Cytokine 62 (2013) 302–309

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Cytokines signatures in short and long-term stable renal transplanted patients

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ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form 27 February 2013
Accepted 3 March 2013
Available online 2 April 2013

Keywords:
Renal transplantation
Cytokines
Stable graft function

ABSTRACT

Despite the evidences showing the relevance of regulatory immune-mediated mechanisms to guarantee the stable graft function in renal transplanted patients, studies focusing on the immune response observed over a long-term period after renal transplantation are still limited. Several efforts have been done to establish novel biomarkers with relevant predictive values that could be used as prognostic laboratorial tools to monitor the complex network triggered through time after kidney transplantation. In this study, we have evaluated the pro-inflammatory and regulatory patterns of plasma cytokines in a group of 120 renal transplanted patients with stable graft function ranging from 1 to 160 months. Our data demonstrated an overall predominance of regulatory cytokines short-term after renal transplantation (1–24 months) with peaks of IL-4, IL-5 and IL-10. Moreover, a slight peak of TNF-α was observed 25–60 months after renal transplantation. Following a gap of stable cytokine profile (61–120 months), peaks of pro-inflammatory cytokines IL-8, IL-6, IL1β, TNF-α and IL-12 were observed later on (>120 months) after renal transplantation. Additionally, the categorical analysis of "low" or "high" cytokine producers re-enforce the occurrence of an overall regulatory status early-after stable renal graft function with a predominant pro-inflammatory pattern later on long-term renal transplantation. Taken together, our data suggest that IL-5 is a good biomarker associated with short-term stable renal function, whereas IL-12 seems to be a relevant pro-inflammatory element in long-term renal transplanted patients.

1. Introduction

Renal transplantation is the better choice for treatment of end stage renal disease. Nevertheless, after renal transplantation patients need permanent immunosuppressive treatment to prevent graft rejection and loss. Despite great improvements in renal allograft survival over the last three decades, long-term graft loss, particularly through antibody-mediated rejection, remains the great challenge of renal transplantation [1–3].

Recent studies have suggested that cytokines plasma assess after renal transplantation could allow a better understanding of the renal allograft rejection pathogenesis, as well as to predict future rejection process [2–7]. Besides, cytokines gene polymorphisms have been correlated with increased production of these cytokines [8,9].

Cytokines are generally responsible for TH1 and TH2 responses in renal transplantation. TH1 lymphocytes produce IL-2, TNF-α and IFN-γ. By activation of macrophages, these lymphocytes participate in delayed hypersensitivity and cytotoxic activity. TH2 lymphocytes produce IL-4, IL-5, IL-6, IL-10, IL-13 and participate in the production of antibodies. Deregulated production of pro-inflammatory or regulatory cytokines plays an important role in the disease susceptibility and progression of renal transplantation [5–7]. Therefore, induction of specific immunologic tolerance with suppression of IL-6, IL-12, TNF-α and others proinflammatory cytokines remains as an important goal of organ transplantation [10–12].

The aim of this study was to evaluate the cytokines plasma levels in renal transplanted patients with stable graft function. It should be highlighted that this is the first study evaluating a wide cytokine profile in distinct periods post-transplant. This study can contribute to expanding the understanding about the immune response after renal transplantation and provide novel biomarkers with relevant predictive values that could be used as laboratorial tools for prognostic and monitoring purposes.

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1043-4666
http://dx.doi.org/10.1016/j.cyto.2013.03.001

2. Materials and methods

2.1. Patients

A total of 120 renal transplanted patients were selected at two Renal Transplant Units, in Belo Horizonte, Minas Gerais, Brazil from 2010 to 2011. The study population comprises 82 males and 38 females with age ranging from 19 to 73 years and time post-transplantation ranging from 1 to 160 months. All patients have received kidney from living organ donors. Pre-transplant panel reactive anti-HLA antibody was negative in 49 receptors, positive in 8 and not available for 63 patients. All patients were under corticosteroid therapy at their inclusion in this study. Triple immunosuppressive protocol with tacrolimus + mycophenolate mofetil + prednisone was given to 51.6% of patients and ciclosporin + mycophenolate mofetil + prednisone to 35.0% of patients. A small group of patients (13.4%) were treated with prednisone associated with other immunosuppressive drugs. Seven out of 120 patients presented previous CMV infection short-term after renal transplant. These patients were included in the present investigation since they showed negative CMV antigenemia at the blood collection. The inclusion criterion was stable graft function and the exclusion criteria were acute or chronic allograft rejection. Rejection was defined by an increase in creatinine plasma levels by 0.3 mg/dL from baseline that was not attributed to other causes with subsequent return to baseline after treatment with pulse steroids or anti-lymphocytic antibodies [13]. The study population was categorized into four subgroups according to the time after transplantation and included G1: patients within 1–24 months; G2: patients within 25–60 months; G3: patients within 61–120 months and G4: patients 121–160 months post-transplant. These subgroups typify distinct posttransplantation phases including early (G1), intermediate (G2 and G3) and long-term (G4) survival with stable graft function. Four out of 29 patients from the G4 subgroup presented previous and sporadic episodes of renal dysfunction after renal transplantation, all of them were considered to present stable graft function at the time of their inclusion in the present study. The major demographic and clinical features of these subgroups are presented in Table 1.

2.2. Ethics

This study was approved by the Ethics Committee at Federal University of Minas Gerais and informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescriptions.

2.3. Plasma samples

Five mL whole blood samples were drawn in EDTA-K_3 1.8 mg/mL (Vacuette®) and centrifuged at 1300*g* for 20 min at 4 °C to obtain the plasma samples. Plasma aliquots were stored at −70 °C until use for flow cytometric cytokine measurements.

2.4. Cytometric beads array for cytokine measurements

Cytokine plasma levels were determined using commercially available kits, including Human Th1/Th2 Cytometric Beads Array – CBA (BD Biosciences Pharmingen, USA) to quantify IFN-γ, IL-4 and IL-5 along with the Human Inflammation kit to quantify IL-1β, IL-6, IL-8, IL-10, TNF-α and IL-12.

The CBA immunoassay uses 7.5 μm polystyrene microbeads, assembled in distinct fluorescent sets, unique on their type 4 fluorescence intensity (FL-4). Each microbead is coupled to monoclonal antibody (MAb) against a given cytokine. Following incubation with the test sample, the bead-captured cytokines were detected by direct immunoassay using a "detection cocktail" of distinct MAbs labeled with type 2 fluorescence, phycoerythrin-PE (FL-2).

The method was carried out as recommended by the manufacturer, modified as follows: briefly, 25 μL of undiluted plasma samples or standards (previously diluted) were added to 15 μL of bead-mix and incubated for 90 min at room temperature in the dark. The cytokine standard curves were run daily for each assay. After incubation, the samples and standards were washed with 500 μL of wash buffer and centrifuged at 600*g* for 7 min at room temperature. Subsequently, 20 μL of detection cocktail were added to each tube and the bead-mix re-incubated for 90 min at room temperature in the dark. Following incubation, the samples and standards were washed again with 500 μL of wash buffer and centrifuged at 600*g* for 7 min at room temperature to remove unbound detector reagent. After washing, 250 μL of wash buffer was added to each tube. Data acquisition and analysis was performed in dual-laser FACScalibur™ flow cytometer (BD Biosciences Pharmingen, San Jose, CA, USA), using the BD Bioscience CBA software. Although the fluorescently labeled particles in the BD CBA immunoassay are designed to be excited by the 488 nm and 532 nm lasers on other BD flow cytometers, they can also be excited by the red diode laser 633 nm on dual-laser BD FACSCalibur instruments. The detection of beads emission at FL-4 channel simplifies the instrument set-up procedure and reduces the need for fluorescence compensation. Thus, a total of 1800 beads/tube were acquired after proper set-up of a flow cytometer. Results were expressed as mean fluorescence intensity (MFI) for each cytokine.

2.5. Analysis of "overall cytokine patterns" and "cytokine signatures"

The plasma cytokine levels were initially analyzed as the mean fluorescent intensity provided by the CBA immunoassays and referred as "overall cytokine patterns". Each cytokine pattern was first expressed as moving average of plasma cytokines according to the time after renal transplantation. Following study population sub grouping categorization, the median overall cytokine patterns were compared amongst G1, G2, G3 and G4 subgroups. Additional analysis referred as "cytokine signatures" were also performed as previously proposed by Luiza-Silva et al. [14]. Briefly, the global median value for each cytokine was calculated taking the whole data universe from all renal transplanted patients. The global median cut off were used as the cut-off edge to tag each patient as they display "Low levels" (▭ for all cytokines), high levels of pro-inflammatory (■ for IL-8, IL-6, IL-1β, TNF-α, IL-12, IFN-γ) or high levels of regulatory (▬ for IL-4, IL5 and IL-10) cytokines. After assembling of gray-scale diagrams for each study subgroup, the frequency (%) of patients showing "High cytokine levels" was calculated. This strategy allowed for computation of the percentage

Table 1
Demographic and clinical features of the study population[a].

Groups	Time post-transplant (months)	*N*	Creatinine levels (mg/dL)	Gender (M/F)	Age (years)
G1	1–24	31	1.7 ± 0.9	22/09	36 (19–63)
G2	25–60	30	1.5 ± 0.6	19/11	45 (21–66)
G3	61–120	30	1.5 ± 0.5	21/09	49 (33–73)
G4	121–160	29	1.6 ± 0.5	20/09	47 (33–64)
Total	1–160	120	1.6 ± 0.7	82/38	44 (19–73)

[a] Time post-transplant are expressed as min–max, creatinine levels as mean ± SD and age as median (min–max).