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Systemic over-release of interleukin-17 in acute kidney injury after septic shock: Clinical and experimental evidence

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ABSTRACT

In order to investigate the role of T-helper 17 (Th17) cell activation in acute kidney injury (AKI) after septic shock, a two-stage approach was used. Firstly, peripheral blood mononuclear cells (PBMCs) and CD4-lymphocytes were isolated the first 24 h after septic shock from 26 patients with AKI and 18 patients with chronic renal disease (CRD) without AKI and stimulated for the release of tumour necrosis factor-alpha (TNFα), interleukin (IL)-10, IL-17, IL-22 and interferon-gamma (IFNγ). Results were compared with 15 healthy volunteers and 13 patients with uncomplicated sepsis. Secondly, a murine model of multiple organ dysfunction (MODS) complicated with AKI and bacterial gut translocation was studied, and IL-10, IL-17, IL-22 and IFNγ were measured in kidney homogenates. IL-17 was the only cytokine produced at greater quantities from PBMCs and CD4-lymphocytes of patients with septic shock and AKI than comparators. When PBMCs of patients with septic shock and AKI were ex-vivo stimulated, intracellular staining for IL-17 was greater in CD3(+)/CD4(+)/CD196(+) cells compared to patients with septic shock and CRD. IL-17 was released at greater amounts from PBMCs of non-survivors by septic shock and AKI but not of septic shock and CRD. In the murine model of MODS, a gradual decrease of IL-17, but not of IL-10, IL-22 and IFNγ, of kidney homogenates was found indicating over-consumption. These results suggest that AKI after septic shock is driven through IL-17 release by Th17 cells; this is gradually consumed in the kidney.

1. Introduction

Acute kidney injury (AKI) is a common complication of sepsis. It appears within the first 24 h in almost 65% of cases of septic shock [1]. AKI imposes considerably on the outcome of septic shock while reversal of AKI is associated with reduction of the risk of death [2].

Kidney infiltration by neutrophils and the subsequent release of free radicals is one major mechanism leading to AKI. The contribution of neutrophils in the pathogenesis of AKI is indirectly shown in a survey of 998 patients with chronic renal disease (CRD) undergoing surgery; increase of circulating neutrophils after surgery was related with the chance of developing AKI so that neutrophil counts postoperatively were an early prognostic sign for the development of AKI in the field of CRD [3]. Recruitment of neutrophils in the kidney in AKI is mediated through chemokines that prime chemotaxis. Interleukin (IL)-17 is one pro-inflammatory cytokine that acts as a chemokine; it is released by Th17 lymphocytes and it induces neutrophil migration [4].

Several recent reports have shown an implication of IL-17 in the development of AKI either in the field of auto-immune disorders [5] or of non-bacterial infections [6]. However, no evidence exists for the implication or not of IL-17 in AKI developing after bacterial septic shock. The present study is using a two stage approach to investigate the role of Th17 lymphocytes in AKI complicating septic shock: firstly, we assessed the activation of circulating Th17 lymphocytes in septic shock patients who develop AKI; secondly, we investigated Th17 cell-related cytokines in the kidney of mice with multiple organ dysfunction (MODS) and AKI.

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2. Patients and methods

2.1. Clinical study

The study was conducted during the period January 2011 to March 2014 and March 2016 to July 2016 in patients admitted to the 4th Department of Internal Medicine of ATTIKON University. The study protocol was approved by the Ethics Committee of the hospital. Patients were enrolled after written informed consent provided by themselves or by their first-degree relatives in case of patients unable to consent.

Inclusion criteria were: a) age more than or equal to 18 years old; b) community-acquired infection. The included infections were acute pyelonephritis (UTI), community-acquired pneumonia (CAP), intraabdominal infections (IAI) and bloodstream infections (BSI). c) presence of septic shock defined as infection aggravated by systolic blood pressure below 90 mmHg despite the intravenous administration of fluids necessitating the administration of vasopressors; d) presence of at least two signs of the systemic inflammatory response syndrome (SIRS) or septic shock aggravated by AKI in a patient without pre-existing CRD or septic shock developing in a patient with pre-existing CRD without signs of AKI; and e) blood sampling within less than 12 h from the onset of septic shock.

A retrospective evaluation of the enrolled study population showed that all patients had SOFA (sequential organ failure assessment) score equal to or above 2 so as to be classified as sepsis using the newly proposed Sepsis-3 definitions. Furthermore, all patients with septic shock had plasma lactate more than 2 mmol/l and were under the need for vasopressors so as to be classified also as septic shock with the new Sepsis-3 definitions [7].

UTI, CAP, IAI, BSI and SIRS were defined as reported elsewhere [8,9]. AKI was defined as urinary output below 0.5 ml/kg/h for two continuous hours provided that the negative fluid balance was corrected [9,10]. CRD was defined as creatinine clearance below 30 ml/min with a medical history of at least two years of onset. Creatinine clearance was calculated by the Cockroft-Gault formula [11].

Exclusion criteria were: a) age below 18 years; b) deny of written informed consent; c) infection by the human immunodeficiency virus; d) neutropenia defined as an absolute neutrophil count less than 1000/mm^3; and d) chronic intake of corticosteroids defined as any more than 0.4 mg/kg daily intake of equivalent prednisone for more than 15 days. The last three exclusion criteria applied because their presence can modulate cytokine production.

Twenty ml of heparinized blood was sampled by venipuncture of one forearm vein under aseptic conditions and processed within less than one hour. A complete work-out was done comprising blood cultures, quantitative cultures of tracheobronchial secretions, chest X-ray and chest and abdominal computed tomography if considered necessary. A similar amount of blood for isolation of PBMCs was sampled from 15 healthy controls well-matched for age and gender.

Heparinized venous blood was layered over Ficoll Hypaque (Biochrom, Berlin, Germany) and centrifuged for 20 min at 1400g. Separated PBMCs were washed three times with ice-cold PBS (phosphate buffered saline) (pH: 7.2) (Biochrom) and counted in a Neubauer chamber. Their viability was more than 99% as assessed by trypan blue exclusion of dead cells. They were then diluted in RPMI 1640 enriched with 2 mM of L-glutamine, 10% of fetal bovine serum (Biochrom), 100 U/ml of penicillin G and 100 μg/ml of gentamicin and suspended in wells of a 96-well plate (Biochrom). The final volume per well was 200 μl with a density of 2×10^6 cells/ml. PBMCs were incubated for five days at 37 °C and 5% CO_2 in duplicate with the following stimuli: 5 μg/ml of the lymphocyte agonist phytohemagglutin (PHA) of *Phaselolus vulgaris* (PHA-L, Roche Diagnostics GMBH, Mannheim, Germany); 5×10^5 colony-forming units (CFU)/ml of one heat-killed isolate of *Candida albicans*; and 5×10^5 colony-forming units (CFU)/ml of one heat-killed isolate of methicillin-resistant *Staphylococcus aureus*. These were clinical isolates from the bloodstream of patients with severe sepsis. Cytokine stimulation for five days by PHA was used as a read-out for non-selective lymphocyte activation, by heat-killed *C. albicans* for Th17 cell activation and by heat-killed *S.aureus* for monocyte activation [12,13].

In order to focus on IL-17 release by Th17 cells, purified CD4-lymphocytes were studied. For this purpose, in a subset of patient PBMCs were labeled with anti-CD4 micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD4-lymphocytes were isolated by positive magnetic separation using a MiniMacs magnet (Miltenyi) according to the instructions of the manufacturer. The isolated cells were counted using a Neubauer chamber with trypan blue exclusion of death cells and were incubated at a density of 5×10^6/ml for five days at 37 °C and 5% CO_2 in RPMI 1640 supplemented with 2 mM glutamine, 10% Fetal Bovine Serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin in the absence/presence of 5×10^5 cfu/ml of heat-killed *C. albicans*.

After five days of incubation at 37 °C in 5% CO_2, the plates containing PBMCs or purified CD4-lymphocytes were centrifuged. Supernatants were kept stored at −70 °C until assayed for tumour necrosis factor-alpha (TNFα), interleukin (IL)-10, IL-17A, IL-22 and interferon-gamma (IFNγ). All cytokines were measured in duplicate by an enzyme immunoassay (R&D Minneapolis, USA). The lower detection limits were: 20 pg/ml for TNFα and IL-10, 40 pg/ml for IL-17A; 20 pg/ml for IL-22; and 16 pg/ml for IFNγ.

Finally, to further investigate the implication of Th17 cells as a cell source of IL-17, washed PBMCs from a subset of patients were incubated for four hours into 24 well plates at a density of 5×10^6/ml at 37 °C and 5%CO_2 in RPMI 1640 supplemented with 2 mM glutamine, 10% Fetal Bovine Serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin in the absence/presence of 5×10^5 cfu/ml of heat-killed *C. albicans*. Thirty minutes after the start of the incubation 1 μl of Protein-transport inhibitor containing brefeldin (GolgiPlug, BD Bioscience, San José, California) was added into wells. At the end of the incubation, non-adherent lymphocytes were collected and stained for 15 min in the dark with anti-CD4 at the fluorochrome fluorescein isothiocyanate (FITC, emission 525 nm, Immunotech, clone 13B8.2, Marseille, France), anti-CD3 at the fluorochrome Phycoerythrin-Texas Red-x (ECD, emission 480 nm, clone UCHT1, Immunotech) and anti-CD196 at the fluorochrome Peridinin-Phykocyanin (PerCP-Cy5.5, emission 695 nm, clone 11A9, BD Bioscience). Cells were then fixed and permeabilized using Leucocytic permeabilization reagents according to the instructions of the manufacturer (IntraPrep, Beckman Coulter, Miami, Florida USA). Intracellular staining with anti-IL-17 at the fluorochrome phycoerythrin (PE, emission 575 nm, cloneN49-653, BD Bioscience) was then performed and cells were analyzed through the CYTOMICS FC-500 flow cytometer (Beckman Coulter) with FS/SS scattering; 100,000 events were analyzed and cells staining positive for CD3(+)/CD4(+)/CD169(+)/cytIL-17(+) were considered Th-17 releasing IL-17. Results were expressed as mean fluorescence intensity (MFI) per cell.

Based on the hypothesis that IL-17 release by circulating PBMCs as an indicator of activation of Th17 cells would be greater in at least 70% of patients with septic shock and AKI compared to healthy volunteers, 13 patients should be enrolled in each study group to achieve 80% power at the 10% level of significance.

2.2. Animal study

A total of 49 male C57BL/6 outbred mice 20–25 g of body weight and 7–9 weeks old (Hellenic Pasteur Institute) were studied. Experiments were performed in the Laboratory for Experimental