Journal of Biochemistry Advance Access published December 30, 2014

The form of the paper: Regular Paper / Field: Biochemistry / Topic: Biochemical Pharmacology

Glucocorticoid augments lipopolysaccharide-induced activation of the IκBζ-dependent genes encoding the anti-microbial glycoproteins lipocalin 2 and pentraxin 3

Soh Yamazaki [1,*], Shizuo Akira [2,3], Hideki Sumimoto [1,†]

[1]Department of Biochemistry, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; [2]Laboratory of Host Defense, World Premier International Immunology Frontier Research;Center, Osaka University, Suita, Osaka 565-0871, Japan; and [3]Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

Running title: Dex enhances LPS-induced activation of *Lcn2* and *Ptx3*

Correspondence

*Soh Yamazaki, Department of Biochemistry, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81-92-642-6101, Fax: +81-92-642-6103, email: syamaz@mailserver.med.kyushu-u.ac.jp

†Hideki Sumimoto, Department of Biochemistry, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81-92-642-6096, Fax: +81-92-642-6103, email: hsumi@med.kyushu-u.ac.jp

Abbreviations: BMM, bone marrow-derived macrophage; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; Dex, dexamethasone; ELISA, enzyme-linkedimmunosorbent assay; GR, glucocorticoid receptor; GRE, glucocorticoid response element; Lcn2, lipocalin 2; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblast; PTX3, pentraxin 3; qRT-PCR, quantitative reverse transcription-real time PCR; TLR(s), toll-like receptor(s); and TSS, transcription start site.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

© The Authors 2014. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved.

Summary

Bacterial lipopolysaccharide (LPS), one of the most potent inducers of inflammation, activates the transcription factor NF-κB to induce expression of both proinflammatory mediators and anti-microbial glycoproteins such as lipocalin 2 (Lcn2) and pentraxin 3 (PTX3) in macrophages. Glucocorticoids are known to inhibit LPS-induced expression of proinflammatory cytokines via glucocorticoid receptor (GR)-mediated transrepression of NF-κB, whereas their effect on induction of anti-microbial effectors has remained to be elucidated. Here we show that the synthetic glucocorticoid dexamethasone (Dex) strongly enhances LPS-induced transcription of *Lcn2* and *Ptx3*, although Dex by itself fails to trigger their transcription. In macrophages deficient in IκBζ (an inducible coactivator of NF-κB), *Lcn2* and *Ptx3* are not activated by LPS either alone or in combination with Dex. Association of GR as well as Brg1 (a subunit of the chromatin remodeling Swi/Snf complex) with a functional glucocorticoid response element in *Lcn2* requires both the costimulation with LPS and the presence of IκBζ. Although *Ptx3* does not contain the element, LPS induces recruitment of Dex-liganded GR to NF-κB-binding sites in regulatory regions of *Ptx3*, an event that does not occur in IκBζ-deficient macrophages. Thus glucocorticoids likely regulate infection-induced inflammation by increasing anti-microbial effectors in an IκBζ-dependent manner, while repressing proinflammatory genes.

Keywords: signal transduction; glucocorticoid; dexamethasone; lipocalin 2; pentraxin 3

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Introduction

Infection is accompanied by inflammation, a biological process that recruits immune cells and is considered essential for wound repair (*1*, *2*). During microbial infection, macrophages function as a crucial cellular mediator of the inflammatory response by recognizing a variety of pathogen-derived molecules such as bacterial lipopolysaccharide (LPS) via Toll-like receptors (TLRs) (*3–7*). A crucial point of control of inflammation is considered to be at the level of gene transcription. Indeed LPS ligation to TLR4 elicits activation of the transcription factor NF-κB in macrophages, which leads to gene expression of both proinflammatory mediators and anti-microbial effectors. NF-κB also induces expression of IκBζ (also known as MAIL or INAP) (*8–10*), a transcriptional coactivator that is required for induction of a subset of NF-κB-inducible genes such as *Lcn2* (encoding lipocalin 2) and *Csf3* (encoding granulocyte colony-stimulating factor) (*11–13*). The inducible coactivator IκBζ appears to act at least partly by recruiting the transcription factors NF-κB and C/EBPβ and the Brg1-containing Swi/Snf chromatin remodeling complex (*13*, *14*). On the other hand, LPS induces activation of proinflammatory genes encoding TNF-α, IL-1β, the inducible NO synthetase NOS2, and the chemokine CXCL10, in an IκBζ-independent manner (*8*).

The anti-microbial proteins that are synthesized and released by LPS-stimulated macrophages include lipocalin 2 (Lcn2) and pentraxin 3 (PTX3), which are also expressed in other types of cell such as fibroblasts, but to a lesser extent (*15*, *16*). Lcn2 exerts its crucial bacteriostatic activity by depriving of irons though binding to siderophores (ion-chelating small compounds) produced by bacteria (*17–19*). The soluble pattern recognition molecule PTX3 activates the complement system and phagocytosis for killing microbes (*20–22*). Although inflammation can greatly compromise cellular function and tissue architecture if not controlled (*1*, *2*), genes encoding these anti-microbial effectors do not seem to have the potential to cause tissue damage, and thus should be regulated to provide continuous protection from infection.

On the other hand, expression of proinflammatory genes should be selectively repressed to limit tissue damage. Elevation in blood levels of proinflammatory cytokines, such as TNF-α, IL-6, and

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

IL-1β, leads to release of the anti-inflammatory hormone glucocorticoid via the hypothalamic-pituitary-adrenal axis; and glucocorticoid released in turn inhibits cytokine expression in a negative-feedback manner (*23*, *24*). The significance of glucocorticoid is evident from an observation that administration of dexamethasone (Dex), a synthetic glucocorticoid, protects mice from LPS-triggered endotoxin shock, a state that is mediated by proinflammatory cytokines (*25*). It is generally known that glucocorticoid receptor (GR) in a ligand-bound form directly binds to glucocorticoid response elements (GREs) in regulatory regions of target genes, thereby activating transcription (*26*). In transcriptional regulation of proinflammatory cytokines, ligand-bound GR appears to directly interact with NF-κB without binding to DNA, and thus represses NF-κB-driven transactivation (*23*, *24*). In contrast to intensive studies about GR-mediated regulation of proinflammatory cytokine genes, little is known about the role for GR in expression of anti-microbial effector proteins in macrophages.

In the present study, we show that Dex-liganded GR strongly enhances LPS-stimulated transcription of *Lcn2* and *Ptx3* in an IκBζ-dependent manner, although Dex alone fails to activate these genes. The costimulation with LPS induces recruitment of Dex-liganded GR as well as Brg1 to a functional GRE in *Lcn2*, which depends on the presence of IκBζ. Although *Ptx3* does not contain a GRE, LPS induces association of Dex-activated GR with NF-κB-binding sites in *Ptx3* in wild-type macrophages but not in IκBζ-deficient cells. Also in fibroblasts, Dex and LPS cooperatively activates *Lcn2* in an IκBζ-dependent manner. Thus IκBζ appears to play a crucial role in infection-induced inflammation by regulating glucocorticoid-mediated enhancement in activation of antimicrobial effector genes.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Experimental Procedures

Cells, mice and reagents

Mouse macrophage-like RAW264.7 cells and NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (*10*). IκBζ-deficient mice were generated as described previously (*11*). Mouse embryonic fibroblasts (MEFs) and bone marrow-derived macrophages (BMMs) were prepared and maintained as previously described (*12*). All animals were housed and maintained in a specific pathogen-free animal facility at Kyushu University. All experiments were performed in strict accordance with the guidelines for Proper Conduct of Animal Experiments (Science Council of Japan). The experimental protocol was approved by the Animal Care and Use Committee of Kyushu University (Permit Numbers: A22-005 and A24-042). All efforts were made to minimize the numbers of animals and their suffering. Dex and RU486 (Mifepristone) were purchased from Sigma-Aldrich; LPS (*E. coli* 0111:B4) from List Biological Laboratories; cycloheximide (CHX) from and Nakalai Tesque; MG-132 from Calbiochem; Mouse recombinant TNF-α protein from Wako Pure Chemical Industries; and Helenalin from Alexis Biochemicals. All antibodies used in the present study were obtained from Santa Cruz Biotechnology.

Determination of mRNA expression levels by quantitative reverse transcription-real time PCR (qRT-PCR)

Total RNA extracted with TRIsure reagent (BIOLINE) was reverse transcribed by ReverTra Ace system (TOYOBO). The cDNA was analyzed by real-time PCR using SYBR Premix Ex Taq (TaKaRa Bio) on the Roter-Gene 6200 system (Corbett Life Science). The sequences of the primers used for qRT-PCR are as follows: *Lcn2*, 5'-AAGGAGCTGTCCCCTGAACT-3' and 5'-GGTGGGGACAGAGAAGATGA-3'; *Ptx3*, 5'-TAACAAAACAAGCTCTGTTGCC-3' and 5'-TTCGTTAATAAGTCACTTTGTGG-3'; *Il6*, 5'- CACAGAGGATACCACTCCCAA-3' and 5'-TCCACGATTTCCCAGAGAACA-3'; *Il12b*, 5'- AAGTATTCAGTGTCCTGCCAGGA-3' and 5'-

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

TGCTTCCAACGCCAGTTCA-3'; *Cxcl2*, 5'-GGATTTCAATGTAATGTTGTGAG-3' and 5'-CAAAACAATTGCTAAGCAAGGC-3'; *Sgk*, 5'-AGGAGCCGGAGCTTATGAAC-3' and 5'-AGTGAAAGTCGGAGGGTTTGG-3'.

Determination of protein concentration by enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out using Quantikine immunoassay kits for mouse lipocalin 2/NGAL, pentraxin 3/TSG-14, IL-6, and IL-12 p40 according to the manufacture's instruction (R&D Systems).

Immunoblot analysis of nuclear and total cellular proteins

Nuclear proteins were prepared as previously described (*27*, *28*). Briefly, after cell lysis in a hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM dithiothoreitol, and 10 mM HEPES, pH8.0), nuclei were precipitated by centrifugation for 5 min at 4°C at 2,500 × *g*. For extraction of nuclear proteins, the nuclear pellet was incubated for 30 min on ice in a high-salt buffer (600 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and 10 mM HEPES, pH8.0). Immunoblot analysis was performed as previously described (*10*).

Chromatin immunoprecipitation (ChIP) analysis

ChIP was conducted as described previously (*28*, *29*). Cells were fixed for 10 min at 25°C with 1% formaldehyde, and washed with ice-cold phosphate-buffered saline. After sonication, a chromatin-containing solution was precleared with Protein G-Sepharose 4 Fast Flow (GE Healthcare), and incubated overnight at 4°C with the indicated antibody with continuous rotation. Antigen-antibody complexes on the resin were washed sequentially with a low-salt wash buffer (150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, and 20 mM Tris-HCl, pH 8.0); a high-salt wash buffer (500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, and 20 mM Tris-HCl, pH 8.0); a LiCl wash buffer (250 mM LiCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Nonidet-P40, and 10 mM Tris-HCl, pH 8.0); and TE (1 mM EDTA and 10 mM Tris-HCl, pH 8.0). DNA-protein complexes were eluted with the elution buffer containing 1% SDS and 100 mM sodium bicarbonate. After cross-links were

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

reversed by overnight incubation at 65°C, Proteinase K (0.1 mg/ml) was added and incubated for 3 h at 45°C. Purified DNA was subjected to PCR using the specific primers as follows: *Lcn2*NFκB/CEBP, 5'-TGGGAATGTCCCTCTGGTCC-3' and 5'-CCATCCTTTACCAAGTCCAGG-3'; *Cxcl2* (NF-κB), 5'-CAACAGTGTACTTACGCAGACG-3' and 5'-CTAGCTGCCTGCCTCATTCTAC-3'; *Lcn2*GRE-2, 5'-ACACTTCCAGGATAATCTTTTGG-3' and 5'-CAGACCCTGTGCAGCTTCC-3'; *Sgk* (GRE), 5'-TCTAACTCGCCACCTCCTCA-3' and 5'-CCAACTAATCTCCGAGAACA-3'; *Ptx3*NFκB-1, 5'-CTCGGTGGTTTAACATTGTGC-3' and 5'-CGGGATGAATGACTAATGTCG-3'; *Ptx3*NFκB-2/CEBP-3, 5'-TTACTACTATCTCCAACAAAGGC-3' and 5'-TATGTGGGCTGGAGACATTCG-3'.

Plasmid construction and luciferase reporter assay

The genomic DNA fragments corresponding to 5'-upstream regions of murine *Lcn2* and *Ptx3* were amplified by PCR and inserted into pGL3-basic vector (Promega). Mutations in the 5'-upstream regions of the two genes were generated by PCR-mediated site-directed mutagenesis. The identity of all constructs was confirmed by DNA sequencing. The sequences of the mutations are as follows (mutated nucleotides are underlined): *Lcn2*CEBP, 5'-CTGTTGCTCAACCTT-3' to 5'-CTGCCACTCAACCTT-3'; *Lcn2*NFκB, 5'-CTGGGAATGTCCCTC-3' to 5'-CTAATAATGTCCCTC-3'; *Lcn2*GRE-1, 5'-AGTGGACAGGCAGTCCAGA-3' to 5'-AGTGTCAAGGCAGTCCAGA-3'; *Lcn2*GRE-2, 5'-AGGGTGTCCTGTTCTTC-3' to 5'-AGGGTGTCCCAGCCTTC-3'; *Lcn2*GRE-3, 5'-GCTGCCCTGTCTGTTCCTG-3' to 5'-GCTGCCCTGTCTACGCCTG-3'; *Ptx3*NFκB-1, 5'-AGGGGAACTCCCTC-3' to 5'-AGATAGACTCCCTC-3'; *Ptx3*GRE-1, 5'-CAGAGGCTCTCTGTACTGG-3' to 5'-CAGAGGCTCTCTTGGATGG-3'; *Ptx3*CEBP-1, 5'-AACTTGCCCAATAGG-3' to 5'-AACGCATCCAATAGG-3'; *Ptx3*GRE-2, 5'-AAAGAACAGTAAGCATTTT-3' to 5'-AAAAGCTAGTAAGCATTTT-3'; *Ptx3*CEBP-2, 5'-CATTTACACAATGCG-3' to 5'-CATGCGAACAATGCG-3'; *Ptx3*CEBP-3, 5'-CTGATGAAGAAATCT-3' to 5'-CTGATGAATGCGTCT-3'; *Ptx3*NFκB-2, 5'-AGGGGAAATCCCTA-3' to 5'-

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

AGATTCAATCCCTA-3’. Cells were transfected with the *Lcn2* or *Ptx3* reporter plasmid and the internal control plasmid pRL-TK (Promega) using FuGene6 (Roche Applied Science). After cultured for two days, cells were treated with the indicated stimulant, and luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega).

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Results

Dex enhances LPS-induced expression of Lcn2 and PTX3 in macrophages

Consistent with previous observations (*30*, *31*), Dex suppressed expression of the NF-κB-regulated cytokine genes *Il6* (encoding IL-6) and *Il12b* (encoding IL-12 p40) in LPS-stimulated BMMs at both mRNA (Fig. 1A) and protein levels (Fig. 1B). Although LPS elicits expression of *Lcn2* and *Ptx3* via NF-κB activation in macrophages (*11*, *13*, *18*, *32*, *33*), little is known whether Dex affects LPS-induced expression of *Lcn2* and *Ptx3*. To address this question, we tested expression of *Lcn2* and *Ptx3* when BMMs were stimulated with LPS, Dex, or both. As shown in Fig. 1A, Dex by itself failed to induce mRNA synthesis for *Lcn2* and *Ptx3*, but it markedly enhanced LPS-induced expression of the two genes. Dex also increased the amounts of the Lcn2 and PTX3 proteins released from LPS-stimulated BMMs, whereas these proteins were not produced in response to Dex alone (Fig. 1B). Thus Dex augments LPS-induced expression of *Lcn2* and *Ptx3* at the transcriptional level.

Cis-elements for Lcn2 transcription by LPS and Dex in macrophages

To know the mechanism for transcriptional regulation of *Lcn2* in macrophages, we examined *cis*-acting elements upstream of the transcription start sites (TSSs) (Fig. 2A). In RAW264.7 macrophages, LPS activated a luciferase reporter that contains a mouse *Lcn2* genomic sequence from positions -1,031 to +54 relative to the TSS (Fig. 2B). The reporter activity was intensified by costimulation with Dex. Because a reporter activity of an *Lcn2* construct from positions -731 to +54 is similar to that of the -1,031/+54 construct (Fig. 2C), we focused on the region from positions -731 to +54. A search using the Genomatix MatInspector program (Genomatix Software GmbH, Munich Germany) revealed that this region contains one C/EBP-binding site (-188/-174, designated as *Lcn2*CEBP), one NF-κB-binding site (-232/-218, *Lcn2*NFκB), and three GREs (-303/-285, *Lcn2*GRE-1; -522/-499, *Lcn2*GRE-2; -653/-635, *Lcn2*GRE-3) (Fig. 2A). Mutation in *Lcn2*CEBP and *Lcn2*NFκB prevented the LPS-induced promoter activation even in the presence of Dex (Fig. 2B),

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

indicating an indispensable role of the two sites in *Lcn2* transcription in macrophages. Disruption of *Lcn2*GRE-2 abrogated the enhancement by Dex without affecting *Lcn2* activation by LPS alone (Fig. 2B). On the other hand, mutation in *Lcn2*GRE-1 or *Lcn2*GRE-3 did not affect any promoter activities.

Cis-elements for Ptx3 transcription by LPS and Dex in macrophages

We also examined *cis*-acting elements in mouse *Ptx3* gene, which contains two NF-κB binding sites (-41/-48, *Ptx3*NFκB-1; -2,471/-2,458, *Ptx3*NFκB-2), two GREs (-1,358/-1,340, *Ptx3*GRE-1; -1,465/-1,447, *Ptx3*GRE-2), and three C/EBP binding sites (-1,396/-1,382, *Ptx3*CEBP-1; -1,660/-1,646, *Ptx3*CEBP-2; -2,370/-2,358, *Ptx3*CEBP-3) (Fig. 3A). We first tested a reporter activity of the -1,570/+136 construct, which covers the region (-1,374/+84) that can be activated by TNF-α (*34*). Although this reporter was upregulated by LPS in RAW264.7 macrophages, Dex did not affect the promoter activity (Fig. 3B). In contrast, a longer *Ptx3* reporter (-2,555/+136) was much more strongly activated by LPS (Fig. 3B), and the activation was further augmented by Dex (Fig. 3B). Mutation analysis revealed a requirement of *Ptx3*NFκB-1, *Ptx3*CEBP-3, and *Ptx3*NFκB-2 (Fig. 3C). On the other hand, mutations of the two GREs (*Ptx3*GRE-1 and *Ptx3*GRE-2) did not abrogate Dex enhancement of the LPS-induced reporter activity.

Role of NF-κB and GR in expression of Lcn2 and Ptx3 in macrophages

Because NF-κB binding sites are involved in *Lcn2* and *Ptx3* expression by LPS alone and in combination with Dex in macrophages (Fig. 2B), we tested the role for NF-κB using the proteasome inhibitor MG-132, which blocks degradation of the NF-κB inhibitor IκBα to prevent NF-κB activation (*35*), and Helenalin, an alkylating agent that selectively inhibits the p65 subunit of NF-κB (*36*). As shown in Figure 4A, treatment of BMMs with MG-132 resulted in a loss of *Lcn2* expression in the presence of both LPS and Dex. The expression was also inhibited by Helenalin. Similarly, these inhibitors almost completely interfered with *Ptx3* expression in response to both LPS

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

and Dex (Fig. 4A). The findings suggest that NF-κB participates in cooperative expression of the two genes by Dex and LPS in macrophages.

We next used RU486, a GR antagonist, to know the role of GR in Dex-mediated activation of *Lcn2* and *Ptx3*. Because mutation of *Lcn2*GRE-2, a GR-binding site in *Lcn2*, ablates the effect of Dex (Fig. 2B), Dex-bound GR is expected to function in *Lcn2* activation. Indeed RU486 abrogated Dex enhancement of LPS-induced *Lcn2* expression, although it marginally affected LPS induction *per se* (Fig. 4B). Thus Dex appears to function in *Lcn2* activation via binding to GR. On the other hand, *Ptx3* activation by Dex is not affected by mutations of the two putative GR-binding sites *Ptx3*GRE-1 and *Ptx3*GRE-2 (Figure 3C, *GRE-1m* and *GRE-2m*). Intriguingly, RU486 prevented Dex enhancement of LPS-induced *Ptx3* expression but not the induction by LPS alone (Fig. 4B). Given the dispensability of the putative GR-binding sites, Dex-liganded GR likely enhances *Ptx3* activation without directly binding to DNA.

LPS and Dex cooperatively induce recruitment of NF-κB, C/EBPβ, and GR to Lcn2 regulatory regions

Gene expression is often regulated at the step of nuclear translocation of transcription factors that normally localize to the cytoplasm, which include NF-κB and Dex-liganded GR (*26*). To examine nuclear translocation of NF-κB p65 and GR, we performed immunoblot analysis of the nuclear fraction prepared from RAW264.7 macrophages. As shown in Figure 5A, LPS but not Dex induced accumulation of p65 to the nucleus; Dex did not affect LPS-induced nuclear translocation of p65. In Dex-treated macrophages GR moved to the nucleus, whereas LPS neither induced nuclear localization of GR on its own, nor enhanced nuclear accumulation of Dex-bound GR (Fig. 5A). Thus LPS and Dex independently regulate nuclear translocation of p65 and GR, respectively.

We next performed ChIP experiments to analyze the step after nuclear translocation, *i.e.*, recruitment of p65, C/EBPβ, and GR to specific gene regions required for transcriptional regulation of *Lcn2* and *Ptx3* by LPS and Dex. For detection of binding of p65 and of C/EBPβ to *Lcn2*NFκB and *Lcn2*CEBP, respectively, a single pair of primers was used because these sites are very close to each

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

other; the region containing both binding sites is designated as *Lcn2*NFκB/CEBP (Fig. 2A). As shown in Figure 5B, LPS-induced association of p65 and C/EBPβ with *Lcn2*NFκB/CEBP was only slightly facilitated by Dex. On the other hand, Dex affected neither LPS-induced expression of the chemokine gene *Cxcl2* (Fig. 5D) nor LPS-triggered association of p65 with an NF-κB-binding site of *Cxcl2* (Fig. 5B).

We also examined GR recruitment to *Lcn2*GRE-2, a site responsible for Dex-enhanced activation of *Lcn2* (Fig. 2B). Stimulation with Dex alone did not induce GR targeting to *Lcn2*GRE-2 (Fig. 5C), although Dex efficiently activated the protein kinase-encoding gene *Sgk* (Fig. 5D) and induced GR targeting to a GRE of *Sgk* (Fig. 5C). Importantly, costimulation with LPS did induce association of Dex-liganded GR with *Lcn2*GRE-2 (Fig. 5C). Thus LPS and Dex appear to activate *Lcn2* transcription by cooperatively recruiting GR to its regulatory region.

LPS and Dex cooperatively induce recruitment of NF-κB, C/EBPβ, and GR to Ptx3 regulatory regions

Activation of *Ptx3* requires *Ptx3*NFκB-2 and *Ptx3*CEBP-3; they are combined here and thus designated as *Ptx3*NFκB-2/CEBP-3 (Fig. 3A). LPS slightly induced p65 recruitment to *Ptx3*NFκB-1 and *Ptx3*NFκB-2/CEBP-3, and the recruitment was markedly augmented by co-stimulation with Dex (Fig. 5E). Dex also facilitated LPS-induced association of C/EBPβ with *Ptx3*NFκB-2/CEBP-3 (Fig. 5E). Although Dex enhanced an LPS-induced reporter activity of the *Ptx3* construct of positions from -2,555 to +136, this region does not contain recognizable GREs (Fig. 3C). Intriguingly, simultaneous stimulation with Dex and LPS resulted in recruitment of GR to the GRE-free sites *Ptx3*NFκB-1 and *Ptx3*NFκB-2/CEBP-3 (Fig. 5E). The finding suggests that Dex-bound GR is recruited to *Ptx3* regulatory regions via interaction with NF-κB and/or C/EBPβ on DNA (*37–40*).

IκBζ regulates synergistic activation of Lcn2 and Ptx3 by LPS and Dex in macrophages

It is well known that the coactivator IκBζ is required for LPS-induced expression of *Lcn2* in macrophages (*11*, *13*, *14*). To clarify whether IκBζ participates in synergistic activation of *Lcn2* by

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

LPS-activated NF-κB and Dex-liganded GR, we tested the effect of the stimulants in IκBζ-deficient BMMs. In these cells, *Lcn2* expression was not induced by LPS either alone or in combination with Dex (Fig. 6A). LPS-induced expression of *Ptx3* was also impaired by IκBζ deficiency in both the presence and absence of Dex (Fig. 6B). Thus Dex likely activates *Lcn2* and *Ptx3* together with LPS in an IκBζ-dependent manner. Furthermore, IκBζ was required for LPS-induced association of p65, C/EBPβ, and Brg1 with *Lcn2*NFκB-1/CEBP-1, irrespective of the presence of Dex (Fig. 6C). GR as well as Brg1 was recruited to *Lcn2*GRE-2 solely in BMMs stimulated simultaneously with LPS and Dex, which recruitment was abolished in IκBζ-deficient BMMs (Fig. 6D). These findings indicate that IκBζ participates in GR recruitment to the inherently inactive GRE. In contrast, IκBζ was not required for LPS-triggered association of p65 with a NF-κB site in the Dex-insensitive gene *Cxcl2* (Fig. 6C) or for Dex-induced recruitment of GR (and Brg1) to a GRE of the NF-κB-independent gene *Sgk* (Fig. 6D). Targeting of transcription regulators to *Ptx3* regulatory regions also appears to involve IκBζ. As shown in Figure 6E, ablation of IκBζ decreased LPS-induced association of p65 and Brg1 with *Ptx3*NFκB-1, and that of p65, C/EBPβ, and Brg1 with *Ptx3*NFκB-2/CEBP-3. GR recruitment to these sites by LPS and Dex was also abrogated in IκBζ-deficient BMMs (Fig. 6E), suggesting that IκBζ may tether Dex-liganded GR to the NF-κB sites.

IκBζ regulates synergistic activation of Lcn2 by LPS and Dex in fibroblasts

Although Dex is incapable of activating *Lcn2* in the absence of LPS in macrophages (Fig. 1), Dex by itself is known to induce *Lcn2* expression in mammary epithelial adenocarcinoma and hepatocyte cell lines (*41*). As shown in Figure 7A, treatment of NIH3T3 fibroblasts with Dex alone led to expression of *Lcn2*. LPS also induced *Lcn2* expression, but to a lesser extent. It is known that *Ptx3* is activated by Dex in human fibroblasts (*42*). The activation also seems to be regulated by LPS and Dex in mouse NIH3T3 fibroblasts: Dex and LPS each activated *Ptx3*, which was further enhanced by a combined treatment (Fig. 7B).

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Like in macrophages, LPS and Dex synergistically activated *Lcn2* expression in NIH 3T3 fibroblasts (Fig. 7C) and MEFs (Fig. 7D). To investigate the mechanism for the combinatorial effect of LPS and Dex, we examined the activity of the *Lcn2* -1,031/+54 reporter in NIH3T3 cells. As shown in Figure 7E, LPS and Dex each activated the *Lcn2* reporter in fibroblasts; the reporter activity was enhanced by simultaneous treatment with the two stimulants. Mutation in the C/EBP-binding site (*Lcn2*CEBP) or the NF-κB-binding site (*Lcn2*NFκB) abrogated reporter activation by LPS but not by Dex (Fig. 7E). On the other hand, disruption in *Lcn2*GRE-2 but not in the other two potential GREs (*Lcn2*GRE-1 and *Lcn2*GRE-3) abrogated the effect of Dex on the *Lcn2* reporter (Fig. 7E). Thus the elements crucial in macrophages (Figure 2) also play an important role in synergistic activation of *Lcn2* by LPS and Dex in fibroblasts. The synergism was potently impaired in IκBζ-deficient cells (Fig. 7D). These findings indicate that Dex and LPS synergistically activate *Lcn2* in fibroblasts in an IκBζ-dependent manner.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Discussion

In the present study, we show that the anti-microbial effector genes *Lcn2* and *Ptx3* exhibit synergistic response to the synthetic glucocorticoid Dex and the bacterial pathogen LPS: Dex enhances LPS-induced transcriptional activation of *Lcn2* and *Ptx3* in an IκBζ-dependent manner, leading to an increased production of their respective proteins in macrophages (Fig. 1). Dex and LPS appear to cooperatively activate *Lcn2* and *Ptx3* at least partly by recruiting GR to enhancer regions; the recruitment does not occur in the absence of LPS (Fig. 5). Intriguingly, GR recruitment requires the presence of IκBζ: IκBζ deficiency results in a loss of GR targeting to a GRE in *Lcn2* and to an NF-κB-binding site in *Ptx3* (Fig. 6). Dex also facilitates LPS-induced enhancer association of NF-κB, C/EBPβ, and the Swi/Snf complex, suggesting a substantial role for cooperative actions of these transcription factors. In fibroblasts, Dex alone is capable of activating *Lcn2* and *Ptx3*, and Dex functions more effectively in combination with LPS, which is largely dependent on IκBζ (Fig. 7). On the basis of these findings, we conclude that IκBζ regulates glucocorticoid-mediated transcriptional enhancement of anti-microbial effector genes such as *Lcn2* and *Ptx3*, thereby playing a crucial role in infection-induced inflammation.

Although inflammation is a complex immune response that is triggered by infection and tissue injury (*1*, *2*), glucocorticoids regulate inflammation to protect the host from detrimental consequences of an overactive inflammatory immune response (*26*). For this purpose, the hormones not only suppress inflammatory responses but also should maintain or rather activate expression of anti-microbial genes, especially when microbes are not completely excluded from the host. Indeed the present study demonstrates that the synthetic glucocorticoid Dex enhances the bacterial component LPS-mediated expression of the anti-microbial effector proteins Lcn2 and PTX3. In addition to anti-microbial effector functions, Lcn2 and PTX3 have been reported to participate in suppression of inflammatory reactions: Lcn2 interferes with LPS-induced expression of the proinflammatory cytokines IL-6 and IL-1β in macrophages (*43*); PTX3 inhibits neutrophil adhesion to endothelial cells via directly interacting with P-selectin to attenuate neutrophil recruitment to sites of inflammation (*44*).

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

It seems thus possible that GR-mediated transactivation of *Lcn2* and *Ptx3* contributes to negative control of inflammatory responses as well as upregulation of anti-microbial activities.

As shown in the present study, Dex is almost inactive in *Ptx3* expression on its own but strongly enhances LPS-elicited activation of *Ptx3* in macrophages, whereas Dex and LPS are each capable of weakly inducing *Ptx3* expression and synergistically activate this gene in mouse NIH3T3 fibroblasts and MEFs. A similar synergistic effect of Dex on *Ptx3* activation in nonhematopoietic cells has been demonstrated by Doni *et al.* (*42*): Dex superinduces *Ptx3* expression in combination with TNF-α in human fibroblasts and with IL-1β in human vascular endothelial cells. They also have reported that Dex inhibits LPS-induced production of the *Ptx3* protein in mouse monocyte-derived dendritic cells (detected by ELISA) and that of the *Ptx3* mRNA in BMMs (detected by qRT-PCR) (*42*), which is in contrast with the present finding, obtained by qRT-PCR, that Dex enhances LPS-induced activation of *Ptx3* in mouse BMMs and RAW264.7 macrophages. Although the reason for this discrepancy is presently unknown, it might be due to the difference in the culture conditions used: in the present study, BMMs were cultured with 100 ng/ml of LPS in Dulbecco's modified Eagle's medium supplemented with 10% serum, instead of 10 ng/ml of LPS in RPMI 1640 medium supplemented with 2% serum (*42*). On the other hand, consistent with the present finding, a study using experiments of gene-expression microarray profiling has shown that Dex enhances LPS-induced expression of *Ptx3* in mouse peritoneal macrophages (*45*), and a recent similar analysis has revealed that LPS-induced activation of *Ptx3* is further augmented by Dex in mouse BMMs (*46*). The latter analysis has also supported the present finding that LPS-induced expression of *Lcn2* is enhanced by Dex in mouse BMMs.

GR exerts its regulatory activity through complex mechanism; it directly engages consensus DNA sequences (GREs) in the regulatory regions for gene activation, or it represses gene transcription via a protein–protein interaction: GR-mediated transrepression is generally ascribed to indirect "tethered" interaction with other DNA-binding transcription factors such as NF-κB and AP-1. On the other hand, agonist-liganded GR is also known to act as a repressor via directly binding to "repressing" negative GREs (nGREs), which are unrelated to GREs (*47*). Our computer search has revealed that

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

the nGRE sequence is absent in the regulatory regions of *Lcn2* and *Ptx3* (unpublished observation); the absence seems to agree with the present finding that these two genes are not repressed by Dex in macrophages or fibroblasts. In contrast to intensive studies on the mechanism for transcriptional repression by GR, there exist a few examples in which GR enhances gene expression in combination with other transcription factors. Among them, it is known that synergy between GR and Stat family transcription factors that are activated upon cell stimulation with cytokines (*48*). GR activates transcription of a subset of target genes in cooperation with members of the Stat family (*49–51*). Stat5 binds to its cognate DNA sequences and likely uses hormone-liganded GR as a coactivator to induce gene expression (*52*, *53*); on the other hand, GR appears to act in synergy with Stat3, when Stat3 is tethered to GRE-bound GR (*54*).

Although it is well known that hormone-liganded GR directly interacts with NF-κB, this interaction has been considered to be mainly involved in GR-mediated transrepression, but not transactivation, in NF-κB-induced gene expression (*26*). The present study demonstrates that GR in the Dex-liganded form activates *Lcn2* and *Ptx3* synergistically with LPS-activated NF-κB. In the regulatory region of *Ptx3*, the two NF-κB-binding sites (*Ptx3*NFκB-1 and *Ptx3*NFκB-2) are crucial for *Ptx3* activation by LPS either alone or in combination with GR (Fig. 3). NF-κB association with these sites is enhanced by Dex-liganded GR; and intriguingly, in spite of the absence of GREs, GR is also recruited in a manner dependent on both Dex and LPS (Fig. 5). These findings suggest that GR may be tethered to NF-κB and function as a coactivator in *Ptx3* gene expression, which is similar to the case of synergy between GR and Stat5 as described above (*52*, *53*). On the other hand, LPS-induced recruitment of NF-κB to its cognate site in the *Lcn2* gene is not further facilitated by Dex-bound GR (Fig. 5), although this site is indispensable for *Lcn2* expression by LPS in the absence or presence of Dex (Fig.2). Instead, a functional GRE in *Lcn2* (*Lcn2*GRE-2) becomes accessible to Dex-ligated GR in the presence of LPS (Fig. 5), which may play a crucial role in Dex-mediated enhancement of *Lcn2* activation. Thus *Lcn2* and *Ptx3* appear to be activated cooperatively by the two transcription factors GR and NF-κB, but in a different manner.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Despite the difference, *Lcn2* and *Ptx3* each require IκBζ for their activation (Fig. 6), indicating the role for IκBζ as a crucial coactivator of NF-κB in synergistic activation of the genes by Dex and LPS. IκBζ, absent in resting cells, becomes rapidly induced upon cell stimulation with LPS, and interacts with NF-κB to activate a subset of NF-κB-dependent genes (*10*, *13*). The present finding that IκBζ is required for access of NF-κB to its binding sites in both *Lcn2* and *Ptx3* (Fig. 6) suggests a pivotal role for IκBζ in determining the accessibility of NF-κB sites. Interestingly, recruitment of Dex-ligated GR to the functional GRE of *Lcn2* also requires the presence of IκBζ (Fig. 6). It seems thus possible that IκBζ (possibly together with NF-κB) stabilizes GR association with the GRE to form an active transcription complex. In addition, because Brg1, an ATPase subunit of the Swi/Snf remodeling complex, is also recruited to the GRE and the NF-κB sites in an IκBζ-dependent manner (Fig. 6), chromatin remodeling may play a role in activation of *Lcn2* and *Ptx3* possibly via increasing promoter accessibility. On the basis of these findings, we conclude that IκBζ plays a crucial role in glucocorticoid-mediated transcriptional enhancement of NF-κB-inducible genes such as *Lcn2* and *Ptx3*. The conclusion appears to be supported by the observations that LPS-induced activation of another IκBζ-dependent genes, *Sla* and *Dtx2* (*11*), is enhanced by simultaneous treatment of macrophages with Dex (*45*, *46*) and that Dex, albeit inactive on its own, facilitates LPS-triggered transcription *Csf3* (unpublished observation), which requires IκBζ (*13*). On the other hand, LPS-induced activation of IκBζ-independent genes such as one encoding TNF-α, IL-1β, NOS2, or CXCL10 (*11*) is repressed by Dex (*45*, *46*). Future studies should be addressed to clarify a detailed molecular mechanism by which IκBζ functions together with glucocorticoid-liganded GR.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Acknowledgements

We thank Yohko Kage (Kyushu University) and Namiko Kubo (Kyushu University) for technical assistance, and Shoko Miura (Kyushu University) for secretarial assistance. We are also grateful to technical supports from Research Support Center, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan.

Funding

This work was supported in part by MEXT (the Ministry of Education, Culture, Sports, Science and Technology) KAKENHI Grant Number 26111009, by grants from The Uehara Memorial Foundation and Sankyo Foundation of Life Science, and by Kyushu University Interdisciplinary Programs in Education and Projects in Research Development. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Conflict of Interest

None declared.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

References

1. Nathan, C. (2002) Points of control in inflammation. *Nature* **420,** 846–852
2. Medzhitov, R. (2008) Origin and physiological roles of inflammation. *Nature* **454,** 428–435
3. Medzhitov, R. and Horng, T. (2009) Transcriptional control of the inflammatory response. *Nat. Rev. Immunol.* **9,** 692–703
4. Smale, S. T. (2010) Selective transcription in response to an inflammatory stimulus. *Cell* **140,** 833–844
5. Kawai, T. and Akira, S. (2011) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11,** 373–384
6. Natoli, G., Ghisletti, S., and Barozzi, I. (2011) The genomic landscapes of inflammation. *Genes Dev.* **25,** 101–106
7. Foster, S.L., Hargreaves, D.C., and Medzhitov, R. (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447,** 972–978
8. Kitamura, H., Kanehira, K., Okita, K., Morimatsu, M., and Saito, M. (2000) MAIL, a novel nuclear IκB protein that potentiates LPS-induced IL-6 production. *FEBS Lett.* **485,** 53–56
9. Haruta, H., Kato, A., and Todokoro, K. (2001) Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. *J. Biol. Chem.* **276,** 2485–12488
10. Yamazaki, S., Muta, T., and Takeshige, K. (2001) A novel IκB protein, IκB-ζ, induced by proinflammatory stimuli, negatively regulates nuclear factor-κB in the nuclei. *J. Biol. Chem.* **276,** 27657–27662
11. Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K., and Akira, S. (2004) Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ. *Nature* **430,** 218–222

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

12. Yamazaki, S., Muta, T., Matsuo, S., and Takeshige, K. (2005) Stimulus-specific induction of a novel nuclear factor-κB regulator, IκB-ζ, via Toll/Interleukin-1 receptor is mediated by mRNA stabilization. *J. Biol. Chem.* **280,** 1678–1687

13. Yamazaki, S., Matsuo, S., Muta, T., Yamamoto, M., Akira, S., and Takeshige K. (2008) Gene-specific requirement of a nuclear protein, IκB-ζ, for promoter association of inflammatory transcription regulators. *J. Biol. Chem.* **283,** 32404–32411

14. Kayama, H., Ramirez-Carrozzi, V. R., Yamamoto, M., Mizutani, T., Kuwata, H., Iba, H., Matsumoto, M., Honda, K., Smale, S. T., and Takeda, K. (2008) Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IκBζ. *J. Biol. Chem.* **283,** 12468–12477

15. Liu, Q. and Nilsen-Hamilton, M. (1995) Identification of a new acute phase protein. *J. Biol. Chem.* **270,** 22565–22570

16. Lee, G.W., Lee, T.H., and Vilček, J. (1993) TSG-14, a tumor necrosis factor- and IL-1-inducible protein, is a novel member of the pentaxin family of acute phase proteins. *J. Immunol.* **150,** 1804–1812

17. Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N., and Strong, R.K. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol. Cell* **10,** 1033–1043

18. Flo, T.H., Smith, K. D., Sato, S., Rodriguez, D.J., Holmes, M.A., Strong, R.K., Akira, S., and Aderem, A. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432,** 917–921

19.Berger, T., Togawa, A., Duncan, G.S., Elia, A.J., You-Ten, A., Wakeham, A., Fong. H.E., Cheung, C.C., Mak, T.W. (2006) Lipocalin 2-deficient mice exhibit increased sensitivity to Escherichia coli infection but not to ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 1834–1839

20. Garlanda, C., Hirsch, E., Bozza, S., Salustri, A., De Acetis, M., Nota, R., Maccagno, A., Riva, F., Bottazzi, B., Peri, G., Doni, A., Vago, L., Botto, M., De Santis, R., Carminati, P., Siracusa, G., Altruda, F., Vecchi, A., Romani, L., and Mantovani, A. (2002) Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* **420,** 182–186

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

21. Garlanda, C., Bottazzi, B., Bastone, A., and Mantovani, A. (2005) Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu. Rev. Immunol.* **23,** 337–366

22. Bottazzi, B., Garlanda, C., Cotena, A., Moalli, F., Jaillon, S., Deban, L., and Mantovani, A. (2009) The long pentraxin PTX3 as a prototypic humoral pattern recognition receptor: interplay with cellular innate immunity. *Immunol. Rev.* **227,** 9–18

23. Sternberg, E.M. (2006) Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat. Rev. Immunol.* **6,** 318–328

24. Glass, C.K. and Saijo, K. (2010) Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat. Rev. Immunol.* **10,** 365–376

25. Bertini, R., Bianchi, M., and Ghezzi, P. (1988) Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. *J. Exp. Med.* **167,** 1708–1712

26. Glass, C.K. and Ogawa, S. (2006) Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat. Rev. Immunol.* **6,** 44–55

27. Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R., and Cheng, G. (2002) IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17,** 251–263

28. Yamazaki, S. and Takeshige, K. (2008) Protein synthesis inhibitors enhance the expression of mRNAs for early inducible inflammatory genes via mRNA stabilization. *Biochim. Biophys. Acta* **1779,** 108–114

29. Saccani, S., Pantano, S., and Natoli, G. (2001) Two waves of nuclear factor κB recruitment to target promoters. *J. Exp. Med.* **193,** 1351–1359

30. De Bosscher, K., Vanden Berghe, W., and Haegeman, G. (2006) Cross-talk between nuclear receptors and nuclear factor κB. *Oncogene* **25,** 6868–6886

31. Kassel, O. and Herrlich, P. (2007) Crosstalk between the glucocorticoid receptor and other transcription factors: molecular aspects. *Mol. Cell. Endocrinol.* **275,** 13–29

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

32. Alles, V.V., Bottazzi, B., Peri, G., Golay, J., Introna, M., and Mantovani, A. (1994) Inducible expression of PTX3, a new member of the pentraxin family, in human mononuclear phagocytes. *Blood* **84,** 3483–3493

33. Introna, M., Alles, V.V., Castellano, M., Picardi, G., De Gioia, L., Bottazzai, B., Peri, G., Breviario, F., Salmona, M., De Gregorio, L., Dragani, T.A., Srinivasan, N., Blundell, T.L., Hamilton, T.A., and Mantovani, A. (1996) Cloning of mouse ptx3, a new member of the pentraxin gene family expressed at extrahepatic sites. *Blood* **87,** 1862–1872

34. Altmeyer, A., Klampfer, L., Goodman, A.R., and Vilček, J. (1995) Promoter structure and transcriptional activation of the murine TSG-14 gene encoding a tumor necrosis factor/interleukin-1-inducible pentraxin protein. *J. Biol. Chem.* **270,** 25584–25590

35. Palombella, V.J., Rando, O.J., Goldberg, A.L., and Maniatis, T. (1994.) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78,** 773–785

36. Lyß, G., Knorre, A., Schmidt, T.J., Pahl, H.L., and Merfort, I. (1998) The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-κB by directly targeting p65. *J. Biol. Chem.* **273,** 33508–33516

37. Ray, A. and Prefontaine, K.E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 752–756

38. Scheinman, R.I., Gualberto, A., Jewell, C.M., Cidlowski, J.A., and Baldwin Jr, A.S. (1995) Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. *Mol. Cell. Biol.* **15,** 943–953

39. Boruk, M., Savory, J.G., and Hache, R.J. (1998) AF-2-dependent potentiation of CCAAT enhancer binding protein β-mediated transcriptional activation by glucocorticoid receptor. *Mol. Endocrinol.* **12,** 1749–1763

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

40. Rudiger, J.J., Roth, M., Bihl, M.P., Cornelius, B.C., Johnson, M., Ziesche, R., and Block, L.H. (2002) Interaction of C/EBPα and the glucocorticoid receptor in vivo and in nontransformed human cells. *FASEB J.* **16,** 177–184

41. Hakim, O., John, S., Ling, J.Q., Biddie, S.C., Hoffman, A.R., Hager, G.L. (2009) Glucocorticoid receptor activation of the Ciz1-Lcn2 locus by long range interactions. *J. Biol. Chem.* **284,** 6048–6052

42. Doni, A., Mantovani, G., Porta, C., Tuckermann, J., Reichardt, H. M., Kleiman, A., Sironi, M., Rubino, L., Pasqualini, F., Nebuloni, M., Signorini, S., Peri, G., Sica, A., Beck-Peccoz, P., Bottazzi, B., and Mantovani, A. (2008) Cell-specific regulation of PTX3 by glucocorticoid hormones in hematopoietic and nonhematopoietic cells. *J. Biol. Chem.* 283, 29983–29992

43. Zhang, J., Wu, Y., Zhang, Y., Leroith, D., Bernlohr, D.A., and Chen, X. (2008) The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. *Mol. Endocrinol.* **22,** 1416–1426

44.Deban, L., Russo, R.C., Sironi, M., Moalli, F., Scanziani, M., Zambelli, V., Cuccovillo, I., Bastone, A., Gobbi, M., Valentino, S., Doni, A., Garlanda, C., Danese, S., Salvatori, G., Sassano, M., Evangelista, V., Rossi, B., Zenaro, E., Constantin, G., Laudanna, C., Bottazzi, B., and Mantovani, A. (2010) Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nat. Immunol.* **11,** 328–334

45. Ogawa, S., Lozach, J., Benner, C., Pascual, G., Tangirala, R.K., Westin, S., Hoffmann, A., Subramaniam, S., David, M., Rosenfeld, M.G., and Glass, C.K. (2005) Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* **9,** 707–721

46. Uhlenhaut, N.H., Barish, G.D., Yu, R.T., Downes, M., Karunasiri, M., Liddle, C., Schwalie, P., Hübner, N., and Evans, R.M. (2013) Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. *Mol. Cell* **49,** 158–171

47. Surjit, M., Ganti, K.P., Mukherji, A., Ye, T., Hua, G., Metzger, D., Li, M., and Chambon, P. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* **145,** 224–241

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

48. Schindler, C., Levy, D.E., and Decker T. (2007) JAK-STAT signaling: from interferons to cytokines. *J. Biol. Chem.* **282,** 20059–20063

49. Stöcklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1996) Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* **383,** 726–728

50. Zhang, Z., Jones, S., Hagood, J.S., Fuentes, N.L., and Fuller, G.M. (1997) STAT3 acts as a co-activator of glucocorticoid receptor signaling. *J. Biol. Chem.* **272,** 30607–30610

51. Aittomäki, S., Pesu, M., Groner, B., Jänne, O. A., Palvimo, J. J., and Silvennoinen, O. (2000) Cooperation among Stat1, glucocorticoid receptor, and PU.1 in transcriptional activation of the high-affinity Fcγ receptor I in monocytes. *J. Immunol.* **164,** 5689–5697

52. Stoecklin, E., Wissler, M., Moriggl, R., and Groner, B. (1997) Specific DNA binding of Stat5, but not of glucocorticoid receptor, is required for their functional cooperation in the regulation of gene transcription. *Mol. Cell. Biol.* **17,** 6708–6716

53.Engblom, D., Kornfeld, J. W., Schwake, L., Tronche, F., Reimann, A., Beug, H., Hennighausen, L., Moriggl, R., and Schütz, G. (2007) Direct glucocorticoid receptor-Stat5 interaction in hepatocytes controls body size and maturation-related gene expression. *Genes Dev.* **21,** 1157–1162

54. Langlais, D., Couture, C., Balsalobre, A., and Drouin, J. (2012) The Stat3/GR interaction code: predictive value of direct/indirect DNA recruitment for transcription outcome. *Mol. Cell* **47,** 38–49

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Figure Legends

Fig. 1 Dex enhances LPS-induced production of the anti-microbial proteins lipocalin 2 and pentraxin 3 in macrophages. (**A**) BMMs were stimulated with LPS (100 ng/ml), Dex (1 μM), or LPS plus Dex for the indicated times. mRNA expression levels for *Lcn2*, *Ptx3*, *Il6*, and *Il12b* were determined by qRT-PCR using total RNA extracted, and normalized to those of the housekeeping gene *Rpl32* (ribosomal protein L32). The results are representative of three independent experiments. (**B**) BMMs were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) for 8 h. Protein concentrations in cultured supernatants collected were determined by ELISA. The results are mean ± S.E. of duplicate samples from representative of at least two independent experiments.

Fig. 2 Identification of *cis*-elements responsible for transcriptional activation of *Lcn2* in macrophages. (**A**) A schematic diagram of the 5'-upstream region of mouse *Lcn2*. Potential binding sites for the transcription factors are shown in ovals. *cis*-elements found to be required for the transcriptional activation are shown in hatched ovals. (**B** and **C**) RAW264.7 macrophages were transiently transfected with the indicated luciferase reporter plasmids and the control plasmid pRL-TK. Two days after transfection, cells were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) for 8 h, and the luciferase activities were measured. Luciferase activities were normalized to the control *Renilla* luciferase activities. The results are mean ± S.E. of duplicate transfections from representative of three independent experiments.

Fig. 3 Identification of *cis*-elements responsible for transcriptional activation of *Ptx3* in macrophages. (**A**) A schematic diagram of the 5'-upstream region of mouse *Ptx3*. Potential binding sites for the transcription factors are shown as in Figure 2A. (**B** and **C**) Luciferase reporter analyses were carried out as in Figure 2B. (**B**) The left and the middle graphs present the same data in different scales of y-axis; the middle and the right graphs are depicted in the same scale of y-axis.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 4 Inhibition of NF-κB and GR abrogates synergistic expression of *Lcn2* and *Ptx3* in macrophages. (**A**) BMMs were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) in the presence of MG132 (5 μM) or Helenalin (10 μM). BMMs were stimulated for 8 h (for *Lcn2*) or 4 h (for *Ptx3*), and mRNA expression levels were analyzed by qRT-PCR. (**B**) mRNA expression analyses were performed as in (**A**) in the presence of RU486 (1 μM). The results are mean ± S.E. of duplicate samples from representative of at least two independent experiments.

Fig. 5 LPS and Dex cooperatively induce recruitment of p65, C/EBPβ, and GR to *Lcn2* and *Ptx3*. (**A**) RAW264.7 macrophages were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) for the indicated times. Nuclear proteins were prepared and subjected to immunoblot analyses using the indicated antibodies. USF2 is a loading control for nuclear proteins. (**B** and **C**) BMMs were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) for the indicated periods. Formaldehyde-fixed chromatin was subjected to ChIP assay using the indicated antibodies. Precipitated DNA was analyzed by PCR using primers corresponding to the indicated genomic loci. The primer locations in the *Lcn2* gene are shown in Figure 2A. The results are representative of experiments from at least three independent experiments. (**D**) RAW264.7 macrophages were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) for 4 h, and mRNA expression levels of *Cxcl2* and *Sgk* were determined by qRT-PCR. The results are mean ± S.E. of duplicate samples from representative of at least two independent experiments. (**E**) ChIP analyses were performed as in (**B**) and (**C**). The primer locations for the *Ptx3* gene are shown in Figure 3A.

Fig. 6 IκBζ is required for the synergistic expression of *Lcn2* and *Ptx3* in macrophages. BMMs prepared from gender-matched littermate mice with the indicated *Nfkbiz* (IκBζ) genotypes were stimulated with LPS (100 ng/ml) and/or Dex (1 μM) for the indicated times. (**A** and **B**) mRNA levels of *Lcn2* (**A**) and *Ptx3* (**B**) were determined by qRT-PCR. The results are representative of three independent experiments. (**C** to **E**) Formaldehyde-fixed chromatin was subjected to ChIP assay using the indicated antibodies. Precipitated DNA was analyzed by PCR using primers corresponding

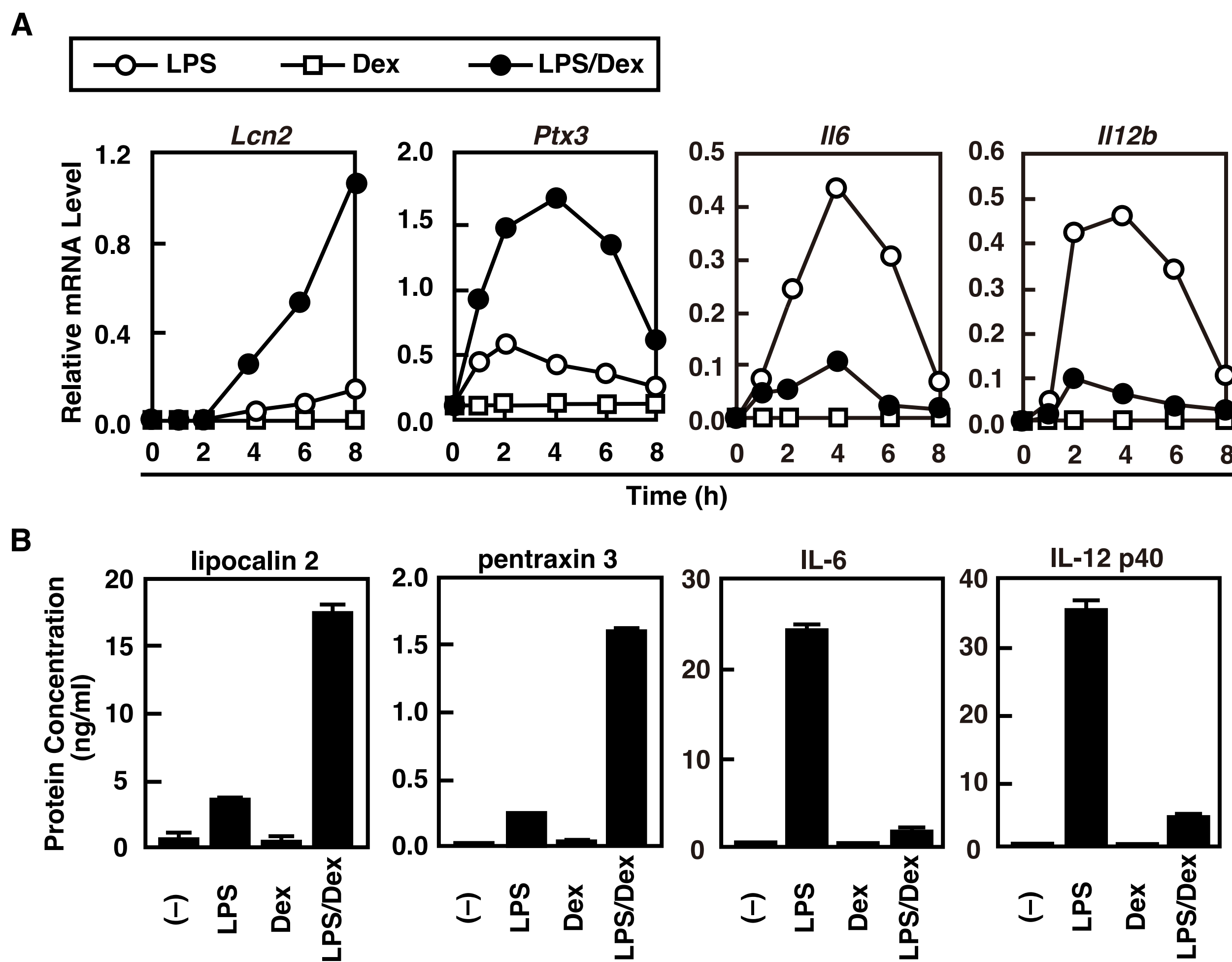
Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

to the indicated genomic loci, which are shown in Figures 2A and 3A. The results are representative of experiments at least three independent experiments.

Fig. 7 IκBζ is required for synergistic expression of *Lcn2* in fibroblasts. (**A**) NIH3T3 fibroblasts were stimulated with LPS (100 ng/ml) or Dex (1 μM) for the indicated times. mRNA expression levels for *Lcn2* were analyzed by qRT-PCR. (**B**) NIH3T3 fibroblasts were stimulated with LPS (1 μg/ml) and/or Dex (100 nM) for the indicated times. mRNA expression levels for *Ptx3* were determined by qRT-PCR. The results are representative of at least two independent experiments. (**C**) NIH3T3 fibroblasts were stimulated with LPS (1 μg/ml) and/or Dex (100 nM) for 4 or 8 h. mRNA expression levels for *Lcn2* were determined by qRT-PCR. (**D**) MEFs prepared from littermate embryos with the indicated *Nfkbiz* genotypes were stimulated with LPS (1 μg/ml) and/or Dex (100 nM) for 4 or 8 h. *Lcn2* mRNA levels were determined by qRT-PCR. The results are mean ± S.E. of duplicate samples from representative of two independent experiments. (**E**) NIH3T3 fibroblasts were transiently transfected with the indicated luciferase reporter plasmids. Luciferase reporter assays were conducted as in Figure 2. The results are mean ± S.E. of duplicate transfections from representative of two independent experiments.

Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

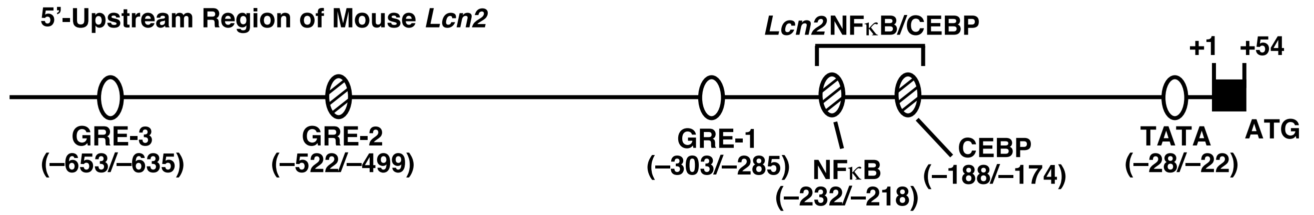
Fig. 1



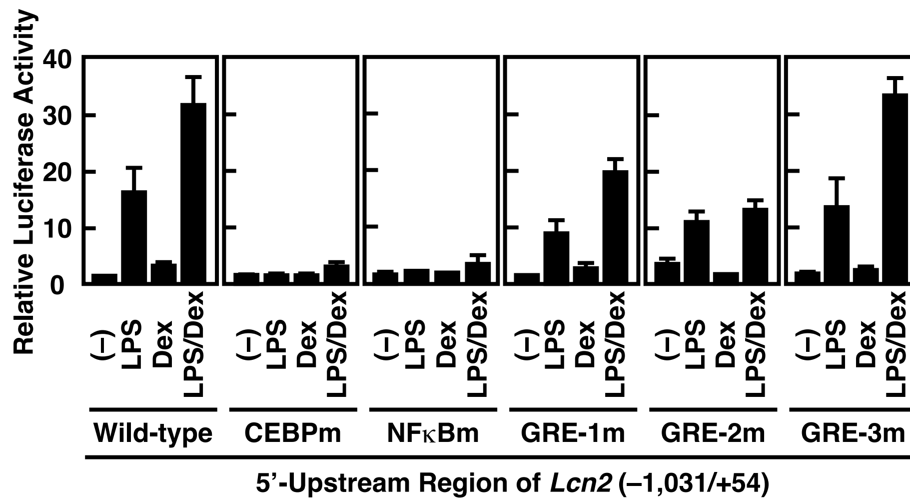
Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 2

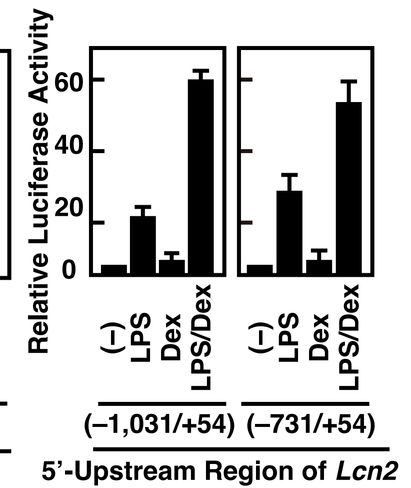
A



B

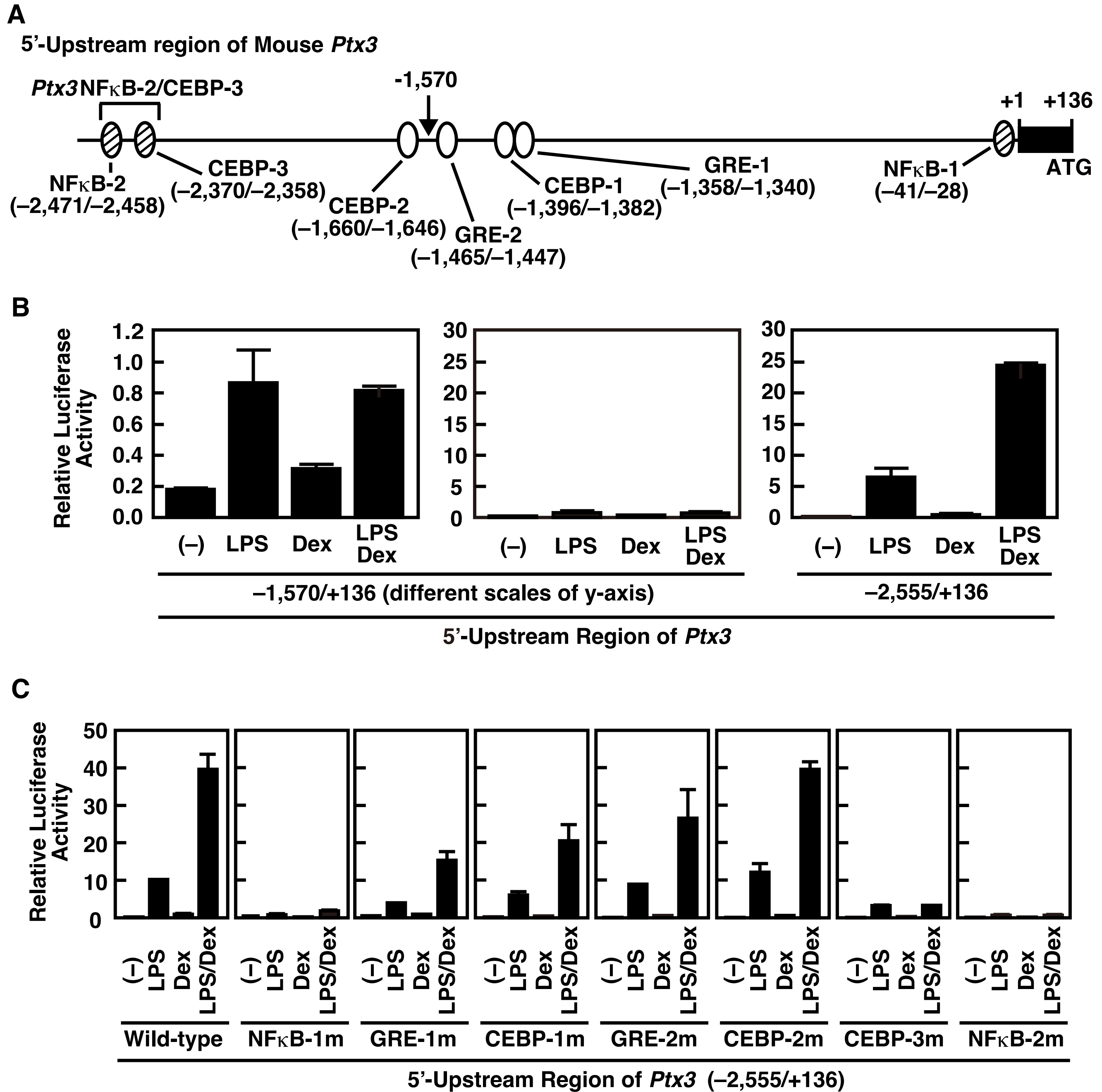


C



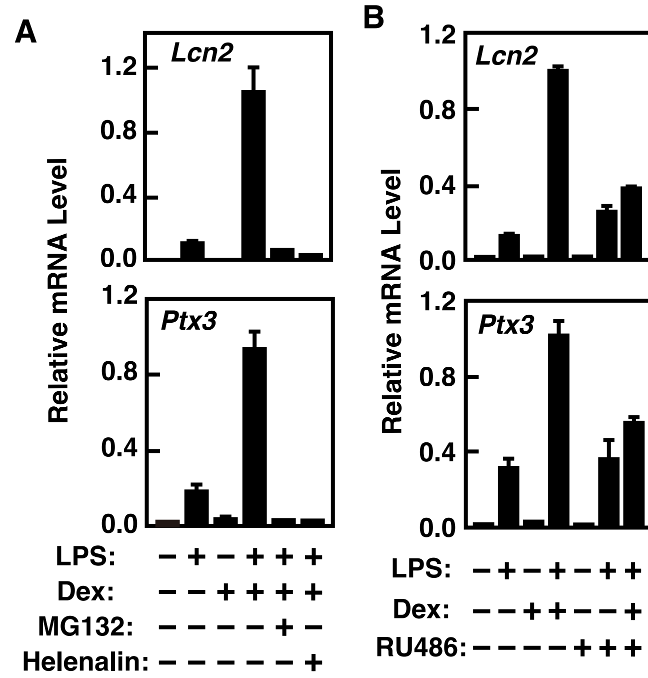
Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 3



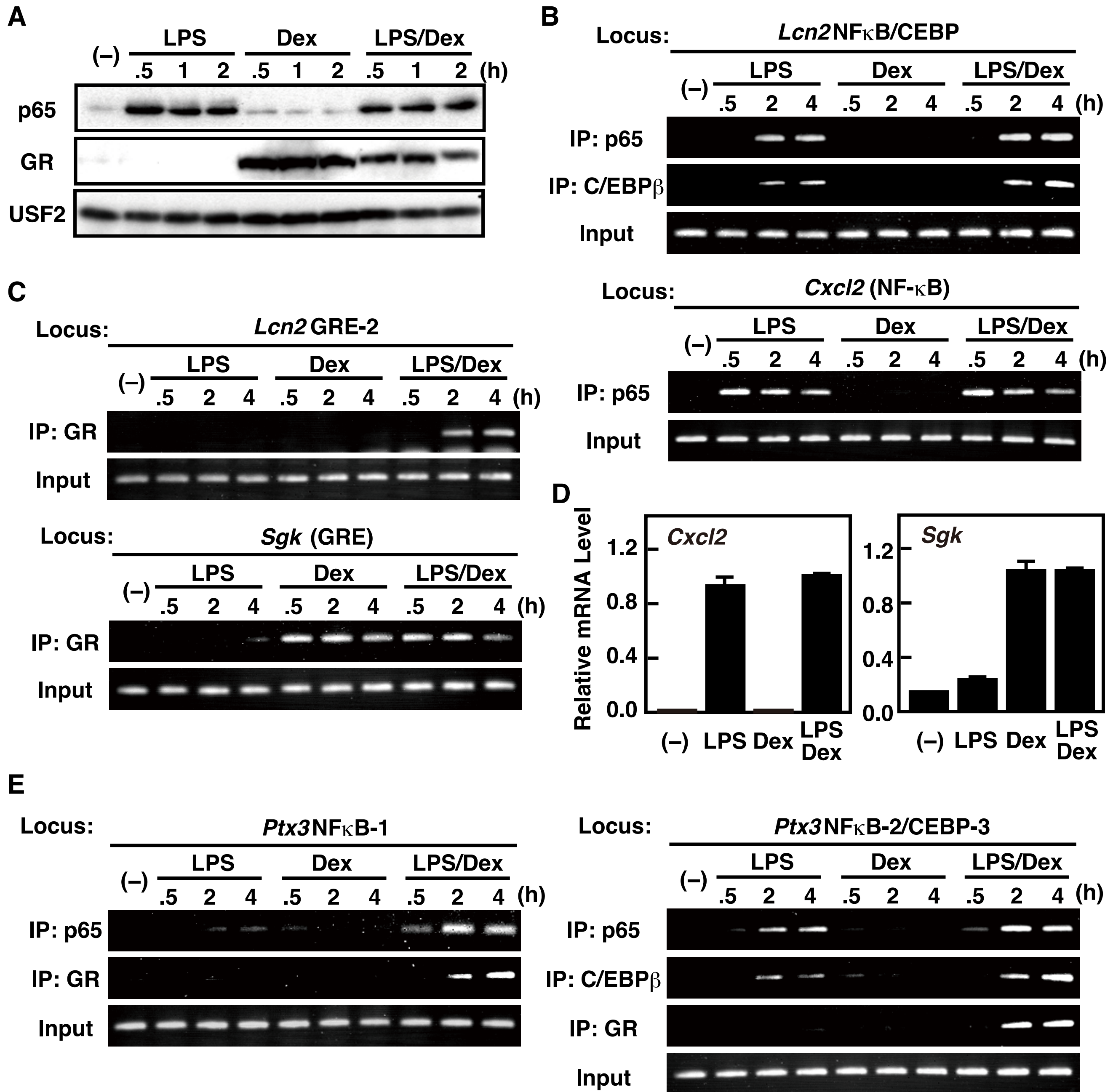
Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 4



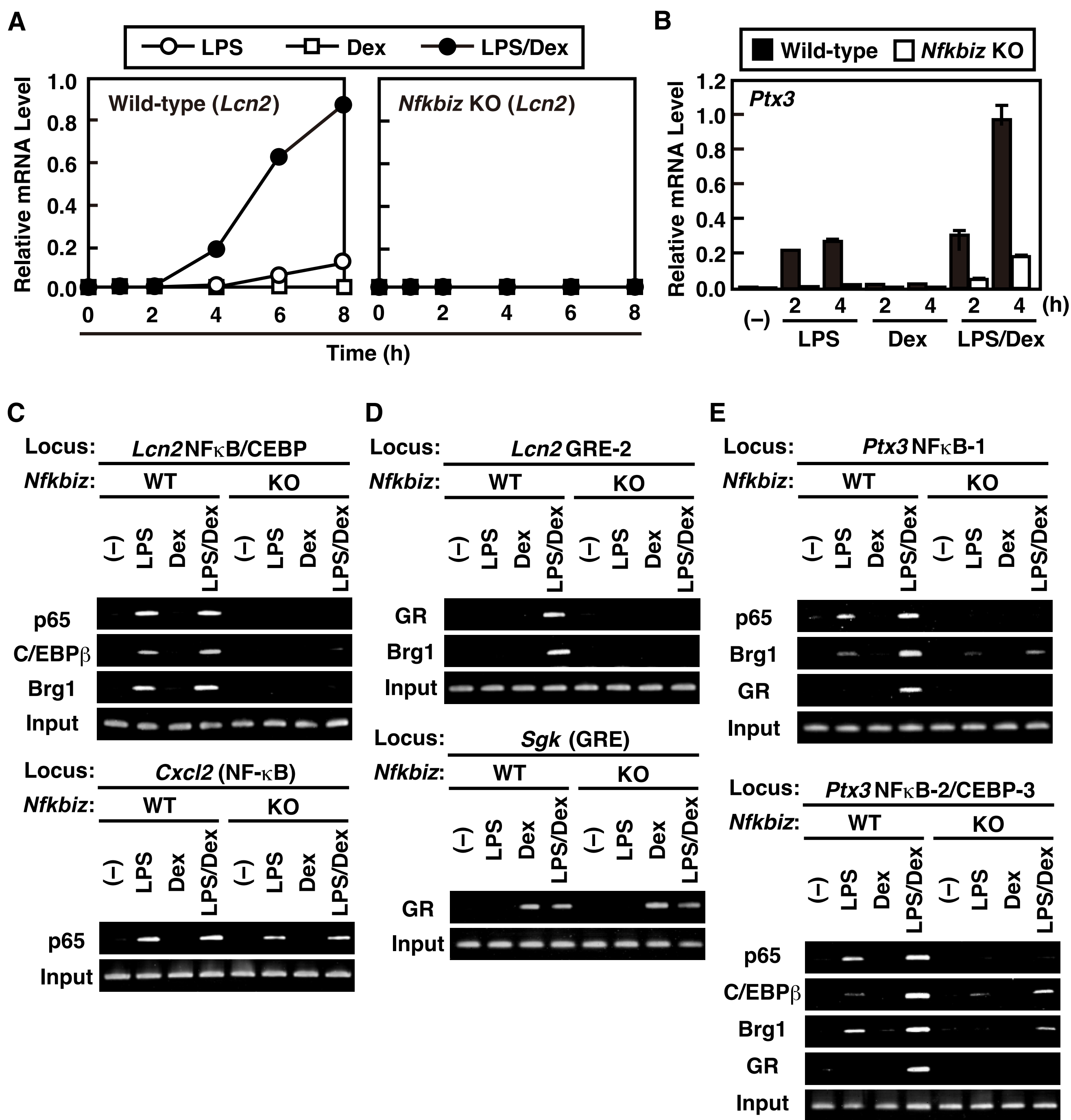
Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 5



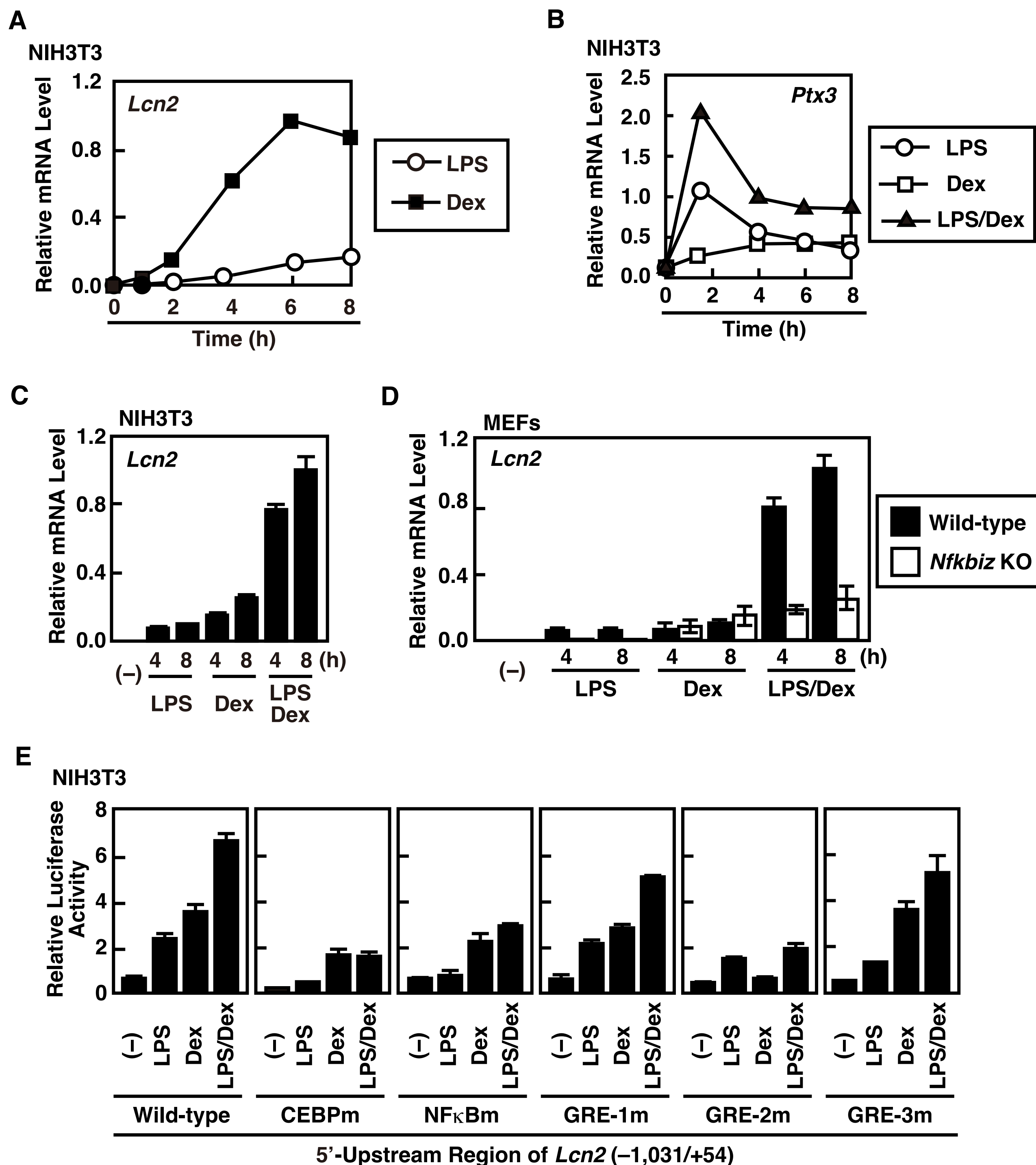
Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 6



Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015

Fig. 7



Downloaded from http://jb.oxfordjournals.org/ at Main Library of Gazi University on January 5, 2015