RMSD values for HLA-B*35:01 and B2M remained stable around 2 Å, but the RMSD values of the peptide reached almost 4 Å around 5 ns and remained around that value during the entire simulation (Figure 12A) providing a significantly higher RMSD values as compared to the binder LVLNYGVC**F** (Figure 12B). The analogous analysis of the binding of the two peptides to HLA-A*02:01 (Figure 12C,D) showed higher RMSDs for HLA-A*02:01 and peptides as compared to the HLA-B*35:01-peptide complexes. Notably, RMSD values were higher for HLA-A*02:01 molecule when in complex with LVLNYGVC**F** as compared to the complex with VLNYGVC**F**C (Figure 12D). The same held through for the peptides themselves, i.e. LVLNYGVC**F** had higher RMSD values than VLNYGVC**F**C (Figure 12D).

Collectively, these data suggest that the neoantigen LVLNYGVC**F** fulfills all the requirements for effective antigen processing and presentation by the specific HLA-B*35:01 molecule. HLA-A*02:01 is likely to present VLNYGVC**F**C, which however is not less efficiently produced after proteasomal cleavage and poorly transported to the ER.

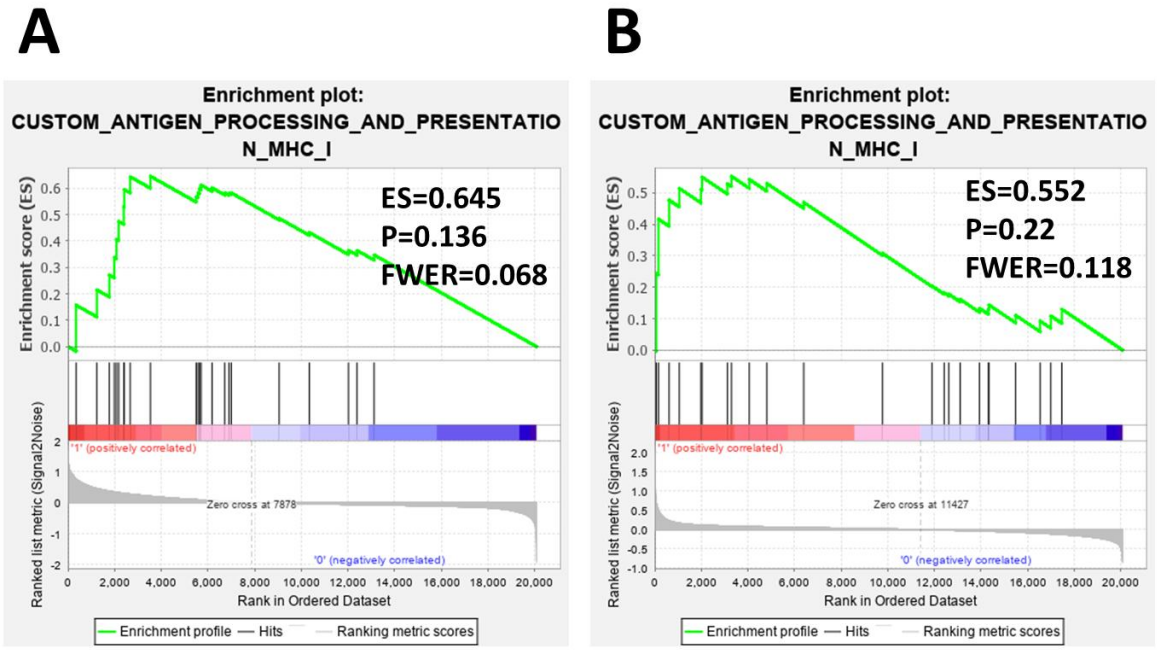

Figure 13. GSEA analysis of MHC-I antigen processing and presentation pathway genes in CD34+ SCs from MPN patients and healthy controls. (A) PB CD34+ SCs from PMF patients vs. PB CD34+ SCs from healthy controls; **(B)** BM CD34+ SCs from PV/ET patients vs. BM CD34+ SCs from healthy controls.

JAK2 V617F positive CD34+ cell down-regulate MHC class I pathway molecules

As our top predicted protective allele *HLA-B*35:01* was not absolutely depleted in JAK2 V617F-positive MPN patients, we reasoned that several mechanisms responsible for the escape of the transformed JAK2 V617F+ HSCs from *HLA-B*35:01*-mediated immunoediting might exist. The most reasonable explanation would be that, on many occasions, MPN stem cells (MPN-SCs) downregulate the expression of molecules involved in antigen processing and presentation via the HLA-I pathway. Therefore, we specifically investigated the expression of HLA-I molecules by CD34+ cells in PMF, PV,

and ET patients versus healthy controls. We used publically available data sets and selected MHC class I antigen processing and presentation pathway genes (KEGG database).

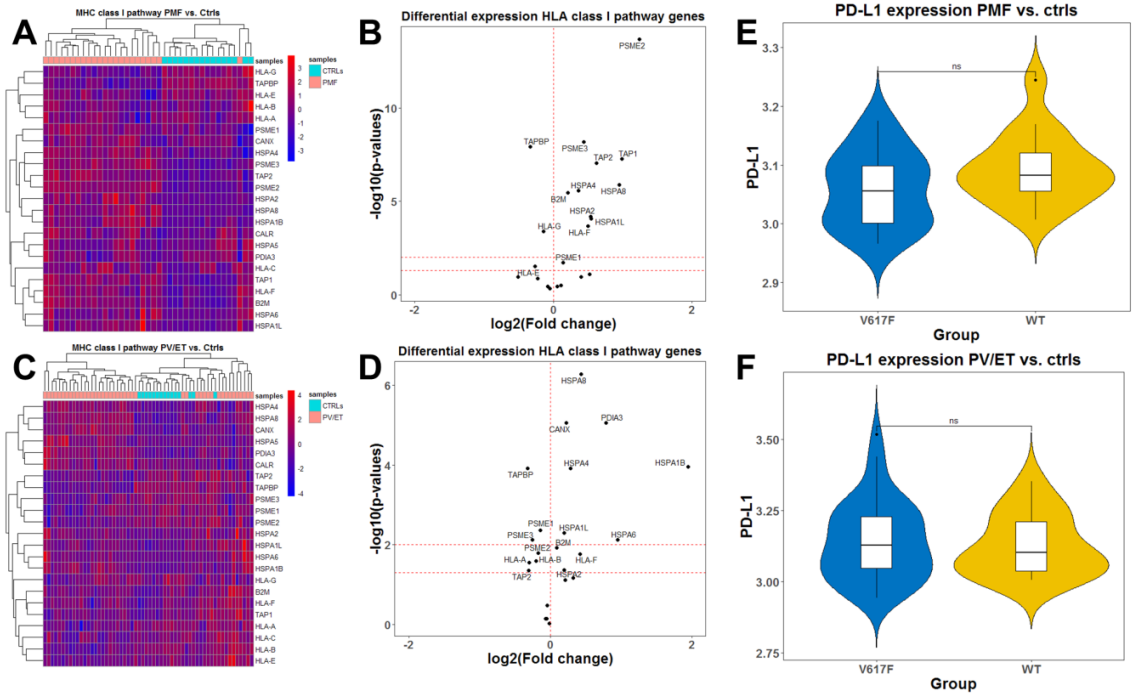

Figure 14. Gene expression profiles of CD34+ cells from MPN patient and healthy controls. (A) Heatmap of MHC class I pathway genes in CD34+ cells from PMF patients and controls. **(B)** Volcano plot of the differentially expressed MHC class I pathway genes in CD34+ cells from PMF patients and controls. **(C)** Heatmap of MHC class I pathway genes in CD34+ cells from PV/ET patients and controls. **(D)** Volcano plot of the differentially expressed MHC class I pathway genes in CD34+ cells from PV/ET patients and controls. **(E)** Comparison of the level of expression of *PD-L1* in CD34+ cells from PMF patients vs. controls. P-values are from a two-sided t-test. **(F)** Comparison of the level of expression of *PD-L1* in CD34+ cells from PV/ET patients vs. controls. P-values are from two-sided t-tests. Horizontal red dashed lines in **(B)** and **(D)** represent significance levels of 0.05 and 0.01. P-values designation in **(E)** and **(F)**: ns – $p > 0.05$, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$.

GSEA analysis did not show any statistically significant enrichment of the gene expression profile of peripheral blood (PB) CD34+ of PMF patients in comparison to the gene expression profile of PB CD34+ from healthy controls (Fig. 13A). These findings were confirmed in regards to the expression of individual *HLA-A, -B* and *–C* genes using the standard analysis of the differential gene expression through the *limma* algorithm (Fig. 14A,B). The identical analysis showed statistically

significant up-regulation of only one gene, which is directly involved in the antigen presentation by non-classical HLA-I molecules, namely tapasin (*TAPBP*) (Fig. 14A,B).

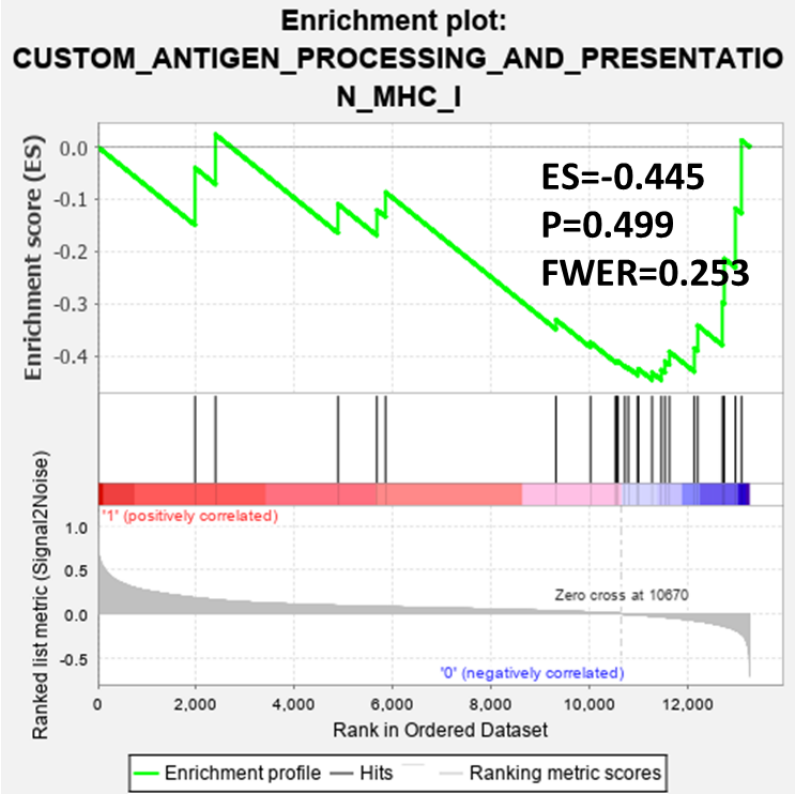

Figure 15. GSEA analysis of MHC-I antigen processing and presentation pathway genes in PB granulocytes from MPN patients and healthy controls.

On the other hand, bone marrow (BM)-derived CD34+ cells from PV and ET had a downregulation of key components of antigen processing, transport, loading, and presentation machinery, such as *PSME1, PSME2, PSME3, TAP1, TAPBP, HLA-A*, and *HLA-B* (Fig. 14C,D). Gene expression profile, however, was not statistically enriched with genes from that pathway (Fig. 13B). Finally, PB CD34+ from PV patients did not exhibit a differential expression of HLA-I pathway genes compared with normal bone marrow cells when another data set was analyzed (data not shown).

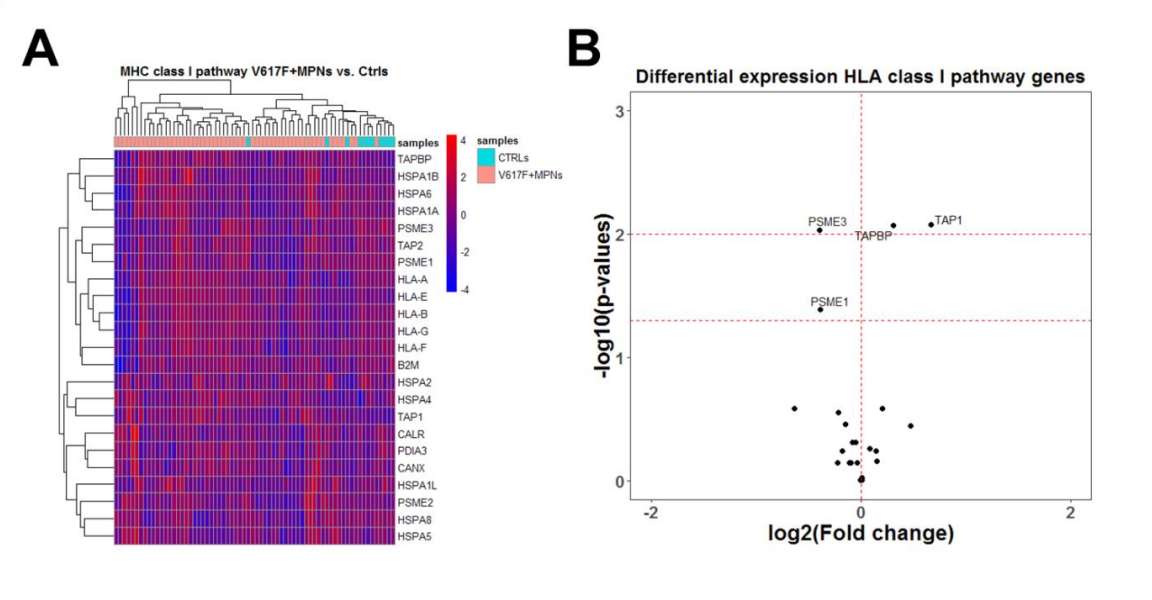

Figure 16. Gene expression profiles of granulocytes from JAK2 V617F+ MPN patient and healthy controls. (A) Heatmap of HLA-I pathway genes in granulocytes from MPN patients and controls; (B) Volcano plot of the differentially expressed HLA-I pathway genes in granulocytes from MPN patients and controls.

Analogous GSEA analysis of PB granulocytes from JAK2 V617F+ MPN patients vs. normal controls did not show significant enrichment with genes from the MHC-I antigen processing and presentation pathway (Fig. 15). Differential gene expression analysis using limma revealed an up-regulation of only two HLA-I pathway genes (*TAP1* and *TAPBP*) (Fig. 16.). Prestipino et al. (2018) reported that JAK2 V617F+ megakaryocytes, monocytes, and platelets can escape immunoediting because of up-regulation of *PD-L1* gene.

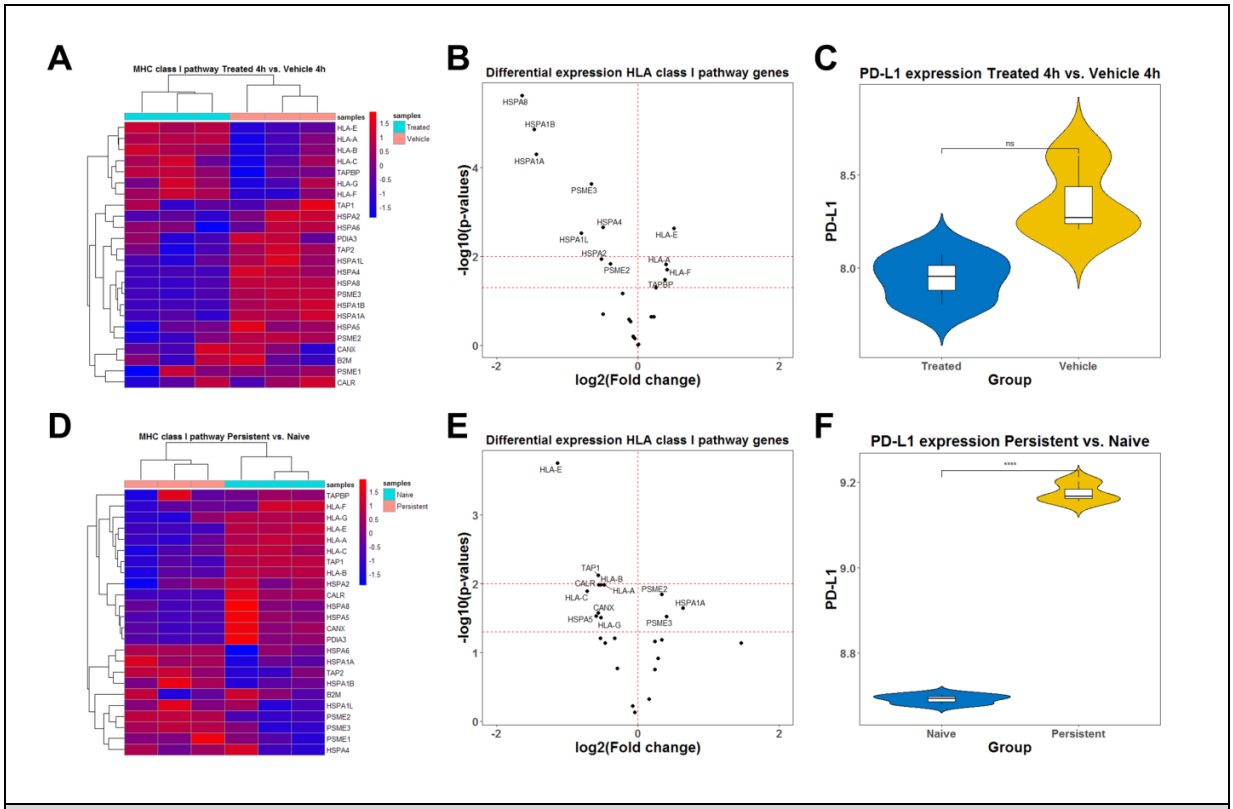

Figure 17. Effects of treatment with ruxolitinib (INC424) on gene expression profiles in SET-2 cell line. (A) Heatmap of MHC class I pathway genes expression in SET-2 cells treated with ruxolitinib vs. vehicle for 4 hours. **(B)** Volcano plot of the differentially expressed MHC class I pathway genes in SET-2 cells treated with ruxolitinib vs. vehicle for 4 hours. **(C)** Comparison of the level of expression of *PD-L1* in SET-2 cells treated with ruxolitinib vs. vehicle for 4 hours. P-values are from a two-sided t-test. **(D)** Heatmap of MHC class I pathway genes in ruxolitinib persistent vs. naïve SET-2 cells. **(E)** Volcano plot of the differentially expressed MHC class I pathway genes in ruxolitinib persistent vs. naïve SET-2 cells. **(F)** Comparison of the level of expression of *PD-L1* in ruxolitinib persistent vs. naïve SET-2 cells. P-values are from two-sided t-tests. Horizontal red dashed lines in **(B)** and **(E)** represent significance levels of 0.05 and 0.01. P-values designation in **(C)** and **(F)**: ns – $p > 0.05$, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$.

We did not find significant JAK2 V617F-dependent expression of *PD-L1* in either PMF CD34+ cells or bone marrow PV/ET CD34+ cells *versus* normal controls (Fig. 14E,F). These findings suggest that at least part of the JAK2 V617F+ clone may escape cytotoxic T cell-mediated immunoediting at the MPN-SC level, irrespective of their *HLA-I* genotype, because of the downregulation of the HLA-I antigen processing and presentation machinery.

Resistance to ruxolitinib might be associated with down-regulation of HLA-I molecules and up-regulation of PD-L1

We further analyzed the differential gene expression profiles of naïve, short-term ruxolitinib treated and long-term treated (persistent) human CD34+ SET-2 cell line harboring heterozygous JAK2 V617F mutation. Notably, short-term ruxolitinib treatment was associated with up-regulation of some of genes from HLA-I antigen processing and presentation pathway (*HLA-A*, *HLA-E*, *HLA-F* and *TAPBP*) (Fig. 17A, B) and statistically insignificant increase of *PD-L1* expression (Fig. 17C). On the other hand, SET-2 cells persistent to long-term ruxolitinib treatment showed down-regulation of *HLA-A*, *HLA-B*, *HLA-C*, *HLA-E*, *HLA-G*, *CALR*, *TAP1* and *HSPA5* (Fig. 17D, E) and significant *PD-L1* up-regulation as compared to the untreated cell line (Fig. 17F).

Effects of IFN-α on MHC-I molecules and Pdl1 in mouse cells

We further analyzed publically available dataset for the effect of IFN-α on gene expression in JAK2 V617F mouse stem cells. Czech et al. (2019) deposited 2 samples of mouse 32D cells with artificial JAK2 V617F expression treated with either IFN-α or vehicle. Short-term treated cells showed up-regulation of most of the genes of the MHC-I pathway and *Cd274* (*Pdl1*) (Fig. 6A). Mullaly et al. (2013) provided gene expression data of mouse long-term HSCs after long-term with IFN-α or vehicle *in vivo*. Notably, there was no significant change in the gene expression of MHC-I genes and *Cd274* (*Pdl1*) (Fig. 6B-D).

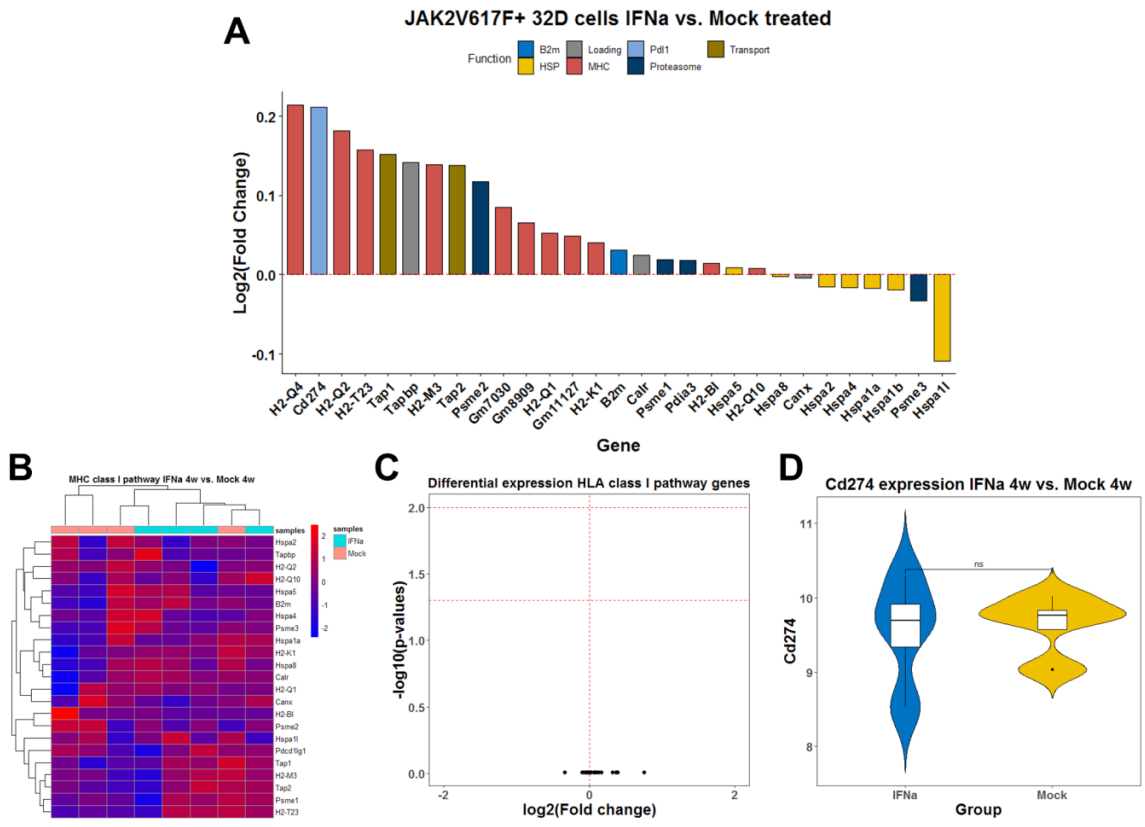

Figure 18. Effects of IFN-α on gene expression profiles in mouse JAK2 V617F+ HSCs *ex vivo* and *in vivo*. (A) Waterfall plot of the difference (calculated as log2 (fold change)) in MHC class I pathway genes expression in JAK2V617F+ 32D cell line treated with IFN-α vs. mock treated. No statistical inference tests were performed as this plot is based on only 2 publically available samples. **(B)** Heatmap of MHC class I pathway genes expression in mouse Jak2V617F+ long-term HSC isolated after 4 weeks treatment with mouse IFN-α or vehicle of chimeric recipient mice. **(C)** Volcano plot of the differentially expressed MHC class I pathway genes in mouse long-term HSC isolated after 4 weeks treatment with mouse IFN-α or vehicle of chimeric recipient mice. **(D)** Comparison of the level of expression of *Pdl1* (*Cd274*) gene in mouse long-term HSC isolated after 4 weeks treatment with mouse IFN-α or vehicle of chimeric recipient mice. P-values are from a two-sided t-test. P-values designation: ns – $p > 0.05$, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$.

V. Discussion

A growing body of evidence suggests that a number of tumors might be rejected through elucidation of adaptive T cell mediated responses against neoantigens. This implies that the HLA genotype of a given individual may shape cancer genome as has been shown in a number of malignancies with specific mutations. The viable immunoediting mechanism of early oncogenesis also implies the existence of MHC alleles protective for the development of cancers harboring specific mutations. A notable example for protective MHC alleles is the restriction of virus driven cancers in mammalian species. A similar example in human cancers is the well documented association of HLA-A*02 with decreased risk for Epstein-Barr virus-positive classical Hodgkin lymphoma. However, in general hematological malignancies are characterized by a relatively lower mutational burden thereby being less likely to produce immunogenic neoepitopes. Spontaneous CD8+ T cell immune responses have been documented in several studies for NPM1 mutated acute myeloid leukemia (AML) and in chronic myelogenous leukemia (CML). Several studies proposed the existence of protective HLA-I alleles for CML oncogenesis presumably because of their ability to present neoantigens resulting from the BCR-ABL1 fusion. However, it was recently shown that BCR-ABL1 derived peptides could not be detected in the HLA-I ligandome of patients with overt CML, which might be interpreted as a lack of presentation of BCR-ABL1 derived peptides or as an evidence for foregoing immunoediting.

An interesting question is whether JAK2 V617F-driven oncogenesis follows our concept regarding cancer hallmarks. Indeed MPNs and CHIP are characterized by different levels of phenotypic heterogeneity, which is assumed to be due to several groups of factors including the type of the driver mutation, presence and the order of acquisition of additional driver mutations, mutant allele burden (at least for JAK2 V617F mutation), the inherent heterogeneity of the affected pool HSCs pool, microenvironmental factors and germ-line predisposition. The firmly established germ-

line genetic factors predisposing to the development of JAK2 V617F+ MPNs is the so called JAK2 46/1 genotype. However, the mechanism through which this genotype contributes to the acquisition of JAK2 V617F mutation remains unclear, but in many cases the acquisition of JAK2 V617F mutation takes place early during ontogenesis.

The subsequent evolution of the JAK2 V617F+ cell clone to a large extend follows the principles for diversification and selection of the acquired hallmarks, which ensure competitive advantage until the capability to sustain long-term self-renewal is achieved. The initial genetic diversification most probably is a consequence of the slightly enhanced replicative stress due to the mildly increased proliferative signaling conferred by the mutated JAK2 V617F tyrosine kinase. Besides it has been demonstrated that JAK2 V617F causes DNA replication fork stalling and suppression of the apoptotic response to the increased genotoxic stress via negative regulation of p53. In that way a pool of genetically diverse myeloid stem and progenitor cells can be established, which depending on internal or external stimuli can differentiate into different lineages and thus lead to the variable phenotypic presentations of overt MPNs. A number of studies support this model due to the demonstrated clonal diversification in myelofibrosis evolution. Obviously, the process of clonal evolution can be influenced by germ-line immunogenetic factors such as the genotype of the canonical and non-canonical histocompatibility genes.

Here we set out to provide evidence for immunoediting in JAK2 V617F driven MPNs as proposed by the existence of immune response directed against mutant calreticulin in healthy individuals. We hypothesized that if immunoediting in JAK2 V617F positive MPNs is taking place during the early stages of leukemogenesis then there should exist protective HLA-I alleles able to present JAK2 V617F derived peptides. To address this question we analyzed the HLA-I genotype of 139 MPN patients and 622 healthy controls of Caucasian origin from the Bulgarian population. As it has been recently shown that the entire HLA-I genotype could contribute to protection from cancers harboring specific mutations we extensively studied the diversity of the HLA genotypes of MPN

patients and controls. There was no difference in the frequency of homozygosity at any locus between patients and controls. Furthermore, we assessed quantitatively the evolutionary divergence between the alleles at any locus as well as the mean HED for all three loci for each subject. There appeared to be no significant difference in locus specific and mean HEDs between patients and controls. This metrics, however, may not reflect the true contribution to the restriction of tumor growth driven by a single specific mutation. Therefore, we assessed the predicted ability of the HLA-I genotype of any subject to present peptides derived from JAK2 V617F following exactly the procedure developed by Marty et al. (2017). There was no difference in the estimated PHBRs in patients and healthy controls. Collectively, these data suggested that the global HLA-I genotype and its diversity have little if any protective role for development of JAK2 V617F driven MPNs.

We further analyzed the frequency of specific HLA-I alleles in MPN patients and controls. We sought to control for the imbalance in the age distribution between the two groups and the gender as a known factor for development of MPNs. In order to achieve this, we fitted additive generalized linear models with age and gender as covariates. Using this approach we were able to identify 3 HLA-I alleles (*HLA-A**02:01, *HLA-B**35:01 and *HLA-C**15:02) that were significantly less frequent in MPNs as compared to the controls. This finding does not contradict the recent meta-analysis of genome wide association studies (GWAS), which did not identify protective SNPs for MPN within the MHC locus [49] as GWAS studies are known for their lower sensitivity to detect associations with highly polymorphic loci such as HLA. Besides, our data cannot be compared with data from several previous small studies which used serological techniques to investigate the association between HLA and MPNs.

The HLA-B*35:01 molecule was predicted to specifically bind a 9-mer peptide (LVLNYGVC**F**) derived from JAK2 V617F protein. The identification of *HLA-A**02:01 as a protective allele for JAK2 V617F driven MPN may not be considered surprising. Recently, Holmstrom et al. (2017) demonstrated the induction of specific CD8+ T cells HLA-A2 positive healthy individual by the

VLNYGVC**F**C peptide. Of note, another group using an RNA sequencing and *in silico* prediction of binding peptides to the most frequent HLA alleles failed to identify JAK2 V617F-derived neoepitopes in an MPN cohort of mixed genetic background. However, their MPN cohort showed depletion of HLA-B*35:01 carriers, which is consistent with our HLA-genotyping data. Another indirect evidence supporting the protective role of HLA-B*35:01 is its absence in all JAK2 V617F+ cell lines from the Cancer Cell Line Encyclopedia. Finally, we used an MDS approach to demonstrate that HLA-B*35:01 can bind LVLNYGVC**F** but not VLNYGVC**F**C, whereas the HLA-A*02:01 seems to be more stable in complex with VLNYGVC**F**C than with LVLNYGVC**F**. Prediction tools also showed that LVLNYGVC**F** can be derived after proteasomal degradation of the native JAK2 V617F protein and can be efficiently transported the TAP system thus fulfilling all the prerequisites for a neoantigen. These analyses collectively suggest that *HLA-B**35:01 may be a protective allele for development of JAK2 V617F driven MPNs by the presentation of the neoantigen LVLNYGVC**F**. Probably HLA-A*02:01 could also present a JAK2 V671F derived peptide but that peptide is less likely to be produced by the proteasome and transported to the ER. Besides, HLA-B*35:01 may exert its protective role because of other properties. For instance, Geng et al. (2018) showed that HLA-B*35:01 can be expressed on cell surface as empty conformers and therefore can enhance the strength of the immunological synapses between antigen presenting cells and CD8+ T cells. Interestingly some studies showed increased expression of open *HLA-I* conformers on the cell surface of lymphocytes and monocytes from PV patients. Another intrinsic feature of HLA-B*35:01 and HLA-C*15:02 alleles is their relative independence of tapasin mediated peptide loading making them less amenable in terms of antigen presentation to low levels of tapasin. The latter property might be of particular importance as many cancer types evade immune surveillance through mutation MHC-I molecules or down-regulation of various members of MHC-I mediated antigen processing and presentation pathway. This effect might be even more pronounced for some HLA alleles including *HLA-B**35:01 with intrinsically lower cell surface expression. In line with this a previous study demonstrated more frequent down-regulation of HLA-A and HLA-Bw6 (such as HLA-B*35:01 and HLA-B*41:01) but not of HLA-Bw4 (such as HLA-

B*44:03) allo-specificities by leukemia cells. Furthermore, MPN patients have been shown to have low levels of HLA class I and class II molecules on PBMCs but they are up-regulated after treatment with interferon-alpha2. Such interferon-dependent increase in *HLA-I* and *-II* genes expression has been demonstrated in AML and ALL blasts as well. Previous studies showed that JAK2 V617F mutations occur at the level of HSCs compartment. Therefore, the putative active immunosurveillance in JAK2 V617F+ MPNs should also take place at that level, which prompted us to specifically assess the levels of expression of MHC-I pathway genes in CD34+ in PMF, PV and ET patients. Of note, bone marrow CD34+ cells from PV and ET patients showed significant down-regulation of a number of genes including *HLA-A*, *HLA-B* and *TAPBP*. This observation suggests that indeed in the settings of low expression alleles with other properties such as HLA-B*35:01 would be more likely to play protective role. However, it remains unclear how many HLA molecules are expressed on the surface of malignant CD34+ cells as recent studies showed a broad range. Furthermore, it is likely that CD34+ cell from peripheral blood and bone marrow and spleen have variable expression of HLA-I pathway genes as demonstrated previously and are therefore differentially targeted by cytotoxic lymphocytes. It is also unclear how many HLA molecules need to express a specific neoantigen to be effectively recognized by a T cell with cognate TCR but some experimental evidence suggests that the number might be truly minimal. Some reports proposed that MPNs can escape immune surveillance through direct up-regulation of PD-L1. Prestipino et al. (2018) demonstrated such JAK2 V617F mediated up-regulation in megakaryocytes and monocytes as well as depletion of JAK2 V617F+ CD34+ in a single MPN patient treated with nivolumab after allogeneic transplantation. We did not observe significant up-regulation of PD-L1 in CD34+ cells from MPN patients, which, however, does not preclude the existence of such mechanism for immune escape in MPNs.

Positive association of the presence of JAK2 V617F was another finding of our analyses. A plausible explanation for this observation would be that these HLA molecules mediate immunoediting in MPNS through interaction with their respective NK cell receptors such as inhibitory

or activating killer immunoglobulin like receptors (KIR) in a manner similar to that demonstrated for CML. The initial observations that INF-α treatment can reduce JAK2 V617F mutant allele burden bring hope for the successful development of immunotherapeutic approaches in MPNs including combinatorial approaches and specific vaccination. Notably, a recent phase 2 study showed that combination therapy with JAK2 inhibitor (ruxolitinib) and IFN-α led to significant reduction of JAK2 V617F allele burden presumably because of non-overlapping effects on MPN-SCs. This is consistent with our analyses demonstrating that short term treatment of human and mouse JAK2 V617F+ cells with either ruxolitinib or IFN-α caused up-regulation of multiples MHC-I pathway genes. However, cells persistent to ruxolitinib treatment down-regulated HLA genes and up-regulated *PD-L1* expression consistent with a previous report. It is unclear whether this effect is contributing to ruxolitinib resistance *in vivo* as other pathways may be more important. The same principle holds true for IFN-α treatment, i.e. short-term treatment may cause up-regulation of MHC-I pathway genes rendering MPN-SCs susceptible to immunoediting, whereas resistant MPN-SC fail to up-regulate these genes and escape T cell killing. Based on our findings we can propose a model for specific HLA-I alleles-mediated restriction of JAK2 V617F-driven oncogenesis in MPNs (Fig. 19).

Finally, our observations have application to the development of exogenous proteins based vaccines in JAK2 V617F positive MPNs. Effective cross-presentation of exogenous peptides might be limited to a subset of patients with specific HLA alleles as shown in recent trials in which calreticulin mutant peptides were observed to elicit predominantly CD4+ T cell responses. It is rational to propose that in such patients, administration of IFN-α or even ruxolitinib might elicit a more pronounced immune response initially. Furthermore, the presence of *HLA-B**35:01 and *HLA-A**02:01 might appear to be a predictive marker for response to immunomodulatory therapy.

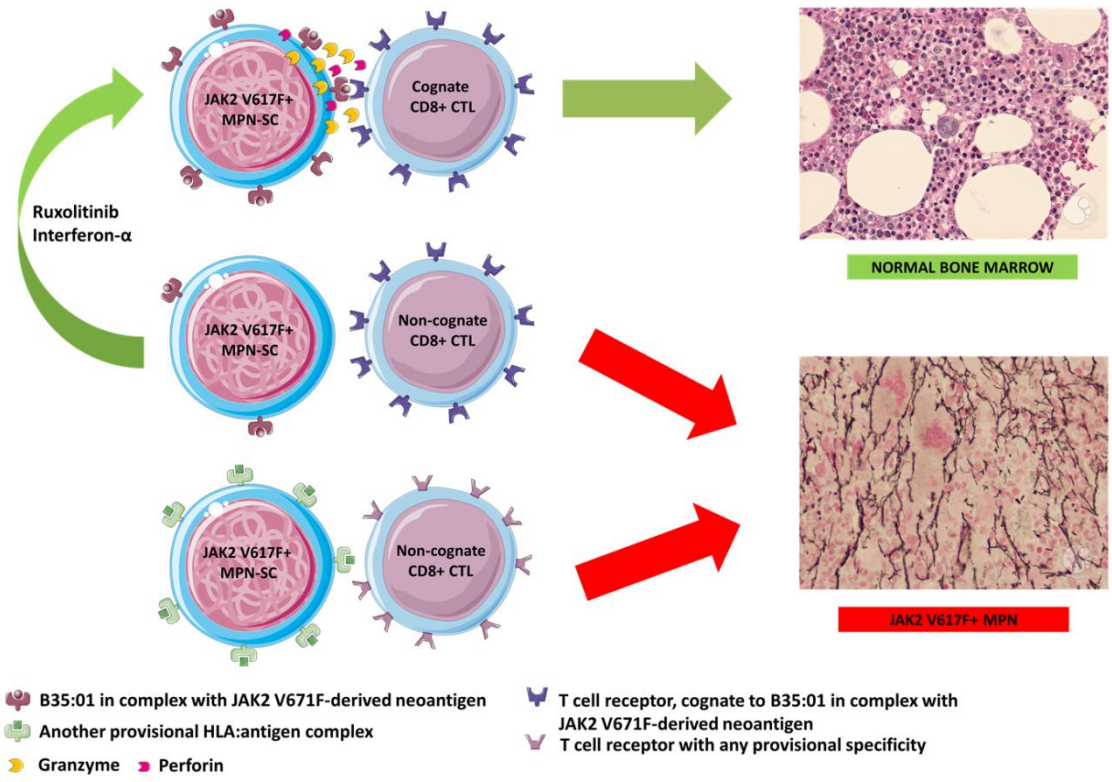

Figure 19. Proposed model for HLA-mediated immunoediting in JAK2 V617F+ MPNs. JAK2 V617F-mutated MPN-SCs, which express HLA-B*35:01 molecules on their surface can present the LVLNYGVCF peptide to cognate T-cytotoxic lymphocytes with specific T cell receptors, which recognize the complex HLA-B*35:01: LVLNYGVCF and can establish immune synapse. This leads to activation and degranulation of the cognate T cells leading to the killing of the respective transformed MPN-SCs and no JAK2 V617F+ MPN develops. If the JAK2 V617F+ MPN-SC expresses low levels of HLA-B*35:01 it is likely that it would not be recognized by a cognate T cell and would not be eliminated and thereby a clinically overt JAK2 V617F+ MPN will develop. Interferon-alfa and ruxolitinib treatment can lead to increased expression of HLA-I molecules and can eventually lead to clinical improvement and restriction of the neoplastic clone in the bone marrow or the sites of extramedullary hematopoiesis. Finally, MPN-SCs, which express HLA-I molecules not able to present mutant JAK2 V617F-derived peptides are not recognized by cognate T cytotoxic lymphocytes and a clinically overt disease develops. Bone marrow images are from ASH Image Bank.

VI. Conclusion

Pursuing the goal of this work we completed the preset research tasks and based on that one can conclude the following:

- Some HLA class I alleles (such as *HLA-B*35:01*) can restrict the development of JAK2 V617F-driven myeloproliferative neoplasms;
- Stem cells in MPNs can escape HLA-mediated immunoediting through down-regulation of genes from HLA-I antigen processing and presentation pathway;
- Some medications can up-regulate the expression of genes from HLA-I antigen processing and presentation pathway in MPN-SCs thereby enhancing the primary therapeutic response.

Our findings raise a number of questions, which could be addressed in future investigations. Some of those questions are:

- Can such association be detected in other cohorts of patients and healthy controls?
- Do protective HLA alleles exist for other MPN subtypes such as those with mutations in *CALR* and *MPL* genes?
- What is the role of other immunogenetic factors (e.g. *HLA-II* genes, *KIR* genes, NKG2D ligands genes, etc.) in MPNs?
- Do immunogenetic factors have predictive power in MPNs?
- How can be the elucidated mechanisms for immunosurveillance escape be harnessed to design immunotherapeutic approached in MPNs?

VII. Contributions

Original contributions

1. Evidence that heterozygosity and evolutionary divergence of the personal HLA genotype are not associated with the development of JAK2 V617F-positive MPNs.
2. Evidence that the theoretical ability of the individual HLA-I genotype to present mutant JAK2 V617F-derived epitopes does not differ between MPN patients and healthy subjects.
3. Identification of some HLA-I alleles and HLA-I haplotypes, which are depleted (potentially protective) or enriched (potentially predisposing) in JAK2 V617F+ MPN patients in comparison to healthy controls.
4. A series of bioinformatics evidence in support of the feasible and probable presentation of the neoantigen LVLNYGVCF in the context of HLA-B35:01 complexes.
5. Evidence that in some cases JAK2 V617F+ MPN-SCs downregulate the expression of key genes from MHC-I antigen processing and presentation pathway.
6. Evidence that ruxolitinib treatment persistent JAK2 V617F+ CD34+ cell lines downregulate the expression of key genes from MHC-I antigen processing and presentation pathway.
7. Proposed model for HLA-I-mediated immunoediting of the early stage oncogenesis in JAK2 V617F+ MPNs.

Confirmatory findings

1. Confirmation that short-term treatment of JAK2 V617F+ MPN-SCs with interferon-alfa leads to increased expression of key genes from the MHC-I antigen processing and presentation pathway.
2. Confirmation that ruxolitinib treatment persistent JAK2 V617F+ CD34+ cell lines show up-regulation of *PD-L1* gene expression.

VIII. Declaration of original work

The author of this PhD thesis declares that all described data is original and obtained due to his research work as an Associate Professor in Genetics at Sofia University on the following research projects:

„Immunogenetic factors in the cancer immunosurveillance“, Faculty of Medicine, Medical University – Sofia, Bulgaria (supported be NSF grant, Contract КП-06-Н41/2 from 30.11.2020).

„Immunomodulation of neoplastic diseases by non-classical HLA molecules – MIC“, Faculty of Medicine, Medical University – Sofia, Bulgaria (supported be NSF grant, Contract КП-06-Н23/4 from 17.11.2018).

“Multidisciplinary approach to the development of a novel high-throughput, liquid, bead-based “array” method for detection of mutations associated with myeloproliferative neoplasms”, Faculty of Medicine, Medical University – Sofia, Bulgaria (supported be NSF grant, Contract ДИД 02/5 from 2009).

All data and its interpretation were published in the accompanying scientific publications. All used bibliographic sources were cited accordingly.

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IX. Publications

Journal articles

1. Ivanova M, Shivarov V. HLA genotyping meets response to immune checkpoint inhibitors prediction: A story just started. Int J Immunogenet. 2021 Apr;48(2):193-200.

https://onlinelibrary.wiley.com/doi/full/10.1111/iji.12517

2. Ivanova M, Tsvetkova G, Lukanov T, Stoimenov A, Hadjiev E, Shivarov V. Probable HLA-mediated immunoediting of JAK2 V617F-driven oncogenesis. Exp Hematol. 2020 Dec;92:75-88.e10.

https://www.sciencedirect.com/science/article/pii/S0301472X20305658

3. Alkhazraji A, Elgamal M, Hui Ang S, Shivarov V, All cancer hallmarks lead to diversity, International Journal of Clinical and Experimental Medicine, 2019 Jan; 1 (12):132-157.

https://e-century.us/files/ijcem/12/1/ijcem0072581.pdf

Book chapters

1. Shivarov, V., 2022. Asking Existing Data the Right Questions: Data Mining as a Research Option in Low-and Middle-Income Countries. In *Improving Oncology Worldwide* (pp. 69-74). Springer, Cham.

Scientific meetings

1. V. Shivarov, G. Tzvetkova, T. Lukanov, A. Stoimenov, E. Hadjiev,M. Ivanova. HLA CLASS I GENOTYPE SHAPES JAK2 V617F DRIVEN LEUKEMOGENESIS. 25th Congress of the European Hematology Association. Virtual Edition. 2020.

2. Milena Ivanova , Gergana Tzvetkova, Tzvetelin Lukanov, Antoaneta Nedyalkova, Angel Stoimenov, Elissaveta Naumova, Evgueniy Hadjiev, Velizar Shivarov. MHC CLASS I GENOTYPE SHAPES JAK2 V617F DRIVEN LEUKEMOGENESIS Joint 34th European Immunogenetics and Histocompatibility and 31st British Society for Histocompatibility and Immunogenetics Conference Glasgow, Scotland, United Kingdom, April 26-29, 2020

3. Иванова М, Цветкова Г., Луканов Ц., Алхадра Б., Стоименов А., Хаджиев Е., Шиваров В. ОТКРИВАНЕ НА HLA КЛАС I АЛЕЛИ, ПРОТЕКТИВНИ ЗА РАЗВИТИЕТО НА JAK2 V617F ПОЛОЖИТЕЛНИ МИЕЛОПРОЛИФЕРАТИВНИ НЕОПЛАЗМИ. Единадесети национален конгрес по хематология. 10-13 октомври 2019.