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Investigation of Functional IL-10 Gene Polymorphism and IL-10 Levels in Acute Graft-Versus-Host Disease

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Abstract
Background Graft-versus-host disease (GVHD) represents a major complication in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients. Although studies have been conducted concerning the investigation of cytokine polymorphisms in the development of acute GVHD (aGVHD), the contribution of recipients and donors as regards cytokine levels has not yet been thoroughly assessed.
Objective The aim of this study was to investigate the impact of IL-10 polymorphisms on cytokine levels in blood and saliva, in addition to the occurrence and severity of aGVHD.
Methods Fifty-eight consecutive allo-HSCT recipients and their donors were included in this prospective study. Saliva and/or blood samples were obtained from recipients and donors to determine *IL10* polymorphisms. The IL-10 levels in the blood and saliva were also assessed. The samples were collected from seven days before transplant (day −7) to 100 days after allo-HSCT (day +100), once a week or until the death of recipient.
Results No association was found between recipient and donor *IL10* polymorphism and IL-10 levels in the saliva with aGVHD. In contrast, IL-10 levels in the blood were associated with the occurrence of aGVHD. The high producer phenotype in the recipient was also associated with high levels of IL-10 in the blood and saliva.
Conclusion Although *IL10* polymorphisms were not associated with the occurrence and severity of aGVHD, the genetic background of the recipient did in fact influence the production of the cytokine. Furthermore, as IL-10 levels in the blood were associated with the disease development, this parameter may well be a useful predictor of aGVHD development.

Keywords Hematopoietic stem cell transplant · graft-versus-host disease · acute graft-versus-host disease · polymorphisms · cytokines

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Introduction

Hematopoietic stem cell transplant (HSCT) is currently an important curative treatment for many patients with malignant and non-malignant hematological diseases. Graft-versus-host disease (GVHD) develops in many allogeneic HSTC recipients and is one of the main factors affecting the success of transplants, as its occurrence is associated with high morbidity and mortality rates [1–4]. GVHD occurs as result of complex immunological interactions between host cells and transplanted donor cells. Acute GVHD (aGVHD) most commonly begins within 100 days after allo-HSCT and is characterized by lympho-

cyte infiltration and epithelial cell apoptosis within target organs, typically in the gastrointestinal tract, in the liver, and on the skin. aGVHD is clinically ranked from grades I to IV according to the extension of organ damage [2, 4, 5].

Cytokines play an important role in the pathological damage of aGVHD, influencing two out of the three stages of aGVHD development (the so-called "cytokine storm"), as described by Ferrara and Yanik [6]. In the step 1, the conditioning regimen leads to the secretion of inflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). In step 2, donor T cells proliferate and secrete IL-2 and interferon-γ (IFN-γ). In step 3, activated cytotoxic T lymphocytes and natural killer cells induce target tissue through cell-mediated cytotoxicity.

Cytokine interleukin 10 (IL-10) is produced by a variety of different cells of both hematopoietic and non-hematopoietic lineages. IL-10 is a potent suppressor of the immune responses and hence it is thought to be useful in preventing GVHD. However, it has also been shown to have some immunostimulatory effects [7, 8].

In addition to immunological stimulus, it is well-known that cytokine production also remains under genetic control due to the polymorphisms of several cytokine genes. Allelic variants of cytokine genes are typically related to either a higher or lower production of cytokines [7]. Previous studies have shown an association between genetic variations in cytokine-related genes in recipients and donors and the occurrence of aGVHD [9–11]. However, the impact of genetic polymorphisms on cytokine levels in blood and saliva were not assessed in these subjects. The aim of the present study was to investigate the impact of *IL10* polymorphisms on cytokine levels in blood and saliva as well as to test the hypothesis if both were in fact associated with the occurrence and severity of aGVHD.

Methods

Subjects and Sample Collection

Fifty-eight consecutive allo-HSCT recipients and related donors from Hospital das Clínicas at Universidade Federal de Minas Gerais (HC-UFMG), between October 2006 and October 2008, were deemed eligible and included in this prospective study. Recipients were conditioned for allo-HSCT according to the specific protocols from the Stem Cell Transplant Unit at HC-UFMG and varied according to the type of the disease, disease status and the previous treatment at the time of transplantation. Cyclosporin, in combination with methotrexate or mycophenolate mofetil, was used for GVHD prophylaxis, whereas 2 mg/kg of methylprednisolone, in combination with Cyclosporin, was used for GVHD treatment. Clinical data from patients and donors are described in Table I. Saliva and blood samples were collected from recipients and donors one week before the HSTC and were submitted for DNA analysis. Saliva samples were collected using cotton swabs on the floor of the mouth, tongue, and labial and buccal normal oral mucosa of the HSCT subjects. Sites with localized injuries were not included. The cotton swabs were removed with a sterile cytobrush, placed immediately in sterile tubes containing 500 μl of Krebs buffer (NaCl, 20%; KCl, 2%; CaCl22H2O, 2%; MgSO4, KH2-PO4, and C6H12O6), and stored at −20°C until processing. Peripheral blood (4 ml) was collected in vacutainer tubes containing EDTA and stored at −70°C until processing.

To determine the cytokine levels, one blood and one saliva sample were obtained once a week from seven days before transplant (day −7) to 100 days after allo-HSCT (day +100). The saliva sample was colleted in Salivette tubes (Sarstedt AG & Co, Numbrecht, Germany) according to the manufacturer's instructions. The saliva samples were subsequently diluted (1:1) in a PBS solution containing protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20 and subsequently stored at −20°C until analysis. The serum samples were obtained from venous blood samples and were centrifuged within 2 h after blood collection and stored at −20°C.

The total protein content in the saliva was determined using the Bradford Reagent (Sigma, Saint Louis, MO, USA) and the BSA standard (Fermentas Life Sciences, Vilnius, Lithuania). The total protein content was used to correct the IL-10 values for each sample.

DNA Isolation

Total genomic DNA was extracted from saliva and blood samples using a QIAamp DNA Blood Mini Kit (Quiagen, Valencia, CA, USA) in accordance with to the manufacturer's instructions. The final elution of saliva and blood DNA was performed in 50 μl of a specific AE buffer from the Kit and stored at −20°C until use.

IL10 Gene Polymorphism Analysis

Recipient and donor *IL10* G>A at −1082 polymorphism was assessed by means of polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers were 5′ CCAAGACAACACTACTAAGGCTCCTTT 3′ and 5′ GCTTCTTATATGCTAGTCAGGTA 3′, with an expected PCR product size of 377 pb, as previously described by Koch et al. [12] The PCR was carried out in a total volume of 50 μ, containing approximately 400 ng of DNA, primers (20 pmol⁄reaction), and 25 μ of Pre-mix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the Pre-mix buffer contained 50 mMKCl,

Table I Clinical Characteristics of Allo-HSCT Patients and Donors (*n*=58)

Parameters	Total (*n*=58)
Recipient median age in years (range)	31.5 (5–56)
Female gender	25 (43.2%)
Primary disease	
Malignant	
Chronic myeloid leukemia	9 (15.5%)
Acute myeloid leukemia	15 (26%)
Acute lymphoid leukemia	5 (8.6%)
Non-Hodgkin's lymphoma	4 (6.9%)
Hodgkin's lymphoma	3 (5.1%)
Other malignancies[a]	4 (6.9%)
Bone marrow failure syndrome[b]	18 (31%)
HLA match	
HLA-matched related	52 (89.6%)
HLA-matched unrelated	4 (6.8%)
HLA-mismatched related	2 (3.6%)
Donor median age in years (range)	35.4 (6–69)
Donor female gender	21(36.3%)
Conditioning regimen	
BU/CY	20 (34.5%)
CY±ATG or Alemtuzumab	14 (24.2%)
BU+FLU±Alemtuzumab	9 (15.5%)
CY+FLU±Alemzutumab	8 (13.8%)
MEL+FLU±Campath	5 (8.6%)
Others[c]	2 (3.4%)
Ethnic group	Brazilian mixed population
Source of stem	
Bone marrow	32 (55.2%)
Peripheral blood stem cells	25 (43.1%)
Umbilical cord blood	1 (1.7%)

BU busulfan, *CY* cyclophosphamide, *FLUD* fludarabine, *MEL* melphalan, *ATG* antithymoglobulins

[a] Myelodisplastic syndrome (*n*=1), myelofibrosis (*n*=1), and multiple mieloma (*n*=2)

[b] Paroxysmal noctural hemaglobinuria (*n*=2), severe aplastic anemia (*n*=14), and Fanconi anemia (*n*=2)

[c] BU/MEL (*n*=1); cytarabine/campath/FLUD (*n*=1)

10 mM Tris–HCl pH 8.4, 0.1% Triton X-100, 1.5 mMMgCl2, deoxynucleoside triphosphates, and 1.25 units of *Taq* DNA polymerase. The conditions for amplification consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 35 s, and 72°C for 30 s. The run was terminated by final elongation at 72°C for 5 min. In all steps, the lid temperature was 103°C. The products all contained 5U XagI of the restriction enzyme (Promega, Madison, WI, USA) at 37°C for 4 h and obtained 280+97 bp for the AA genotype, 280+253+97+27 bp for the GA genotype, and 253+97+27 bp for the GG genotype. Visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis stained with silver.

Detection of IL-10 Levels

Cytokine concentration was determined by means of a quantitative sandwich ELISA technique using a Human IL-10 DuoSet Kit (DY217B, R&D Systems, Minneapolis, MN, USA).

Acute GVHD Grades

All patients were classified from grade I to grade IV for acute GVHD, as described by Glucksberg et al. [5].

Statistical Analysis

Univariate analyses were performed using the Chi-square, Mann–Whitney, and Friedman tests within an SPSS software (SPSS Inc., version 16.0, Chicago, IL). A p value≤0.05 was considered statistically significant.

Results

Clinical Outcomes

Acute GVHD could be observed in 27 patients. All patients were classified from grades 0 to IV for acute GVHD: 38 subjects were within grades 0–I (65.5%),

whereas grades II–IV were observed in 20 individuals (34.5%).

IL10 Gene Polymorphisms in Recipient and Donor of Allo-HSCT and aGVHD

Results of recipient and donor *IL10* gene polymorphism, their prevalence and corresponding phenotypes, and the impact of the polymorphisms on the incidence and severity of aGVHD are presented in Table II. According to previous studies [13] in addition to due to the low number of subjects with the GG genotype, the individuals were grouped into low (AA genotype) and high producer phenotypes (GA and GG genotypes). The frequencies of the gene polymorphisms found were similar to those reported in white population [9]. In the present study, we did not find association between recipient and donor *IL10* polymorphism and the occurrence or severity of aGVHD.

IL-10 Levels

Tables III and IV summarize the mean, minimum, and maximum levels of blood and saliva IL-10 levels during the 100 days following allo-HSCT. In each week, the number of valid samples analyzed suffered variations according to the availability of the samples and depending on the complications which arise from the post-HSCT treatment of the individuals. Week 1 represents day −7 before transplant.

IL-10 Levels in Blood and Saliva and *IL10* Polymorphisms in Recipients of HSCT

IL-10 blood levels were associated with *IL10* polymorphism in recipients at week 7, where an increased level of this cytokine in the high producer phenotype can be observed (Table III). An increased level of IL-10 cytokine in the saliva was also found in recipients with the IL-10 high producer phenotype at week 12 (Table IV).

IL-10 Levels in Blood and Saliva and *IL10* Polymorphisms in Donors of HSCT

Donor genotypes were analyzed according to IL-10 levels in the blood and saliva of recipients during the first 100 days following HSCT. The donor IL-10 genotype was not associated with IL-10 levels in blood or saliva (data not shown).

IL-10 Levels in Blood and Saliva and the Incidence of aGVHD

An increased level of IL-10 was observed in the blood of patients with aGVHD (mean 504.05 pg/ml) compared to individuals without aGVHD (mean 275.50 pg/ml) at the week 5 (p=0.01). No association was found between IL-10 levels in saliva and the development of aGVHD. As there was not enough valid cases for processing the Friedman test in all weeks, the weeks 1 to 8 were used for the blood analysis in patients without aGVHD and the weeks 1 to 11 for patients with aGVHD. In the saliva, the analysis was done for the weeks 1 to 16 (patients without aGVHD) and for the weeks 1 to 7 (patients with aGVHD). IL-10 levels did not vary significantly in the blood of patients without and with aGVHD and in saliva IL-10 of patients without and with aGVHD (Figs. 1 and 2).

IL-10 Levels in Blood and Saliva According to Date of Diagnosis of aGVHD

Samples were divided into the following groups: From 30 days to 07 days before, day of diagnosis, and 30 days after the diagnosis of aGVHD. The median of IL-10 blood and saliva levels among these periods were not statistically different (data not shown).

Discussion

The importance of the cytokine cascade in the different phases of GVHD is well established. Earlier studies have

Table II Association Between Recipient and Donor Cytokine Genotypes and Occurrence and Grade of aGVHD (n=58)

Gene	Genotype	Associated phenotype	Number (%)	GVHD		p^a	GVHD		p^a
				A	P		0–I	II–IV	
IL10 (–1082) (patient)	AA	Low	22 (37.9)	12	10	NS	16	06	NS
	GA/GG	High	36 (62.1)	19	17		22	14	
IL10 (–1082) (donor)	AA	Low	02 (3.4)	01	01	NS	01	01	NS
	GA/GG	High	56 (96.6)	30	26		37	19	

NS not significant, *P* present, *A* absent

[a] Chi-square test

Table III Association Between Blood IL-10 Levels and Recipient IL-10 Genotypes (n=58)

Weeks	Genotype	Associated phenotype	*n* valid	IL-10 Levels (pg/ml)			p^a
				mean	minimum	maximum	
1	AA	Low	19	283.39	0.00	2,954.94	–
	GA/GG	High	24	589.08	0.00	7,265.10	
2	AA	Low	19	256.39	0.00	2,954.94	NS
	GA/GG	High	26	633.11	0.00	7,265.10	
3	AA	Low	21	287.89	0.00	1,825.88	NS
	GA/GG	High	24	486.48	0.00	5,809.48	
4	AA	Low	22	212.99	0.00	1,477.94	NS
	GA/GG	High	23	457.44	0.00	4,954.05	
5	AA	Low	14	197.57	0.00	671.61	NS
	GA/GG	High	28	445.06	0.00	5,296.79	
6	AA	Low	15	140.99	0.00	1,065.20	NS
	GA/GG	High	20	759.45	0.00	5,954.02	
7	AA	Low	18	210.05	0.00	941.77	0.03
	GA/GG	High	20	232.14	0.00	2,915.15	
8	AA	Low	16	142.44	0.00	730.33	NS
	GA/GG	High	25	269.90	0.00	1,793.38	
9	AA	Low	14	50.72	0.00	257.46	NS
	GA/GG	High	21	199.31	0.00	1,352.23	
10	AA	Low	16	118.67	0.00	756.29	NS
	GA/GG	High	21	263.67	0.00	2,837.37	
11	AA	Low	19	130.98	0.00	954.70	NS
	GA/GG	High	20	196.06	0.00	1,959.88	
12	AA	Low	12	56.23	0.00	250.70	NS
	GA/GG	High	18	250.70	0.00	1,808.37	
13	AA	Low	13	189.84	0.00	1,387.26	NS
	GA/GG	High	18	194.28	0.00	1,978.28	
14	AA	Low	12	46.95	0.00	305.70	NS
	GA/GG	High	14	211.17	0.00	2,180.92	
15	AA	Low	14	119.19	0.00	768.36	NS
	GA/GG	High	10	376.87	0.00	1,991.56	
16	AA	Low	14	62.28	0.00	752.80	NS
	GA/GG	High	10	294.47	0.00	1,374.33	

NS not significant

[a] Mann–Whitney test

shown that the genetic make-up of recipients and donors can influence the success or failure of HSCT, and it has been suggested that the existence of high or low cytokine production is considered a significant risk factor associated with transplant results. Some authors have reported that genetic polymorphisms can in fact be responsible for phenotypic variations and transplant results, including aGVHD [14, 15].

IL-10 is a key immunomodulatory cytokine that is produced by B cells, regulatory T-cells, monocytes, and dendritic cells [16]. It plays an important role in the downregulation of TNF and Th1 cytokine production, as well as in the inhibition of T-cell proliferation. *In vitro* results show that administration of IL-10 decreases alloreactivity of T cells in mixed lymphocyte culture assays [17]. However, one *in vivo* study showed that IL-10 exacerbates GVHD at higher levels but provides protection within physiological doses [12]. Although the role of IL-10 cytokine in the pathogenesis of GVHD is not well-established, it does appear to have an impact on the fatal outcome of patients after transplants [2]. The gene of IL-10 cytokine maps to chromosome 1 (1q31-32) and is highly polymorphic, presenting several microsatellites and five SNPs that form haplotypes associated with high (GCC),

Table IV Association Between Saliva IL-10 Levels and Recipient IL-10 Genotypes (n=58)

Weeks	Genotype	Associated phenotype	*n* valid	IL-10 Levels (pg/mg protein)			p^a
				mean	minimum	maximum	
1	AA	Low	16	503.17	0.00	7,528.51	–
	GA/GG	High	22	117.58	0.00	816.89	
2	AA	Low	16	1,041.67	0.00	11,439.56	NS
	GA/GG	High	21	143.41	0.00	1,614.31	
3	AA	Low	15	517.36	0.00	6,578.82	NS
	GA/GG	High	21	84.79	0.00	529.02	
4	AA	Low	14	387.56	0.00	2,581.22	NS
	GA/GG	High	15	54.08	0.00	271.55	
5	AA	Low	13	146.66	0.00	866.15	NS
	GA/GG	High	16	181.41	0.00	1,245.73	
6	AA	Low	13	205.75	0.00	955.43	NS
	GA/GG	High	18	127.81	0.00	482.41	
7	AA	Low	12	100.21	0.00	341.40	0.03
	GA/GG	High	15	205.19	0.00	901.46	
8	AA	Low	10	92.03	0.00	428.93	NS
	GA/GG	High	16	156.79	0.00	558.11	
9	AA	Low	14	63.76	0.00	228.03	NS
	GA/GG	High	15	847.05	0.00	8,204.74	
10	AA	Low	12	99.99	0.00	326.71	NS
	GA/GG	High	18	243.78	0.00	932.58	
11	AA	Low	12	470.62	0.00	4,105.29	NS
	GA/GG	High	16	184.29	0.00	1,064.35	
12	AA	Low	09	21.02	0.00	74.61	0.03
	GA/GG	High	14	279.65	0.00	1,903.60	
13	AA	Low	10	33.09	0.00	104.66	NS
	GA/GG	High	17	176.77	0.00	1,011.25	
14	AA	Low	09	152.94	0.00	591.96	NS
	GA/GG	High	18	134.80	0.00	683.12	
15	AA	Low	08	60.10	0.00	268.89	NS
	GA/GG	High	13	168.94	0.00	556.15	
16	AA	Low	07	72.94	0.00	343.69	NS
	GA/GG	High	16	131.76	0.00	406.19	

NS not significant

[a] Mann–Whitney test

intermediate (ATA), and low (ACC) IL-10 production [3]. Nucleotide variations in genes that encode this molecule may affect the transcription or translation of the gene, the secretion or the function of the correspondent protein and may play an important role in the pathogenesis of GVHD [18].

One of the aims of the present study was to determine whether recipient and donor IL-10 (-1082 G/A) gene polymorphism contributes to the development and severity of aGVHD after allo-HSCT. The present study found no association between recipient and donor *IL10* polymorphism and aGVHD development and severity. Other studies involving *IL10* polymorphisms and transplant outcomes showed divergent results. The low producer (ACC) haplotype in recipient was associated with severe acute GVHD grades III-IV in ciclosporin alone [14] and ciclosporin plus MTX-treated HLA-matched sibling HSCT cohorts [19]. The intermediate producer IL-10 haplotype (ATA) was confirmed to play a role in severe aGVHD in two large cohorts, where almost 1000 patients were analyzed [18]. Kögler et al. [20] studied HLA-mismatched cord blood transplant and did not find association between *IL10* gene polymorphism with GVHD. Middleton et al. [9] reported that the alleles of the IL10-1064 promoter region micro-

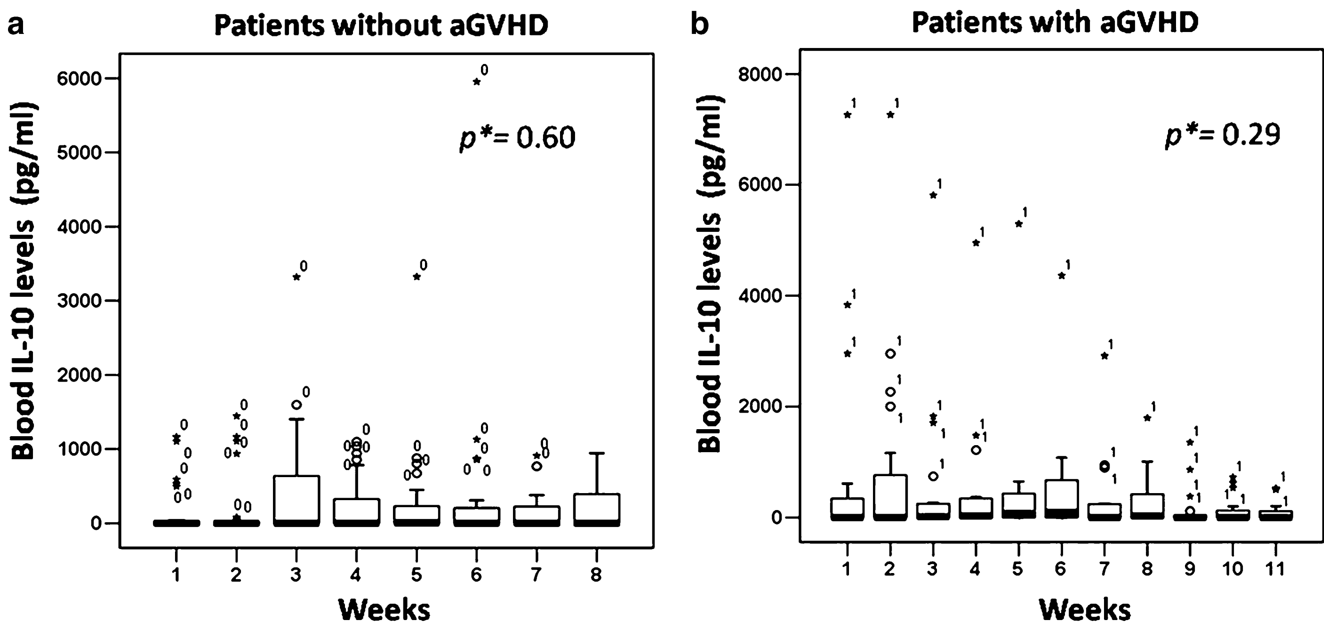

Fig. 1 IL-10 levels in the blood during the weeks after HSCT in patients who develop aGVDH (**a**) and in patients who did not develop aGVHD (**b**) (*asterisks* Friedman test)

satellite polymorphism possess greater numbers of dinucleotide (CA) repeats that are associated with more severity of GVHD. However, other authors reported an association between high producer IL-10 haplotype (GCC) and the occurrence of grade 2 to 4 aGVHD [21]. In addition, the donor *IL10* genotype has also been associated with a lower risk of aGVHD. Socié et al. [21] reported that the presence of donor IL-10 GCC/GCC homozygous genotype protects the patients from aGVHD. Other authors reported that the presence of high number of CA repeats in the donors was associated with an increased risk for aGVHD [14], while Karabon et al. [22] did not find significant association between *IL10* polymorphisms in donors and the incidence of acute GVHD. These discrepant findings may be partly explained by the differences in transplant conditioning regimens and initial release of cytokine by the recipient [9, 14]. We are aware that the relatively small numbers of patients in our study do not allow a definitive conclusion regarding the impact of cytokine genotype-associated GVHD risk. Thus, more studies are necessary to confirm our data.

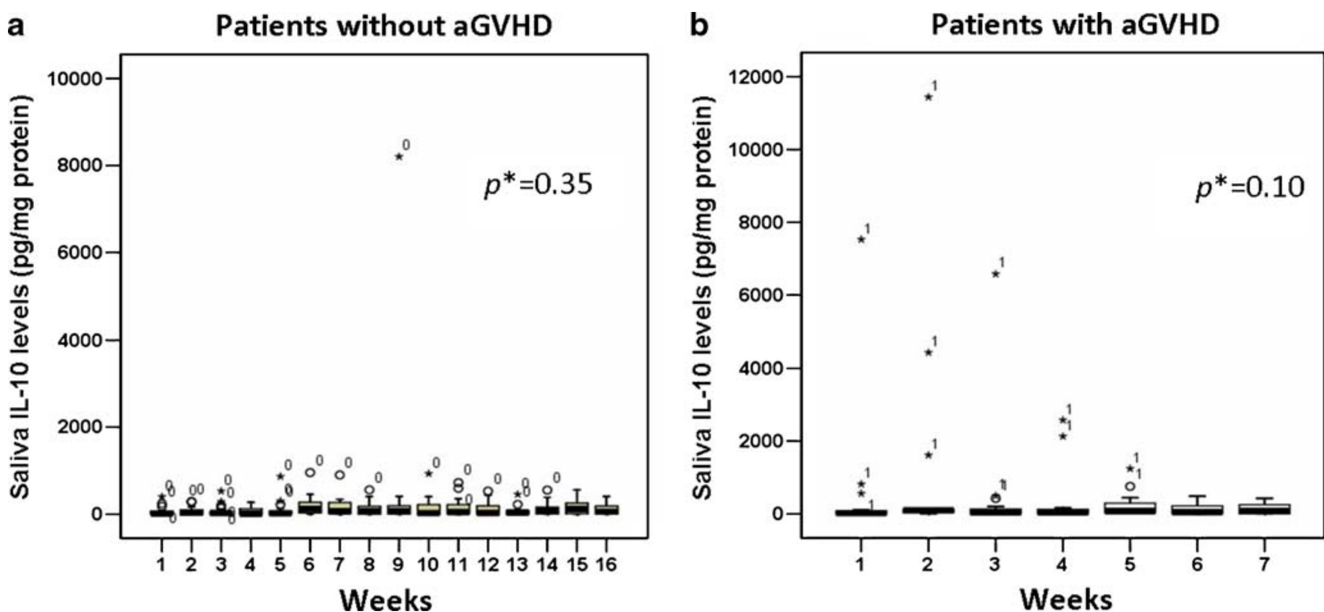

Fig. 2 IL-10 levels in the saliva during the weeks after HSCT in patients who develop aGVDH (**a**) and in patients who did not develop aGVHD (**b**) (*asterisks* Friedman test)

Measurements of cytokine levels in the serum of patients with GVHD have been used to investigate the impact of these cytokines on the development and course of the disease. To assess the impact of the *IL10* polymorphism on cytokine levels of HSCT patients, blood and saliva levels of IL-10 were analyzed, attempting to associate it with the occurrence of aGVDH. It could be observed that the donor IL-10 genotype did not influence IL-10 levels in blood and saliva; however, recipients with a high producer phenotype did show higher levels of cytokine in the blood and saliva. It could also be observed that high IL-10 levels in the blood were associated with the presence of the aGVHD. Hempel et al. [23] reported that the high IL-10 serum levels in patients in post-HSCT treatment were significantly associated with a fatal outcome. Similar findings were reported by Min et al. [24], where high IL-10 levels in the blood at week +4 after the transplant were associated with fever, severe stomatitis, and aGVHD. Although high IL-10 production can lead to immunodeficiency, and consequently to the development of complications after HSCT [6], its increased production may be an attempt to suppress the various inflammatory cytokines that tend to increase during the leukocyte recovery phase [25]. Therefore, an increased level of IL-10 is a negative indicator of the cytokine storm that mediates aGVHD development.

Conclusion

Although *IL10* polymorphisms were not associated with the occurrence and severity of aGVHD, the genetic background of the recipient does in fact influence the production of the cytokine. In addition, as IL-10 levels in the blood were associated with disease development, this parameter may well be a useful predictor of aGVHD development.

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