Ann Hematol (2017) 96:1287–1295
DOI 10.1007/s00277-017-3036-4

ORIGINAL ARTICLE

Influence of *TNF* and *IL6* gene polymorphisms on the severity of cytopenias in Argentine patients with myelodysplastic syndromes

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Received: 22 March 2017 / Accepted: 26 May 2017 / Published online: 10 June 2017
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Abstract Myelodysplastic syndromes (MDS) represent a heterogeneous group of hematologic disorders characterized by cytopenia(s) and predisposition to leukemic progression. An immune dysregulation and an aberrant bone marrow microenvironment seem to be key elements in the physiopathological process of MDS. In order to evaluate a possible association between susceptibility and clinicpathologic features, we genotyped 153 MDS patients for functional cytokine polymorphisms: *TNF* (−308 G/A), *IFNG* (+874 A/T and +875 CAn), *IL6* (−174 G/C), and *TGFB1* (+869 C/T and +915 G/C). The frequency of *TNF* and *IL6* polymorphisms was different between patients and healthy controls (n = 131), suggesting a relatedness to MDS susceptibility in our population. Furthermore, the presence of each or both high-producing genotypes [*TNF*: $p = 0.048$, odds ratio (OR): 3.979; *IL6:* $p = 0.001$, OR: 6.835; both: $p = 0.010$, OR: 6.068] and thrombocytopenia at platelet counts of <50,000/μL ($p = 0.004$, OR: 4.857) were independently associated with an increased risk of manifesting a hemoglobin level of <8 g/dL at diagnosis. In particular, a severe bicytopenia was more frequently observed in patients with the *TNF* (high)_*IL6* (high) combined genotype ($p = 0.004$, OR: 8.357), who consistently became transfusion dependent earlier (2.9 vs. 34.6 months; $p = 0.001$); and this likelihood was more evident in patients with lower bone marrow blast counts. The contribution of the remaining functional polymorphisms to the disease phenotype was less relevant. Our results demonstrate that *TNF* and *IL6* gene polymorphisms, as underlying host features, are likely to play a key role in influencing the severity of the cytopenias in MDS and they may be instrumental for tailoring cytokine-target therapies.

Keywords *TNF* · *IL6* · Polymorphisms · Myelodysplastic syndromes · Cytopenia

Electronic supplementary material The online version of this article (doi:10.1007/s00277-017-3036-4) contains supplementary material, which is available to authorized users.

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Introduction

Myelodysplastic syndromes (MDS) are a group of heterogeneous hematopoietic clonal stem cell disorders characterized by peripheral cytopenia(s), myeloid dysplasia, and an increased risk of progression to acute myeloid leukemia (AML) [1–5]. Despite the recent advances in the last 5 years in describing a panel of molecular abnormalities that might contribute to the multistep process in the pathogenesis of MDS [6], the pathologic sequence remains poorly understood and likely depends on an interplay between aberrant hematopoietic cells and their microenvironment [7–9].

Several findings account for an immune dysregulation whose nature differs between MDS-risk groups. In the lower-

risk population, the immune system is characterized by an activated proinflammatory state leading to high levels of apoptosis of hematopoietic progenitors, thus releasing dysplasia-associated antigens that may evoke an adaptive immune response. In advanced MDS, an immunosuppressive environment with an increased number of regulatory T cells occurs, enabling dysplastic clones to expand and evade immune surveillance. The disturbed differentiation is accompanied by apoptosis resistance and an increased proliferation [9]. Accumulated evidence has indicated that marrow failure, in some MDS, is associated with autoimmunity, T-cell mediated myelosuppression, and cytokine-induced cytopenias [8]. In addition, a previous history in patients of any autoimmune disease has been associated with an increased risk of MDS, and –vice versa– clinical manifestations of autoimmune disease are frequently described in patients with MDS and chronic myelomonocytic leukemia [10].

Cytokines are key elements in the interaction of the malignant clone with the microenvironment along with the ineffective hematopoiesis in MDS and may influence a variety of clinical manifestations that characterize the disease. The role of the tumor necrosis factor-α (TNF-α) in affecting the rates of intramedullary apoptosis [11], the outcome of MDS patients [12], and the response to erythropoietin (EPO) treatment has been widely confirmed [13]. Higher levels of interferon-γ (IFN-γ) have also been demonstrated in trephine biopsies and in the bone marrow (BM) plasma [14]. Adherent cell layers from MDS patients in culture produced higher levels of interleukin-6 (IL-6) and TNF-α [7], while increased plasma levels of IL-6 were independently associated with an inferior outcome [15]. A higher production of the transforming growth factor-β (TGF-β) by mesenchymal stem cells in the high-risk MDS group contributes to the increase in regulatory T cells in those patients [16].

Several reports have identified polymorphic variants in human leukocyte antigen genes and in cytokine and cytokine-receptor genes that are linked to susceptibility to cancer and autoimmunity and that contribute differentially to disease development, progression, and the response to therapy [17–27]. Functional variants such as single nucleotide polymorphisms (SNPs) localized in the promoter or regulatory regions of relevant cytokine genes may influence their genetic expression, thus skewing the functioning of immune cells through the action of their effector molecules, the cytokines and chemokines. Previous studies have proven that the presence of the *TNF* –308A, *IFNG* +875 12 CA-repeat, *IL6* –174G, and *TGFB1* +869C or +915G alleles increase the expression of the transcript and/or the protein of the encoded cytokines. Although the influence of these variants has been confirmed in vitro (i.e., mitogen stimulation of cells in culture, isolation of individual alleles of gene promoters by cloning adjacent to a reporter gene, and/or performing a mobility shift assay in the presence of nuclear extracts), certain data reinforce the notion of an in vivo modulation as well [28–30].

Since the level of cytokine secretion is under the influence of genetics and environmental conditions, in the present study, we were interested in evaluating a possible association between the above detailed cytokine gene polymorphisms and both disease susceptibility and clinic-pathologic features in a series of Argentine MDS patients.

Subjects, materials and methods

Study population

The study was a multicenter retrospective and prospective analysis of 153 MDS patients from the registry of the *Laboratorio de Genética Hematológica, Instituto de Medicina Experimental (IMEX-CONICET)/ Academia Nacional de Medicina*, Buenos Aires. Hematologists from the participating institutions completed a standard registration form for each patient detailing the clinical and hematologic features at presentation and during the follow-up. The ethics committee of the *Institutos de la Academia Nacional de Medicina* has evaluated and approved this research.

Inclusion in the study required a confirmed diagnosis of de novo MDS, excluding those patients with treatment-related MDS or a previous history of toxic substance exposure. MDS patients were diagnosed from September 1981 through May 2014 with a median overall survival of 44.4 months. During the follow-up period of 26.1 (1.0–174.6) months, 45 (29%) evolved to AML and 87 (57%) died including 44 patients with previous progression to AML. Patients were classified according to the French-American-British (FAB) [1] and the World-Health-Organization (WHO) [3] diagnostic criteria and were distributed as defined by the International Prognostic Scoring System (IPSS) and the System's revised version (IPSS-R) (Table 1) [2, 4].

Samples from 131 unrelated healthy blood donors who donated between 2000 and 2003 in a different ward from the one used for the patients' blood withdrawal (Transfusion Blood Bank from the *Servicio de Hemoterapia y Hemofilia*, *Instituto de Investigaciones Hematológicas*, Buenos Aires) were studied as the control population. The donors presented with a median age of 38 years (range: 20–64) and a male/female ratio of 1.6 not being statistically different from the patients' sex ratio (Fisher's exact test, $p = 0.539$).

Genotyping of cytokine polymorphisms

Genomic DNA was isolated from peripheral blood or BM mononuclear cells by a standard phenol-chloroform method.

The *TNF* (−308 G/A, rs1800629) study was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphisms (PCR-RFLP, *Nco*I digestion) and visualized by silver staining [31]. The *IFNG* microsatellite region (+875

Table 1 Clinical characteristics and IPSS, IPSS-R, FAB, and WHO classifications of the MDS population studied

Characteristics	
Age (years), *n* = 153	
Median (range)	66 (14–89)
Gender, *n* = 153	
Male/female	87/66: 1.3
FAB classification, *n* = 153 (%)	
RA	78 (51)
RARS	13 (9)
RAEB	34 (22)
RAEBt	9 (6)
CMML	19 (12)
WHO 2008 classification, *n* = 131 (%)	
RCUD/−RS	25 (19)
5q- syndrome	5 (4)
RCMD/−RS	54 (41)
RAEB-1	14 (11)
RAEB-2	26 (20)
RC with missing data of dysplasia	7 (5)
IPSS, *n* = 139 (%)	
Low	35 (25)
Intermediate-1	72 (52)
Intermediate-2	24 (17)
High	8 (6)
IPSS-R, *n* = 136 (%)	
Very low	21 (15)
Low	57 (42)
Intermediate	27 (20)
High	18 (13)
Very high	13 (10)
Karyotype, *n* = 144 (%)	
Normal	76 (53)
Abnormal	68 (47)

FAB French-American-British criteria, *WHO* World Health Organization, *RA* refractory anemia, *RARS* RA with ring sideroblasts, *RAEB* RA with excess blasts, *RAEBt* RAEB in transformation, *CMML* chronic myelomonocytic leukemia, *RC* refractory cytopenia, *RCUD* RC with unilineage dysplasia, *RCMD* RC with multilineage dysplasia, *IPSS* International Prognostic Scoring System, *IPSS-R* IPSS revised version

CAn, rs587776821) was amplified by PCR, resolved by nondenaturing polyacrylamide gel electrophoresis (PAGE-PCR), and visualized by silver staining [20]. The *IFNG* (+874 A/T, rs2430561), *IL6* (−174 G/C, rs1800795), and *TGFB1* (+869 C/T, rs1800470; +915 G/C, rs1800471) genotypes were determined by PCR through the use of sequence-specific primers (SSP-PCR) and resolved on ethidium bromide stained agarose gels [19, 29, 32]. Table 2 lists the PCR primers used. The genotypes obtained for each cytokine polymorphism were confirmed by automated DNA sequencing.

Statistical analysis

Differences in genotypic or allelic frequencies and in genotype-related clinical parameters at diagnosis regarding genotype were evaluated by the chi-square or Fisher's exact test, and the Mann-Whitney test or Student t test for continuous data. In the multivariate analysis, variables with a p value <0.2 were included, and the logistic regression model was performed to calculate the odds ratio (OR) and the respective 95% confidence interval (CI). The logistic regression model was internally validated by a bootstrap algorithm based on 1000 replications. The results were presented as bootstrap CIs and significance tests for the correlation coefficient (B). Pairwise linkage disequilibrium (D' value) between the rs2430561 SNP and the rs587776821 microsatellite were estimated according to Maynard [33].

Overall survival (OS), leukemia-free survival (LFS) and transfusion dependence-free survival (TDFS) were defined as the interval from diagnosis to the date of death, progression to AML, and transfusion dependence, respectively. Patients undergoing hematopoietic stem cell transplantation (3.9%) were censored up to the moment of the procedure. OS, LFS, and TDFS were estimated by the Kaplan-Meier method and compared with the log-rank test (Mantel-Cox). Analyses were performed with SPSS V24 software (IBM, New York, USA) and InfoStat Version 2016 (Grupo InfoStat, Universidad Nacional de Córdoba, Argentina). And, the correspondence analysis was performed according to Version 1.1, Data Theory Scaling System Group (DTSS) (DTSS, Faculty of Social and Behavioral Sciences, Leiden University, The Netherlands). All statistical tests were two-sided, and the level of statistical significance was defined as a p value <0.05.

Results

Analysis in the overall MDS population

Genotypic and allelic frequencies of cytokine polymorphisms

As described in Table 3, both the genotypic and allelic frequencies of the *TNF* −308 G/A and *IL6* –174 G/C SNPs were different between the MDS patients and the control subjects. That the high-expressing *TNF* genotype (G/A + A/A) was overrepresented in patients suggests an increased susceptibility of 2.7-fold to develop MDS (p = 0.005). The high-expressing *IL6* genotype (G/G) was less frequently observed, thus inferring a protective role of 0.5-fold (p = 0.017). With respect to *IFNG* and *TGFB1* polymorphisms, only the +869 C/T heterozygote genotype tended to be increased (p = 0.094, OR: 1.658). These results were confirmed by bootstrap analysis as the method of internal validation (Online Resource

Table 2 PCR primers used for genotyping of cytokine polymorphisms

Gene	*Polymorphism*	*Primer sequences (5′ → 3′)*	*PCR size*	*Reference*
TNF	-308 G/A rs1800629	Fw: AGGCAATAGGTTTTGAGGGCCAT Rv: TCCTCCCTGCTCCGATTCCG	107 bp	Wilson et al. [31]
IFNG	+874 A/T rs2430561	Fw–A: TTCTTACAACACAAAATCAAATCA Fw–T: TTCTTACAACACAAAATCAAATCT Rv: TCAACAAAGCTGATACTCCA	264 bp	Pravica et al. [29]
	+875 CAn rs587776821	Fw: GCTGTCATAATAATATTCAGAC Rv: CGAGCTTTAAAAGATAGTTCC	123 bp (119–131 bp)	Dufour et al. [20]
IL6	-174 G/C rs1800795	Fw–G: CCCTAGTTGTGTCTTGCG Fw–C: CCCTAGTTGTGTCTTGCC Rv: GAGCTTCTCTTTCGTTCC	230 bp	Fishman et al. [19]
TGFB1	+869 C/T rs1800470	Fw: TCCGTGGGATACTGAGACAC Rv–C: GCAGCGGTAGCAGCAGCG Rv–T: AGCAGCGGTAGCAGCAGCA	240 bp	Perrey et al. [32]
	+915 G/C rs1800471	Fw–G: GTGCTGACGCCTGGCCG Fw–C: GTGCTGACGCCTGGCCC Rv: GGCTCCGGTTCTGCACTC	233 bp	

Fw forward, *Rv* reverse, *bp* base pairs

Table S2a). The genotype distributions for cytokine polymorphisms in MDS patients and controls were in Hardy-Weinberg equilibrium, except for the *IL6* polymorphism.

Association of cytokine polymorphisms with clinical variables

Relevant MDS clinical variables were analyzed according the respective level of expression associated with each cytokine polymorphism (Online Resource Table S3). As we previously described [22], the presence of the genotype of high (G/A + A/A) as opposed to low (G/G) *TNF* expression was associated with a lower hemoglobin level (8.8 vs. 9.7 g/dL; $p = 0.034$), reduced platelet counts (90,000 vs. 131,000/μL; $p = 0.010$), and a shorter TDFS (7.9 vs. 27.0 months; $p = 0.016$). In addition, this group ran a 3.9-fold higher risk to succumb to non-AML related death (73 vs. 41%; $p = 0.013$). Similarly, the genotype of high (G/G) opposed to intermediate + low (G/C + C/C) *IL6* expression was correlated with a lower hemoglobin level (8.8 vs. 9.9 g/dL; $p = 0.009$), with a 3.9-fold risk of a lower basal hemoglobin level <8 g/dL (40 vs. 14%; $p = 0.001$), and accordingly, with a shorter TDFS (6.4 vs. 39.4 months; $p = 0.044$). Therefore, we found a significant association between the presence of high-producing *TNF* and *IL6* variants and a severer anemic phenotype within the context of the disease.

The analysis of the *IFNG* +875 CAn microsatellite polymorphism revealed that MDS patients carrying the high-expressing homozygote *IFNG* genotype presented with lower neutrophil counts [12/12 CA (high): 1140 vs. 12/non-12CA + non-12/non-12 CA (intermediate + low): 1777/μL; $p = 0.029$]. A similar association with a more profound neutropenia was manifested with the *IFNG* +874 T/T genotype (data not shown) owing to the strong linkage disequilibrium between the two functional alleles ($D' = 0.99$), as we previously described [25].

As to *TGFB1* polymorphisms, the high-producing +869 genotype was associated with a younger age at diagnosis [C/C (high): 62 vs. C/T + T/T (intermediate + low): 68 years; $p = 0.034$], while the high-producing +915 genotype with <5% BM blasts [G/G (high): 72% vs. G/C + C/C (intermediate + low): 48%; $p = 0.032$, OR: 2.790]. No other relevant associations were found (Online Resource Table S3).

Relevant combined genotypes affecting clinical features

Since *TNF* and *IL6* SNPs were associated with abnormalities in the hemoglobin level in the univariate analysis, we evaluated the influence of the combined genotypes on the severity of the anemia at diagnosis along with other clinical variables. The presence of each or both high-producing genotypes [*TNF* (high): $p = 0.048$, OR: 3.979; *IL6* (high): $p = 0.001$, OR: 6.835; *TNF* (high)_*IL6* (high): $p = 0.010$, OR: 6.068] and platelet counts <50,000/μL ($p = 0.004$, OR: 4.857) were independently associated with an increased risk to manifest MDS with hemoglobin levels <8 g/dL at diagnosis (Table 4). These results were internally validated by bootstrap analysis (Online Resource Table S2b). Patients with the *TNF* (high)_*IL6* (high) genotype presented more frequently with severe bicytopenia at diagnosis than those with other genotypic combinations ($p = 0.010$, Fig. 1) along with an associated risk of 8.4-fold (5/15, 33% vs. 7/124, 6%; $p = 0.004$, 95%CI: 2.240–31.185) and consistently became transfusion dependent earlier (2.9 vs. 34.6 months; $p = 0.001$) (Fig. 2a). The *TNF* (high)_*IL6* (high) genotype manifested a 3.4-fold higher susceptibility to develop MDS with respect to the control group ($p = 0.015$).

Table 3 Distribution of genotypic and allelic frequencies of cytokine polymorphisms in MDS patients and control subjects

Polymorphisms	Controls *n* (%)	MDS *n* (%)	*Univariate analysis*		*Multivariate analysis*[d]	
			p value	OR (95% CI)	p value	OR (95% CI)
TNF –308 G/A	*n* = 131	*n* = 152				
G/G (low)	115 (88)	111 (73)	0.003[b]	2.655	0.005	2.706
G/A + A/A (high)	14 + 2 (12)	38 + 3 (27)		(1.408–5.004)		(1.350–5.424)
G allele	244 (93)	260 (86)	0.004[b]	2.294		
A allele	18 (7)	44 (14)		(1.290–4.080)		
IFNG +875 CAn[a]	*n* = 128	*n* = 153				
Non-12/non-12 CA (low)	56 (44)	57 (37)	0.532[c]			
12/non-12 CA (intermediate)	53 (41)	72 (47)				
12/12 CA (high)	19 (15)	24 (16)				
Non-12 CA allele	165 (64)	186 (61)	0.383[b]			
12 CA allele	91 (36)	120 (39)				
IL6 –174 G/C	*n* = 126	*n* = 140				
G/C + C/C (intermediate + low)	61 + 2 (50)	85 + 5 (64)	0.025[b]	0.556	0.017	0.538
G/G (high)	63 (50)	50 (36)		(0.340–0.908)		(0.324–0.895)
C allele	65 (26)	95 (34)	0.047[b]	0.677		
G allele	187 (74)	185 (66)		(0.465–0.985)		
TGFB1 +869 C/T	*n* = 131	*n* = 142				
T/T (low)	41 (31)	37 (26)	0.152[c]			
C/T (intermediate)	53 (41)	74 (52)			0.094	1.658 (0.917–2.997)
C/C (high)	37 (28)	31 (22)			0.899	
T allele	135 (52)	148 (52)	0.932[b]			
C allele	127 (48)	136 (48)				
TGFB1 +915 G/C	*n* = 127	*n* = 143				
G/C + C/C (intermediate + low)	13 + 2 (12)	23 + 2 (17)	0.230[b]			
G/G (high)	112 (88)	118 (83)				
C allele	17 (7)	27 (9)	0.272[b]			
G allele	237 (93)	259 (91)				

OR odds radio, *CI* confidence interval

[a] Genotypes were grouped according to the presence of the high-producing *IFNG* 12 CA-repeat allele (distribution of the +875 CAn microsatellite is detailed in Online Resource Table S1).

[b] Fisher's exact test.

[c] Pearson's chi-square test.

[d] Logistic regression model (MDS *n* = 137, controls *n* = 126) [T/T (low) were considered reference genotype in the analysis of *TGFB1* +869 C/T polymorphism].

TNF-α and IFN-γ are typical cytokines mediating a Th1 immune response. The *TNF* (high)_*IFNG* (high) genotype, observed in eight patients, was associated with a reduced platelet count relative to those of other genetic combinations (88,000 vs. 124,000/μL; $p = 0.042$) along with lower neutrophil counts (702 vs. 1763/μL; $p = 0.056$) at diagnosis.

Analysis in MDS patients with <5% BM blasts

The relationship between these cytokine gene polymorphisms and MDS susceptibility in the group of patients that presented with <5% BM blasts ($n = 102$) was similar to that of the overall MDS population (logistic regression, *TNF*: $p = 0.003$, OR: 3.080, 95%CI: 1.468–6.461; *IL6*: $p = 0.037$, OR: 0.547, 95%CI: 0.311–0.963, and *TGFB1* +869: $p = 0.099$, OR: 1.754, 95%CI: 0.899–3.422, as internally validated through bootstrap analysis).

In this restricted population, the presence of the genotype of high (G/A + A/A) as opposed to low (G/G) *TNF* expression was also associated with a lower hemoglobin level (8.9 vs. 10.1 g/dL; $p = 0.020$), reduced platelet counts (100,500 vs. 141,500/μL; $p = 0.021$), a shorter TDFS (8.5 vs. 173.6 months; $p < 0.001$), and with a 9.5-fold increased risk to succumb to death unrelated to AML evolution (12/13, 92% vs. 19/34, 56%;

Table 4 Associations of *TNF* and *IL6* gene polymorphisms and clinical variables with the degree of anemia at diagnosis in MDS patients

Polymorphisms/variables	Hb ≥8 g/dL n (%)	Hb <8 g/dL n (%)	*Univariate analysis*		*Multivariate analysis*[c]	
			p value	OR (95% CI)	*p* value	OR (95% CI)
TNF_IL6 combined genotypes	*n* = 107	*n* = 32				
TNF (low)_*IL6* (intermediate + low)	64 (60)	7 (22)	0.001[b]			
TNF (high)_*IL6* (intermediate + low)	22 (21)	13 (41)			0.048	3.979 (1.013–15.633)
TNF (low)_*IL6* (high)	13 (12)	5 (16)			0.001	6.835 (2.237–20.883)
TNF (high)_*IL6* (high)	8 (7)	7 (22)			0.010	6.068 (1.536–23.976)
Platelet counts (/μL)	*n* = 117	*n* = 36				
≥50,000	101 (86)	22 (61)	0.002[a]	4.017 (1.733–9.309)	0.004	4.857 (1.674–14.096)
<50,000	16 (14)	14 (39)				
Neutrophil counts (/μL)	*n* = 117	*n* = 36				
≥800	100 (86)	28 (78)	0.305[a]			
<800	17 (14)	8 (22)				
BM blast (%)	*n* = 117	*n* = 36				
<5	80 (68)	22 (61)	0.426[a]			
≥5	37 (32)	14 (39)				
Karyotype	*n* = 111	*n* = 33				
Normal	57 (51)	19 (58)	0.557[a]			
Abnormal	54 (49)	14 (42)				
Age (years)	*n* = 117	*n* = 36				
≤66	55 (47)	23 (64)	0.088[a]	0.501 (0.234–1.073)	0.343	
>66	62 (53)	13 (36)				

OR odds radio, *CI* confidence interval, *BM* bone marrow.

[a] Fisher's exact test.

[b] Pearson's chi-square test.

[c] Logistic regression model (MDS, *n* = 139) [*TNF* (low)_*IL6* (intermediate + low) was considered the reference combined genotype in the analysis].

p = 0.036, 95%CI: 1.535–58.484). Furthermore, the genotype of high (G/G) as opposed to intermediate + low (G/C + C/C) *IL6* expression was associated with a 2.9-fold increased risk of presenting with hemoglobin levels <8 g/dL (12/36, 33% vs. 9/61, 15%; *p* = 0.042, 95%CI: 1.073–7.778). The *TNF* (high)_*IL6* (high) vs. the other combined genotypes was associated with a 4.5-fold higher risk of manifesting hemoglobin levels <8 g/dL (logistic regression, *p* = 0.037, 95%CI: 1.091–18.408, as internally validated by bootstrap analysis), a 16-fold proclivity to manifest a severe bicytopenia (4/14, 29% vs. 2/88, 2%; *p* = 0.004, 95%CI: 2.592–98.773) at diagnosis, and a shorter TDFS (2.9 vs. 173.6 months; *p* < 0.001) (Fig. 2b).

Because of the relevance of IL-6 to promote Th17 development within the context of TGF-β1, we combined both genotypes. The *IL6* (high)_*TGFB1* +869 (high) vs. the other combined genotypes was associated with increased risks of 4.4- and 5.6-fold of manifesting hemoglobin levels <8 g/dL (5/10, 50% vs. 16/86, 19%; *p* = 0.038, 95%CI: 1.195–16.018) and platelet counts <50,000/μL (4/9, 44% vs. 10/82, 12%; *p* = 0.029, 95%CI: 1.415–23.450), respectively. This genotypic combination was also associated with a 7-fold increased risk of presenting with a severe bicytopenia at diagnosis (3/10, 30% vs. 5/86, 6%; *p* = 0.035, 95%CI: 1.496–32.216).

Discussion

Polymorphisms in the immunomodulatory genes are known to affect the immune functions and to be closely correlated with the susceptibility to autoimmune disease. Although an immune dysregulation seems to be a key player in the pathologic process in MDS development, to the best of our knowledge, there are few articles to date have been focused on isolated cytokine polymorphisms and their relationship to susceptibility. Furthermore, studies that evaluate possible associations with the clinical characteristics in MDS patients are infrequent, restricted to only *TNF* variants [21, 34], or are otherwise absent.

Since our previous work was focused on only the *TNF* –308 G/A SNP, we increased the population size

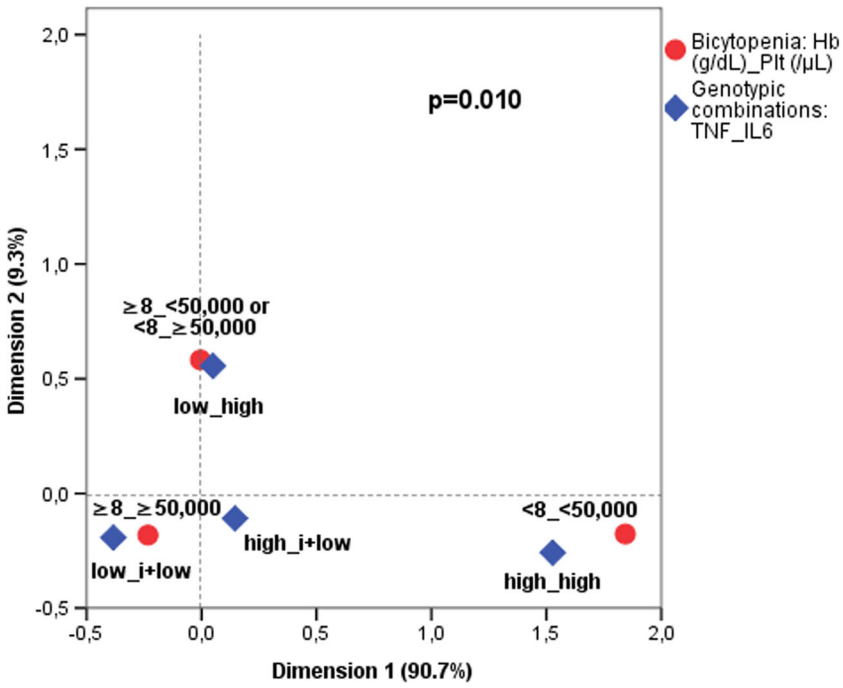

Fig. 1 Simple correspondence analysis. Distribution of MDS patients according to the *TNF_IL6* combined genotypes and bicytopenia at diagnosis, the latter defined as hemoglobin level (Hb) <8 g/dL and platelet counts (Plt) <50,000/μL. Dimension 1 (abscissa) explains 90.7% of variance, while dimension 2 (ordinate) 9.3%. The *TNF* (high)_*IL6* (high) and <8 g/dL_<50,000/μL categories are positively associated owing to the greater distance from the origin

and extended the study to five other candidate polymorphisms in the *IFNG*, *IL6,* and *TGFB1* genes based on published data that indicated the relevance of these cytokines to the MDS aberrant microenvironment. We were then able to confirm that the high-expressing *TNF* genotype (A/A + G/A) was related to an increased chance of developing MDS, in accord with other previous reports [23, 35], and that this genotype was associated with anemia and thrombocytopenia at diagnosis [22]. Consistent with the cytopenia, these patients proved to be transfusion dependent and died of non-AML related causes. MDS patients express high levels of TNF-α in the BM [7, 12], a phenotype related to the rates of intramedullary apoptosis and a worse patient outcome [12]. Because TNF-α exerts a negative regulation on endogenous EPO, the expression of that cytokine directly affects hemoglobin levels [12] and the response to EPO treatment [13].

TNF-α and IFN-γ are the main cytokines involved in Th1-lymphocyte immune-mediated suppression of hematopoiesis through different pathways. In agreement with the previous study of Serio et al. analyzing the *IFNG* +874 A/T SNP [23], which variation shows a strong linkage disequilibrium with *IFNG* +875 CAn microsatellite (D′ = 0.99) [25], no relationship of these two polymorphisms to susceptibility was found in our population. Nevertheless, MDS patients carrying the high-expressing *IFNG* genotype (12/12 CA) presented with a severer neutropenia at diagnosis. IFN-γ reduces the neutrophil differentiation induced by the granulocyte-colony stimulating factor [36] and is also overexpressed in MDS patients [14] whose CD34+ cells manifest an up-regulation of antiproliferative *IFNG*-stimulated genes [37]. Although the *TNF* (high)_*IFNG* (high) combined genotype may contribute to the cytopenic state, that genetic pairing was observed in only eight patients.

We were able to observe a direct relationship between the *IL6* –174 G/C SNP and MDS susceptibility, in contrast to previous reports [23, 26]; furthermore, the high-expressing *IL6* genotype (G/G) was associated with a severe anemia and a shorter TDFS. The contribution to MDS pathophysiology of IL-6, another multifunctional cytokine, is not completely understood: whether elevated levels of IL-6 represent a reactive response or an intrinsic pathophysiologic component. This cytokine is the principal mechanism underlying the anemia of inflammation that induces the synthesis of hepcidin, which dysregulation constitutes a complication in MDS [38]. Fishman et al. demonstrated that a genetically determined difference exists in the degree of the IL-6 response between individuals within an in vitro inflammatory context [19], and that the influence of the high-expressing variant may perturb the hemoglobin levels of MDS patients. An overproduction of IL-6 has been described in MDS [7, 15] that was associated with a lower OS and LFS [15]. Furthermore, the presence of IL-6, within the context of TGF-β1, induces differentiation in favor of the Th17 development, thus contributing to the immune dysregulation as an additional characteristic of low-risk MDS [9]. In this regard, our results indicated that the *IL6* (high)_*TGFB1* +869 (high) combined genotype may intensify the severity of bicytopenia in MDS patients with low (<5%) BM blast counts.

As defined above, *TNF* and *IL6* polymorphisms were independently associated with the hemoglobin level at diagnosis

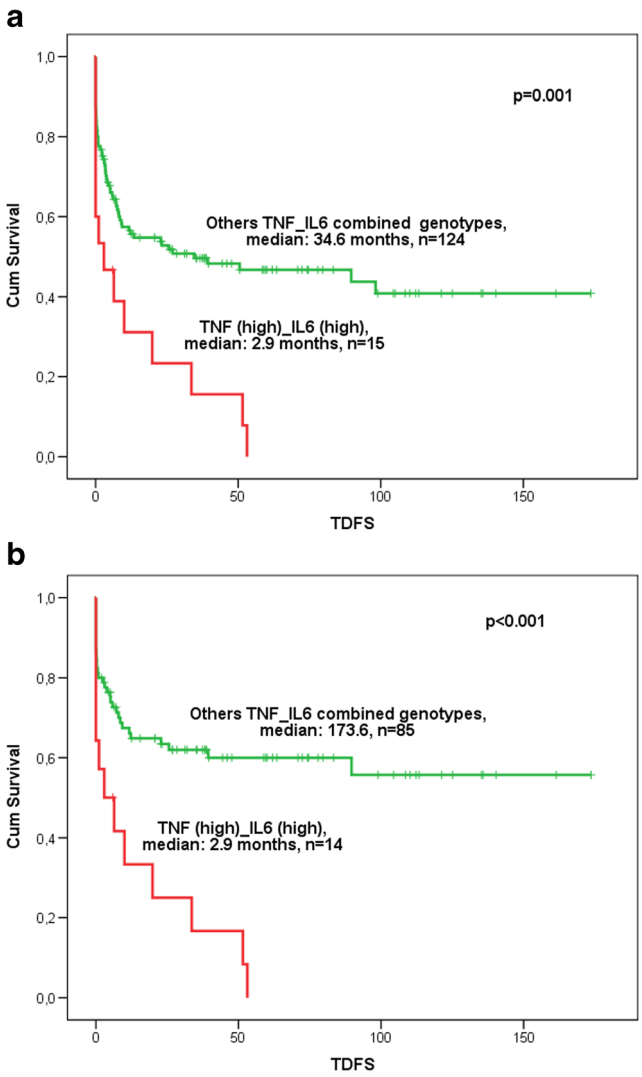

Fig. 2 Transfusion dependence-free survival (TDFS) and combined genotypes. Kaplan-Meier curves indicating the median TDFS with respect to *TNF_IL6* combined genotypes for (a) the overall MDS population and (b) patients with <5% bone marrow blasts

in our series of patients, thus influencing the MDS phenotype. The present analysis of combined genotypes confirmed the association between the high-producing *TNF* and *IL6* genotypes and the severity of anemia. Especially, patients carrying the *TNF* (high)_*IL6* (high) combined genotype presented with a severer bicytopenia and consistently experienced a shorter TDFS. Since both cytokines are directly linked to hematopoietic failure in MDS, the two have been targeted through the production of their respective inhibitors. Approved TNF-α inhibitors have shown a varying degree of efficacy in improving blood cell counts in MDS patients [39–41]. Despite the therapeutic efficiency observed in Castleman's disease and rheumatoid arthritis, an anti-IL-6 therapy did not reduce the need for transfusions in transfusion-dependent patients with low-risk MDS [42]. An association has been recognized between genetic polymorphisms and the response to anti-TNF-α of patients with inflammatory diseases. López-Hernández et al. found an increased frequency of the high-expressing *TNF* allele (−308A) and genotype (−308G/A) in patients not responding to TNF-α inhibitors [24]. The relationship between *TNF* –308A and anti-TNF-α refractoriness as well as the influence of this allele on the clinical features of our patients may constitute the rational bases for designing future clinical trials in MDS.

Our results point out that a patient's genetic background likely contributes to the T cell-mediated inhibition of hematopoiesis that influences the degree of cytopenias in MDS, which effect becomes more evident in patients with lower BM blast counts. *TNF* and *IL6* polymorphisms, as underlying host factors, are likely to play an essential role in influencing the susceptibility and clinical characteristics in MDS patients. These findings would argue that the genotyping of functional cytokine polymorphisms might be instrumental to the tailoring of cytokine-target therapies in MDS.

Acknowledgements The authors would like to thank all members of Argentine participating hematological services for their involvement in the present study. All the authors gave significant intellectual contributions to the composition of the manuscript, critically reviewed the content, and approved the final version.

This paper was supported by Argentine grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 0088 and PIP 0056) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 1677 and PID 0044).

Dr. Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

Compliance with ethical standards The ethics committee of the *Institutos de la Academia Nacional de Medicina* has evaluated and approved this research. Since this investigation is a retrospective and prospective study; therefore, a formal consent was required in the last cases.

Conflict of interest The authors declare that they have no conflict of interest.

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