Impact of Immunogenetic Polymorphisms in Bone Marrow Failure Syndromes

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Abstract: *Aim*: To explore whether predisposition to bone marrow failure syndromes (BMF), such aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH) and myelosysplastic syndromes (MDS), is found in killer cell immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA) ligand (KIR-L) gene variations or cytokine polymorphisms.

Patients: We studied a cohort of 77 patients with AA, 129 with MDS and 285 healthy controls for the frequencies of KIR-L and KIR genotypes and 22 selected single nucleotide polymorphisms (SNPs) located within 10 cytokine (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL12, IFN-γ, TNF-α, TGF-β) and 3 cytokine receptor (IL-1R, IL-1RA, IL-4Rα) genes.

Results: In AA we found a decreased frequency of inhibitory KIR-2DL3 genes. In MDS, no difference in the frequency of KIR genotype was identified; however, a decreased frequency of 2DL3 was found in hypocellular MDS. Analysis of the KIR genotype in correlation with the corresponding KIR-L profile, revealed a decreased frequency of stimulatory 2DS1/C2 mismatch both in AA and MDS. In AA and MDS cohorts, compared to controls, we found a higher frequency of TT codon 10 variant and of GG codon 25 variant of TGF-β gene, consistent with a high secretory phenotype. This relationship was even more pronounced in PNH and hypocellular MDS. We confirm that the hypersecretory genotype T/T at position -874 of INF-γ gene was overrepresented only in AA and correlates with presence of a PNH clone. Instead in MDS patients, the frequency of G/A polymorphism at position –308 on the TNF-α gene promoter, which correlates with higher TNF-α production, was found significantly higher. Moreover, hypocellular MDS was characterized by a higher prevalence of IL-10 GCC/GCC haplotype, which is functionally associated with a low secretor phenotype.

Conclusion: Our findings suggest that alterations in KIR/KIR-L matching, such as increased 3DL2 and decreased 2DS1 mismatch, and in the polymorphisms of TGFβ1, IFN-γ, TNF-α and IL-10 may account for the propensity to immune-mediated killing of hematopoietic stem cells and/or ineffective hematopoiesis characteristic of AA and MDS. Further studies are needed to elucidate whether these immunogenetic traits may be involved in increased risk of developing immune-mediated BMF.

Keywords: Aplastic anemia, myelodysplastic syndromes, cytokine polymorphisms, KIR-KIR-L.

INTRODUCTION

Idiopathic aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH) and some variants of myelodysplastic syndrome (MDS) share similar pathogenic mechanisms underlying hematopoietic stem cell (HSC) injury [1-5]. Cytotoxic T lymphocytes (CTLs), T helper 1 (Th_1) lymphocytes and their cytokine products such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) or Fas-induced apoptosis are considered the main effector mechanisms of immune-mediated suppression of hematopoiesis in bone marrow failure (BMF) syndromes [6-14].

Despite progress in the understanding of the immune pathophysiology of AA, PNH and MDS, there is still considerable conflict and uncertainty concerning the immunogenetic background responsible for susceptibility to these diseases. It is well established that highly polymorphic genes of the major histocompatibility complex (MHC), of killer-cell immunoglobulin-like receptor (KIR) and various cytokines and cytokine receptors may influence both genetic susceptibility and resistance to several autoimmune diseases, including BMF syndromes [15-24]. An association of AA, PNH and MDS with specific HLA alleles, such as HLA-DR15, has been reported in patients responsive to immunosuppressive therapy, suggesting that HSC-linked autoantigen may trigger HLA-restricted immune reaction eventually leading to BMF [9, 25,26].

Interactions between HLA class I molecules and KIR regulate the development and response of NK cells to kill infected and transformed cells while sparing normal self cells. They mediate these activities through direct killing of transformed or infected cells and production of cytokines such as IFN-γ and TNF-α [27]. KIRs are a family of about 15 closely linked genes located on chromosome 19q13.4, which

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encode inhibitory and activating receptors expressed by NK cells and by subpopulations of gammadelta$^+$ and alfa/beta$^+$ T cells [28,29]. KIRs contain 2 or 3 Ig-like domains with long (2DL, 3DL) or short (2DS, 3DS) cytoplasmic tails which mediate inhibitory and stimulatory signals, respectively. A higher propensity to autoimmunity has been linked to fewer KIR ligands and inhibitory KIR receptors [30-33]. Consistent with these findings, decreased frequency of KIR-2DS1 and KIR-2DS5 genotypes was found in BMF patients [34]. The interaction of inhibitory or stimulatory KIR variants with their matching KIR-L modulates the immune cytotoxic responses. Combinations of certain KIR–L with KIRs have been linked with susceptibility to autoimmune diseases such as rheumatoid arthritis, scleroderma and diabetes [35]. In the setting of transplantation of HLA-nonidentical hematopoietic stem cells, strong graft versus leukemia reaction occurs when none of the KIRs in a subset of donor NK cells is bound by the host KIR-L, because the host's HLA repertoire lacks the appropriate KIR-L [36].

Single-nucleotide polymorphisms (SNPs) and a more limited number of microsatellite polymorphisms have been recently identified within promoter and/or coding region of genes encoding for cytokines. Such polymorphisms may affect cytokine production and perturbation of the Th1/Th2 balance as seen in various immune-mediated diseases [19,23]. It has also been recently documented that some patients with acquired AA and MDS have a higher frequency of polymorphisms linked to high production of proinflammatory cytokines such as TNF-α, TGF-β and IFN-γ [37-39].

To test the hypothesis that similar immunogenetic predisposition may be found in all or specific subgroups of immune-mediated BMF, we investigated specific inhibitory and activating KIR genotypes as well as SNPs of cytokine promoter and receptor genes controlling Th1/Th2 balance in 206 patients with AA and/or PNH and MDS.

MATERIALS AND METHODS

Patients

Heparinized blood samples were obtained after informed consent according to the protocols approved by the Institutional Review Board of the Cleveland Clinic Foundation from 77 patients with AA (mean age 47.1, range 4–79) and 129 patients with MDS (mean age 61.8, range 25–80). Diagnosis of AA was established by bone marrow biopsy and peripheral blood counts according to criteria of the international study of AA and agranulocytosis [40]. Diagnosis of PNH was determined by flow cytometric detection based on a significant glycosylphosphatidyl inositol anchor-deficient clone according to previously outlined criteria. Among the 77 patients with AA (overall AA cohort was designed as AA all in Fig. **1**), 44 had a typical AA (designed as AA alone), 19 had a co-presence of a small PNH clone (designed as AA + PNH) and 14 had typical PNH. According to the World Health Organization (WHO) criteria, MDS patients were diagnosed as follows: 21 with refractory anemia and refractory cytopenia with multilineage dysplasia (RA/RCMD), 37 with refractory anemia with ringed sideroblasts and refractory cytopenia with multilineage dysplasia and ringed syderoblasts (RARS/RCMD-RS), 44 with refractory anemia with blast excess (RAEB) or secondary AML (sAML), 11 with chronic myelomonocytic leukemia (CMML), 3 with chromosome 5q deletion syndrome and 13 with unclassified MDS (overall MDS cohort was designed as MDS all in Fig. **2**) The hypoplastic features (n=10) in MDS (designed as hypocellular MDS) were supported in all cases by bone marrow histological evaluation. Adequate cytogenetic data and/or FISH analysis were available for 107 out of 129 MDS patients (normal karyotype, n= 21; complex karyotype, n=37; trisomy 8, n=44; 5q-, n=3). The control group comprised 60 internal healthy controls and a large historical control cohort.

KIR-L and KIR Genotyping and Assignment

Genomic DNA was isolated from whole blood by using a modified salting out technique (GentraPUREGENE DNA purification kit, Minneapolis, MN, USA). HLA class I and II typing was performed by polymerase chain reaction (PCR)-sequence-specific primers (Allogen Laboratories, Cleveland, OH, USA), as previously described. Patients were categorized according to their HLA KIR-L motifs by determining whether or not they expressed HLA-A3 or HLA-A11, HLA-Bw4 or HLA-Bw6, HLA-C1 (consisting of Cw1, Cw3, Cw7, Cw8, Cw12, Cw13, Cw14, and Cw16 alleles) or HLA-C2 (consisting of Cw2, Cw4, Cw5, Cw6, Cw15, Cw17, and Cw18 alleles) groups. For HLA-A haplotypes, A3/A11 heterozygosity was defined as the presence of either the A3 or A11 allele while homozygosity as the absence/presence of both A3 and A11 alleles; for HLA-B haplotypes, typing results were subdivided in Bw4 or Bw6 groups and the results were shown as homozygous for either or heterozygous; for HLA-C haplotypes, heterozigosity was indicated by the presence of one HLA-C allele from each group (C1/C2) and homozigosity by the presence of two group 1 or two group 2 HLA-C alleles (C1/C1 or C2/C2).

Molecular KIR genotyping was performed using PCR-SSP technology and a commercial KIR typing kit ((Pel-Freez, Brown Deer, WI, USA), as previously described. Following amplification with KIR primers based on conserved regions specific to each KIR locus, amplified product was electrophoresed in agarose gels containing ethidium bromide and the genotype determined by the presence or absence of specifically amplified KIR products in each lane containing individual allele-specific KIR primers. The following inhibitory and stimulatory KIR genes were determined: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4 (001, 002, and 003), 2DS5, 3DL1, 3DL2, 3DL3, and 3DS1 along with the pseudogenes 2DP1 and 3DP1.

The specificity of the inhibitory KIR 2DL1, 2DL2, 2DL3, 3DL2, 3DL1 and of stimulatory KIR2 2DS1, 2DS2 for their KIR-L have been clearly defined. KIR2DL1 binds group 2 HLA-C allotype with lysine at position 80 of the heavy chain (HLA-C^{Lys80}), KIR2DL2/L3 binds group 1 HLA-C allotype which have asparagine at position 80 (HLA-C^{Asn80}), KIR3DL1 binds Bw4 and KIR3DL2 binds HLA-A3 and -A11. KIR2DS1 and KIR2DS2 have a much lower

Table 1. Frequency of KIR-L in AA and MDS Patients

Genotype	Controls (n = 126)		AA (n = 91)			MDS (n = 56)		
	(n)	%	(n)	%	p	(n)	%	p
HLA-A								
A3+/A11+	(11)	8.7	(0)	0	0.01	(0)	0	0.008
A3+/A11-	(46)	36.6	(40)	43.9		(13)	23.3	
A3-/A11-	(69)	54.7	(51)	56.1		(43)	76.7	
HLA-B								
Bw4/Bw4	(18)	14.2	(9)	9.8	0.5	(14)	25.0	0.5
Bw4/Bw6	(57)	45.3	(47)	51.8		(29)	51.8	
Bw6/Bw6	(51)	40.5	(35)	38.4		(13)	23.2	
HLA-C								
C1/C1	(42)	33.3	(41)	45.0	0.1	(25)	44.6	0.3
C1/C2	(54)	42.9	(34)	38.6		(17)	30.4	
C2/C2	(30)	23.8	(15)	16.4		(14)	25.0	

Abbreviations: KIR-L, killer immunoglobulin-like receptor-ligand; AA: aplastic anemia; MDS: myelodysplastic syndromes.

affinity for the same HLA class I ligands as KIR2DL1 and KIR2DL2. KIR mismatch, for the purpose of this study, has been defined as the presence of a certain KIR gene and the lack of its corresponding KIR-L, according to the method proposed by Parham *et al.* [36,43].

Genotyping of Cytokine and Cytokine Receptor Gene Polymorphisms

Twenty-two SNPs distributed in 13 cytokine and cytokine receptor genes were investigated: IL-1α (-889, T/C), IL-1β (–511, T/C), IL-1β (+3962, T/C), IL-1R (pst1 1970, T/C), IL-1RA (mspa1, 11100, T/C), IL-4Rα (+1902, G/A), IL-12 (–1188, C/A), IFN-γ (+874, A/T), TGF-β (codon 10, C/T and codon 25, G/C), TNF-α (–308, A/G and –238, A/G), IL-2 (+166, G/T and –330, T/G), IL-4 (–1098, T/G; –590, T/C; –33, T/C), IL-6 (–174, C/G and nt565, G/A), IL-10 (–1082, G/A; -819, C/T; -592, A/C). All genotyping was performed using PCR-SSP using the Cytokine Genotyping Kit (Dynal Biotech, Invitrogen Corporation, Brown Deer, WI, USA). Primer pairs for the amplification of a target sequence were provided for a total of 16 PCR reactions per sample per assay. The lyophilized primer mixes, reagents contained in the kit and PCR amplifications were carried out exactly according to manufacturer's manual. The thermal cycling programme was as follows: initial denaturation 94°C for 2 min, then 10 cycles of 94°C 15 s and 65°C 60 s, and then 20 cycles of 94°C 15s, 61°C 50 s and 72°C 30s. After the cycling was completed, the PCR products were loaded onto a 2% agarose gel stained with ethidium bromide for electrophoresis (5 V/cm). Positive reactions for a specific allele were discerned by the presence of a band between the larger internal control band and the smaller primer dimer band.

Statistical Analysis

HLA, cytokine and cytokine receptor allele frequency and KIR gene frequency were calculated by direct counting. The degree of significance was determined by the chi-squared and Fisher's exact tests. *P*-values <0.05 were considered significant. The *t*-test and the non-parametric Wilcoxon rank sum test, based on the normality assumption, were used to compare the various clinical parameters with the aforementioned laboratory characteristics.

RESULTS

Analysis of HLA-KIR-L and KIR Genotypes

It is well established the remarkable influence of certain KIR/HLA combinations on the development of several autoimmune diseases [15-23]. When we compared AA and MDS patients with controls for the distribution of KIR binding HLA motifs, we found that there were no AA and MDS patients positive for both the A3 and A11 alleles which are ligands for KIR3DL2 (Table **1**).

To establish frequencies of KIRs responsible for inhibitory functions (2DL1, 2DL2, 2DL3 and 3DL1) and activating signals (2DS1, 2DS2, 2DS3, 2DS4, 2DS5 and 3DS1), we performed KIR genotyping of patients and controls (Table **2**). All of the KIR genes tested were present in both patients and controls. KIR gene frequencies in our internal control cohort were similar to those described for previous cohorts [41]. Except for decreased frequency of the inhibitory KIR2DL1 (80.3% *vs* 94.3% in AA and controls, respectively; *p*=.001), KIR2DL3 (66.6% *vs* 88.1%; *p*=.002) and KIR3DL1 (80.3% *vs* 96.5%; *p*=.004), the frequencies for all other KIRs did not differ in AA, MDS and controls (Table **2**). However, similar decreased frequency of the

Table 2. Frequency of KIR Genes in AA and MDS Patients

Genotype	Controls			AA (n = 51)		MDS (n = 85)		
	(n/N)	%	(n)	%	p	(n)	%	p
Inhibitory								
2DL1	(265/281)	94.3	(41)	80.3	0.001	(82)	96.4	0.4
2DL2	(83/157)	52.8	(27)	52.9	0.1	(47)	55.2	0.6
2DL3	(231/262)	88.1	(34)	66.6	0.0002	(74)	87.0	0.7
3DL1	(277/287)	96.5	(41)	80.3	0.004	(80)	94.1	0.6
Activating								
2DS1	(36/104)	34.6	(15)	29.4	0.5	(41)	48.2	0.06
2DS2	(68/142)	47.8	(27)	52.9	0.5	(45)	52.9	0.4
2DS3	(1772)	23.6	(17)	33.3	0.2	(24)	28.2	0.5
2DS4	(273/285)	95.7	(47)	92.1	0.2	(77)	90.5	0.09
2DS5	(1976)	25.0	(15)	29.4	0.7	(29)	34.1	0.3
3DS1	(34/101)	33.6	(24)	47.0	0.1	(32)	37.6	0.5

Abbreviations: KIR-L, killer immunoglobulin-like receptor; AA: aplastic anemia; MDS: myelodysplastic syndromes; (n): number of positive controls or patients; (N): total number of controls or patients studied.

inhibitory KIR2DL3 was also found in a subgroup of MDS patients showing bone marrow hypoplasia and cytopenia (63% *vs* 88.1% in MDS and controls, respectively; p=.002), which also presented increased frequency of the activating KIR2DS5 (66% *vs* 25%, p=.001) (not shown).

The frequency distributions for the various KIR/KIR-L combinations in AA and MDS patients, showed that the mismatch between the activating KIR2DS1 and its ligand C2 was less frequent than in the control group (13.7% *vs* 44% in AA and controls, respectively; p = .003; 15.7% *vs* 44% in MDS and controls, respectively; p = .01). However, for both AA and MDS the frequency for genotypic mismatch between the other activating KIR2DS2 and its ligand C1 was not different than in controls (Table **3**). In AA patients, no significant difference was also observed for KIR/KIR-L mismatch between any of the inhibitory KIR both binding HLA-C1 (2DL2, 2DL3, 2DS2) and BW4 and HLAA3 or A11 (Table **3**).

In contrast, in MDS patients a decreased frequency for the inhibitory KIR3DL1/BW4 mismatch compared with those found in controls was found (Table **3**). When we examined frequencies for the various KIR/KIR-L

Table 3. KIR mismatches for each KIR in AA and MDS patients.

KIR/KIR-L	Mismatched Controls			Mismatched AA (n = 51)			Mismatched MDS	
	(n/N)	%	n	%	p	n	%	P
Inhibitory								
2DL1/C2	(31/85)	36.4	28	54.9	0.3	21	36.8	0.7
2DL2/C1	(6/53)	11.3	2	3.9	0.1	7	12.2	0.8
2DL3/C1	(13/79)	16.4	4	7.8	0.1	13	22.8	0.2
3DL1/Bw4	(43/92)	46.7	29	39.2	0.3	10	17.5	0.0003
3DL2/A3 or A11	(54/93)	58.0	28	54.9	0.7	42	73.6	0.05
Activating								
2DS1/C2	(11/25)	44.0	7	13.7	0.003	9	15.7	0.01
2DS2//C1	(5/52)	9.6	1	1.9	0.3	7	12.2	0.6

Abbreviations: KIR-L, killer immunoglobulin-like receptor; AA: aplastic anemia; MDS: myelodysplastic syndromes; n: number of positive controls or patients; N: total number of controls or patients studied.

mismatches in high-risk and low-risk MDS according to IPSS scores, they differed only for an increased frequency of the activating KIR2DL3/C1 mismatch in high-risk MDS (43% *vs* 16.4% in MDS and controls; *p*=.006) (not shown).

Cytokine and Cytokine Receptor SNPs in AA and MDS

In order to determine possible influences of proinflammatory and anti-inflammatory cytokine polymorphisms on susceptibility, clinical phenotype and severity of AA and MDS, we systematically screened the frequencies of 22 selected SNPs located within 10 cytokine (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL12, IFN-γ, TNF-α, TGF-β) and 3 cytokine receptor (IL-1R, IL-1RA, IL-4Rα) genes involved in the regulation of Th1/Th2 balance.

In AA cohort, no association was found for the SNPs in IL-1α, IL-1β, IL-1R, IL-2, IL-4, IL-4Rα, IL-6, IL-10, IL-12 and TNF-α (not shown). When we examined the frequency of TGF-β genotypes, increased frequency of GG variant on codon 25, consistent with high secretor phenotype, was found in the AA population (61% in AA vs 35% in controls, p=.03). Moreover, the TGF-β high secretor genotype was more frequent among AA with PNH clone (76% in AA vs 35% in controls, p=.01) (Fig. **1A**). In addition, we found that the frequency of the hypersecretor genotype T/T of the IFN-γ was most over represented in the AA cohort compared to normal controls (28% vs 10%, p=.02). The frequency of IFN-γ 874 T/T genotype was significantly higher in AA with PNH clone than that of controls (35% vs 14%, p=.01) (Fig. **1B**). In addition, we found a lower prevalence of TT genotypes for the IL1RA gene in AA patients (33% vs 62% p=.02) (not shown).

In MDS cohort, no significant difference, nor in any of subgroups compared to control and each other, was found for the SNPs in IL-1α, IL-1β, IL-1R, IL-2, IL-4, IL-4Rα, IL-6, IL-12 and IFN-γ (data not shown). As above documented in AA cohort, when we examined the frequency of TGF-β genotype, MDS population showed a higher rate of TT codon 10 variant (59% vs. 32% in controls, p=.002) and of GG codon 25 variant (71% vs. 35% in controls, p=.001), consistent with a high secretor phenotype (Fig. **2A**). When MDS patients were subdivided according to marrow cellularity, we found that hypoplastic variant of MDS (n=10) were characterized by a higher prevalence for T/T genotype of TGF- β (80% vs 32% in controls, p= .0001) (Fig. **2A**).

A G/A combination at position –238 in the promoter of the TNF-α gene has been associated with increased TNF-α production *in vitro*. Subgroup analysis of MDS patients revealed a higher prevalence of the high secretor phenotype for the TNF-α gene only in MDS patients, consistent with high secretor phenotype (9% vs 1%, p=.02) (Fig. **2B**).

Several polymorphic sites within the promoter region of IL-10 have been described, including three biallelic polymorphisms at positions −1082 (G/A), −819 (C/T), and −592 (C/A) upstream from the transcription start site, which have been associated with variability in IL-10 production [24-26].

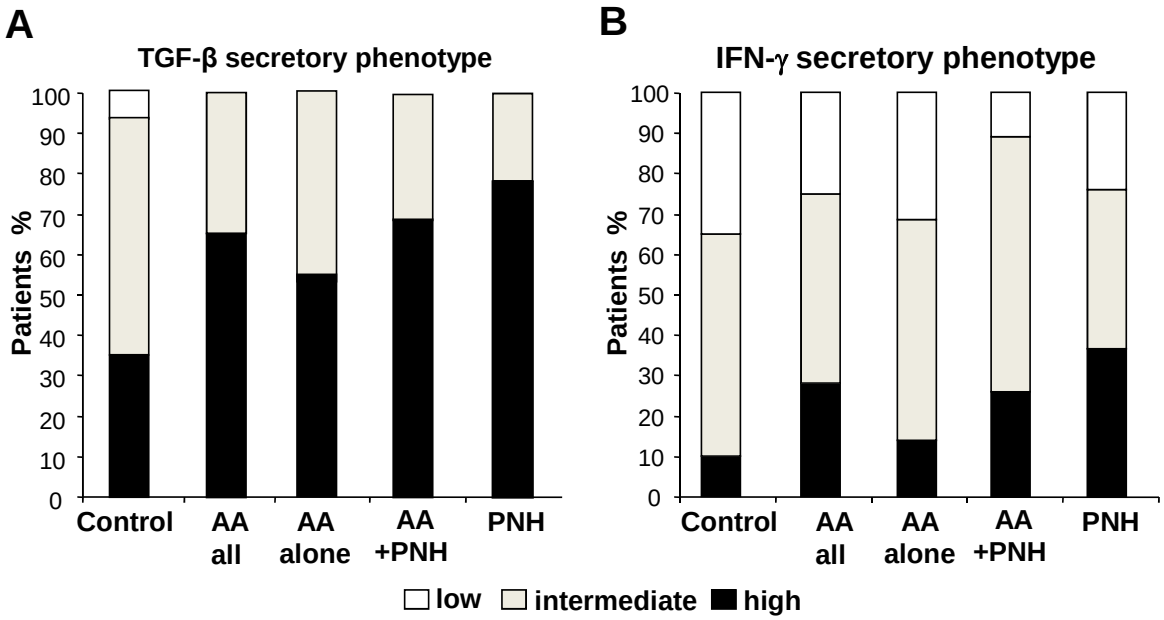

Fig. (1). Increased frequency of high secretory TGF-β1 and IFN-γ genotypes in AA patients.

Low, intermediate and high producing phenotypes for codon 25 of TGF-β1 were defined by the genotypes C/C, G/C and G/G, respectively. Significant differences were found only for the high producing phenotype (GG) both in overall AA population and in AA alone, AA + PNH and PNH cohorts (p=.03, p=.04, p=.02 and p=.01 in comparison to controls, respectively).

The A/A, T/A, and T/T genotypes for base -874 IFN-γ gene design low, intermediate, and high IFN-γ secretory phenotypes, respectively. High IFN-γ secretory phenotype was different between overall AA, AA + PNH and PNH cohorts and controls (p=.02, p=.05, p=.02 p=.01 in comparison to controls, respectively).

Abbreviations: AA: aplastic anemia; AA all: overall AA cohort; AA alone: only AA; AA + PNH: AA with PNH clone; PNH: paroxysmal nocturnal hemoglobinuria.

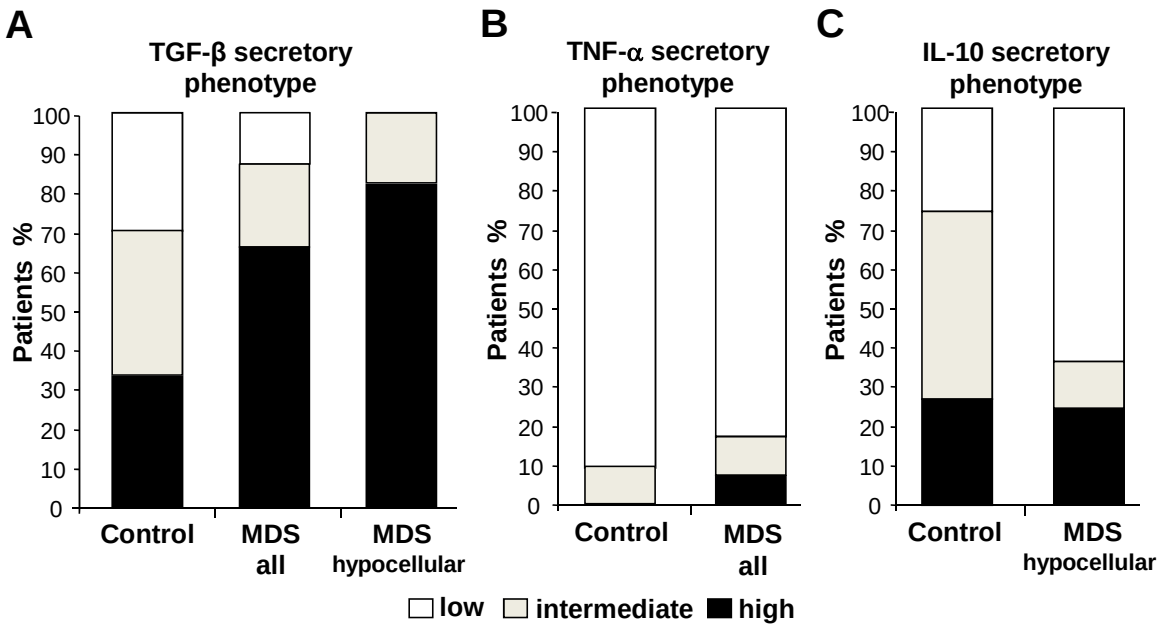

Fig. (2). MDS patients show increased frequency of high secretory TGF-β1 and TNF-α and of low secretory IL-10 phenotypes.

Low, intermediate and high producing phenotypes for codon 25 of TGF-β1 were defined by the genotypes C/C, G/C and G/G, respectively. Low, intermediate and high producing phenotypes for codon 10 of TGF-β1 were defined by the genotypes C/C, T/C and T/T, respectively. Significant differences were found only for the high producing phenotypes (codon 25 G/G and codon 10 T/T) in overall MDS and in hypocellular MDS (p=.001 and p=.0001 in comparison to controls, respectively). Low, intermediate and high producing phenotypes for base -238 TNF-α were defined by the genotypes G/G, G/A, and AA, respectively. Significant differences were found only for the high producing phenotype in overall MDS in comparison to controls ((9% vs 1% p=.02). Producing phenotypes for IL-10 haplotypes derived from the combination of three different genotypes (-1082 A/G; -819T/C, -592 A/C) were defined as low (ATA/ATA, ACC/ACC, ACC/ATA), intermediate (GCC/ATA and GCC/ACC) and high (GCC/GCC and GCC/ATC). Significant differences were found only for the low producing phenotype in hypocellular MDS (p=.03 in comparison to controls).

Abbreviations: MDS: myelodysplastic syndromes; MDS all: overall MDS cohort.

A complete linkage disequilibrium exists between the alleles present at −819 and −592 positions; so these polymorphisms occurred in tandem and only three haplotypes have been found (GCC, ACC and ATA). Carriers of the GCC/GCC and GCC/ATC genotypes are considered as genetically high producers, whereas carriers of ACC/ACC, ATA/ATA and ACC/ATA genotypes are considered as genetically low producers. Hypocellular MDS were characterized by a higher prevalence of IL-10 low secretor phenotype (58% vs 20%, p=.03) (Fig. **2C**).

DISCUSSION

It is well established that various immunogenetic factors, including HLA type, KIR genotype and cytokine and cytokine receptor gene SNPs may play an important predisposition to exaggerated and or decreased immune responses, such those observed in autoimmune diseases [15-24]. Based on this notion, when we have compared AA and MDS patients with controls for the distribution of KIR-L groups, we found that the frequencies of both A3 and A11 alleles were decreased in AA and MDS patients, thus contributing to the increased occurrence of mismatch between KIR3DL2 and its ligands. Because binding of inhibitory KIR3DL2 to its corresponding ligand results in suppression of cytotoxicity, it is conceivable that this KIR/KIR-L lacking combination may result in a greater susceptibility to self-intolerance and could be responsible for the triggering of the pathological cytotoxic response underlying pathophysiology of AA and MDS [1-13,27,43]. Similar effects may be related to stimulatory KIR, such as 2DS1 and 2DS2 and their ligand HLA-C2 imbalance. The observed lower frequency of 2DS1-C2 mismatch in AA and MDS patients could result in reduced silencing of cytotoxic activity [44-47].

In addition to HLA and KIR, another group of immunogenetic factors that are becoming of great interest for the understanding of several autoimmune diseases are represented by cytokine and cytokine receptor SNPs, preferentially located in non-coding regions containing regulatory sequences. Polymorphisms within regulatory sequences of cytokines and cytokine receptors may per se affect the secretion or function of the corresponding proteins, and thus influence immune responses [17-24,51]. For example, homozygous genotypes for high producer alleles are generally associated with high cytokine production, heterozygotes with intermediate production, and homozygotes for the low producer alleles with low cytokine production. In addition, cytokine promoter polymorphisms may also block the binding of several transcription factors, such as NF-κB, Jak, STAT and IRF, to regulatory regions further affecting their function [17-24,51]. These polymorphisms segregate independently and thus each

individual is a mosaic of high, intermediate-, and low-producing phenotypes.

TGF-β is a pleiotropic cytokine involved in many immune and hematopoietic regulation processes [52]. TGF-β plays a major role under inflammatory conditions; TGF-β, together with other pro-inflammatory cytokines, promotes T helper 17 (Th17) cells differentiation, which can further induce inflammation and amplify autoimmune conditions [53]. In addition, TGF-β in combination with IL-4 stimulates the differentiation of IL-9- and IL-10-producing T cells, which lack suppressive function, and thus promoting tissue inflammation [54]. In a recent study, it was shown that TGF-β may enhance survival of memory $CD8^+$ T cells and increased the production of IL-17 and IFN-γ [52-54]. TGF-β is produced by a single gene for which have been described various polymorphisms. In this study, we analyzed two polymorphic sites of the TGF- β1 gene: T869C (codon 10) and G915C (codon 25). The polymorphisms in the codon 10 and 25 are the most studied polymorphism of the TGF-β1 gene because of their position in the signal sequence and consequent potential role in controlling TGF-β1 production. Although the functional significance of these polymorphisms are not entirely clear, a number of studies have shown that polymorphisms in codons 10 and 25 result in high producer phenotype and are associated with an increased prevalence of several immune-mediated diseases and worse outcome following organ and hematopoietic stem cell transplantation [56,57]. In our study when AA and MDS patients were compared to controls, a higher frequency of TGF-β high secretor genotype was detected. We have also documented that the presence of PNH clone was associated with a higher frequency of TGF-β high secretory genotype consistent with the hypothesis that the expansion of the PNH clone requires a concomitant immune-mediated damage of the normal hematopoiesis sparing PNH stem cells [39,41,56]. Consistent with the observations in AA, when MDS patients were subdivided according to marrow cellularity, we found that hypoplastic MDS patients showed a higher prevalence of codon 10 T/T genotype of TGF-β further suggesting that immune-mediated mechanisms similar to those seen in AA patients may also operate in hypoplastic MDS patients, leading to depletion of early and late hematopoietic progenitor cells.

The immune effector mechanisms involved in AA and some types of MDS mainly include, in addition to direct cell-mediated killing, release of TH1 cytokines with inhibitory activity on hematopoietic progenitors such as IFN-γ and TNF-α [1-13]. We examined the intronic IFN-γ polymorphism at position +874 and at the promoter region TNF-α polymorphisms at positions -238 and -308, which confer high IFN-γ and TNF-α expression. The A/A, T/A, and T/T IFN-γ genotypes for base -874 have been reported to correspond to low, intermediate, and high *in vitro* cytokine production, respectively [59-64]. According with the data already reported, we confirmed that the frequency of the hypersecreting genotype T/T of the IFN-γ was most over represented in the AA population compared to controls [39,58]. By contrast, there were no significant differences in the polymorphism of IFN-γ at position +874 between all MDS and each subgroup of MDS patients and healthy controls. When we went to compare the subgroup of the AA cohort based on the presence or not of the PNH clone, we interestingly found that the presence of PNH clone seems be related with the T/T genotype of IFN-γ phenotype. Instead, the frequency of G/A polymorphism at position –308 on the TNF-α gene promoter, which correlates with higher TNF-α production, was found significantly higher only in MDS but not in AA patients.

IL-10 is usually regarded as a potent inhibitor of T cell-mediated immune responses by suppressing the expression of proinflammatory cytokines such as TNF*α*, IL-1, IL-6 and IL-12 [65,66] and by inducing IL-10 downregulation of class II MHC [67,68]. Low levels of IL-10 production have been correlated with increased prevalence of both acute and chronic GVHD [69,70]. In our study, hypocellular MDS patients, but not AA patients, showed increased prevalence of IL-10–1082 A/A genotype, associated with decreased IL-10 levels. Defective IL-10 production may allow enhanced production of Th_1 cytokines responsible for increased apoptosis and decreased cell differentiation and proliferation.

Clinically, the strongest evidence for T-cell–mediated hematopoietic suppression in BMF syndromes is the response to immunosuppression, which has been documented more often in AA patients and some types of MDS patients, in particular hypocellular MDS [1,2,71-74]. Recently, it has been reported that IFN-γ and TGF-β1 genes polymorphisms may be associated with response of AA patients to immunosuppression [75]. However, there are no large studies showing that above reported and other cytokine gene polymorphisms may be associated with responsiveness to immunosuppressive therapy.

In conclusion, our study demonstrates the presence of various immunogenetic polymorphisms which in a complex fashion could increase propensity to or modulate the severity or clinical features of immune-mediated bone marrow failure. However, further studies are needed to elucidate whether both alterations in KIR/KIR-L matching and SNPs affecting cytokine expression, implicated in the perturbations of the Th_1/Th_2 balance, are responsible for an increased risk of developing immune-mediated bone marrow failure syndromes and may help to identify patients who will benefit of immunosuppressive treatment.

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