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CD14^{++} CD16^{-} monocytes are the main source of 11β-HSD type 1 after IL-4 stimulation

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ABSTRACT

The anti-inflammatory actions of IL-4 are well established through earlier findings. However, the exact mechanism it uses to downregulate the pro-inflammatory cytokine production through monocytes and macrophages is poorly understood. In this study, we examined the effect of IL-4 in the induction of 11β-HSD1 in the two main classes of monocytes, CD14^{++} CD16^{-} (CD14) and CD14^{+} CD16^{+} (CD16). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from 17 healthy donors and were sorted into CD14 and CD16 subpopulations using cell sorting. Effect of IL-4 on 11β-HSD1-enzyme activity was measured in sorted and unsorted monocytes using Homogeneous Time-Resolved Fluorescence (HTRF) and M1/M2 polarization analysis was performed by flow cytometry. Our results indicate that CD14 cells are the major source of 11β-HSD1 enzyme after IL-4 stimulation and that M2 phenotype is not a pre-requisite for its synthesis.

1. Introduction

The peripheral interconversion of pharmacologically active cortisol and inactive cortisone is accomplished by two independent 11β-hydroxysteroid dehydrogenases (11β- HSDs) that exhibit tissue-specific expression [1]. 11β-HSD1 generates active cortisol from inactive cortisone and thereby enhances the activation of the glucocorticoid receptor while 11β-HSD2 is a powerful glucocorticoid inactivator. The two 11β-HSDs also interconvert inactive prednisone with active prednisolone, commonly used for the treatment of inflammatory diseases. 11β-HSD1 is broadly distributed among tissues, with predominant expression occurring in hepatic, adipose, gonadal, and central nervous system tissues. The peripheral control of inflammation involves also the conversion of inactive cortisone to cortisol (or of inactive prednisone to active prednisolone) by 11β-hydroxysteroid dehydrogenases expressed also in some immune cells [2] including dendritic cells and macrophages [3], while its expression is negligible in human blood monocytes. However, when these latter cells are exposed to IL-4 (regardless of whether the monocytes were maintained in suspension culture in Teflon beakers or as an adherent monocyte layer in plastic tissue culture dishes) the enzyme activity is tremendously enhanced, an effect abrogated by pro-inflammatory IFN-γ [3].

It has been demonstrated that IL-4 has anti-inflammatory properties, with an ability to suppress the production of tumour necrosis factor (TNF)-α and IL-1β by lipopolysaccharide (LPS)-activated human monocytes [4]. IL-4 has been successfully used for the treatment of inflammatory disease in animal models [5,6]. Recent studies suggest that therapies that cause an increase in IL-4 concentrations in tissues may be useful in the treatment of inflammation [7,8]. However, the exact mechanisms by which IL-4 exerts its anti-inflammatory effects are poorly understood.

In human peripheral blood there are two main classes of monocytes, the classical CD14^{++} CD16^{-} (CD14) and the pro-inflammatory CD14^{+} CD16^{+} (CD16) [9]. The CD16-negative classical monocytes form about 90% of the population, whereas the CD16-positive cells include only 10% of all monocytes under physiological conditions at rest [10]. These two kinds of monocytes have different cell surface markers and express

Abbreviations: CD14, CD14^{++} CD16^{-}; CD16, CD14^{+} CD16^{+}; HTRF, Homogeneous Time-Resolved Fluorescence; 11β-HSDs, 11β-hydroxysteroid dehydrogenases; CFSE, carboxyfluorescein-diacetate-succimidyl-ester method; rMFI, median fluorescence intensity.

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differentially specific genes such as HLA-DR, Ig-like transcript 4 and others. The preferential induction of pro-inflammatory molecules from CD16 monocytes after induction with TLR2–4 ligands or LPS suggests that CD16 are the pro-inflammatory monocytes. In contrast, transcript for interleukin 10 (IL-10), are present in CD14 monocytes and almost absent in CD16 monocytes suggesting that CD14 monocytes are involved in anti-inflammatory processes [11]. 11β-HSD1 activity is tightly related to the inflammatory status of human macrophages. 11β-HSD1 gene expression is higher in pro-inflammatory M1 and anti-inflammatory M2 macrophages than in resting macrophages, whereas its activity is highest in M2 macrophages. Elevated 11β-HSD1 expression in polarized M2 macrophages might contribute to their function in the anti-inflammatory response and the resolution of inflammation, with potential consequences on inflammatory diseases [12]. It is already established that M2 macrophages show elevated levels of 11β-HSD1, but it is still unclear is the M2 phenotype is a pre-requisite for this phenomenon.

Aim of this study was to determine if IL-4 induces HSD1 activity preferentially in one the two circulating monocytes subpopulations and if this observation is influenced by their polarization towards the M2 phenotype.

2. Materials and methods

2.1. Monocyte cultures

Blood samples from 17 healthy donors provided by the blood transfusion center of Verona University Hospital were collected under a protocol approved by the local Ethics Committee (Comitato Etico per la Sperimentazione – AOUI) and have been performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standard. The freshly harvested buffy coats were processed immediately to obtain PBMCs. PBMCs were isolated by Ficoll-Paque (Stemcell technologies, Vancouver, Canada) density gradient separation. The isolated PBMCs were plated at a density 700,000 cells/well in 96 well culture plates (BD Biosciences, New Jersey, USA) and cultured for two hours in serum free RPMI-1640 (Lonza, Basel, Switzerland) medium supplemented with 2 mM L-glutamine (Gibco, Life technologies) and 50 μg/ml gentamycin (Gibco, Massachusetts, USA). After incubation cells were washed with PBS to remove lymphocytes and non-adherent cells.

2.2. Isolation of monocyte sub-populations

Isolated PBMCs were stained for 15 min in the dark with a mix of the following monoclonal antibodies: anti-CD33 PE-C7 (Clone P67.6, BD Biosciences, New Jersey, USA), mouse anti-Human CD123 PE (Clone 9F5, BD Biosciences, New Jersey, USA), anti-CD16 PE (clone NKP15, BD Biosciences, New Jersey, USA) and anti-CD14 FITC (clone MϕP9, BD Biosciences, New Jersey, USA). Using BD FACS Aria II (BD Biosciences, New Jersey, USA) cells were sorted into CD14++/CD16− (CD14, classical) and CD16+/CD14++ (CD16) subpopulations. Cell purity was confirmed through flow cytometry using FACS Canto II (BD Biosciences, New Jersey, USA).

2.3. Culture of monocyte sub-populations

Monocyte subpopulations were plated at a density of 70,000 cells/well in 96-well culture plates (BD Biosciences, New Jersey, USA) and cultured overnight in RPMI-1640 complete culture medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin and 10% FBS (Gibco, Massachusetts, USA).

2.4. IL-4 stimulation

Adherent monocytes and monocyte subpopulations were left untreated or were incubated with various concentrations of recombinant human IL-4 (CellGro, Freiburg, Germany) ranging from 1 to 50 ng/ml in RPMI-1640 complete culture medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin and 10% FBS and cultured for various time points from 24 h to seven days.

2.5. 11B-HSD1 activity

11β-HSD1 activity was measured in terms of conversion of cortisone to cortisol by the HTRF cortisol assay kit (Cisbio Assays, Codolet, France). Cortisone (Sigma, Missouri, USA) was added to the culture medium 24 h prior to every time point to a final concentration of 100 nM and incubated for another 24 h. Cell culture supernatants were harvested and centrifuged and the clear supernatant was used for enzyme activity measurements by Time-Resolved Fluorescence Technology. HTRF technology is a competitive immunoassay in which native cortisol produced by cells and d2-labeled cortisol compete for binding to a monoclonal anti-cortisol antibody labeled with europium-Cryptate. The specific signal is inversely proportional to the concentration of cortisol in the calibrator or in the sample. The HTRF kit was used according to manufacturer's instruction and the fluorescence signals were read on a microplate reader (Victor X, Perkin Elmer, Massachusetts, USA) with an excitation filter at 340 nm and emission filters at 615 nm and 665 nm. The direct involvement of 11β-HSD1 in the conversion of cortisone to cortisol was tested by co-incubating monocytes and monocyte subpopulations with IL-4 (15 ng/ml) and a known inhibitor of 11β-HSD1, Carbenoxolone [13,14] in concentrations ranging from 0.1 to 20 μM for 48 h. The fold response of 11β-HSD1 activity to IL-4 stimulation in each individual was calculated using the following formula: Fold increase = cortisol produced by IL-4 + cortisone stimulated cells / cortisol produced by cortisone stimulated cells.

2.6. 11β-HSD1 expression

Gene expression analysis was performed on lysates of adherent cells obtained after collecting the supernatants for enzyme activity using Trizol reagent (Life technologies, California, USA). Total RNA was extracted from Trizol homogenates according to manufacturer's instruction. To remove potential contamination by genomic DNA, total RNA was treated with Turbo DNA-free kit (Life Technologies, California, USA). Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad, CA, USA) according to manufacturer's instruction. Gene expression was determined by real time RT-PCR using Sybr Green performed with the following primer sequences: HSD1 sense, 5′-AAGCAGAGCAATGGAAGCAT-3′; antisense, 5′-GAAGAACCCATCCAAAGCAA-3′; TATA box binding protein (TBP) and HPRT gene were chosen as the endogenous controls to normalize target genes: TBP sense, 5′-TGTATCCACAGTGAATCTTGG-3′; TBP antisense, 5′-ATGATTACCGCAGCAAACC-3′ and HPRT sense, 5′-TGACACTGGCAAAACAATGCA; antisense, 5′-GGTCCTTTTCACCAG CAAGCT-3′. The specificity of the Sybr green fluorescence was tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. RT-PCR reactions were performed in 10 μl, containing 5 μl sso advanced sybergreen supermix (Bio-Rad, CA, USA), 15 ng of cDNA template and 200 nM of forward and reverse primer. The condition for PCR was set up as follows: an initial activation step at 95 °C for 30 s, followed by 40 cycles of denaturing at 95 °C for 5 s, annealing at 60 °C for 20 s. Amplifications were performed using a CFX connect Real-Time PCR detection system. Each sample was amplified in triplicate and for each procedure negative controls without template were included. Amplification efficiency was calculated for each assay by a standard curve made out of four serial dilution of a pool of cDNA samples of all time points. Comparative quantification of gene expression was determined using Pfaffl's efficiency corrected calculation.