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Retinoic acid dampens LPS-induced NF-κB activity: results from human monoblasts and in vivo imaging of NF-κB reporter mice☆

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Abstract

Bacterial lipopolysaccharide (LPS) is a major inducer of systemic inflammatory reactions and oxidative stress in response to microbial infections and may cause sepsis. In the present study, we demonstrate that retinoic acid inhibits LPS-induced activation in transgenic reporter mice and human monoblasts through inhibition of nuclear factor κB (NF-κB). By using noninvasive molecular imaging of NF-κB luciferase reporter mice, we showed that administration of retinoic acid repressed LPS-induced whole-body luminescence, demonstrating in vivo the dynamics of retinoic acid's ability to repress physiologic response to LPS. Retinoic acid also inhibited LPS-induced NF-κB activity in the human myeloblastic cell line U937. Retinoic-acid-receptor-selective agonists mimicked — while specific antagonists inhibited — the effects of retinoic acid, suggesting the involvement of nuclear retinoic acid receptors. Retinoic acid also repressed LPS-induced transcription of NF-κB target genes such as *IL-6*, *MCP-1* and *COX-2*. The effect of retinoic acid was dependent on new protein synthesis, was obstructed by a deacetylase inhibitor and was partly eliminated by a signal transducer and activator of transcription-1 (STAT1)/methyltransferase inhibitor, indicating that retinoic acid induces a new protein, possibly STAT1, that is involved in inhibiting NF-κB. This provides more evidence for retinoic acid's anti-inflammatory potential, which may have clinical implications in terms of fighting microbial infections.

Keywords: Inflammation; LPS; Retinoic acid; U937 cells; Transgenic luciferase reporter mice; In vivo imaging; STAT1

1. Introduction

Pathogenic microorganisms promote inflammatory responses through recruitment and activation of phagocytic cells such as macrophages and neutrophils. This activation results in increased transcription of proinflammatory cytokines and many other genes involved in immune functions, cell proliferation and apoptosis [1,2], as well as in oxidative stress and damage [3,4].

The nuclear factor κB (NF-κB) family of transcription factors are central players in inflammatory response and include p65 (RelA), p50, RelB, c-Rel and p52 in mammalian cells, forming homodimers and heterodimers [5]. NF-κB is normally sequestered in the cytosol by a protein called inhibitor κB (IκB). Upon activation by proinflammatory stimuli such as lipopolysaccharide (LPS), growth factors, UV light or viral proteins, IκB is phosphorylated, resulting in degradation of IκB by proteasome, and NF-κB translocates to the nucleus, binds to DNA target sequences and activates transcription [2,5]. Dysregulation of NF-κB activity is, however, a feature of chronic inflammatory diseases, atherosclerosis and some cancers [6–8]. As a consequence of its key role in several pathologic conditions, NF-κB is a major drug target in a variety of diseases.

☆ The University of Oslo has transferred the commercial rights of the NF-κB luciferase reporter mice to Cgene, a company in which R.B., H.C. and the University of Oslo are shareholders.

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Several reports have indicated that vitamin A deficiency promotes inflammatory reactions [9–13], hyperplasias and cancer [14–16]. Furthermore, retinoic acid (i.e., the molecule that mediates most effects of vitamin A) has recently been shown to inhibit several types of inflammatory reactions in experimental model systems [17,18].

The majority of the effects of retinoic acid are mediated by the binding of retinoic acid to specific nuclear receptors. These nuclear receptors, which are all transcription factors, consist of retinoic acid receptors (RARs; i.e., RAR-α, RAR-β and RAR-γ) and retinoid X receptors (RXRs; i.e., RXR-α, RXR-β and RXR-γ). The RARs and RXRs form heterodimers that bind to RAR response elements in regulatory regions of target genes and, upon ligand binding, regulate the expression of target genes [19].

We have recently generated a transgenic NF-κB reporter mice model that enables noninvasive molecular imaging of NF-κB in vivo [10,20–22]; by using this model, we have shown that vitamin A suppresses basal NF-κB activity [10]. In the present study, we have elucidated the effect of retinoic acid, as well as a human myeloblastic cell line, on LPS-induced activation in NF-κB reporter mice.

2. Materials and methods

2.1. Materials

Retinoic acid was purchased from Fluka Biochimika (Buchs, Switzerland). Corn oil, cycloheximide (CHX), 12-0-tetradecanoyl-phorbol 13-acetate (TPA), methyl-thioadenosine (MTA), RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), sterile phosphate-buffered saline (PBS), L-glutamine, penicillin/streptomycin-stabilized solution and fetal bovine serum were purchased from Sigma Aldrich (St. Louis, MO). The luciferase reporter assay was obtained from Promega (Madison, WI). Hygromycin B and pMEP4 were purchased from Invitrogen Life Technologies (Carlsbad, CA). D-Luciferin was obtained from Biosynth (Staad, Switzerland). Lipofectamine 2000 was obtained from Invitrogen Life Technologies. The RAR pan-antagonist AGN194310 was a kind gift from Dr. R. Chandraratna at Allergan (Irvine, CA). The p65 antibody (sc-372) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody utilized, horseradish-peroxidase-conjugated goat anti-rabbit, was obtained from Bio-Rad (Hercules, CA). The surface of Gram-negative bacteria is composed of numerous ligands for TLRs (toll-like receptors), including flagella, lipoproteins, peptidoglycan and LPS. Lipid A or endotoxin is the only region of LPS to be recognized by the innate immune system. Although lipid A is an essential component of all Gram-negative bacterial membranes, it is a highly diverse molecule. Lipid A from the enteric commensal and occasional pathogen *Escherichia coli* is highly immune stimulatory. We have therefore used *E. coli* LPS serotype 055:B5 from Fluka Biochemica (Buchs, Switzerland) in the present study.

2.2. U937 cells, U937-3xκB-LUC cells and mouse embryonic fibroblasts

U937 cells (ATCC, Manassas, VA) were stably transfected with an NF-κB reporter construct. The reporter construct contained the luciferase reporter gene regulated by a promoter containing three NF-κB sites (5′-GGGACTTTCC-′3) derived from the Igκ light-chain enhancer region 3xκB-LUC [20]. The plasmid pMEP4 containing a hygromycin B resistance gene was cotransfected into the cells, and the cells were grown in the presence of hygromycin B (75 μg/ml) to ensure selection of cells that retained the construct. The cells were grown in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (50 U/ml)/streptomycin (0.05 mg/ml)-stabilized solution and 10% fetal bovine serum. The cells were diluted to a concentration of 0.4 million cells/ml on the day before the experiments were performed.

Embryonic fibroblasts were isolated from the NF-κB reporter mice and grown in DMEM supplemented as above and split 1:3 every other day. Experiments were performed on confluent cells.

2.3. Measurement of luciferase activity

Luciferase activity from the cells was measured in accordance with the manufacturer's protocol for the luciferase reporter assay, using a luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity was related to total protein content measured with the Bio-Rad Protein Assay. For some of the experiments (indicated in figure legends), the luciferase activity of the cells was measured directly in cell culture plates using the IVIS Imaging System 100 Series (Xenogen Corp., Alameda, CA). Using 24-well plates for the cell culture experiments, at the end of the experiment, 10 μl of D-luciferin solution (0.13 mg/ml) was added to each well. The plate was placed in a light-proof imaging chamber, a gray-scale image was obtained and, 4 min after the addition of D-luciferin, the plate was imaged for 1 min. The images were processed with the software IgorPro 4.06 (Wavemetrics, Lake Oswego, OR). Quantification of luciferase activity (photons/s/cell culture well/sr) was performed using the Living Image Software (Xenogen Corp.).

2.4. NF-κB-regulated luciferase reporter mice

The mice were kept under conditions compliant with the rules and guidelines of the Federation of European Laboratory Animal Science Associations. In this study, we utilized transgenic reporter mice with the NF-κB reporter construct [20,22]. Administration of retinoic acid by gavage feeding was performed as described previously [10]. LPS (50 μg in 100 μl of PBS) was injected subcutaneously into the ventral side of the mouse.

2.5. In vivo imaging of luciferase activity

Mice were anesthetized with 2.5% isoflurane (Baxter, Deerfield, IL) and placed in a light-proof imaging chamber.

Throughout the imaging period, isoflurane anesthesia was maintained inside the imaging chamber. Four milligrams (~150 mg/kg) of the substrate D-luciferin dissolved in 200 μl of PBS (pH 7.8) was injected intraperitoneally into each mouse. Seven minutes later, the whole mouse was imaged for 1 min on the ventral side. The imaging system and processing software were used as described for the cells mentioned above.

2.6. Transient transfection of U937 cells

U937 cells were transiently transfected using Lipofectamine 2000 in accordance with the manufacturer's instructions. The NF-κB reporter construct was the same as the one mentioned above [20]. The RAR-α-pSG5 and the RXR-α-pSG5 expression plasmid were gifts from P. Chambon's laboratory [23,24], and the p65-Rc/CMV expression plasmid was a gift from Fahri Saatcioglu [25]. The interleukin-6 (IL-6) promoter/LUC reporter plasmid, containing 1168 bp of the human IL-6 promoter, was a gift from W. Vanden Berghe [26].

2.7. mRNA isolation and cDNA synthesis

mRNA was isolated using magnetic oligo-dT beads from Genovision (West Chester, PA) in accordance with the manufacturer's protocol. Sonication of the cell lysate was performed for 10 s, with 30–40% output, using Virsonic 50 (The Virtus Co., Gardiner, NY). cDNA synthesis was performed using Omniscript RT kit (Qiagen, Hilden, Germany).

2.8. Real-time polymerase chain reaction analysis

The mRNA levels of luciferase, IL-6, monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using LightCycler from Roche Diagnostics (Mannheim, Germany). Primers were designed using Roche's LightCycler Probe Design Software version 1.0 or Primer3 (http://www.basic.nwu.edu/biotools/Primer3.html) (Table 1). The protocol for Roche's LightCycler FastStart DNA Master SYBR Green I was used and optimized for each primer set. Melting curve analysis was performed at the end of each reaction to detect the presence of potential nonspecific polymerase chain reaction (PCR) products, and PCR was optimized to eliminate such products. All primers gave a PCR product of expected length. GAPDH was used as reference gene. A standard curve from cDNA template was made for each experiment and each primer set. The amount of target gene relative to the reference gene was quantified using Roche's LightCycler Relative Quantification software.

2.9. Immunoprecipitation and Western blot analysis

Nuclear extraction, immunoprecipitation and Western blot analysis were performed as previously described [27].

2.10. Statistical analysis

Two-tailed Student's *t* test was performed in Microsoft Excel (Microsoft Corp., Redmond, WA). Mann–Whitney test and analysis of variance (ANOVA) were performed using SPSS 11 (SPSS, Inc., Chicago, IL). Differences were considered statistically significant when $P<.05$. Bonferroni correction was applied where appropriate, as indicated in the figure legends.

3. Results

3.1. Retinoic acid reduces LPS-induced activation of U937 cells

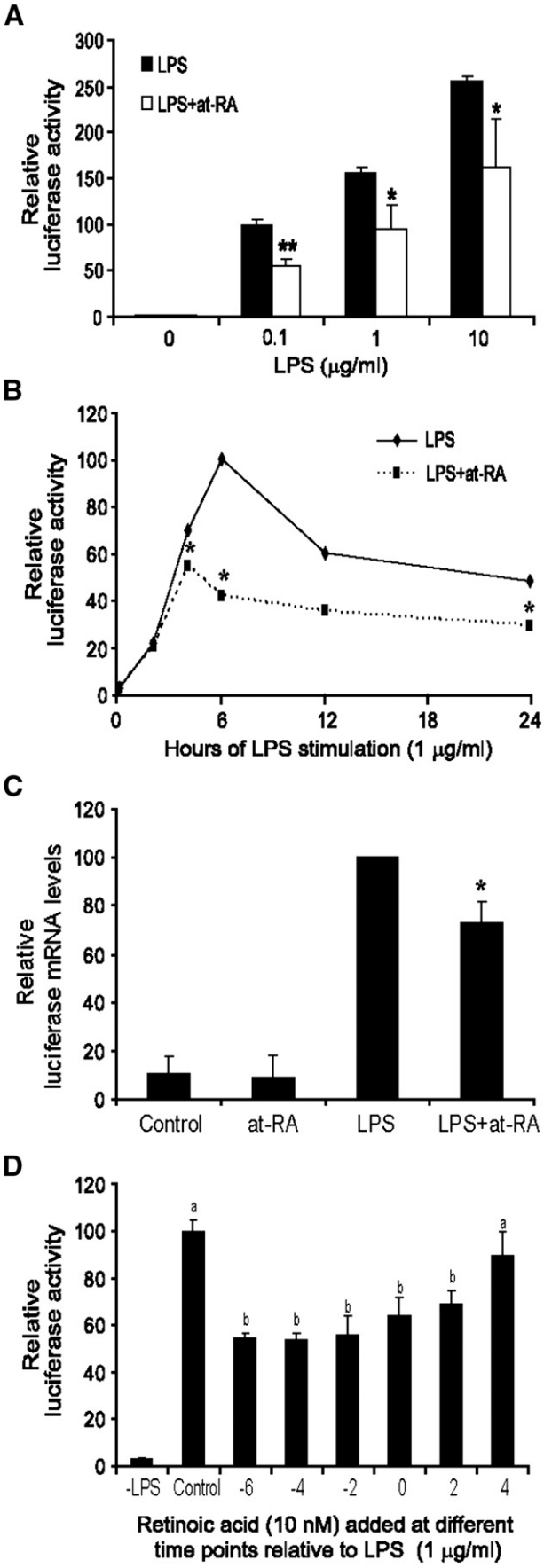
To study the effect of retinoic acid on LPS activation, we used human myeloblast U937 cells stably transfected with a luciferase reporter construct for NF-κB. LPS induced luciferase activity in these cells in a dose-responsive manner (Fig. 1A). Retinoic acid (100 nM) significantly reduced the LPS-induced NF-κB activity by 40±5% (mean±S.D.) at the three LPS concentrations tested (0.1, 1.0 and 10.0 μg/ml) (Fig. 1A).

Retinoic acid (100 nM) inhibited LPS-induced luciferase activity significantly after 4 h of incubation (the activity was reduced by 22±6%); after 6 h of stimulation, luciferase activity was reduced by 59±18%. Significant inhibition by retinoic acid was also present 24 h after LPS stimulation. (Fig. 1B). Induction of luciferase mRNA levels by LPS was also repressed by retinoic acid (69±4%, $P=.00001$) (Fig. 1C).

To study how the time of retinoic acid administration affected inhibition in LPS-induced luciferase activity, retinoic acid (100 nM) was added up to 6 h before or as late as 4 h after LPS (1.0 μg/ml) administration, and cells were harvested after 6 h of LPS stimulation. Retinoic acid added 4 h after LPS stimulation (i.e., 2 h before the cells were harvested) had no effect on LPS-induced NF-κB activity (Fig. 1D), demonstrating that retinoic acid does not affect the

Table 1
Primer sequences, PCR product size and program used for designing the primers

mRNA	Forward primer	Reverse primer	Product size	Primer design program
IL-6	5-CCTTCCAAAGATGGCTGAAA-3	5-TTTCTGCAGGAACTGGATCA-3	196	Primer3
MCP-1	5-AGATGCAATCAATGCCC-3	5-GTTGTGGAGTGAGTGTTC-3	242	LightCycler
COX-2	5-TGAAACCCACTCCAAACACA-3	5-GAGAAGGCTTCCCAGCTTTT-3	187	Primer3
Luciferase	5-GCAGCCTACCGTAGTG-3	5-ACGCAGGCAGTTCTAT-3	288	LightCycler
GAPDH	5-ATCCCAGAGCTGAACG-3	5-GAAGTCGCAGGAGACA-3	198	LightCycler



activity of the luciferase enzyme in itself and that it takes a certain time of between 2 and 4 h (including the time for the synthesis of luciferase) for retinoic acid to mediate its effect. Retinoic acid inhibited the LPS-induced NF-κB activity over a wide concentration range and in a dose-dependent manner from 0.1 nM to 1 μM (data not shown).

3.2. RAR-selective retinoids reduce LPS-induced NF-κB activity in U937 cells

Most — but not all — effects of retinoic acid are mediated through the nuclear receptors RAR and RXR. We therefore tested whether various receptor-selective retinoids (the concentration of all retinoids tested was 100 nM) affected LPS-induced NF-κB activity. All-*trans* and 9-*cis* retinoic acid, the RAR-α-selective ligand AM580 and the RAR-selective pan-agonist TTAB all reduced LPS-induced NF-κB activity to a similar extent (63±5%, 68±14%, 65±12% and 62±3%, respectively, compared with control vehicle). The RXR-selective pan-agonist SR11217 did not have any effect on LPS-induced NF-κB activity (96±4% compared with the control vehicle) (data not shown). Hence, ligand binding of RARs, but not of RXRs, is responsible for the inhibitory effects of retinoic acid on LPS-induced NF-κB activation.

Furthermore, the RAR-α-selective antagonist Ro 40-5253 was able to partially alleviate the inhibitory effect of AM580 on LPS-induced NF-κB activity: Ro 40-5253 (1 μM) significantly decreased the inhibitory effect of Am580 on LPS-induced NF-κB activity (79±3% inhibition with Am580 and Ro 40-5253, compared to 61±6% inhibition with Am580 alone). Ro 40-5253 did not affect LPS-induced NF-κB activity by itself (luciferase activity was 104±6% compared to controls; data not shown).

3.3. Inhibition by retinoic acid also demonstrated in transiently transfected U937 cells and embryonic fibroblasts from 3xκB-LUC mice

The effect of retinoic acid was also observed in U937 cells transiently transfected with the 3xκB-LUC reporter con-

Fig. 1. Retinoic acid inhibits LPS-induced NF-κB activity. (A) U937-3xκB-LUC cells were stimulated with the indicated concentrations of LPS and at-RA (0.1 μM) or vehicle for 6 h and harvested, and luciferase activity was measured. (B) U937-3xκB-LUC cells were stimulated with LPS (1 μg/ml) and at-RA (0.1 μM) or vehicle for the indicated lengths of time. (C) U937-3xκB-LUC cells were stimulated with LPS (1 μg/ml) and at-RA (0.1 μM) or vehicle for 4 h, whereafter the cells were harvested and mRNA levels of luciferase were measured. (D) Retinoic acid was added to U937-3xκB-LUC cells either 6, 4 or 2 h prior to LPS (1 μg/ml) stimulation (marked as −6, −4 and −2, respectively), or 2 or 4 h after LPS stimulation (marked as +2 or +4, respectively). The cells were incubated with LPS for 6 h before they were harvested, and luciferase activity was measured. Bars without identical letters above denote that they are statistically significantly different from each other ($P<.05$). In (A), (B) and (D), one representative experiment out of three or more similar experiments is shown ($n=3$ or more for every experiment). (D) The average of three separate experiments. *$P<.05$ and **$P<.01$ using Student's t test in (A), (B) and (C). In (D), one-way ANOVA with Bonferroni correction was applied, and the level of statistical significance was set to .05.

struct, together with p65, RAR and RXR expression vectors: LPS-induced luciferase activity was repressed by up to 69±11% (P=.0001) by retinoic acid relative to LPS alone in such experiments (data not shown). LPS stimulation was not required for the effect of retinoic acid since, in the absence of LPS, retinoic acid also repressed p65-mediated gene expression by about 27% (P=.02). Also, RAR and RXR were necessary for the repressive effect of retinoic acid.

Embryonic fibroblasts generated from a transgenic reporter mouse containing the 3xκB-LUC reporter construct also demonstrated the ability of retinoic acid to repress LPS-induced NF-κB activity (data not shown).

3.4. Retinoic acid represses LPS-induced NF-κB activity in vivo

To study whether the modulation of NF-κB activity by retinoic acid is relevant to an in vivo model, we treated transgenic NF-κB luciferase reporter mice with LPS and retinoic acid. These experiments demonstrate that LPS

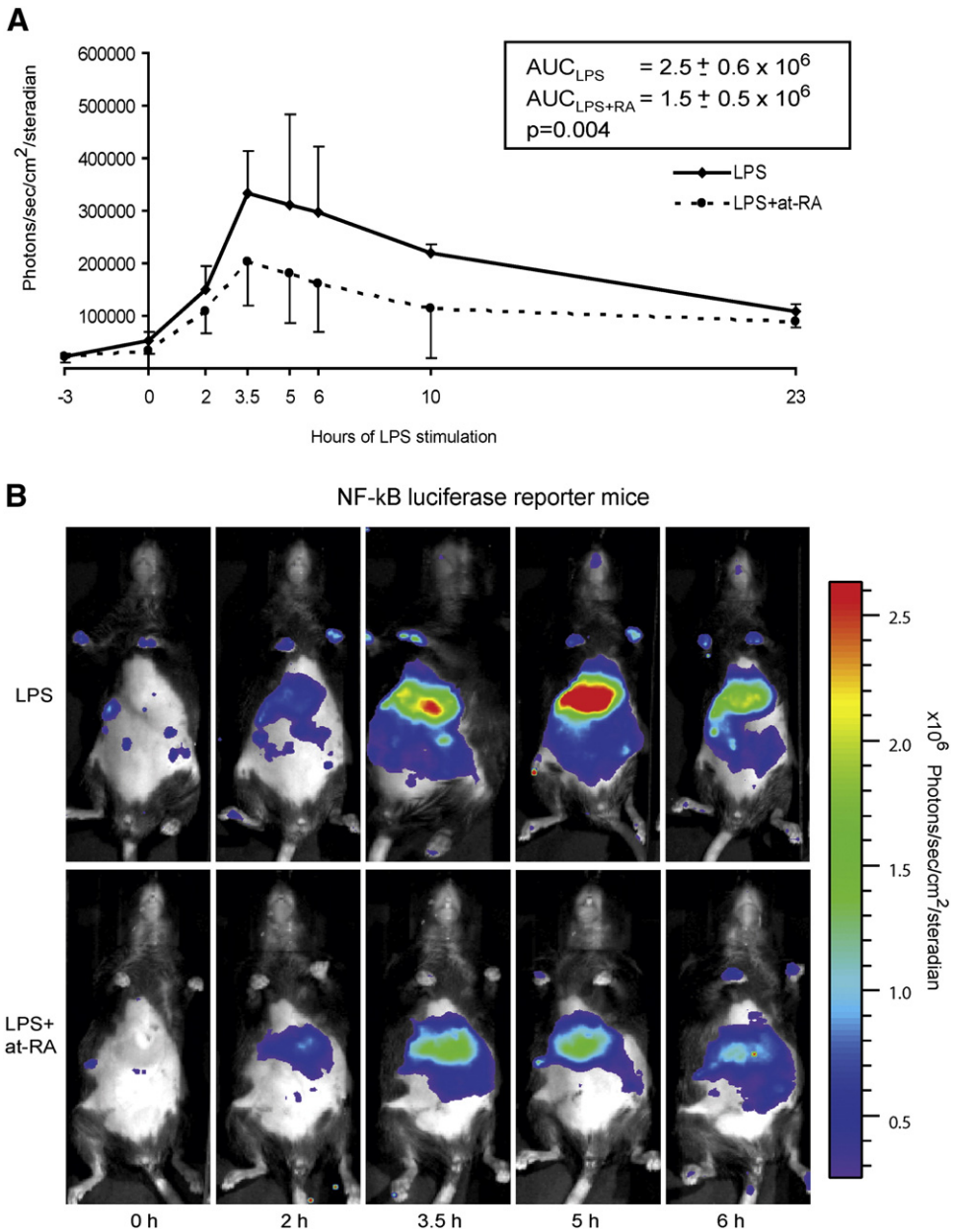


Fig. 2. Retinoic acid represses LPS-induced luciferase activity in NF-κB-LUC reporter mice. (A) The NF-κB-LUC reporter mice were given all-*trans* retinoic acid (50 mg/kg) 3 h prior to the administration of LPS (50 μg). Images were taken before all-*trans* retinoic acid administration, at the time of LPS injection and then after 2, 3.5, 5, 6, 10 and 23 h (A) (n=2 or 3 at 10 and 23 h; n=5–8 at the other time points). The number of photons in the AUC from Time 0 to 10 h after LPS administration was calculated. Nonparametric Mann–Whitney test was used for statistical analysis, and the significance level was set to P<05. (B) Pictures of the in vivo imaging of a mouse 0, 2, 3.5, 5 and 6 h after the administration of LPS only (upper panel) or LPS+at-RA (lower panel).

induces rapid activation of NF-κB in vivo, with peak activity 3–6 h after administration. Administration of retinoic acid (pharmacological concentration, 50 mg/kg body weight) by gavage feeding 3 h prior to LPS stimulation repressed LPS-induced whole-body luminescence (Fig. 2A and B). The number of photons in the area under the curve (AUC) for whole-body luminescence gives a measure of the total impact and cumulative response of the treatment over a certain time period — not only at one specific time point. In the present experiment (from Time 0 to 10 h), the AUC was $2.5\pm0.6\times10^6$ (mean±S.D., n=8) for LPS-treated mice and $1.5\pm0.5\times10^6$ (n=8) for LPS-treated and retinoic-acid-treated mice. Thus, retinoic acid reduced significantly (P=.004) the LPS-induced NF-κB activation in vivo.

3.5. Retinoic acid reduces nuclear levels of p65

One mechanism mediating the inhibition of LPS-induced NF-κB activity by retinoic acid may be through reduced nuclear levels of NF-κB. Looking at nuclear levels of p65 in crude nuclear extract (not shown) or immunoprecipitated p65 from nuclear extracts (Fig. 3A), there was a reduction in nuclear p65 levels as determined by Western blot analysis.

3.6. Effect of retinoic acid is also manifested by inhibition of NF-κB-regulated genes

Retinoic acid strongly reduced the mRNA levels of LPS-induced NF-κB target genes such as *IL-6*, *MCP-1* and *COX-2* (Fig. 3B–D). In parallel experiments, retinoic acid also decreased LPS-induced expression of a reporter construct containing the IL-6 promoter (data not shown).

3.7. The repressive effect of retinoic acid on NF-κB activity depends on new protein synthesis and could be mediated via STAT1

Retinoic acid induces the transcription and activation of signal transducer and activator of transcription-1 (STAT1) [28–30], and STAT1 can inhibit NF-κB nuclear translocation and induction of NF-κB target genes [31]. Thus, in order to determine whether the effect of retinoic acid could be mediated via STAT1, we investigated whether the repressive effect of retinoic acid was dependent on new protein synthesis. An inhibitor of new protein synthesis, CHX, was added to the cells 1 h prior to LPS or LPS+at-RA (all-trans retinoic acid) treatment, induction of luciferase mRNA was performed and MCP-1 levels were determined. CHX treatment abolished the repressive effect of retinoic acid on LPS-induced luciferase mRNA levels (Fig. 4A) and MCP-1 mRNA levels (Fig. 4B), indicating that retinoic acid induces the production of a new protein that mediates the inhibition of NF-κB-induced transcription.

Since the repressive effect of STAT1 on NF-κB is dependent on acetylation of STAT1 [31], we tested whether the effect of retinoic acid is sensitive to acetylation status using trichostatin A (TSA), a histonedeacetylase inhibitor.

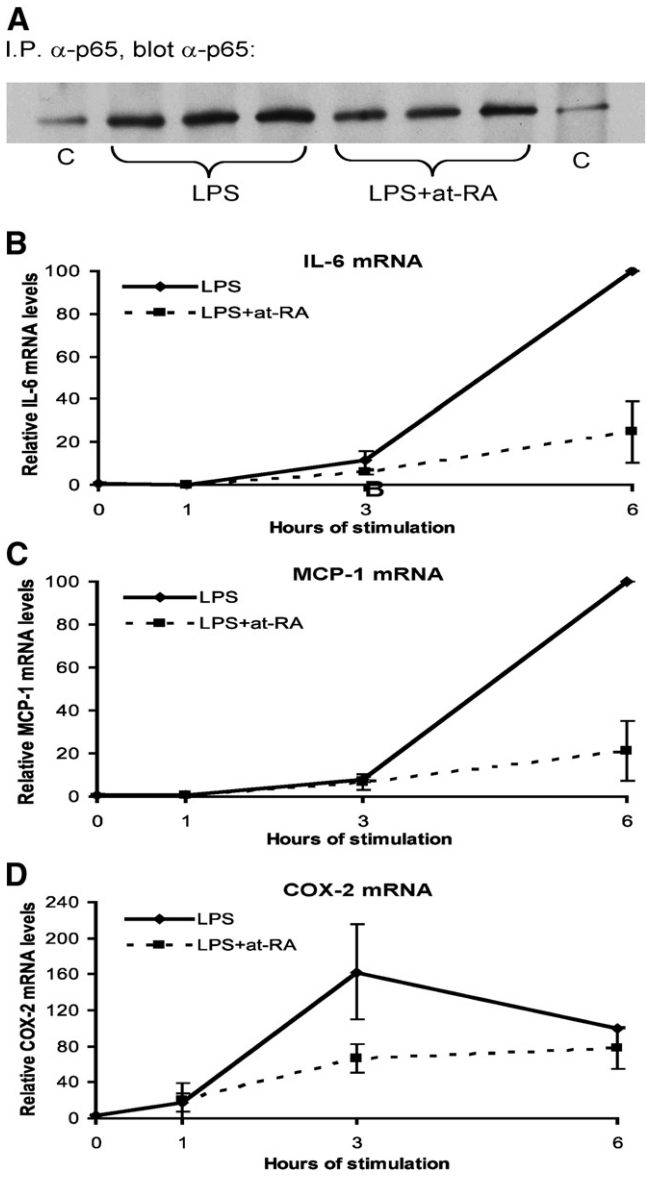


Fig. 3. Retinoic acid causes a small reduction in p65 nuclear levels and represses induction of the mRNA levels of various LPS-induced NF-κB target genes. Immunoprecipitated p65 from nuclear extracts of U937-3xκB-LUC cells treated with LPS or LPS+at-RA for 4 h was blotted against p65 antibody (A). U937-3xκB-LUC cells were treated with LPS and retinoic acid or vehicle for the indicated lengths of time. mRNA was isolated, and mRNA levels of IL-6 (B), MCP-1 (C) and COX-2 (D) were determined by real-time PCR analysis using the LightCycler and normalized to GAPDH mRNA levels using Roche's Relative Quantitation software. The results shown are the mean±S.D. of three separate experiments.

The presence of TSA during stimulation of the U937-3xκB-LUC cells repressed LPS-induced luciferase activity, and retinoic acid had no further repressive effect (Fig. 4C). Also, retinoic acid no longer repressed LPS-induced mRNA levels of luciferase or MCP-1 in the presence of TSA (Fig. 4A and B).

Furthermore, the STAT1 inhibitor MTA, a methyltransferase inhibitor [32], diminished the repressive effect of retinoic acid from a 34% reduction in LPS-induced

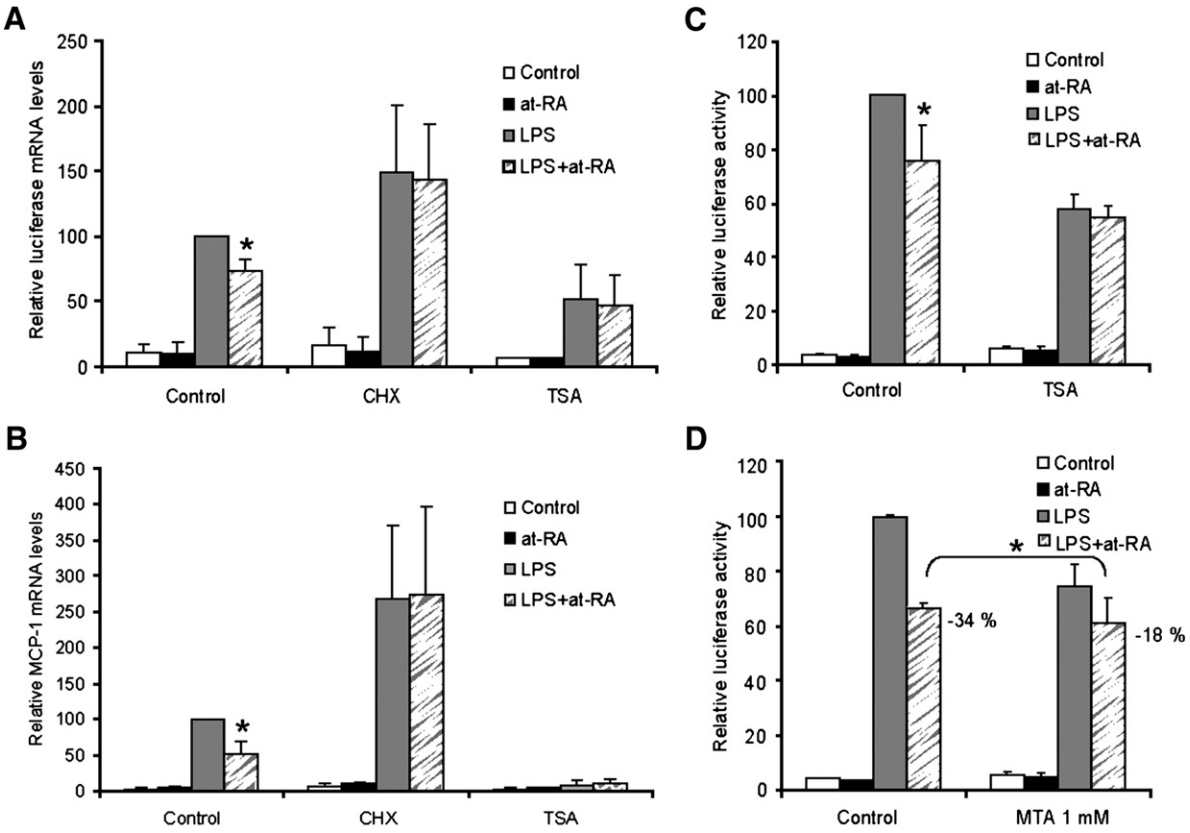


Fig. 4. CHX, TSA and MTA remove the repressive effect of retinoic acid on LPS-induced activation of U937 cells. U937-3xκB-LUC cells were treated with LPS alone or LPS and retinoic acid for 4 h (A and B) or 6 h (C and D), in the presence or in the absence of either CHX, TPA or MTA. CHX was added 1 h prior to LPS/at-RA, whereas TPA and MTA were added simultaneously. For (A) and (B), mRNA was isolated, and the levels of luciferase (A) and MCP-1 (B) were measured. For (C) and (D), luciferase activity was measured. The results shown are the mean±S.D. of at least three separate experiments. Student's *t* test was used for statistical analysis, and the level of significance was set to $P<05$.

luciferase activity to 18% (Fig. 4D). This suggests that STAT1 could be involved in retinoic acid's repressing effect on LPS signaling.

4. Discussion

In the present study, we have shown that retinoic acid is a potent inhibitor of LPS-mediated signaling, and we have demonstrated that this effect is mediated via inhibition of NF-κB. As revealed using specific RAR agonists and antagonists, the repressive effect of retinoic acid on NF-κB transactivation is likely to be mediated through retinoic acid activation of the RAR/RXR heterodimer. Retinoic acid also reduced nuclear translocation of NF-κB. Our results are in agreement with a previous study demonstrating that retinoic acid repressed LPS-induced expression of tumor necrosis factor-α and inducible nitric oxide synthase in cultured rat microglia cells [18] through inhibition of NF-κB activation. Other studies have shown that retinoic acid represses the cellular and physiologic effects of LPS [18,28,33–35].

We observed that the RAR-α-selective agonist AM580 and the RAR-selective pan-agonist TTAB reduced LPS-induced NF-κB signaling similarly to all-*trans* retinoic acid, while the RXR-selective pan-agonist SR11217 was not effective. Furthermore, the RAR-α-selective antagonist Ro 40-5253 was able to alleviate the inhibitory effect of AM580. Thus, these data demonstrate that the binding of all-*trans* retinoic acid to RAR-α — and possibly also to RAR-β and RAR-γ, but not to any of the RXRs — mediates the repressive effects on NF-κB signaling.

To advance our mechanistic understanding of retinoic-acid-induced inhibition of NF-κB signaling, we investigated whether the effect was mediated by STAT1, since it is known that retinoic acid induces STAT1 expression and activation [28–30], and it has been demonstrated that STAT1 represses NF-κB-mediated signaling [31]. Four lines of evidence support a role for STAT1 in the observed effect of retinoic acid. First, we demonstrate that CHX treatment abolishes the effect of retinoic acid, demonstrating that new protein synthesis is required for the effect of retinoic acid. Second, MTA, a STAT1 inhibitor, reduced the ability of retinoic acid to inhibit NF-κB activation from 34% to 18%. Third, the ability of STAT1 to repress NF-κB signaling is dependent on appropriate acetylation of STAT1. Indeed, in the presence of TSA (a deacetylation inhibitor), retinoic acid was not able to inhibit NF-κB signaling any further, consistent with a common mechanism for the effects of TSA and retinoic

acid, since no additive effects were observed. Finally, it is known that STAT1 repression reduces nuclear NF-κB localization, which is exactly what we observed in the presence of retinoic acid. Thus, our observations are therefore consistent with a role of STAT1 in mediating the repressive effect of retinoic acid on NF-κB signaling.

Sepsis is a global burden, and administration of vitamin A to infants and children has been suggested to reduce the burden of sepsis, immunodeficiencies and inflammation in endemic areas with vitamin A deficiency [36,37]. Our finding that retinoic acid reduces the expression of LPS-induced proinflammatory genes such as *IL-6*, *MCP-1* and *COX-2* further supports the anti-inflammatory role of retinoic acid. NF-κB is involved in the induction of all these genes, and the repression of NF-κB's *trans* activity could contribute to the down-regulation of these genes.

Much effort is currently being invested in developing NF-κB inhibitors and in testing their efficacy in various models [38,39]. The most promising NF-κB inhibitors tested to date include sulfasalazine, resveratrol, nonsteroidal anti-inflammatory drugs (e.g., aspirin and sulindac sulphide), cyclopentenone prostaglandins, proteasome inhibitors and glucocorticoids [40,41]. A proper preclinical screening model is important in validating NF-κB inhibitors. The novel transgenic reporter mice, based on an NF-κB-controlled luciferase construct used in this and other recent studies [20–22], were developed for this purpose. Combined with noninvasive biophotonic imaging, these mice are ideal for screening the biological activity of candidate NF-κB inhibitors. Our results suggest that retinoic acid also be included in this list of candidate NF-κB inhibitors, and we propose that pharmacological doses of retinoic acid be utilized to control NF-κB activation associated with sepsis and other microbial infections.

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