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Citrulline and Monocyte-Derived Macrophage Reactivity before Conditioning Predict Acute Graft-versus-Host Disease

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During conditioning, intestinal damage induces microbial translocation which primes macrophage reactivity and leads to donor-derived T cell stimulation. Little is known about the role of intestinal health and macrophage reactivity before conditioning in the development of acute graft-versus-host disease (aGVHD) in patients undergoing allogeneic hematopoietic cell transplantation (allo-HCT). We assessed (1) citrulline, a surrogate marker of functional enterocyte mass and (2) circulating monocyte-derived macrophage reactivity, before allo-HCT. Forty-seven consecutive patients were prospectively included. Citrulline levels from blood samples withdrawn 30 days before transplantation were assessed using liquid chromatography combined with mass spectrometry. Monocyte-derived macrophages were isolated and incubated with 5 pathogen-associated molecular patterns: lipopolysaccharide, PamCSK4, flagellin, muramyl dipeptide, and curdlan. Multiplex fluorescent immunoassay on culture supernatant assessed levels of TNF-α, IL-1β, IL-6, and IL-10 in each condition. Citrulline and cytokine levels were analyzed relatively to aGVHD onset within 100 days after transplantation. Citrulline levels were lower in the aGVHD group (n = 20) than in the no-aGVHD group (n = 27) ($P = .005$). Conversely, IL-6 and IL-10 were greater in aGVHD group, especially after curdlan stimulation ($P = .005$ and $P = .012$). Citrulline levels ≤20 μmol/L, IL-6 ≥ 332 pg/mL, and IL-10 ≥ 90 pg/mL were associated with aGVHD development (log-rank test, $P = .002$, $P = .041$, and $P < .0001$, respectively). In multivariate analysis, IL-10 ≥ 90 pg/mL, myeloablative conditioning, and citrulline ≤20 μmol/L remained independent factors of aGVHD development (hazard ratio [HR], 8.18, $P = .0003$; HR, 4.28, $P = .006$; and HR, 4.43, $P = .01$, respectively). Preconditioning citrulline and monocyte-derived macrophage reactivity are objective surrogate markers suitable to identify patients at risk of developing aGVHD. This work highlights the influence of preconditioning status in aGVHD development.

INTRODUCTION

Acute graft-versus-host disease (aGVHD) remains the major cause of early death after allogeneic hematopoietic cell transplantation (allo-HCT) [1]. Several risk factors associated with aGVHD development have been identified, such as age of recipient, sex mismatch, graft source, cytomegalovirus status, HLA compatibility, and myeloablative conditioning (MAC) [2,3]. To date, the impact of pretransplantation intestinal status and circulating monocyte-derived macrophage reactivity on aGVHD development remains unknown.

In patients undergoing allo-HCT, intestinal exploration is infrequent because of constraints in performing endoscopy to retrieve tissue biopsies. Citrulline, a nonproteinogenic amino-acid produced by enterocytes, is a suitable marker to assess intestinal damage induced by high-dose chemotherapy during the conditioning regimen [4].

The role of the intestinal barrier and microbiota in aGVHD pathogenesis sustains the crucial contribution of the microbial translocation [5]. In the mid 1990s, the discovery of the pathogen-recognized receptors (PRRs) as toll-like receptors highlighted the central role of macrophages in antimicrobial innate immunity [6]. Toll-like receptor-4 is able to recognize lipopolysaccharide (LPS) as a pathogen-associated molecular pattern (PAMP) and triggers proinflammatory innate response leading to cytokine release (eg, TNF-α, IL-1β,

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and IL-6). Such release enhances alloreactivity of donor-derived T cells that are responsible for further tissue damage [7,8]. LPS has been widely investigated during aGVHD, although other PAMPs, such as PAMCSK4, muramyl dipeptide (MDP), flagellin, or β-D-glucan derivatives such as curdlan, may also affect macrophage activation and contribute to aGVHD pathogenesis.

In mouse models, conditioning irradiation represents the first trigger to initiate aGVHD by damaging intestinal mucosa, leading to a host macrophage TNF-α releasing and translocation of microbial products such as LPS through the intestinal epithelium. This phenomenon potentiates alloreactivity of donor-derived T cells and leads to aGVHD development [9,10].

In the present work, we investigated intestinal health through plasmatic citrulline levels and circulating monocyte-derived macrophage reactivity before conditioning in patients undergoing allo-HCT.

PATIENTS AND METHODS

Patients

Forty-seven patients referred to our institution for allo-HCT from October 2014 to August 2015 were prospectively included along with 10 healthy donors (HD) after written informed consent. No anti-inflammatory treatment or antibiotics had been given the week preceding blood drawing. The study was approved by local institutional review board and conducted in agreement with the Declaration of Helsinki.

Transplantation Modalities and Clinical Outcome

Conditionings were myeloablative (n = 15) and reduced-intensity/toxicity (n = 32). Given day 0 as the day of donor cell infusion (Figure 1), i.v. continuous infusion of cyclosporin was administered as prophylaxis against aGVHD from day -1 to day +14 and relayed per os as soon as oral food intake accounted for 60% of daily requirements. Eight patients who received haploidentical allo-HCT were administered mycophenolate mofetil with post-transplantation cyclophosphamide. The 39 others received methotrexate. In addition, all patients were treated with acyclovir, fluconazole, and sulfamethoxazole-trimethoprim as *Pneumocystis carinii* prophylaxis. According to our internal protocol, digestive decontamination was not given. Either ceftazidime or the combination of piperacillin with tazobactam was empirically administered in case of fever and adjusted to the bacterial profile. All patients received enteral nutrition initiated at day +1 via a nasogastric tube, as previously described [11]. C-reactive protein, albumin, renal clearance, and body mass index were evaluated at day -30. We differentiated patients according to the type and the dose of chemotherapy administered within the 6 months before conditioning. For instance, those who received cytarabine >10 g/m^2 or melphalan 200 mg/m^2 were assigned to the "high-dose pretransplantation chemotherapy group." The rest of patients was assigned to the low-dose pretransplantation chemotherapy group (Table 1). Acute GVHD, diagnosed according to clinical and/or histology criteria, was recorded daily during hospital stay and then weekly after discharge until day +100, and it was classified according to severity according to Glucksberg classification [12] (Figure 1). A final visit at 1 year concluded this protocol.

Citrulline Concentration

Fasting plasma citrulline concentration assay (μmol/L) was performed at day -30 by high-performance liquid chromatography method (Shimadzu C18 column, Kyoto, Japan) along with tandem mass spectrometry (AB Sciex 3200 Qtrap, Framingham, MA) using the aTRAQ kit for amino-acid analysis of physiological fluids (AB Sciex) [13]. Acquisition in the mass spectrometer was achieved by multiple reaction monitoring. Data recording and analysis were performed with Analyst software, v.1.6 (AB Sciex).

Macrophage Culture

Venous blood (14 mL) was collected at day -30 and peripheral blood mononuclear cells were isolated by density gradient centrifugation (450*g*, for 20 minutes) with Ficoll-Paque solution (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Cells were cultured in the RPMI 1640 medium (Sigma-Aldrich, St Louis, MO), supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Invitrogen, Strasbourg, France) and plated (10^6 cells/500 μL/well) in 48-well adhesive plates at 37°C in a humidified atmosphere of 5% CO_2 in air. Monocytes, separated from lymphocytes by plastic dish adherence after 2 hours, were washed with PBS and plated overnight to obtain circulating monocyte-derived macrophages [14]. The purity of samples was checked by flow cytometric analysis using CD45 APC and HLA-DR FITC (Beckman Coulter, Villepinte, France) and ranged from 82% to 87% [15]. To run PAMPs assays, cells were incubated for 4 hours in 400 μL of RPMI-FCS (1:10) implemented with a 40 μL-aliquot of LPS (50 ng/mL), PAMCSK4 (1 μg/mL), flagellin (1 μg/mL), MDP (10 μg/mL), or curdlan (100 μg/mL) or PBS as negative control (Invivogen, Toulouse, France) [16]. The culture supernatant was harvested and stored at -80°C.

Cytokine Assay

Supernatant (50 μL) was thawed to assess levels of TNF-α, IL-1β, IL-6, IL-10, and INF-γ by use of Luminex microbead method (R&D System, Minneapolis, MN; detection sensitivity was as low as .4 pg/mL to 1.7 pg/mL). These cytokines had been searched for according to accountability for aGVHD pathophysiology [17]. INF-γ was assessed to confirm the absence of lymphocytes in cell culture. For each patient, all stimulation assays were simultaneously assessed on a 96-well plate. Data

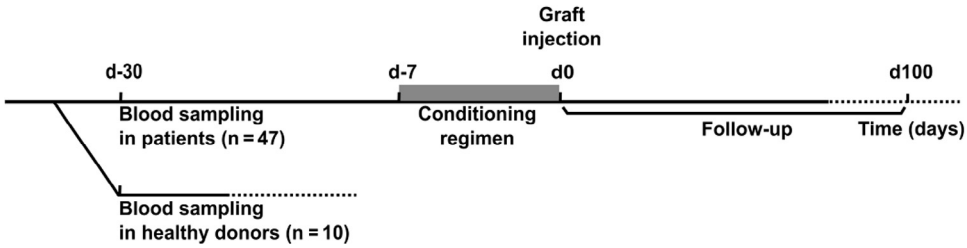

Figure 1. Timeline of study design.