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Interleukin-25 reduces Th17 cells and inflammatory responses in human peripheral blood mononuclear cells

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ABSTRACT

Background: The absence of interleukin-25 (IL-25) favors the induction of Th1 and Th17 immune responses in mice. Th1 immune responses have been associated with the pathology of atherosclerosis, a lipid and inflammation driven disease of the arterial wall.

Purpose of research: To evaluate the effect of IL-25 on human peripheral blood mononuclear cells (hPBMCs) in the presence and absence of oxidized low density lipoprotein (oxLDL), a key player in atherosclerosis development.

Principal results: Human PBMCs were incubated with recombinant human IL-25 (rhIL-25) in the presence and absence of oxLDL and analyzed with flow cytometry while cytokine secretion was measured in cell culture supernatants. The IL-25 receptor, IL-17RB, was mostly expressed on T cells. Incubation of hPBMCs with IL-25 reduced the frequency of Th17 cells. Furthermore, IL-25 inhibited the release of the Th17-inducing cytokine IL-6 from dendritic cells isolated from hPBMCs indicating that the IL-25 mediated Th17 suppression may be indirect. Moreover, IL-25 reduced the secretion of the proinflammatory cytokine IFNγ from hPBMCs. OxLDL decreased IFNγ release from hPBMCs regardless of the presence or absence of IL-25.

Conclusions: IL-25 reduces Th1 and Th17 immune responses in hPBMCs raising the interesting possibility that IL-25 could have a protective role in human atherosclerosis.

1. Introduction

Atherosclerosis is a disease of the arterial wall that is characterized by the deposition of lipids and their subsequent modification and recognition by the immune system. Th1-driven immune responses against oxidized low density lipoprotein (oxLDL) play a key role in the development of atherosclerosis [1,2] and oxLDL reactive T cells are present in the circulation of humans [3]. Regulatory T cells have a protective role [4] and an imbalance in Th17/regulatory T cells has been demonstrated to drive atherosclerosis development [5].

Interleukin-25 (IL-25) is a type 2 inducing cytokine targeting cells such as monocytes/macrophages, dendritic cells (DCs), T cells and type 2 innate lymphoid cells (ILC2s). In experimental studies IL-25 has been shown to promote Th2 [6] and Th9 [7] immune responses and the release of IL-5 and IL-13 via the induction of ILC2s [8–10]. IL-25 has also been shown to regulate autoimmunity by suppressing Th1 and Th17 immune responses. Experimental autoimmune encephalomyelitis (EAE) was accelerated in IL-25-deficient mice and disease progression was associated with an increased frequency of $CD4^+IL17A^+$ T cells indicating that IL-25 affects the induction of Th17 cells [11]. Moreover, IL-25 deficient mice have been found to be more susceptible to parasitic helminths (Trichuris muris and Nippostrongylus brasiliensis) due to reduced Th2 cytokine production [12,13].

In human cells, IL-25 has been shown to potentiate the release of IL-6, IL-10 and the Th2-related cytokines IL-4 and IL-5 from CD3 and CD28 stimulated naïve human T cells [14]. Another study found that human Th2 memory cells expressed high levels of the IL-25 receptor IL17-RB and IL-25 induced expansion and Th2 secretion in these cells upon activation with thymic stromal lymphopoietin (TSLP) stimulated DCs or anti-CD3/CD28 antibodies [15]. Moreover, human blood $CD14^+$ cells have been reported to respond to IL-25 by down-regulating the lipopolysaccharide (LPS) and peptidoglycan induced synthesis of inflammatory cytokines such as IL-1β, IL-6, IL12(p40) and TNFα [16]. Interestingly, treatment of $CD14^+$ cells, isolated from the guts of

Abbreviations: IL, interleukin; hPBMCs, human peripheral blood mononuclear cells; oxLDL, oxidized low density lipoprotein; IL17-RB, interleukin 17 receptor B; Th, T helper; IFNγ, intereferon γ

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patients suffering from Crohn's disease, with IL-25 reduced the secretion of IL-23 and IL12(p70) upon LPS or peptidoglycan stimulation [17]. In normal human arteries IL-25 is reported to be expressed by smooth muscle cells and endothelial cells and in advanced plaques IL-25 is also extensively expressed by mature B cells [18].

Since IL-25 has been shown to regulate autoimmunity by suppressing Th1 and Th17 immune responses in experimental studies, it was of interest to investigate the effect of IL-25 on human peripheral blood mononuclear cell populations (hPBMCs) and also to study whether the addition of oxLDL, which would simulate a pro-inflammatory environment, could modulate the IL-25 induced response. The purpose of the present study was to detect the main IL-25 cell responders in the lineage (Lin^+) cell compartment that exist in the periphery of the human body and to investigate whether IL-25 influences the immune responses in human mononuclear cells in the presence or absence of oxLDL, an atherosclerosis-related pro-inflammatory player.

2. Materials and methods

2.1. Isolation of hPBMCs from leukocyte filters

Human blood leukocyte filters or leukocyte concentrates from anonymous healthy donors were obtained from the regional Center of Blood (permission number 2016:21, Labmedicin, Klinisk Immunologi och Transfusionmedicin, Skåne University Hospital, Lund, Sweden) and hPBMCs were isolated as described previously [19]. Several donors were used for the experiments (Donors I-V, Donors 1-4, Donors i-iii, Donors a-c, Donors A-Γ). Since leukocyte filters and leukocyte concentrates are coming from anonymous healthy donors that cannot be back-traced, ethical approval is not required according to institutional guidelines. Briefly, cells trapped in leukocyte filters were backflushed with 150 mL PBS (without Ca^{2+}, Mg^{2+}) containing EDTA-Na_2 (5 mmol/L) and sucrose 2.5% [w/v] in 3 steps, perfusing the filter with 50 mL of solution each time. The eluted cells or the leukocyte concentrates (the latter resuspended in 150 mL PBS) were layered in Ficoll solution (GE Healthcare) and centrifuged at 445 × *g* at 21 °C for 35 min. At the end of centrifugation the white layer of mononuclear cells was collected and washed with PBS twice. Red blood cells were lysed with Red Blood lysing Buffer (Sigma-Aldrich) and the remaining cells were used for cell culturing after washing with PBS.

2.2. Incubation of cells with IL-25

10^6 hPBMCs/mL were cultured in RPMI 1640 medium containing 10% heat-inactivated human serum (Sigma-Aldrich), 1 mmol/L sodium pyruvate, 10 mmol/L Hepes, 50 U penicillin, 50 μg/mL streptomycin, 0.05 mmol/L β-mercaptoethanol and 2 mmol/L L-glutamine (GIBCO) and incubated with different concentrations (0–100 ng/ml) of recombinant human IL-25 (rhIL-25; R&D Systems) for 20 h. Incubation of cells with 4 mmol/L HCL containing 1% bovine serum albumin (BSA, Sigma-Aldrich), the vehicle for rhIL-25, served as a control. hPBMCs were also incubated with 10 μg/mL Cu^{2+}-induced oxLDL alone or in combination with IL-25 or the control vehicle.

DCs were negatively selected from hPBMCs with a Human Pan-DC Pre-Enrichment Kit according to the company's instructions (Stem cell Technologies). Upon isolation, 0.5 × 10^6 DCs/mL were incubated with 50 ng/mL of rhIL-25 or oxLDL (10 μg/mL) or the combination of both for 20 h and further stimulated for 24 h with 1 μg/mL lipopolysaccharidae (LPS, Sigma-Aldrich). Cell culture supernatants were harvested and stored at −80 °C for cytokine analysis (IL-1β, IL-6, IL-12(p70), IL-17, IL-23, TNFα, IFNγ, Bio-plex Pro Assays, Bio-Rad). Negative immunomagnetic selection of $CD3^+$ T cells was performed with the use of a kit from Stem Cell Technologies according to the company's instructions. The isolated cells (1 × 10^6 cells/mL) were incubated with different concentrations of IL-25 for 20 h and further stimulated with 1 μg/mL Ionomycin, 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 10 μg/mL Brefeldin A for 4 h for flow cytometric analysis.

2.3. Cu^{2+}-induced oxLDL and cytotoxicity assay

LDL was isolated from human blood by sequential ultracentrifugation steps as previously described [20] mixed 1:5 with 50% sucrose in 0.15 mol/L NaCl, 24 mmol/L EDTA, pH = 7.4 and stored at −80 °C [21]. Before oxidation, LDL was cleared up from the freezing medium by elution with PBS through PD10 columns (Bio-Rad) which are packed with a matrix that excludes solutes greater than 6000 Da, allowing them to elute in the void volume. The concentration of LDL was determined with the BCA kit (Thermo Scientific) according to the company's instructions. Next, the concentration of LDL was adjusted at 1 mg/mL by addition of PBS and its oxidation took place by incubation with 5 μmol/L Cu_2Cl_2 for 18 h at 37 °C. The oxidation was stopped by addition of 1 mmol/L EDTA and LDL was stored at 4 °C under nitrogen until use for a maximum period of 2 weeks.

2.4. Flow cytometry

For intracellular cytokine staining, hPBMCs after incubation with IL-25 or vehicle ± Cu^{2+}-oxLDL for 20 h were further incubated with 1 μg/mL Ionomycin, 50 ng/mL PMA and 10 μg/mL Brefeldin A for 4 h, all purchased from Sigma (Sigma-Aldrich). Next, the cells were harvested and stained with fluorochrome conjugated antibodies; CD3-PE/Cy7 (AB_439781), CD3-FITC (AB_314042), CD4-PB (AB_493098), CD4-FITC (AB_571951), CD8-AF700 (AB_528885), CD25-PE (AB_314276), CD19-FITC (AB_314236), CD14-PB (AB_493163) all purchased from Biolegend, IL17-RB-APC (AB_10714829, R&D systems). After removing the unbound extracellular antibodies by washing with cold PBS containing 1% heat inactivated human serum and 0.5 mmol/L EDTA the cells were resuspended in Fix/Perm solution (eBioscience) and incubated for 30 min on ice. Afterwards the cells were washed with permeabilization buffer (eBioscience) and stained with Foxp3-APC (AB_1603280, eBioscience), IL-5-APC (AB_315330), IFN-γ-PE (AB_315234) (all purchased from Biolegend) and IL-17-FITC (AB_10830876, Macs Miltenyi) antibodies for 30 min at 4 °C. Two additional washing steps with permeabilization buffer followed. Finally, the cells were resuspended in PBS containing 1% heat inactivated human serum and 0.5 mmol/L EDTA and analyzed in a CyAn ADP flow cytometer (Beckman Coulter). 1 × 10^5 gated cells were analyzed with FlowJo software (Tree Star, Inc.). In the case of flow cytometric analysis of the IL-17RB expression levels from DCs, cryopreserved hPBMCs were used. Upon thawing, hPBMCs were stained with the extracellular antibodies CD11c-FITC (AB_314173), CD123-PerCP/Cy5.5 (AB_2124258), CD16-PE/Cy7 (AB_314216), CD14-PB (AB_493163), HLA-DR-AF700 (AB_493771), all from Biolegend, IL17-RB-APC (AB_10714829, R&D systems) and with a viability dye (Efluor 506, eBioscience) in order to exclude dead cells. Analysis of the obtained data took place as previously mentioned. All gatings were set according to FMO samples.

2.5. Cytokine release from hPBMCs

hPBMCs (1 × 10^6/mL) after simultaneous incubation with IL-25 and/or Cu^{2+}-oxLDL for 20 h were cultured with 1 μg/mL Ionomycin and 50 ng/mL PMA for 4 h. In parallel experiments, 1 × 10^6 hPBMCs/mL were incubated with different concentrations of IL-25 and/or oxLDL for 20 h and then stimulated with CD3/CD28 beads (7 μl of bead suspension/500 μl of cell suspension) for 4 h. Cells were pelleted and the cell culture supernatants were frozen at −80 °C until further analysis. Cytokine release by the cells was assessed using Luminex technology according to the company's instructions (IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-13, IL-17, Bio-plex Pro Assays, Bio-Rad).