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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).

2.7. Determination of TrxR activity and soluble thiols

The activity of TrxR was determined using the Trx-linked insulin reduction assay as described previously [22,23].

Acid-soluble (low molecular weight) thiols were measured spectrophotometrically after reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). An equal volume of 10% metaphosphoric acid was added to cell extracts, the precipitate was removed by centrifugation, and the supernatant was neutralized with triethanolamine. Thiols were then measured by addition of an equal volume of DTNB solution (1 mM final concentration). The absorbance of Ellman's reagent adduct was measured at 405 nm. Values were derived by comparison with glutathione standards and were normalized to protein concentration in the extract.

3. Results

3.1. Characterization of the effects of auranofin on the activity of thioredoxin reductase and soluble thiols

We began our study by determining the effect of auranofin on the activity of TrxR in J774 macrophages. As shown in Fig. 1A auranofin inhibited total TrxR activity in a time- and dose-dependent manner. Treatment with auranofin at a concentration of 2 μM resulted in ~60% inhibition of TrxR activity within 1 h while having minimal effect on cell viability (Fig. 1A and see below). Based on these results, we chose a concentration of 2 μM of auranofin for further experimentation. Assessing the effects of longer treatments (up to 8 h), we observed that auranofin-mediated inhibition of TrxR was persistent over time (Fig. 1B). LPS treatment for 4 or 8 h resulted in a modest decrease in TrxR activity (~20% less than the activity measured in the control sample, Fig. 1B). We further assessed how these treatments may affect the cellular redox state by measuring the levels of acid soluble thiols (which consist primarily of reduced glutathione). Treatment with auranofin for 4 or 8 h resulted in a progressive decrease in the level of soluble thiols. Treatment with LPS had a similar albeit smaller effect. Combined treatment with LPS and auranofin had the largest effect, decreasing thiol content by ~45% at 8 h (Fig. 1B). These data indicate that prolonged exposure of macrophages to LPS and auranofin induce a gradual shift to a more oxidizing cellular environment.

3.2. Auranofin exerts a selective effect on the LPS-dependent transcriptional response

To assess the effect of inhibition of TrxR activity on the TLR4-dependent transcriptional response we performed microarray analysis using J774 cells stimulated for 4 h with LPS in the presence or absence of auranofin. RNA extracted from three independent experiments was hybridized to the Mouse WG-6 v2.0 Expression BeadChip (Illumina, San Diego, CA). Hybridization and signal quantitation were performed according to Illumina's instructions by the Genomics Core Facility of the Faculty of Medicine at the Technion (Haifa, Israel). Differentially expressed genes were defined as genes in which the normalized ratios were altered by >2 fold and adjusted p-value < 0.05 (see Materials and methods). As expected, analysis of the microarray data showed that LPS stimulation exerted a profound effect on the macrophage

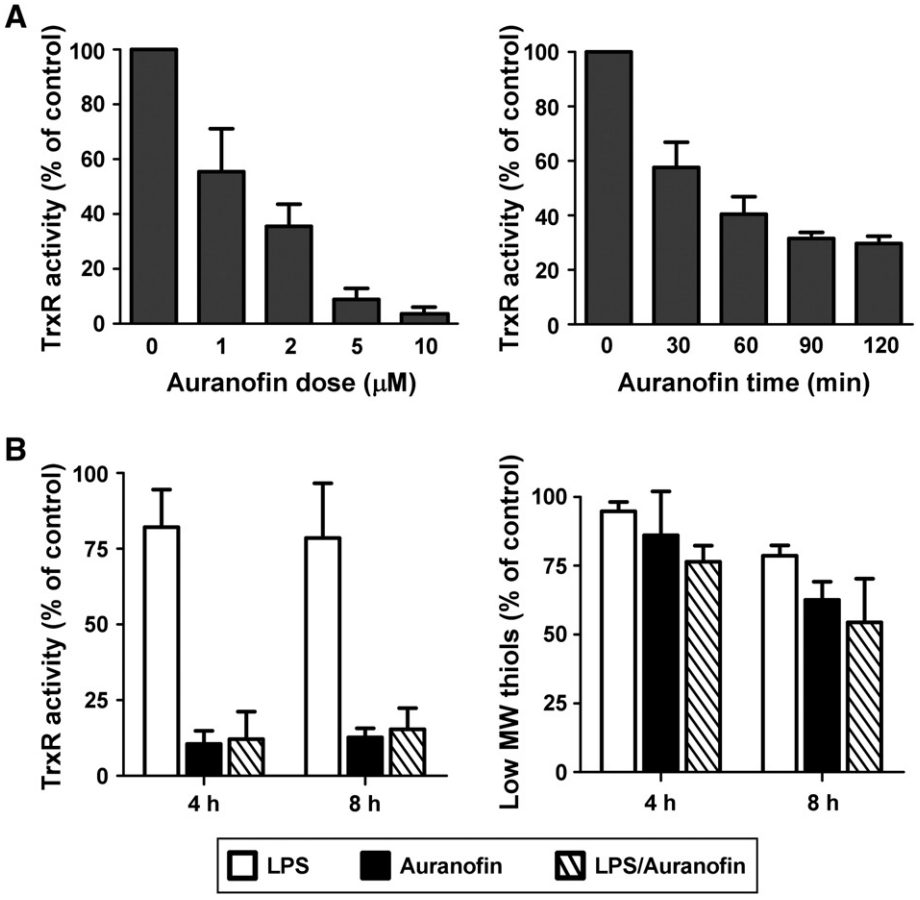

Fig. 1. Determination of the effect of auranofin and LPS on the activity of thioredoxin reductase and the amount of soluble thiols in J774 cells. (A) J774 cells were treated for 1 h with the indicated doses of auranofin (left) or with 2 μM auranofin for the indicated times (right) followed by determination of total TrxR activity in whole cell lysates. (B) J774 cells were treated with LPS (0.5 μg/ml) and/or auranofin (2 μM) for 4 or 8 h. Total cellular TrxR activity and the amount of acid-soluble (low molecular weight) thiols in cell lysates were determined. Data are presented as mean ± S.D. (n = 3).

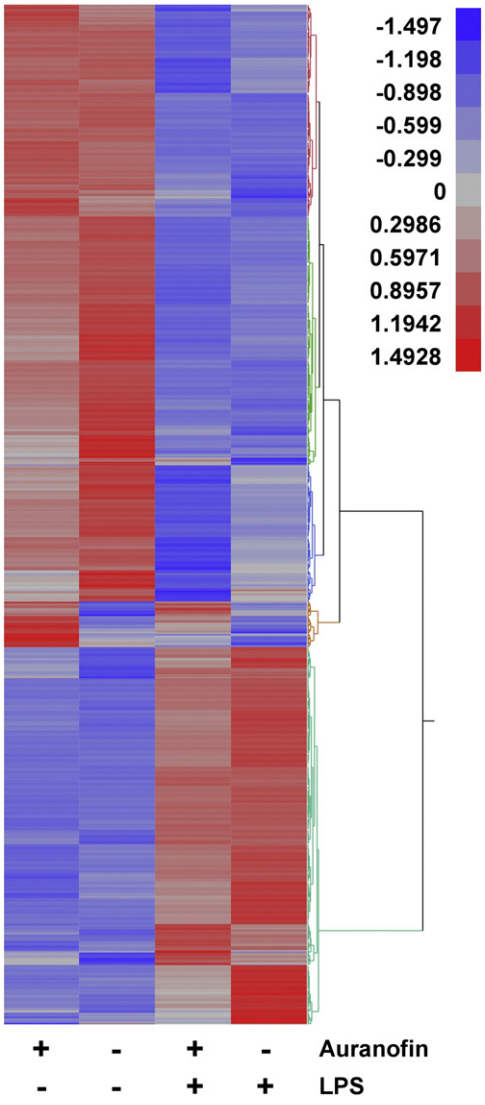

Fig. 2. Cluster analysis of differentially expressed genes in J774 cells. J774 cells were stimulated for 4 h with or without LPS in the presence or absence of auranofin. RNA extracted from these cells was tested in Illumina gene-array. The dendrogram depicts hierarchical clustering patterns among the different treatment groups. Expression values (in log 2 scale) are color coded from low expression in blue to high expression in red.

transcriptome, with 506 transcripts upregulated and 340 transcripts downregulated (Supplemental Table 1). In contrast to the effect of LPS, auranofin had a substantially less pronounced effect on gene expression, with only 30 transcripts displaying expression changes by a factor of twofold or more (Supplemental Table 1). These trends could also be observed upon subjecting the microarray data to hierarchical clustering analysis (Fig. 2). Notably, among the 506 transcripts that were induced by LPS, only 12 were significantly affected by auranofin, in which a suppressive effect was observed for 9 transcripts (Table 1). Most interestingly, within this small group of affected transcripts were three encoding for members of the interleukin-1 (IL-1) family of genes, namely, IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1RN).

3.3. Validation of the microarray results

Focusing on the IL-1 family, we proceeded to carry out quantitative RT-PCR (qPCR) experiments in order to validate the microarray data. Macrophages were treated as before and subjected to qPCR analysis using appropriate primers, which resulted in the amplification of the selected genes. In agreement with our microarray data, we found that auranofin effectively inhibited the LPS-mediated induction of IL-1α, IL-1β, and IL-1RN, with inhibition amounting to 41%, 72%, and 56%, respectively (Fig. 3). These values agree well with those derived from the microarray analysis (Table 1). The TLR4 target gene TNFα was found by our microarray data not to be affected by auranofin. In accordance with this, qPCR experiments also showed that auranofin did not alter induction of TNFα (Fig. 3). Overall, these results validate the microarray data and they support the notion that inhibition of TrxR selectively affects a small group of TLR4-induced genes, particularly the three members of the IL-1 family.

3.4. Auranofin attenuates the induction of both pro-IL-1β and NLRP3

IL-1β plays a key role in many immune and inflammatory responses [24]. Given this fact and due to the robust effect of auranofin on IL-1β mRNA expression noted above we were interested to study further how inhibition of TrxR might affect the activation of IL-1β. In macrophages, generation of biologically active IL-1β requires both a 'priming' signal (such as TLR4 activation) to induce the synthesis of the pro-IL-1β and a second signal to stimulate the assembly and activation of multimeric protein platforms called the inflammasomes, which activate caspase-1 thereby promoting the processing and secretion of IL-1β [25]. The well-studied NLRP3 inflammasome, which is composed of the NOD-like receptor protein (NLRP) 3, the adaptor protein, ASC, and the protease, caspase-1, is activated by a range of infectious and sterile stimuli including extracellular ATP and nigericin [25]. Of note, recent studies in macrophages showed that TLR stimulation upregulates the expression of NLRP3, an event that might serve as a control point in the process of inflammasome activation [26–28]. It was also reported that among different Nod-like receptors, the expression of only NLRP3 is TLR-dependent [29]. Unfortunately, our microarray data did not contain information pertaining to the NLRP3 gene. Nonetheless, by performing qPCR experiments we found that treatment with auranofin

Table 1
Repressive effects of auranofin on LPS-induced genes in J774 macrophages.

Gene name	Description	Accession	Fold change (vs. untreated)		Effect on LPS
			LPS	Auranofin + LPS	
Ccl7	Chemokine (C–C motif) ligand 7	NM_013654.2	22.3	10.6	−52%
Cd69	CD69 antigen	NM_001033122.3	35.9	15.4	−57%
Ikzf1	IKAROS family zinc finger 1	AK088636	3.3	1.6	−49%
Il1a	Interleukin 1 alpha	NM_010554.4	4.6	1.7	−63%
Il1b	Interleukin 1 beta	NM_008361	58.5	12.6	−78%
Il1rn	Interleukin 1 receptor antagonist	NM_001039701.2	3.5	1.8	−49%
Serpina3g	Serine (or cysteine) peptidase inhibitor, clade A, member 3G	NM_009251.1	8.8	2.8	−68%
Serpina3h	Serine (or cysteine) peptidase inhibitor, clade A, member 3H	NM_001034870.2	3.5	1.6	−53%
Gm14446	Predicted gene 14446	XM_992268.1	4.2	2.1	−50%

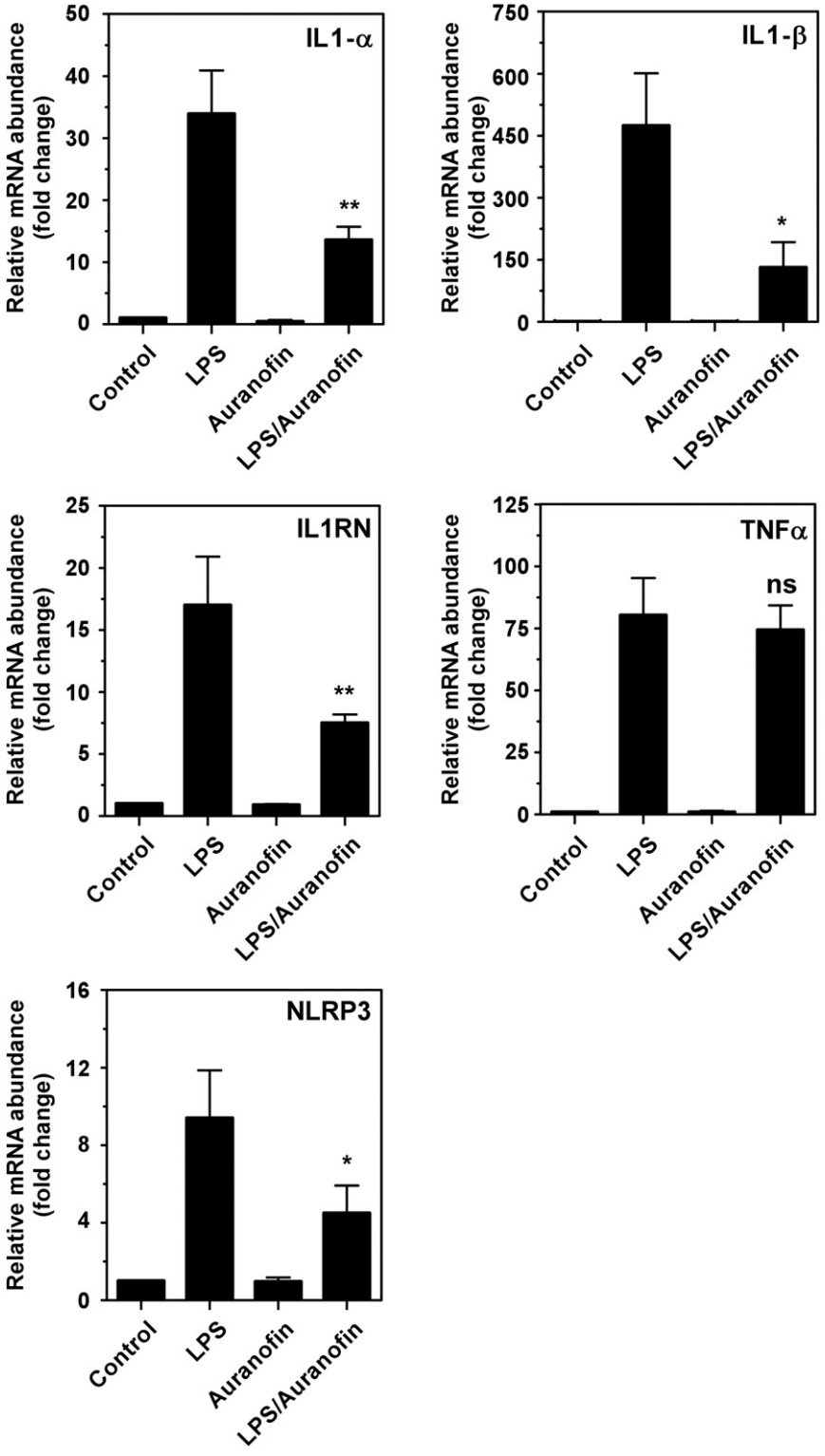

Fig. 3. Quantitative real-time PCR (qPCR) analysis confirms changes in mRNA expression of the selected genes in activated macrophages in response to administration of auranofin. J774 cells were treated for 4 h with or without LPS in the presence or absence of auranofin. qPCR was carried out in triplicate on cDNA prepared from three independent sets of samples. Gene expression was normalized to GAPDH expression, and fold changes between control (set at 1) and treated cells were determined using the $2^{-\Delta\Delta CT}$ method. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus cells treated with LPS alone; ns, non-significant versus cells treated with LPS alone by one-way analysis of variance.

resulted in 2-fold inhibition of the LPS-mediated induction of NLRP3 as compared with vehicle-treated control (Fig. 3).

Next we examined if the effects of auranofin on pro-IL-1β and NLRP3, as revealed by the microarray and qPCR analyses, extend to the protein level. To this end, J774 cells were stimulated with LPS in the presence or absence of auranofin and the protein levels were then determined by Western blot analysis. Similar to the results seen at the mRNA level, we found that auranofin treatment robustly attenuated the LPS-mediated induction of pro-IL-1β. The inhibitory effect of auranofin was evident as early as 2 h post LPS-stimulation and persisted up to 8 h (Fig. 4A and B). The induction in the expression of NLRP3, evident at 4 and 8 h post LPS stimulation, was also markedly attenuated

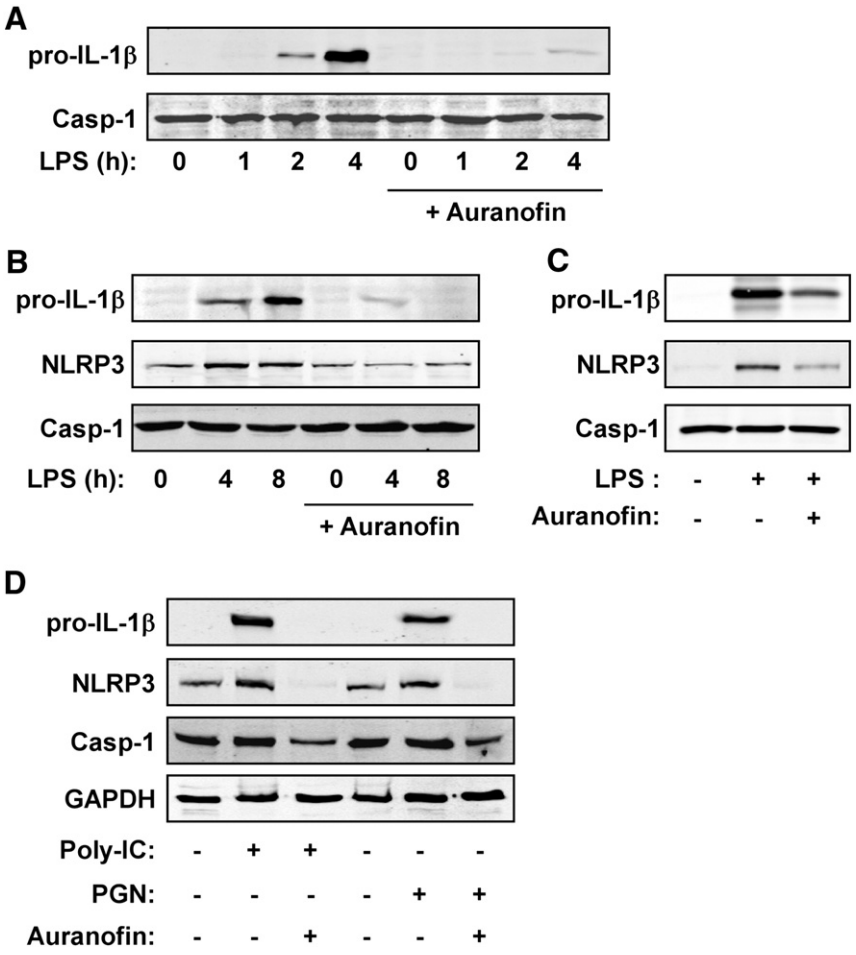

Fig. 4. Analysis of the effect of auranofin on the expression of NLRP3 inflammasome-related proteins. (A–B) J774 cells were treated with LPS for the indicated times in the absence or presence of auranofin. The expression of pro-IL-1β, NLRP3 and caspase-1 was determined by immunoblotting. (C) Mouse peritoneal macrophages were treated with LPS for 4 h in the absence or presence of auranofin. The expression of pro-IL-1β, NLRP3 and caspase-1 was determined by immunoblotting. (D) J774 cells were treated with poly-IC or peptidoglycan (PGN) for 4 h in the absence or presence of auranofin. The expression of pro-IL-1β, NLRP3, caspase-1 and GAPDH was determined by immunoblotting. Blots shown are representative of at least three independent experiments.

by treatment with auranofin (Fig. 4B). Similar to the findings in J774 cells, auranofin also suppressed the LPS-dependent induction of pro-IL-1β and NLRP3 proteins in primary mouse peritoneal macrophages (Fig. 4C).

We wondered if TrxR inhibition influences TLR signaling pathways other than TLR4. To address this point, we examined the effect of auranofin treatment on the induction of pro-IL-1β and NLRP3 induced by the TLR3 ligand poly-IC or the TLR2 ligand peptidoglycan (PGN). Similar to the observations with LPS, auranofin blocked poly-IC- and PGN-induced induction of pro-IL-1β and NLRP3 (Fig. 4D). Intriguingly, the results showed that in the case of NLRP3, auranofin not only blocked its induction but actually decreased the protein expression to a level below that seen in resting cells. A partial downregulation was also seen for caspase-1 protein in auranofin-treated cells (Fig. 4D). Collectively, these data suggest that TrxR activity is needed for the induction of pro-IL-1β and NLRP3 downstream of TLR2 and 3; however, for these TLRs, posttranscriptional mechanisms that control the level of inflammasome proteins may also be influenced by TrxR activity.

3.5. Auranofin attenuates NLRP3-mediated IL-1β processing and macrophage death

The rapid action of auranofin in cells offers the opportunity to interfere with the activity of the Trx system at different time points during cell activation. Accordingly, we next examined the effect of TrxR inhibition that occurs late in the course of the TLR4 response. To this end, J774 cells were treated with LPS for 8 h with auranofin added for the final 2 h. Under these conditions auranofin attenuated the expression of NLRP3 but not of pro-IL-1β (Fig. 5A). To test if this effect on NLRP3 expression was functionally relevant, we measured IL-1β released from cells upon NLRP3 activation with ATP. We found that, in accordance with the reduction of NLRP3 protein, treatment with auranofin resulted in at least 2-fold reduction in the amount of IL-1β released from cells (Fig. 5B). Altogether, our results suggest that TrxR activity is required for the induction of IL-1β bioactivity by regulating the induction of both pro-IL-1β and NLRP3.

Activation of the NLRP3 inflammasome is involved not only in cytokine processing but also in the induction of macrophage death [25]. In particular, a recent study showed that NLRP3 activation promotes macrophage death in response to stimulation with the NLRP3 agonist, nigericin [30]. As we observed that auranofin attenuated that induction of NLRP3, we hypothesized that it may confer protection from nigericin-mediated cell death. Using LDH release assay we observed minimal cell death (<10%) in resting or LPS-stimulated cells that was slightly but insignificantly increased by auranofin (Fig. 6). Consistent with previous data [30] we observed that macrophage treatment with LPS/nigericin induced massive cell death. Notably, treatment with auranofin resulted in significant reduction (over 40%) in LPS/nigericin-mediated cell death (Fig. 6).

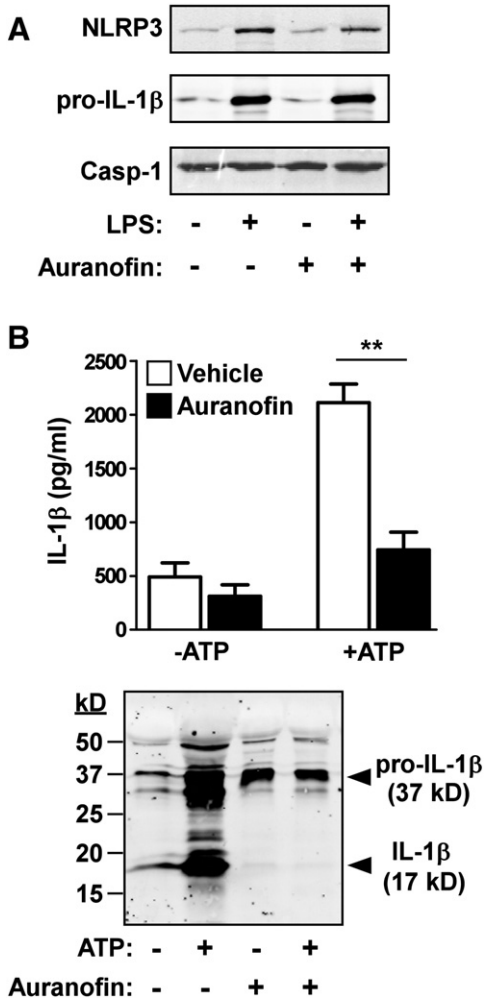

Fig. 5. Analysis of the effect of late and short-term treatment with auranofin on the expression of NLRP3 inflammasome-related proteins and release of interleukin-1β. (A) J774 cells were treated with LPS for 8 h, with auranofin or vehicle added for the final 2 h. The expression of pro-IL-1β, NLRP3 and caspase-1 was determined by immunoblotting. Blots shown are representative of at least three independent experiments. (B) J774 cells were treated as in A followed by stimulation with ATP (5 mM, 1 h). Release of IL-1β from the cells was determined by ELISA (top). The presence of immature pro-IL-1β (37 kDa) and mature IL-1β (17 kDa) in cell culture supernatants was analyzed by immunoblotting (bottom). Data obtained from the ELISA are presented as mean ± S.D. (n = 3). $^{**}P < 0.01$ by one-way analysis of variance.

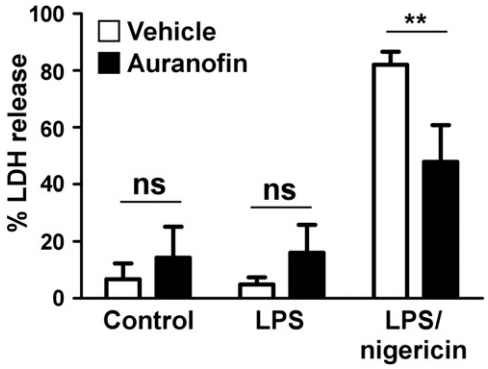

Fig. 6. Auranofin attenuates macrophage death triggered by LPS plus nigericin. J774 cells were stimulated with LPS (4 h) in the absence or presence of auranofin and then treated with or without nigericin (20 μM, 30 min). Cell death was determined by quantifying lactate dehydrogenase (LDH) release to the culture medium. Results are expressed as percentage of total LDH release. Data are shown as mean ± S.D. (n = 3). $^{**}P < 0.01$; ns, non-significant by one-way analysis of variance.

4. Discussion

Recent research has provided increasing evidence for redox regulation of immune and inflammatory responses [3–5] but the underlying molecular mechanisms remain largely unknown. The Trx system, which is a key element in the cellular redox system, has been shown to regulate cellular signaling and related processes [6–8], but how it might be involved in activation of innate immune cells is not clear. In mammals, knockout of the Trx or TrxR genes has been shown to lead to severe cellular and organismal defects that result in lethality during embryonic development [6]. These observations highlight the essential cellular/organismal roles of Trx/TrxR and they underscore the complex and challenging issues involved in studying regulatory roles of Trx/TrxR using genetic knockout approaches. In this regard, the availability of potent and specific TrxR inhibitors, particularly auranofin [12], has greatly facilitated research into the roles of the Trx/TrxR system in various processes in mammalian cells [13–19]. In addition, as exemplified here, the temporal flexibility provided by the rapid action of auranofin allows one to determine the requirement for TrxR activity at different time points after cell stimulation.

It is well known that auranofin exerts anti-inflammatory and anti-tumor effects, however, the molecular mechanisms underlying these effects have been only partly elucidated, as reviewed in [31]. Of particular relevance to the present study, previous investigations have found that auranofin can downregulate the induction of IL-1β in monocytes and macrophages in response to LPS or bacteria [32,33]. The latter study also found that auranofin inhibited the induction of TNFα, a result that differs from our present findings. Auranofin has been reported to exert additional anti-inflammatory effects including the inhibition of prostaglandin synthesis [34]. At the molecular level, auranofin is thought to rapidly form an irreversible covalent bond with the selenocysteine residue in TrxR [12]. It is likely that both the present and the previously reported effects of auranofin are related to its rapid and potent inhibition of the (selenocysteine-dependent) activity of TrxR. However, it cannot be excluded that some of the effects of auranofin are mediated via other selenoproteins or even through non-selenoproteins that contain redox-sensitive thiols.

To our knowledge, this study represents the first attempt to evaluate how manipulation of the Trx/TrxR system might affect activation of a pro-inflammatory transcription program. Specifically, we used transcriptional profiling to examine how TrxR inhibition might influence macrophage gene expression in response to TLR4 stimulation. Using this approach, we found that inhibition of TrxR exerted a selective effect on gene activation, suppressing the induction of a small set of LPS-responsive genes. Strikingly, within this small group of repressed genes were three members of the IL-1 family, namely, IL-1α, IL-1β, and IL-1RN. These and additional findings reported herein suggest that the Trx system might play an important positive regulatory role in IL-1 activation and the resultant immune response. It should be noted however that Trx/TrxR can also exert anti-inflammatory effects [6–8,35,36]. Undoubtedly, elucidation of the molecular mechanisms linking Trx/TrxR to inflammatory signaling will be key to the understanding these pro/anti-inflammatory roles of the Trx system.

IL-1β plays a central role in inflammatory and immune processes [24]. Accordingly, generation of active IL-1β is subject to complex regulation both at the transcriptional and posttranscriptional levels. In addition, the expression level of the NLRP3 protein (but not of other Nod-like receptors) is regulated by TLR signaling [29]. Recent research has provided increasing evidence for redox regulation of the NLRP3/IL-1β pathway. In particular, there has been a lot of interest in the possible role of ROS in regulating the activation of the NLRP3 inflammasome. However, there are conflicting reports regarding whether, how and at which stage do ROS regulate NLRP3 activation, as reviewed in [37]. Other recent studies have linked the Trx system to inflammasome regulation, but again the underlying mechanisms are not well-established [35]. For example, it was shown that in pancreatic β cells, the Trx inhibitor

Txnip promotes activation of the NLRP3 inflammasome [38]. However, it is not clear if this mechanism also operates in macrophages. A direct influence of TrxR on inflammasome activity in macrophages is supported by results of a recent study that reported inhibitory effects of auranofin on the NLRP1 inflammasome [39]. Still, the mechanistic basis for redox regulation of the inflammasome/IL-1β pathway in macrophages remains poorly understood. The above reports notwithstanding, we emphasize that our results indicate that TrxR inhibition affects IL-1β activation at a level upstream of the inflammasome, during the transcriptional induction of pro-IL-1β and NLRP3. As seen for the TLR4 response, TrxR inhibition also repressed the TLR2- and TLR3-mediated induction of NLRP3/pro-IL-1β, though in the case of TLR2/3, inhibition of TrxR appeared to have additional effects on inflammasome protein levels. Overall, our results suggest that TrxR may regulate pro-inflammatory signaling induced by multiple TLRs.

What is the molecular mechanism linking downregulation of TrxR activity to the transcriptional repression of NLRP3 and pro-IL-1β? This question is not easy to answer as TrxR activity impacts the cellular redox system in multiple ways [40] and its inhibition may affect the thiol redox state (and likely the activity) of many proteins, as recently documented in HT-29 cells [41]. In particular, interfering with TrxR activity is likely to increase the proportion of oxidized cellular Trx, though the degree of Trx oxidation may depend on the nature, extent and duration of TrxR inhibition as well as the activity of backup systems [42]. Increased oxidation of Trx may in turn affect several Trx-interacting proteins or dependent enzymes, including ASK-1 and peroxiredoxins [6–8]. TrxR itself can catabolize hydrogen peroxide and lipid hydroperoxides [43]. Whether any of these Trx/TrxR-related biochemical activities and interacting partners are relevant to the present findings on the regulation of the NLRP3/IL-1β pathway is currently unclear and will require further research. In any case, persistent inhibition of TrxR is expected to alter the cellular redox balance and potentiate the oxidant stress elicited by TLR4 activation. Indeed, our analysis of soluble thiols, particularly in response to longer treatments with LPS/auranofin, indicates a shift to a more oxidizing cellular environment, which could modulate the TLR4-induced response.

On the basis of all of our findings, we speculate that the effects of TrxR inhibition reported herein, particularly the repression of pro-IL-1β induction (noted already within 2 h of LPS stimulation) are likely related to a specific signaling event that is regulated by Trx/TrxR, rather than to a major change in the cellular redox state per se. In this regard, prior studies have shown that Trx/TrxR can directly or indirectly promote the transcriptional activity of NF-κB [10,11,44–46], a pivotal transcription factor in TLR4-signaling that is known to be involved in the induction of NLRP3 and IL-1β [26,28,47]. Accordingly, it could be postulated that auranofin-dependent modulation of NF-κB activation is related to our present findings. However, the observations that auranofin affected only a small subset of TLR4-induced genes and did not affect many well-established NF-kB target genes (such as TNFα) suggest that NF-κB is unlikely to play a significant role in the effects of TrxR inhibition herein reported. Rather, our findings are more consistent with a model in which Trx/TrxR activity is needed for the activity of a transcription factor or a signaling protein that has a more limited spectrum of action in the TLR4-induced inflammatory cascade. Further studies are needed to identify this putative redox-sensitive factor and thereby elucidate a novel mechanism for regulating the TLR4/IL-1 pathway.

5. Conclusions

The current study suggests that the Trx system positively regulates the NLRP3/IL-1β pathway. The present findings support the idea that inhibition of the Trx system might exert beneficial effects in pathological conditions associated with excess activation of the NLRP3/IL-1β pathway [25].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.07.012.

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