Research Article

IL-17 Genetic and Immunophenotypic Evaluation in Chronic Graft-versus-Host Disease

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Although interleukin-17 (IL-17) is a recently discovered cytokine associated with several autoimmune diseases, its role in the pathogenesis of chronic graft-versus-host disease (cGVHD) was not established yet. The objective of this study was to investigate the association of \textit{IL17A} and \textit{IL17F} genes polymorphisms and IL-17A and IL-17F levels with cGVHD. IL-17A expression was also investigated in CD4+ T cells of patients with systemic cGVHD. For Part I of the study, fifty-eight allo-HSCT recipients and donors were prospectively studied. Blood samples were obtained to determine \textit{IL17A} and \textit{IL17F} genes polymorphisms. Cytokines levels in blood and saliva were assessed by ELISA at days +35 and +100 after HSCT. In Part II, for the immunophenotypic evaluation, eight patients with systemic cGVHD were selected and the expression of IL-17A was evaluated. We found association between recipient AA genotype with systemic cGVHD. No association was observed between IL-17A levels and cGVHD. Lower IL-17A levels in the blood were associated with AA genotype. In flow cytometry analysis, decreased expression of IL-17A was observed in patients with cGVHD after stimulation. In conclusion, IL-17A may have an important role in the development of systemic cGVHD.

1. Introduction

Hematopoietic stem cell transplant (HSCT) represents the definitive immunotherapy for malignancy and immunologic diseases [1, 2]. However, graft-versus-host disease (GVHD) is an important complication of HSCT that limits its success and can be fatal in approximately 15% of the HSCT patients [3]. Acute GVHD (aGVHD) and chronic GVHD (cGVHD) involve distinct pathological process, where the first one has strong inflammatory components and the second one displays more autoimmune and fibrotic features [4].

T lymphocytes play a critical role in the immune response including allograft rejection, graft failure, and GVHD after transplant; understanding the posttransplant immune regulation might help to generate new therapeutic strategies that might overcome these posttransplant problems [5].
Interleukin-17 (IL-17) is a recently discovered cytokine that can be produced by many cells, although its source from CD4+ T cells is the most investigated [6]. The Th17 cells are a new lineage of CD4+ T helper cells [7] and generate proinflammatory cytokines such as IL-17A, IL-17F, IL-21, IL-22, tumor necrosis factor (TNF), granulocyte macrophage-colony stimulating (GM-CSF), and some chemokines [6]. IL-17A, the original member of this family, was first identified in 1995 [8] and acts predominantly as a chemoattractant. Recent data have shown that Th17 pathway is associated with cGVHD [6]. Another Th17 cytokine is the IL-17F; it shows the highest overall amino acid sequence identity with IL-17A in the IL-17 family. The genes encoding IL-17A and IL-17F are both located on 6p12 [9]. IL-17A and IL-17F share biological functions and have many proinflammatory effects in a wide variety of cells, including macrophage, endothelial cells, and fibroblasts [10].

In few recent studies, IL-17A has been associated with the pathway of the GVHD and some studies have assessed the association between IL17A gene polymorphism and inflammatory diseases, including the cGVHD [6,11,12]. However, the role of IL-17F in the pathogenesis of cGVHD was not previously addressed.

So, the aim of the present study was to investigate the association of the IL17A and IL17F gene polymorphisms and IL-17A and IL-17F levels with cGVHD as well as to determine the relationship between IL-17A expression by CD4+ T cells and the development of the disease.

2. Methods

To achieve the proposed objectives, the work was divided into two parts, which include two groups of individuals. In Part 1 we performed the genotypic and phenotypic analyses of the cytokines, IL-17A and IL-17F, and in Part 2 IL-17A immune marker was assessed. cGVHD was determinate according to NIH Consensus Development Project on Criteria for Clinical Trials [13].

2.1. Part 1: Genotypic e Phenotypic Evaluation

2.1.1. Subjects. Fifty-eight consecutive allo-HSCT recipients and related donors from Hospital das Clinicas at Universidade Federal de Minas Gerais (HC-UFMG) were deemed eligible and included in this prospective study. The patients were followed between the days −7 and +100 after it, in a total of sixteen weeks.

All the patients were followed from pre-HSCT until the development of cGVHD, so the number of samples included in each essay varied because of patient’s death and loss of samples related to the process of collection. 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 transplant.

Cyclosporin, in combination with methotrexate or mycophenolate mofetil, was used for GVHD prophylaxis, whereas 2 mg/kg of methylprednisolone, in combination with cyclosporine, was used for GVHD treatment.

This study protocol was approved by the local Institutional Ethics Committee (ETIC 0124.0.203.000-11). Written informed consent was obtained from all patients.

2.1.2. Subjects and Sample Collection. 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%, CaCl2 H2O 2%, MgSO4, KH2PO4, 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.

2.1.3. DNA Isolation. Total genomic DNA was extracted from saliva and blood samples using a QIAamp DNA blood minikit (Quiagen, Valencia, CA, USA) in accordance with 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.

2.1.4. IL17A and IL17F Gene Polymorphism Analysis. Recipient and donor IL17 197 (A/G) and IL17F 7488 (T/C) polymorphism was assessed by means of polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers for IL17A were reverse 5’ AACAAGTAA-GAATGAAAAAGAGGACATGTT 3’ and anti-sense 5’ CCC-CCAAATGAGGTCACTAAGAGAAGATC 3’ and for IL17F were reverse 5’ GTTCCCATCCAGCAAGAGAC 3’ and anti-sense 5’ AGCTGGGAATGCACACCAAC 3’, as previously described [14,15]. The PCR was carried out in a total volume of 50 µL, containing approximately 400 ng of DNA, primers (20 pmol/reaction), and 25 µL of premix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the premix buffer contained 50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1% Triton X-100, 1.5 mM MgCl2, deoxynucleoside triphosphates, and 1.25 units of Taq DNA polymerase. PCR products were digested overnight at 37°C with Xagl (Fermentas Life Sciences, Vilnius, Lithuania), for IL-17A, and NlaIII (Fermentas Life Sciences, Vilnius, Lithuania), for IL-17F. Visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis stained with silver.

2.1.5. Detection of IL-17A and IL-17F Levels. To determine the cytokine levels saliva and blood samples were collected in days +35 and +100 after allo-HSCT. The saliva sample was collected 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 aprotenin A) and 0.05% Tween-20 and subsequently stored at −20°C until analysis. The total protein content in the saliva
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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-17A values for each sample. The serum samples were obtained from venous blood samples and were centrifuged within 2 hours after blood collection and stored at -20°C.

2.2. Part 2: Immunophenotyping Evaluation

2.2.1. Subjects. For immunophenotypic evaluation, additional patient selection was performed and 3 different subgroups were established: HSCT patients with systemic cGVHD (n = 8), HSCT patients without systemic cGVHD (n = 4), and healthy individuals (n = 3).

2.2.2. Blood Collection and Cell Stimulation. Nine milliliters (9 mL) of blood was collected from each individual in a vacuum tube containing sodium heparin by a qualified healthcare professional, complying with the rules for the use of sharp instruments in an aseptic environment. Whole blood was subjected to 3 different conditions: media, stimulus with superantigen staphylococcal enterotoxin B (SEB) (120 ng/mL), and stimulus with anti-CD3 plus anti-CD28 (αCD3αCD28) monoclonal antibody (Abs) (10 μg/mL) for 20 hours. Brefeldin A (at 1 μg/mL) was added during the last 4 hours of culture. After culture, red blood cell lysis was performed using 1x RBC lysis buffer (eBioscience, San Diego, CA, USA) for flow cytometry, as recommended by the manufacturer.

2.2.3. Cell Surface and Intracellular Staining. After lysis, leukocytes from whole blood were stained with fluorescein isothiocyanate- (FITC-) labeled anti-CD4 monoclonal antibody (eBioscience; BD Pharmingen, San Diego, CA, USA) for 20 minutes at 4°C. The cells were then fixed using 2% formaldehyde (Sigma-Aldrich). The fixed cells were permeabilized and stained, using phycoerythrin- (PE-) labeled anti-cytokine monoclonal antibodies for IL-17A (eBioscience, San Diego, CA, USA), IFN-γ (eBioscience, San Diego, CA, USA), or Foxp3 (eBioscience, San Diego, CA, USA), FITC- or PE-labeled isotype control antibodies and an unstimulated cell control were included in all experiments. Preparations were acquired on a FACSCanto II (Becton & Dickinson, San Jose, CA, USA). A minimum of 50,000 gated events on the lymphocyte population were acquired for analysis due to the low frequency of positive events being analyzed. The acquisition was processed using the Diva software (Becton & Dickinson).

2.2.4. Flow Cytometry Data Analysis. CD4+ T lymphocytes were analyzed for their intracellular expression of IL-17A using the FlowJo program (Tree Star, Ashland, OR, USA). Analyses were performed using a lymphocyte gate (Figure I(a)). We determined the total expression of IL-17 in the lymphocyte gate (Figure I(b), R4); we also determined the frequency of Th1 and Th17 cells by analyzing the frequency of CD4+ IFN-gamma+ and CD4+ IL-17+ cells, respectively (Figure I(b), R3). The expression of IL-17 within the CD4+ T cells was assessed by further gating on the total CD4+ population (Figure I(b), R2) and requesting a histogram analysis of such gating (Figure I(c)). The same strategy was used for IFN-γ and Foxp3. Limits for the quadrant markers were always set based on negative populations and isotype controls.

2.3. Statistics. The Shapiro-Wilk test was used to determine if samples followed normal distribution. Two independent groups were compared by Student’s t-test and by the Wilcoxon test. Univariate analyses were performed using the Chi-square, Mann-Whitney, and Friedman tests and Student’s t-test. The SPSS software was used for the
Table 1: Clinical characteristics of allo-HSCT patients and donors (𝑛= 58).

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

* Myelodysplastic syndrome (𝑛= 1), myelofibrosis (𝑛= 1), and multiple myeloma (𝑛= 2).
** Paroxysmal nocturnal hemoglobinuria (𝑛= 2), severe aplastic anemia (𝑛= 14), and Fanconi anemia (𝑛= 2).
*** BU/MEL (𝑛= 1) Cytarabine/Campath/FLUD (𝑛= 1).
BU: busulfan; CY: cyclophosphamide; FLUD: fludarabine; MEL: melphalan; ATG: antithymoglobulins.

Analyses (SPSS Inc., version 19.0, Chicago, IL). Differences were considered significant at 𝑃< 0.05.

3. Results

3.1. Part I: Genotypic and Phenotypic Evaluation

3.1.1. Clinical Outcomes. Clinical data from patients and donors is shown in Table 1. During the monitoring period, the final "𝑛" value for the analysis related to IL-17A was 34 and related to IL-17F was 42. The initial "𝑛" selected was 58, but over time of patients' followup, losses occurred due to patient death or loss of samples for analysis related to the collection process.

3.1.2. IL17A and IL17F Gene Polymorphisms in Recipient and Donor of Allo-HSCT and cGVHD. Results of recipient and donor IL17A and IL17F gene polymorphism and their association with systemic cGVHD are presented in Table 2. We found association between recipient IL17A AA genotype and the occurrence of systemic cGVHD (𝑃= 0.03).

3.1.3. IL-17A and IL-17F Levels in Blood and Saliva and the Incidence of cGVHD. No association was found between IL-17A or IL-17F levels in the blood or saliva samples and the occurrence of systemic cGVHD (data not shown).

3.1.4. IL-17A Levels in Blood and Saliva and Recipient and Donors IL17A Genotypes. Higher IL-17A levels in the blood were observed at day +35 in recipients with IL17 AG genotype (𝑃= 0.007). No association was found between recipient genotypes and IL-17A levels in saliva or between donor IL17A genotypes and IL-17A levels in blood or saliva (Table 3). No association was also found between IL-17F blood or saliva levels and IL17F genotypes (data not shown).

3.2. Part II: Immunophenotyping Evaluation

3.2.1. Clinical Outcomes. Clinical data from patients and healthy individuals is in Table 4. None of the patients from the group without cGVHD presented the systemic form of the disease.

3.2.2. Expression of IL-17A in Total CD4 and in CD4+ T Cells and cGVHD. No statistical difference was found between the expression of total IL-17A and IL-17A+ CD4+ in total T cells, as well as percentage of IL-17A in CD4+ T cells and patients with cGVHD (data not shown).

3.2.3. Expression of IL-17A in Total CD4 and in CD4+ T Cells and cGVHD after Stimulus. Decreased IL-17A expression was observed in total CD4 T cells of patients with cGVHD after stimulation with SEB (𝑃= 0.008) and αCD3αCD28 (𝑃= 0.008) stimulus, compared to media-stimulated control cells (Figure 2(a)). In patients without cGVHD or in healthy individuals, the expression of IL-17A in total CD4 T cells after stimulation with SEB or αCD3αCD2 was not statistically different than that seen in media-stimulated control cells (data not shown). Overlapping histograms for IL-17A fluorescence are shown in Figure 2(b).

4. Discussion

Several studies have been devoted to analyze the role of IL-17, a new discovered cytokine, in the pathogenesis of GVHD [12, 16–18]. However, this is the first work that evaluated both cytokines IL-17A and IL17F, involving the genetic and phenotypic aspects, and investigated the immunophenotypic aspect of IL-17A regarding systemic cGVHD.
In the current study we investigate the involvement of the \textit{IL17A} and \textit{IL17F} gene polymorphism in the cGVHD and our results showed association of AA recipient genotypes with the occurrence of systemic cGVHD. Previous studies showed association between GI97A polymorphism and several chronic diseases [12], like ulcerative colitis [19] and rheumatoid arthritis [20]. A study reported association between allele A of \textit{IL17A} polymorphism and high risk of cGVHD [12]. Until now, no other work was reported in the literature relating \textit{IL17F} polymorphism and GVHD. We did not find association between \textit{IL17F} genotypes and cGVHD; however other authors showed association between \textit{IL17F} polymorphism (T/C 7488) and Behçet disease [21], gastric cancer [14], and bone mineral density [22].

Although our results did not show association between IL-17A or IL-17F levels and the presence of cGVHD, the importance of both in the context of the disease cannot be excluded because we do not understand all the temporal and dynamic factors involved in cGVHD development. The detection of cytokines in a specific time of the disease provides only a snapshot of a more complex process. Th17 pathway has been implicated in the development of acute
and cGVHD [6]. Previous preclinical and clinical studies have shown the role of IL-17A in pathogenesis of cGVHD, especially in skin [16, 23].

When we investigated the impact of the IL17A polymorphism on the cytokine levels of HSCT patients, we found that recipients with AA genotypes showed lower levels of cytokine in the blood in moment +35, compared to the other genotypes. Several polymorphisms in cytokine genes with transcriptional relevance in vitro have been identified [24]. A modification in cytokine production is associated with genetic variations in the promoter region [24–26]. Just one work until now investigated the relationship between IL17A polymorphism and IL-17A levels and the authors showed that serum concentration of IL-17A was significantly higher in the presence of allele A (AA and AG genotypes) in a population of Chinese patients with atopic dermatitis [27].

In our study we observed decreased percentage of IL-17A+CD4+ T cells after stimulus with SEB and αCD3αCD28. T-cell production of IL-17 induces epithelial, endothelial, and stromal cells to secrete proinflammatory cytokines (IFN-γ, IL-6, TNF-α, IL-1β, and G-CSF) and the levels of these cytokines have been shown to be higher in patients with cGVHD [28]. Therefore, decreased expression of IL-17A in the stimulated cells observed in this work may be caused by the inhibitory effect of others cytokines, like IFN-γ [7], which may be relevant to the pathogenesis of cGVHD.

5. Conclusion

In conclusion, we demonstrated association between AA genotype of recipients with systemic cGVHD and those with lower IL-17A levels and observed that stimulated lymphocytes of patients with cGVHD present decreased expression of IL-17A. Our data indicate that this cytokine could be an important mediator of the cGVHD and may be an important new target for investigation and therapeutics of the disease.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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