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5-Lipoxygenase contributes to PPARγ activation in macrophages in response to apoptotic cells

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ABSTRACT

Macrophage polarization to an anti-inflammatory phenotype upon contact with apoptotic cells is a contributing hallmark to immune suppression during the late phase of sepsis. Although the peroxisome proliferator-activated receptor γ (PPARγ) supports this macrophage phenotype switch, it remains elusive how apoptotic cells activate PPARγ. Assuming that a molecule causing PPARγ activation in macrophages originates in the cell membrane of apoptotic cells we analyzed lipid rafts from apoptotic, necrotic, and living human Jurkat T cells which showed the presence of 5-lipoxygenase (5-LO) in lipid rafts of apoptotic cells only. Incubating macrophages with lipid rafts of apoptotic, but not necrotic or living cells, induced PPAR responsive element (PPRE)-driven mRuby reporter gene expression in RAW 264.7 macrophages stably transduced with a 4xPPRE containing vector. Experiments with lipid rafts of apoptotic murine EL4 T cells revealed similar results. To verify the involvement of 5-LO in activating PPARγ in macrophages, Jurkat T cells were incubated with the 5-LO inhibitor MK-866 prior to induction of apoptosis, which failed to induce mRuby expression. Similar results were obtained with lipid rafts of apoptotic EL4 T cells preexposed to the 5-LO inhibitors zileuton and CJ-13610. Interestingly, Jurkat T cells overexpressing 5-LO failed to activate PPARγ in macrophages, while their 5-LO overexpressing apoptotic counterparts did. Our results suggest that during apoptosis 5-LO gets associated with lipid rafts and synthesizes ligands that in turn stimulate PPARγ in macrophages.

1. Introduction

Sepsis is a major cause of death in intensive care units [1–3]. Although sepsis has been in the focus of research during the last decade, only little progress has been made towards its therapy [4]. The disease is characterized by an initial hyper-inflammatory phase, exemplified by an uncontrolled, excessive release of pro-inflammatory cytokines and mediators such as reactive oxygen species (ROS) or nitric oxide (NO) [5]. Moreover, the complement system and the coagulation cascade get activated [6]. Already starting during the hyper-inflammatory phase, anti-inflammatory principles increase and finally overwhelm pro-inflammatory responses thus, causing a hypo-inflammatory period [7]. Now, the immune response is significantly impaired by T cell depletion via apoptosis as well as macrophage desensitization [8,9]. Interestingly, the removal of apoptotic cells by macrophages known as efferocytosis, causes a macrophage phenotype shift from classically activated (M1) to polarized macrophages (M2 MΦ) [10]. M2-type MΦ, also known as alternatively activated MΦ show an expression profile, which fosters wound healing and resolution of inflammation [11,12]. As a consequence, their pathogen elimination capacity is low, which allows recurrence of the primary or the development of new opportunistic secondary infections in this phase of sepsis [13]. For this reason, therapy approaches shifting the macrophage phenotype from M2 to M1 in the hypo-inflammatory phase are important for a successful treatment regime. Along those lines, knowledge provoking the M1 to M2 phenotype shift may provide new concepts for an optimized therapy protocol.

Macrophage polarization is linked to activation of the ligand-dependent transcription factor PPARγ. Recently, Odegaard et al. demonstrated with a macrophage-specific deletion of PPARγ in mice that alternative macrophage activation is impaired [14]. We provided evidence that contact to apoptotic cells (AC) activates PPARγ, inducing PPRE-driven transactivation in RAW 264.7 MΦ [15]. Because the PPARγ-activating principle remains obscure we analyzed AC to search for the MΦ polarization principle. We established a stable PPRE-driven reporter

Abbreviations: 5-LO, 5-lipoxygenase; AC, apoptotic cell(s); CV, control virus; d/n, dominant negative; F5, fraction 5; FF, firefly; FLAP, 5-LO activating protein; LC, living cell(s); LR, lipid raft(s); Luc, luciferase; M, marker; MΦ, macrophage(s); mßCDT, methyl-ß-cyclodextrin; NAC, N-acetyl-cysteine; NC, necrotic cell(s); NO, nitric oxide; OE, overexpression; PC, positive control; PPARα/β/γ, peroxisome proliferator activating receptor alpha/delta/gamma; PPRE, PPAR response element; ROS, reactive oxygen species.

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system in RAW 264.7 MΦ with mRuby and firefly luciferase as reporter genes. Based on the hypothesis that a potential activating principle/factor most likely is associated with the cell membrane, possibly associated to lipid rafts, we analyzed the expression of potential proteins associated with lipid rafts of AC and identified 5-LO as the most rational candidate. Lipid rafts of AC, containing active 5-LO stimulated PPARγ, whereas lipid rafts of living cells (LC) did not. The association of 5-LO with lipid rafts in AC and its activation are required to polarize MΦ, a process that may be of relevance during the hypo-inflammatory phase of sepsis.

2. Materials and methods

2.1. Cells

We cultured RAW 264.7 MΦ expressing PPARγ wild type as well as RAW 264.7 MΦ stably transduced with a PPARγ dominant negative mutant (PPARγ d/n) [15] in DMEM high glucose (PAA Laboratories, Linz, Austria). Jurkat T cells, EL4 T cells, and MCF7 breast cancer cells were grown in RPMI 1640 (PAA Laboratories). Both media were supplemented with 100 U/ml penicillin (PAA Laboratories), 100 μg/ml streptomycin (PAA Laboratories), and 10% heat inactivated fetal calf serum (FCS, PAA Laboratories).

2.2. Apoptotic and necrotic markers

To induce apoptosis Jurkat T cells and MCF7 cells were treated for 3 h with 0.5 μg/ml staurosporine (Sigma-Aldrich, Munich, Germany) in FCS-free medium. Apoptosis of EL4 T cells was induced by glucose deprivation for 24 h. Necrosis of Jurkat T cells was initiated by heating cells for 30 min to 65 °C. Apoptosis and necrosis were verified by annexin-V/propidium iodide staining (Immunotools, Friesoythe, Germany) and FACS analysis (LSR Fortessa, BD Life Sciences, Heidelberg, Germany).

2.3. Lentiviral transduction

To overexpress 5-lipoxygenase (5-LO OE) in Jurkat T cells, we used a lentiviral vector kindly provided by Dieter Steinhilber (Institute of Pharmaceutical Chemistry, Goethe-University Frankfurt, Frankfurt, Germany). To generate lentiviral particles, $3 * 10^6$ HEK293T cells were seeded in 5 ml complete DMEM medium. The following day, cells were transfected using jetPEI (Biomol, Hamburg, Germany) according to the distributor's instructions. Briefly, 3 μg of the 5-LO OE vector plasmid in combination with 26 μl of the packaging mix (Sigma) and 6 μl jetPEI (Biomol, Hamburg, Germany) were used. Afterwards, cells were cultured for 2 days. Then, the medium was changed and after two more days the supernatant containing the infectious lentiviral particles was recovered. To transduce target cells, lentiviral particles were filtered through a 0.45 μm filter (Millipore, Schwalbach, Germany) and thereafter directly spun twice onto Jurkat T cells (90 min, 500 g, RT). Transduced Jurkat cells were grown for 1 week in complete medium. Expression was verified by Western analysis.

To determine PPARγ transactivation, following different stimulation regimes, RAW 264.7 MΦ were stably transduced with a versatile HIV-based PPRE lentivector (pRF-PPRE-EF1-Puro, System Biosciences, Mountain View, USA). This vector co-expresses mRuby and firefly luciferase as the reporter genes for quantitative PPRE-driven transactivation. For generation of lentiviral particles, $3 * 10^6$ HEK293T cells were seeded in 5 ml complete DMEM medium. The following day, cells were transfected using jetPEI (Biomol, Hamburg, Germany) according to the distributor's instructions. Briefly, 3 μg of the pRF-PPRE-EF1-Puro vector in combination with 26 μl of the packaging mix (Sigma) and 6 μl jetPEI (Biomol, Hamburg, Germany) were used. Afterwards, cells were cultured for 2 days. Then, the medium was changed and after 2 days the supernatant containing the infectious lentiviral particles was harvested. To transduce target cells, lentiviral particles were filtered through a 0.45 μm filter (Millipore, Schwalbach, Germany), and thereafter directly spun twice onto RAW 264.7 MΦ (90 min, 500 g, RT). Transduced RAW 264.7 MΦ were grown for 1 week in complete medium before puromycin was added for antibiotic selection of positive clones. Following 2 weeks of puromycin selection reporter gene activity was analyzed by fluorescence microscopy in response to stimulation with 1 μM rosiglitazone, an established PPARγ agonist.

2.4. Lipid raft isolation and Western blot analysis

To enrich lipid rafts, living, apoptotic, or necrotic Jurkat T cells or EL4 T cells were lysed, basically as described [16]. Briefly, following individual treatments, 1×10^7 cells were harvested and washed three times with ice-cold PBS buffer. Cells were resuspended in 700 μl lysis buffer (25 mM HEPES-HCl, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1× protease inhibitor cocktail, 1% Triton X-100) using a pipet. Cell lysis was achieved using a Teflon-coated dounce homogenizer with 25 strokes. Lysis proceeded for 30 min on ice. Afterwards, lysates were mixed with the same volume (1 ml) of 80% sucrose cushion solution to obtain a final 40% sucrose gradient. Subsequently, the mixture was transferred to a 12 ml polyallomer ultracentrifuge tube for a SW40Ti rotor (Beckman Coulter, Krefeld, Germany), covered with 6.5 ml 30% and 3.5 ml 5% sucrose cushion, respectively. Sucrose cushion solutions were prepared in 25 mM HEPES-HCl, pH 6.5, 150 mM NaCl, and 1 mM EDTA. Ultracentrifugation was performed at 4 °C, 35,000 rpm for 20 h in an Optima L90-K ultracentrifuge (Beckman Coulter, Krefeld, Germany). Following gradient centrifugation 12× 1 ml fractions are recovered to identify the fraction(s) containing the lipid rafts. To identify corresponding fractions, 100 μl of each of the twelve fractions was mixed with 20 μl of a 5× SDS sample buffer by vortexing. After boiling for 5 min at 96 °C, samples were chilled on ice for 5 min, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel, and blotted onto nitrocellulose sheets basically following standard procedures. Filters were incubated with the anti-$p56^{lck}$ antibody (1:500, Transduction Laboratories, BD Biosciences, Heidelberg), the anti-Flotilin antibody (1:500, Transduction Laboratories), the anti-5-lipoxygenase antibody (1:500, Transduction Laboratories), or the anti-actin antibody (1:2000, Amersham Biosciences, Freiburg, Germany) overnight at 4 °C. IRDye700 or IRDye800 polyclonal antibodies (1:5000, Licor, Bad Homburg, Germany) and the Odyssey Infrared Imaging System were used for direct infrared fluorescence detection and quantification.

2.5. Reporter assay and immunofluorescence staining

To follow mRuby expression, RAW 264.7 MΦ were seeded on a slide for 24 h to allow attachment. MΦ were counted and living, apoptotic, or necrotic Jurkat T cells or living as well as apoptotic EL4 T cells were added at a fivefold excess. Incubations went on for 24 h, followed by fixation of cells by incubation with 4% paraformaldehyde at 4 °C for 30 min. Subsequently, cells were washed twice with PBS and cell nuclei were counterstained with DAPI (1 μg/ml in PBS for 15 min). After a final 5 minute wash in PBS, cells were covered with Vectashield mounting medium (Linaris, Wertheim, Germany) and a cover slip. mRuby expression was determined using an AxioScope fluorescence microscope with the ApoTome upgrade (Carl Zeiss, Frankfurt, Germany, lens 63 × 0.6 NA; ocular 10×), documented by a CCD camera (Carl Zeiss) and the AxioVision Software (Carl Zeiss).

To quantify PPRE-driven transactivation, cells were harvested following 24 h co-incubation with living, apoptotic, or necrotic cells and processed as described above. Cells were lysed in passive reporter lysis buffer (Promega, Mannheim, Germany) and luciferase activity was quantified using a single tube luminometer (Berthold, Bad Wildbad, Germany).

Where indicated, lipid raft fractions of cells prepared as described above were used instead of whole cells.