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Suppression of the pro-inflammatory NLRP3/interleukin-1β pathway in macrophages by the thioredoxin reductase inhibitor auranofin

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ABSTRACT

Background: The thioredoxin/thioredoxin reductase system, which is best known for its essential role in antioxidant defense and redox homeostasis, is increasingly implicated in the regulation of multiple cellular signaling pathways. In the present study, we asked if the thioredoxin system in macrophages might regulate toll-like receptor 4 (TLR4)-dependent gene expression and consequent responses.
Methods: Using microarray analysis we analyzed the effect of auranofin, a highly potent and specific inhibitor of thioredoxin reductase, on the transcriptional program activated in J774 macrophages by the TLR4 agonist, lipopolysaccharide (LPS). We used quantitative real-time PCR (qPCR), Western blotting, ELISA and cytotoxicity assays to confirm and extend the microarray results.
Results: Global transcriptional profiling revealed that macrophage treatment with auranofin exerted a selective effect on LPS-induced gene expression, suppressing the induction of a small number of genes. Interestingly, among these suppressed genes were three members of the interleukin-1 (IL-1) family of genes, among which IL-1β was most affected. qPCR analyses confirmed the repressive effects of auranofin on IL-1 genes. In addition, qPCR and Western blot analyses showed that auranofin impaired TLR4-dependent induction of the inflammasome receptor NLRP3, which plays a critical role in IL-1β processing. Consistent with these findings, inflammasome-dependent release of IL-1β from stimulated macrophages was suppressed by auranofin as was inflammasome-mediated cell death.
Conclusions: Our findings suggest a regulatory role for the thioredoxin system in macrophage inflammatory signaling. Inhibition of the thioredoxin system in macrophages exerts an anti-inflammatory effect by repressing the activation of the NLRP3/IL-1β pathway.

1. Introduction

Macrophages act as sentinel cells in host defense by orchestrating innate and adaptive immune responses, particularly in the host response to infectious agents and other inflammatory or danger signals [1]. Macrophage activity is dictated by the nature of the immunologic stimulus. Stimulation by toll-like receptor (TLR) agonists such as lipopolysaccharide (LPS) induces the so-called classical activation of macrophages, a state characterized by robust production of pro-inflammatory cytokines, chemokines and reactive oxygen and nitrogen species (ROS and RNS, respectively). In particular, generation of ROS/RNS plays important roles in promoting the rapid destruction of infectious organisms and induction of protective inflammation and healing [2].

ROS/RNS such as nitric oxide (NO), superoxide anion and hydrogen peroxide are part of the cellular redox system that also includes enzymes that detoxify these reactive species, such as superoxide dismutases, glutathione reductase and peroxidases, thioredoxins and peroxiredoxins. Accumulating evidence suggests that these redox systems play regulatory roles in macrophage activation and innate immune responses [3–5]. Such redox regulation has been implicated in several processes in macrophages, including differentiation, hypoxia signaling, production of pro/anti-inflammatory cytokines, and cell death [3–5]. However, much remains unknown about the significance and nature of these redox-based mechanisms and precisely how they affect specific macrophage functions.

The thioredoxin (Trx) system, which is composed of Trx, the selenoenzyme Trx reductase (TrxR) and NADPH, plays a central role in ROS/RNS metabolism and cellular redox regulation [6–9]. A role for the Trx system in regulating classical activation of

Abbreviations: IL-1, interleukin-1; LPS, lipopolysaccharide; NLRP3, Nod-like receptor protein 3; TLR, toll-like receptor; Trx, thioredoxin; TrxR, thioredoxin reductase

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macrophages appears probable but remains to be elucidated. A previous study showed that treatment of macrophages with TrxR inhibitors suppressed the LPS-induced induction of tumor necrosis factor-α (TNFα) [10]. This effect possibly involved NF-κB, a redox-sensitive transcription factor, or another protein acting upstream to NF-κB [10,11]. Overall, the extent to which the macrophage Trx/TrxR system affects TLR-dependent responses and the underlying mechanisms remains to be established.

S-triethylphosphine-gold(I)-2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (auranofin) and related gold compounds are a class of potent inhibitors of selenoenzymes, particularly, TrxR. Gromer et al. demonstrated that auranofin inhibits purified human TrxR with a K_i in the low nanomolar range [12]. By comparison, a 1000-fold higher concentration of auranofin was required to inhibit another selenoenzyme, glutathione peroxidase or the enzyme glutathione reductase [12]. On the basis of this knowledge auranofin has been widely employed as a tool to investigate the involvement of Trx/TrxR in various cellular processes, including peroxide metabolism [13–16], regulation of S-nitrosylation [17,18] and regulation of growth factor signaling [19].

In the present study, we used auranofin as a tool to explore the potential involvement of Trx/TrxR in the pro-inflammatory response of macrophages to TLR4 activation. Using transcriptional profiling, we found that auranofin exerted a highly specific effect on TLR4-mediated gene activation, blocking the induction of a small set of inducible genes. Notably, induction of the three members of the interleukin (IL-1) family of ligands, including the key pro-inflammatory cytokine IL-1β, was robustly suppressed by auranofin treatment. In addition, auranofin suppressed the induction of the macrophage inflammasome receptor NLRP3, thereby attenuating inflammasome-mediated generation of bioactive IL-1β as well as induction of cell death. The findings reported herein suggest that inhibition of the macrophage Trx system exerts an anti-inflammatory effect by suppressing the activation of the NLRP3/IL-1β pathway.

2. Materials and methods

2.1. Antibodies and reagents

The following antibodies were used throughout this study. Anti-IL-1β (catalog no. BAF401) was from R&D systems. Anti-NLRP3 (AG-20B-0014) was from AdipoGen. Anti-caspase-1 (sc-514) was from Santa Cruz Biotechnology. Anti-GAPDH (MAB374) was from Millipore. Auranofin was from Enzo Life Sciences. Tissue culture media and reagents were from Biological Industries (Beit Haemek, Israel). LPS (*Escherichia coli* serotype O55:B5, catalog no. L4005) and all other materials were from Sigma, unless otherwise indicated.

2.2. Cell culture and treatment

Murine J774 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, L-glutamine and sodium pyruvate at 37 °C in a humidified incubator (5% CO_2, 95% air). Mouse peritoneal macrophages were prepared and cultured as described previously [20]. Cells were subjected to the different treatments in DMEM/10% FBS supplemented with 1 mM L-arginine. Throughout the experiments, LPS was administered to cells at a concentration of 0.5 μg/ml.

2.3. RNA extraction from tissue culture

A total of 10^6 cells seeded in 10-cm dishes were treated with LPS and/or auranofin as described in figure legends. RNA was extracted using the total RNA purification kit (RNeasy Mini Kit; Qiagen) according to the manufacturer's instructions. RNA concentration and purity were determined by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

2.4. Gene expression analysis

Microarray expression profiling was performed in the Genomics Core Facility (Rappaport Research Institute and the Faculty of Medicine, Technion). The quality of total RNA from J774 samples was evaluated using an Experion apparatus (Bio-Rad Laboratories). The RNA was amplified into cRNA and biotinylated by in vitro transcription using the TargetAmp Nano-g Biotin-aRNA Labeling kit for the Illumina system (Epicentre Biotechnologies) according to the manufacturer's protocol, with 100 ng of total RNA as input material. Biotinylated cRNAs were purified, fragmented, and subsequently hybridized to an Illumina MouseWG-6 v2.0 BeadChip according to the Direct Hybridization assay (Illumina Inc.). The hybridized chip was stained with streptavidin-Cy3 (Amersham/GE Healthcare) and scanned with an Illumina HiScan system. The scanned images were imported into GenomeStudio (Illumina Inc.) for extraction and quality control. Using JMP® Genomics V5 software (SAS Institute Inc., Cary, NC), two types of filtering were performed: filtering by signal to remove all probes with signal intensity $\leq 2^6$ (background noise elimination), and filtering by variance to exclude all probes with low variance that is $\leq 2^{0.1}$. Subsequently principal component analysis was performed to detect outliers and identify major trends. Three different sets of treatment were compared with the control, cells treated with either LPS, auranofin, or both. Each set was analyzed using one-way analysis of variance with cutoff for differentially expressed genes (DEG) at an adjusted p-value of 0.05 and a difference of 2-fold change between treated and untreated cells.

2.5. Reverse-transcriptase PCR and quantitative real-time PCR (qPCR)

About 0.5 μg of RNA was reversed transcribed (RT-PCR) into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) in a DNA thermal cycler (Bioer XP cycler). The cDNA was then diluted 1:8 and PCR reactions were performed using the Absolute Blue SYBR-Green ROX mix (Thermo Scientific) according to manufacturer instructions using a Rotor-Gene 6000 (Corbett Life Sciences). Quantification of mRNA was calculated by the comparative C_T method described elsewhere [21] and is shown as fold change of expression ($2^{-\Delta\Delta CT}$). mRNA levels were normalized to GAPDH mRNA levels. Murine NLRP3, TNFα, IL1β, IL1α, IL1RN and GAPDH were amplified using the following primer sets: NLRP3 (forward, 5′- CACGTGGTTTCCTCCTTTTG -3′ and reverse, 5′- TCCGGTTGGTGCTTAGACTT -3′); TNFα (forward, 5′- TCCCTCCAGAAAAG ACACCA -3′ and reverse, 5′- ATGAGA GGGAGGCCATTTG -3′); IL1β (forward, 5′- AGAGCTTCAGGCAGGCAGTAT -3′ and reverse, 5′- GAAGGTGC TCATGTCCTCATC -3′); IL1α (forward, 5′- CAGTTCTGCCATTGACCATC -3′ and reverse, 5′- ATGGACTGCAGGTCATCTTC -3′); IL1RN (forward, 5′- CCTGAGAAACAACCAGCTCA -3′ and reverse, 5′- TCATCTCCAGACTTGG CACA -3′) and GAPDH (forward, 5′- AGGTTGTCTCCTGCGACTTC -3′ and reverse, 5′- ACTCCTTGGAGGCCATGTAG -3′).

2.6. Western blot, ELISA and lactate dehydrogenase cytotoxicity assays

Whole cell lysates were prepared in lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, with protease inhibitors, pH 7.4). Equal amounts of protein (35 μg) were analyzed by SDS-PAGE and Western blotting with specific antibodies to NLRP3, IL-1β, or caspase-1. Blots were visualized and quantified with the Odyssey system and software (LI-COR). Secreted IL-1β was assessed with the DuoSet mouse IL-1β ELISA detection system from R&D Systems (catalog no. DY401). Lactate dehydrogenase (LDH) release was measured using the Cytotox96 cytotoxicity kit as per manufacturer instructions (Promega).