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Annexin-1 Regulates Macrophage IL-6 and TNF via Glucocorticoid-Induced Leucine Zipper

Yuan H. Yang,2 Daniel Aeberli, April Dacumos, Jin R. Xue, and Eric F. Morand

Annexin-1 (ANXA1) is a mediator of the anti-inflammatory actions of endogenous and exogenous glucocorticoids (GC). The mechanism of ANXA1 effects on cytokine production in macrophages is unknown and is here investigated in vivo and in vitro. In response to LPS administration, ANXA1−/− mice exhibited significantly increased serum IL-6 and TNF compared with wild-type (WT) controls. Similarly, LPS-induced IL-6 and TNF were significantly greater in ANXA1−/− than in WT peritoneal macrophages in vitro. In addition, deficiency of ANXA1 was associated with impairment of the inhibitory effects of dexamethasone (DEX) on LPS-induced IL-6 and TNF in macrophages. Increased LPS-induced cytokine expression in the absence of ANXA1 was accompanied by significantly increased LPS-induced activation of ERK and JNK MAPK and was abrogated by inhibition of either of these pathways. No differences in GC effects on MAPK or MAPK phosphatase 1 were observed in ANXA1−/− cells. In contrast, GC-induced expression of the regulatory protein GILZ was significantly reduced in ANXA1−/− cells by silencing of ANXA1 in WT cells and in macrophages of ANXA1−/− mice in vivo. GC-induced GILZ expression and GC inhibition of NF-κB activation were restored by expression of ANXA1 in ANXA1−/− cells, and GILZ overexpression in ANXA1−/− macrophages reduced ERK MAPK phosphorylation and restored sensitivity of cytokine expression and NF-κB activation to GC. These data confirm ANXA1 as a key inhibitor of macrophage cytokine expression and identify GILZ as a previously unrecognized mechanism of the anti-inflammatory effects of ANXA1. The Journal of Immunology, 2009, 183: 1435–1445.

Glucocorticoids (GC) are potent anti-inflammatory and immunosuppressive agents. The ability of endogenous GC to suppress the expression of a variety of proinflammatory genes, and to up-regulate certain anti-inflammatory genes, has been widely exploited in the treatment of inflammatory diseases with exogenous GC (1). Annexin-1 (ANXA1) was originally identified as a GC-regulated protein and was previously termed lipocortin 1. ANXA1 has long been suggested to function as a cellular mediator of the anti-inflammatory effects of GC. ANXA1 expression and secretion in several cell types is induced by GC (2). Exogenous ANXA1, or an N-terminal ANXA1 peptide, mimic many inhibitory effects of GC, including inhibition of leukocyte recruitment at inflammatory sites (3–5), inhibition of proinflammatory mediators such as phospholipase A2, cyclooxygenase-2 (COX2), and NO, induction of apoptosis in inflammatory cells, and induction of the anti-inflammatory cytokine IL-10 (reviewed in Ref. 6). The absence of ANXA1 is associated with increased lethality in experimental endotoxemia (7) and with exacerbation of zymosan-induced acute inflammation (8) and several models of experimental arthritis (9–11). Because of its induction by GC and anti-inflammatory effects, a role of ANXA1 in the regulation of GC sensitivity has been reported in models of acute and chronic inflammation, including carrageenin-induced paw edema, zymosan-induced peritonitis, and Ag-induced arthritis (8, 11, 12).

In several of these models, inhibitory effects of ANXA1 on cytokine expression have been observed. For example, in Ag-induced arthritis, ANXA1 deficiency was associated with increased synovial expression of TNF, IL-6, and other cytokines. In experimental endotoxemia, the absence of ANXA1 is also associated with increased serum IL-6 and TNF (7). In both these examples, the dominant cellular source of cytokines is macrophages, and macrophage TNF and IL-1 release in vitro have been reported to be increased in the absence of ANXA1 (7).

Macrophage cytokine expression is induced via activation of numerous signaling pathways, including MAPK and NF-κB pathways (13), but the mechanisms of ANXA1 effects on cytokine expression, during inflammation and under the influence of GC, are not known. At least two GC-induced regulatory proteins are involved in controlling MAPK- and NF-κB-regulated cytokines, namely MAPK phosphatase 1 (MKP-1; also known as DUSP1) and GC-induced leucine zipper (GILZ) (14, 15). MKP-1 dephosphorylates activated MAPK and acts as a key negative regulator of TLR-induced inflammation in vivo (16–19). Although a stimulatory effect of ANXA1 on ERK activation has been suggested in stably transfected RAW 264.7 cells (20), we recently reported that in cultured fibroblasts, endogenous ANXA1 inhibits MAPK activation via up-regulation of MKP-1(21). GILZ interacts directly with proinflammatory transcription factors, including NF-κB (22) and AP-1(23). The potential effect of ANXA1 on GILZ has not been investigated previously.

The present studies were designed to investigate mechanisms of inflammatory regulation by ANXA1 in macrophages. We demonstrate that ANXA1 exerts tonic inhibitory effects on LPS-induced
macrophage IL-6 and TNF release and MAPK and NF-κB activation and participates in GC induction of GILZ. These data indicate that GILZ is a previously unrecognized target of the anti-inflammatory effects of ANXA1 in macrophages.

Materials and Methods

Animals

ANXA1−/− mice were generated as described previously (8). ANXA1−/− and wild-type (WT) littersmates used in this study are of mixed 129/SvJ × C57BL/6 background. Mice were bred and housed under specific pathogen-free conditions. Litters from heterozygous ANXA1+/− parents were genotyped to select homozygous ANXA1−/− and WT mice. Genotyping was performed using a standard PCR protocol as described previously (8). All experiments were approved by the Monash University Animal Research Ethics Committee.

Endotoxemia and DEX treatment

Endotoxemia was induced in male 8- to 10-wk-old ANXA1−/− and WT mice. Animals were injected i.p. with 10 mg/kg LPS from Escherichia coli O111:B4 (Sigma-Aldrich) dissolved in saline. DEX (Sigma-Aldrich) at a dose of 0.5 mg/kg was injected i.p. 1 h before LPS (1 or 10 mg/kg). Blood was collected at indicated times. Ethical considerations precluded the use of a lethality outcome in these studies.

Measurement of cytokines and NO

Concentrations of IL-6 and TNF in serum and culture supernatants were measured using commercially available ELISA (Quantikine M; R&D Systems). The sensitivity of these assays was 15.6 pg/ml. Supernatant cytokines were also determined by mouse inflammation kit of cytometric bead array (BD Biosciences) as described in the instructions of the manufacturer. The samples were analyzed by flow cytometry with a Cytomation MoFlo flow cytometer (DakoCytomation).

NO was determined by measuring the amount of nitrite in culture supernatants using Griess reagent as described previously (24). Peritoneal macrophages were stimulated with LPS (1 µg/ml) in the presence or absence of DEX for 48 h. The limit of sensitivity of this assay was 1.56 μmol/L, and the absorbance was read at 540 nm. Nitrite concentration was determined using sodium nitrite (Ajax Chemicals).

Peritoneal macrophage culture

Primary peritoneal macrophages were prepared from mice by saline flushing or using thioglycollate elicitation, as described previously (25). Cells were cultured in 5% FCS/DMEM at 37°C for 3 h. After washing off non-adherent cells, adherent monolayer macrophages were cultured overnight in complete medium. Cells were stimulated with LPS and/or DEX, and/or specific MAPK inhibitors (Alexis Biochemicals) of p38 (SB203580), ERK1/2 (PD98059), and JNK (SP600125), in indicated concentrations for 4 h. Macrophages were treated with Boc2 (100 µM), a pan antagonist of formyl peptide receptor (FPR) receptors (Gen Script), in the presence or absence of DEX for 3 h. Cells were also treated with Ac2-26 (100 µg/ml) for 3 h. Supernatants were collected, and cells were then resuspended in TRIzol reagent (Invitrogen) or RNeasy mini kit (Qiagen) for RNA extraction.

Western blot analysis

Western blotting was performed as described previously (26). In brief, total cell protein was measured by BCA Protein assay kit (Quantum Scientific). Forty micrograms of protein was separated on 10% SDS-polyacrylamide...
electrophoresis gels and transferred to Hybond-C extra nitrocellulose membranes (Millipore). Membranes were probed with Abs against phospho-ERK, phospo-p38, or phospho-JNK (mAbs; Cell Signaling Technology), MKP-1 (polyclonal; Cell Signaling Technology), GILZ (polyclonal; provided by Prof. C. Riccardi, University of Perugia, Perugia, Italy), and /H9252-ac-
tin (mAb; Sigma-Aldrich). Anti-mouse and anti-rabbit Abs conjugated to Alexa Fluor 700 (Rockland) and IRDye 800 (Rockland), respectively, were used to probe primary Abs. Protein bands were detected and quantified by Western blotting with the Odyssey system (Li-Cor). Densitometry ratios were normalized to /H9252-actin content and expressed as arbitrary units (AU).

Flow cytometry analysis
Flow cytometry was performed as described previously (21). Briefly, peritoneal macrophages obtained from WT and ANXA1−/− mice treated with vehicle or DEX (0.5 mg/kg) for 3 h, or thioglycollate-elicited macrophages in RPMI 1640 with 5% FBS were stimulated with DEX for 3 h at 37°C before fixation with 2% formaldehyde for 10 min. The cells were then pelleted, resuspended in ice-cold methanol, and incubated for 30 min at 4°C. After washing, cells were stained with control rabbit IgG (Abacus ALS; Vector Laboratories) or rabbit anti-GILZ Ab (Santa Cruz Biotechnology) for 1 h and subsequently incubated with donkey anti-rabbit-FITC (Biolab) for 30 min at room temperature. Samples were washed and re-suspended in media for analysis.

Quantitative RT-PCR
Total RNA (0.5 µg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo(dT)20. PCR amplification was performed on a Rotor-Gene 3000 (Corbett Research). Murine IL-6, TNF, MKP-1, and /H9252-actin primers were used as described previously (27). GILZ primers have been reported previously (22). The primers used for COX2 were 5'-TTG AAG GTG TCG GGC AGC-3' (forward) and 5'-CAG AAC CGC ATT GCC TCT G-3' (reverse) and for 18S were 5'-GTAACCCGT TGAACCC CAATTC-3' (forward) and 5'-GCCTCACTAAACCATC CAATCG-3' (reverse).

For PCR, each of the standard and sample cDNA dilutions was added to individual capillary tubes. Amplification (40 cycles) was conducted in a total volume of 10 µl containing primer concentrations of SYBR Green I (Invitrogen) and M-MLV reverse transcriptase (Promega). Melting curve analysis was performed to confirm the specificity of the PCR products (base pair) by agarose gel electrophoresis. Relative quantification of target mRNA expression was calculated and normalized to /H9252-actin or 18S mRNA expression.
expression. The results are presented as the fold induction of mRNA expression relative to the amount present in control samples.

Small interfering RNA (siRNA) and plasmid transfection

Synthetic murine ANXA1 siRNA oligoribonucleotides with symmetric 3’ TT overhangs were purchased from Santa Cruz Biotechnology. Peritoneal macrophages were transiently transfected with ANXA1 siRNA or control siRNA using oligofectamine (Invitrogen) and incubated at 37°C for 48 h. Cells were then treated with DEX (10⁻⁷ M) and/or LPS (0.1 μg/ml) for indicated times (4 h for mRNA and 24 h for supernatant cytokine beads).

A full-length cDNA encoding ANXA1 (provided by Prof. M. Perretti) and GILZ (provided by Prof. C. Riccardi, William Harvey Research Institute, London, U.K.) was inserted into pcDNA3.1 (Invitrogen) as described previously (28). For peritoneal macrophages, 1 × 10⁶ cells were transfected with 2 μg of plasmid DNA using the AMAXA system (Amaxa Biosystems) with mouse macrophage nucleofector kit. As a control, cells were also transfected with the empty pcDNA3.1 vector. After electroporation, the cells were immediately transferred to 6- and 24-well plates with complete medium and cultured at 37°C for 24 h. Transfected cells were treated with LPS or DEX or a combination as indicated times. Transfection efficiency was determined by quantitative RT-PCR and Western blot analysis.

NF-κB activation assay and luciferase assay

Phosphorylation of NF-κB p65 Ser⁵³⁶ was measured with the ELISA-based PathScan Phospho-NF-κB p65 (Ser⁵³⁶) kit and total-NF-κB p65 kit (Cell Signaling Technology), according to the manufacturer’s instructions. Briefly, whole-cell lysates were prepared from peritoneal macrophages cultured in the presence or absence of DEX and/or LPS for 1 h. Ninety-six-well microtiter plates were coated with anti-NF-κB capture Abs, and Ser⁵³⁶-phosphorylated p65 and total p65 were detected using specific Abs. Results are expressed as absorbance values. All measures were performed in duplicate.

Peritoneal macrophages were transiently cotransfected with an NF-κB luciferase plasmid construct (NF-κB-Luc plasmid) (29), an ANXA1 plasmid, GILZ plasmid, or pcDNA3.1 using Nucleofector kit and the AMAXA system. In other experiments, peritoneal macrophages were cotransfected with GILZ-luc (human GILZ-luc; a gift from Prof. M. Pallardy, Univ-Paris Sud, Châtenay-Malabry, France) (30, 31) and ANXA1 plasmid. Transfected cells were treated with LPS (100 ng/ml) in the presence or absence of DEX (10⁻⁷ M) for 18 h. Luciferase activity was measured using the Luciferase Assay System (Promega), according to the manufacturer’s instructions. Luminescence measured using a Wallac Victor 2 luminometer (PerkinElmer).

Statistical analysis

Data were analyzed using the Mann-Whitney two-sample rank test to determine the level of significance between means of groups or the Student’s t test for comparison of continuous variables. Results are expressed as the mean ± SEM. A p value <0.05 was considered statistically significant.

Results

Role of ANXA1 in the regulation of cytokines and GC sensitivity in vivo

We first sought to confirm the reported effect of ANXA1 on the secretion of inflammatory cytokines in vivo. Administration of LPS (10 mg/kg) induced the release of serum IL-6 and TNF in WT mice over 4 h (Fig. 1, A and B). Significantly greater increases of serum IL-6 and TNF were observed in ANXA1⁻/⁻ mice in comparison with WT mice (p < 0.05). The role of ANXA1 in the inhibitory effects of GC on LPS responses in vivo has not been investigated previously. We treated WT and ANXA1⁻/⁻ mice with DEX (0.5 mg/kg) 1 h before LPS administration for 1.5 h. DEX significantly inhibited LPS-induced serum IL-6 in WT mice at both high (10 mg/kg) and low (1 mg/kg) doses of LPS (Fig. 1, C and D). However, the inhibitory effect of DEX on IL-6 was reached statistical significance only in ANXA1⁻/⁻ mice treated with low-dose LPS (1 mg/kg). In contrast, the effect of DEX on LPS-induced serum TNF was comparable in ANXA1⁻/⁻ and WT mice (Fig. 1, C and D).

Role of ANXA1 in the regulation of cytokines and GC sensitivity in macrophages

To explore the mechanism of the effects of ANXA1 on cytokine release, we first sought to confirm these findings in vitro. We first investigated LPS responses in resting and thioglycollate-elicited peritoneal macrophages from WT and ANXA1⁻/⁻ mice. Compared with WT cells, LPS-induced IL-6 and TNF release was significantly greater in ANXA1⁻/⁻ resting (Fig. 2A) and thioglycollate-elicited (Fig. 2B) macrophages. We also observed that LPS-induced NO production (WT undetectable, ANXA1⁻/⁻ 16.3 ± 7 μM, p < 0.05) and COX2 mRNA expression (WT 12 ±
3, ANXA1−/− 29 ± 5, p < 0.05) were significantly increased in ANXA1−/− macrophages. Taken together with the in vivo findings, these data demonstrate that endogenous ANXA1 exerts a tonic inhibitory effect on macrophage activation in response to LPS.

We next sought to analyze the role of ANXA1 in the actions of GC on macrophages. Resting macrophages were treated with LPS in the presence or absence of DEX. Treatment with DEX at concentrations of 10⁻⁸ to 10⁻⁶ M significantly suppressed LPS-induced cytokine expression and MAPK activation. Because ERK MAPK is not responsible for the observed effects of ANXA1 deficiency on cytokine release, we examined the effects of DEX on IL-6 and TNF release from ANXA1−/− cells in comparison with WT. However, a trend toward lower DEX-induced IL-6 and TNF mRNA (Fig. 2A) suggests ANXA1 contributes to the anti-inflammatory effects of GC on macrophages.

To confirm this effect of ANXA1, we restored ANXA1 expression in ANXA1−/− cells by transient transfection. Overexpression of ANXA1 in ANXA1−/− cells restored DEX inhibition of LPS-induced IL-6 and TNF mRNA (Fig. 2B). Taken together, with the in vivo findings, these results demonstrate a role for ANXA1 in macrophage sensitivity to GC.

Role of ANXA1 in the regulation of ERK and JNK MAPK activation

We have previously reported that endogenous ANXA1 inhibits IL-6 release by fibroblasts by inhibition of MAPK activity (21). We therefore investigated whether MAPK activity was involved in the increased cytokine release and decreased GC sensitivity observed in ANXA1−/− macrophages. MAPK activation in response to LPS, as measured by phosphorylation of ERK, p38, and JNK MAPK, was detected in WT macrophages (Fig. 3A). LPS-induced phosphorylation of ERK and JNK was modestly but significantly increased in ANXA1−/− cells in comparison with WT, but LPS-induced phosphorylation of p38 MAPK was comparable in WT and ANXA1−/− cells (Fig. 3A). The involvement of ERK and JNK MAPK in the regulation of LPS-induced IL-6 and TNF by ANXA1 was further investigated using MAPK inhibitors. Inhibition of either ERK or JNK MAPK inhibited LPS-induced IL-6 and TNF release from ANXA1−/− peritoneal macrophages to levels observed from LPS-stimulated WT cells in the absence of inhibitors (Fig. 3B). Taken together, these data suggest ANXA1-mediated inhibition of ERK and JNK phosphorylation may be important in ANXA1 regulation of macrophage responses to LPS.

We next investigated whether altered MKP-1 expression is involved in ANXA1 regulation of MAPK activation in macrophages. MKP1 is potently induced by GC and is a mechanism through which GC inhibits the p38 and JNK MAPK pathways. As shown in Fig. 3A, LPS-induced MKP1 expression was equivalent in WT and ANXA1−/− cells. As shown in Fig. 4A, MKP1 expression was dose-dependently induced by DEX in WT macrophages, and parallel effects were observed in ANXA1−/− cells, with no significant differences observed. These results suggested MKP-1 was not responsible for the observed effects of ANXA1 deficiency on cytokine expression and MAPK activation. Because ERK MAPK is not significantly inhibited by MKP-1, we examined the effects of DEX and ANXA1 on ERK activation directly. Macrophages were treated with DEX for 3 h before stimulation with LPS for 30 and 60 min, and phospho-ERK activity was detected by Western blotting. As noted above, in the absence of ANXA1, LPS-induced phospho-ERK was significantly increased (Fig. 4B). No significant inhibitory effect of DEX on LPS-induced ERK activity was observed in either WT or ANXA1−/− cells, suggesting that although MAPK-dependent effects of ANXA1 were critical to increased LPS responses they were not involved in the effects of ANXA1 on GC sensitivity. We therefore examined an alternate GC-induced modulator of macrophage activation, GILZ. GILZ expression was dose-dependently induced by DEX in WT macrophages, but the effect of DEX on GILZ expression in ANXA1-deficient cells was markedly and significantly impaired (Fig. 4C).

We next sought to confirm the requirement for ANXA1 in the regulation of macrophage GILZ expression. Western blotting was insufficiently sensitive to detect significant differences in GILZ protein in ANXA1 macrophages compared with WT cells (Fig. 5A). However, a trend toward lower DEX-induced GILZ protein was observed, so GC-induced GILZ protein expression was therefore further analyzed more quantitatively by

FIGURE 4. Role of ANXA1 in GC regulation of MKP-1, ERK phosphorylation, and GILZ. A, Macrophages were treated with DEX for 4 h, and MKP-1 mRNA was measured by real-time PCR. Each bar represents mean ± SEM of three separate experiments. B, Macrophages were treated with LPS in the presence or absence of DEX, and cell lysates were analyzed for ERK activation. Top panel: Western blots representative of three experiments. Bottom panel: Each bar represents mean ± SEM of three separate experiments. C, Cells were treated with DEX for 4 h, and GILZ mRNA were measured by real-time PCR. Each bar represents mean ± SEM of three separate experiments performed in duplicate. *, p < 0.05 ANXA1−/− vs WT.
flow cytometry. Using this technique, DEX (3 h) was observed to significantly increase GILZ protein in WT but not ANXA1/macrophages (Fig. 5B). To confirm the effects of ANXA1 on GILZ in vivo, mice were treated with i.p. injection of vehicle or DEX for 3 h, and GILZ protein was detected in peritoneal macrophages by flow cytometry. Treatment with DEX in vivo significantly increased GILZ protein in WT but not ANXA1/macrophages (Fig. 5C).

To further confirm the role of ANXA1 in GC regulation of GILZ, silencing of ANXA1 expression was performed in WT macrophages by siRNA. Silencing efficiency was demonstrated by detecting ANXA1 protein and mRNA expression, which were reduced by 75% compared with cells transfected with control siRNA (Fig. 6, A and B). The biological effect of silencing ANXA1 expression was confirmed, with significantly increased LPS-induced TNF measured in ANXA1 siRNA-transfected cells (Fig. 6C). ANXA1 siRNA significantly reduced DEX-induced GILZ mRNA in WT cells (Fig. 6D). In keeping with this, transient transfection of ANXA1 in ANXA1−/− macrophages increased GILZ protein to the level of WT cells (Fig. 6E). Restoring ANXA1 expression also restored DEX responsiveness with respect to GILZ mRNA induction (Fig. 6F).

**Functional effects of GILZ in ANXA1−/− macrophages**

Major anti-inflammatory effects of GILZ are mediated through inhibition of the NF-κB pathway. This was therefore examined by analysis of NF-κB p65 (Ser^536) phosphorylation and NF-κB reporter gene assays. LPS significantly increased NF-κB p65 Ser^536 phosphorylation in both WT and ANXA1−/− cells (Fig. 6G). A greater increase of p65 phosphorylation in ANXA1−/− cells was observed basally and in response to LPS. DEX partially but significantly inhibited LPS-induced p65 Ser^536 phosphorylation in WT cells at 1 h; however, this action of DEX was impaired in ANXA1−/− cells. LPS activation of an NF-κB luciferase reporter
gene assay was completely inhibited by DEX in WT cells (Fig. 6H). LPS-induced NF-κB reporter activity was significantly increased in ANXA1−/− cells, and DEX inhibition of NF-κB activity was significantly abrogated. Overexpression of ANXA1 in ANXA1−/− cells inhibited LPS-induced NF-κB reporter activity to WT levels, and no further inhibition was observed with DEX treatment of ANXA1 overexpressing cells (Fig. 6H).

To confirm the contribution of GILZ to the phenotype of ANXA1-deficient macrophages, we transiently overexpressed GILZ in ANXA1−/− macrophages. GILZ overexpression in ANXA1−/− macrophages inhibited LPS-induced IL-6 to levels comparable to that in DEX-treated control-transfected cells and restored the ability of DEX to significantly suppress IL-6 and TNF release (Fig. 7, A and B). Examination of IL-6 and TNF mRNA by real-time PCR revealed similar trends. Overexpression of GILZ in ANXA1−/− macrophages resulted in reduction of LPS-induced IL-6 and TNF mRNA comparable to the expression observed in DEX-treated control-transfected cells, and this was not further suppressible by DEX (Fig. 7, C and D). Overexpression of GILZ in ANXA1−/− macrophages significantly reduced LPS-induced ERK MAPK phosphorylation (Fig. 7E) but failed to inhibit LPS-induced NF-κB p65 phosphorylation (Fig. 7F). In contrast, GILZ overexpression in ANXA1−/− macrophages significantly reduced NF-κB reporter activity basally and in response to LPS and DEX (Fig. 7G).

**ANXA1 regulation of GILZ promoter**

To examine how ANXA1 regulates GILZ expression, we first assessed whether ANXA1 regulates GILZ via effects on the GILZ
promoter. Transfection of ANXA1 in ANXA1−/− cells significantly increased GILZ reporter luciferase activity in comparison with control plasmid (Fig. 8A), confirming a transcriptional effect of ANXA1 on GILZ expression. Whether usage of ANXA1 receptors is required for ANXA1 regulation of GILZ was next investigated. In the mouse, the FPR family is complex, with multiple FPR-related receptors (32). Blocking murine FPR using the pan antagonist Boc2 (33) did not significantly reduce DEX-induced GILZ mRNA in WT or ANXA1−/− cells. The biologically active ANXA1-derived N-terminal peptide Ac2-26 interacts with FPR.

**FIGURE 7.** Effect of GILZ overexpression in ANXA1−/− macrophages. ANXA1−/− macrophages were transfected with PcDNA3.1 or murine GILZ plasmid. Transfected cells were treated with LPS and DEX for 24 h. Supernatant IL-6 (A) and TNF (B) concentrations were measured by flow cytometric bead kits. Transfected cells were treated with LPS (0.1 μg/ml) for 4 h in the presence or absence of DEX (10−7 M). IL-6 (C) and TNF mRNA (D) were measured using real-time PCR. GILZ expression cells were treated with LPS over 1 h for ERK (E) and NF-κB p65 activity (F). ANXA1−/− cells were cotransfected with NFκB-luc and GILZ plasmid or PcDNA 3.1, and then NF-κB luciferase reporter activity was measured (G). Bars represent mean ± SEM of four to seven experiments. *, p < 0.05 and **, p < 0.01 ANXA1−/− vs WT.

**FIGURE 8.** Regulation of GILZ promoter by ANXA1. A, ANXA1−/− cells were cotransfected with GILZ promoter-luc and ANXA1 plasmid or pcDNA3.1 using AMAXA system. GILZ luciferase activity was measured after 48 h. B, WT and ANXA1−/− macrophages were pretreated with Boc2 (100 μM) in the presence or absence of DEX (0.1 μM) for 3 h. GILZ mRNA was analyzed by quantitative PCR. C, GILZ mRNA was also examined in ANXA1−/− cells treated with ANXA1 peptide Ac2-26 (100 μg/ml) for 3 h. Bars represent mean ± SEM of three duplicate experiments. *, p < 0.05.
MAPK activation is inhibitory. ANXA1-deficient fibroblasts, we believe the current data demonstrate that ANXA1, and our previous report of increased MAPK activation in the setting of reduced expression of ANXA1 on the one hand and deletion of endogenous ANXA1 on the other hand are difficult to reconcile. However, given the confirmation here of the full inhibitory effect of GC on macrophages in vitro and providing for the first time a mechanism for these observations.

The mechanism(s) through which ANXA1 regulates cytokine expression, either in the setting of LPS stimulation or GC inhibition, has not been previously established. We recently reported that cultured fibroblasts from ANXA1-deficient mice demonstrated markedly increased spontaneous IL-6 release, secondary to increased MAPK activation in the setting of reduced expression of the MAPK inhibitory phosphatase MKP-1 (21). We here demonstrate the involvement of ERK and JNK MAPK in the amplified IL-6 and TNF, demonstrating that ANXA1 is required for the full inhibitory effect of GC on macrophages in vitro and providing for the first time a mechanism for these observations.

Unexpectedly, we were unable to demonstrate an effect of ANXA1 on MKP-1 expression in macrophages and found no evidence of altered GC regulation of MAPK activation in the absence of ANXA1. MKP-1 is a critical GC-induced negative regulator of MAPK activity and innate immune responses, with increased LPS-induced MAPK activation and cytokine production observed in MKP-1-deficient mice (16–19). MKP-1 has chiefly been reported to dephosphorylate activated p38 and JNK MAPK, with lesser effects on ERK, and through this mechanism, MKP1 is thought to be a dominant mediator of the effects of GC on MAPK pathways. We observed no effect of ANXA1 deficiency on macrophage MKP-1 expression basically, under LPS stimulation, or in response to GC and, in parallel with this, observed no evidence for differences in GC effects on ERK MAPK in ANXA1−/− cells. These findings are in contrast to previous findings in fibroblasts and suggest that ANXA1 does not regulate MKP-1 expression in macrophages. It is possible that other members of the DUSP family may be regulated by ANXA1, but this has not been investigated here. However, these findings suggest that notwithstanding the involvement of increased MAPK phosphorylation in response to LPS in explaining increased LPS-induced cytokine release in ANXA1−/− cells, a separate pathway must be involved in ANXA1 mediation of GC effects on macrophage cytokine release.

We observed that in addition to increased ERK MAPK phosphorylation, ANXA1−/− macrophages exhibited increased NF-κB activity in response to LPS. GILZ is a recently described GC-induced anti-inflammatory protein, the reported actions of which include effects on ERK MAPK and NF-κB pathways, suggesting it as a candidate to explain these effects of ANXA1. GILZ binds directly to the p65 subunit of NF-κB (22), impacts via protein-protein interactions with c-fos and c-Jun to affect AP-1 transcriptional activation (23), and inhibits ERK1/2 phosphorylation via interaction with the upstream MAPK pathway (38, 39). The current study provides evidence that GILZ is a previously unrecognized target of ANXA1. We observed reduced basal GILZ expression in ANXA1−/− macrophages, consistent with the increased ERK activation and ERK-dependent cytokine expression observed. ANXA1 regulation of GILZ expression was confirmed using ANXA1 silencing by siRNA in WT macrophages, and confirmation of reduced GC-induced GILZ expression in the absence of ANXA1 was obtained in vivo. Restoring ANXA1 in ANXA1−/− macrophages reconstituted GC inducibility of GILZ, in parallel with restoration of the inhibitory effects of GC on IL-6 and TNF.

The involvement of GILZ in increased ERK MAPK activation in the absence of ANXA1 was supported by inhibition of ERK activation in response to GILZ overexpression in ANXA1−/− cells. Reduced GC-induced GILZ expression in ANXA1−/− macrophages also paralleled reduced GC sensitivity in these cells, and overexpression of GILZ restored GC inhibitory effects on LPS-induced IL-6 and TNF in ANXA1−/− macrophages. Finally, increased NF-κB reporter activation observed in ANXA1−/− macrophages was inhibited by overexpression of either ANXA1 or GILZ. Ser536 phosphorylation of NF-κB p65 is required for NF-κB nuclear translocation and is shown here to be modestly inhibited by endogenous ANXA1. No evidence for involvement of GILZ in this phenomenon was adduced, suggesting it may be secondary to increased cytokine expression in the absence of ANXA1 rather than a GILZ-dependent effect. This is not inconsistent with the observations that GILZ binds directly to NF-κB p65 (22, 39), although an odd study suggest that GILZ influences NF-κB p65 phosphorylation state per se in T cells (40).

The mechanism of ANXA1 regulation of GILZ expression remains unknown. The current data indicate that the effect of GILZ expression is transcriptional, but no effect of the ANXA1 receptor...
lignand Ac2-26 or the ANXA1 receptor antagonist Boc2 was observed, suggesting that ANXA1 effects on GILZ transcription do not require ANXA1 binding with FPR family members.

The findings of this study context can at this stage only be applied to the in vivo with caution. In vivo modulation of GILZ expression by DEX was impaired in ANXA1−/− mice, consistent with this mechanism applying in vivo, and DEX inhibition of LPS-induced IL-6 was impaired in ANXA1−/− mice. However, no difference in DEX inhibition of LPS-induced TNF was observed in ANXA1−/− mice in vivo. Multiple factors may contribute to this, including the fact that in vivo cytokine measurements were made on samples obtained at a single time point, whereas assays of in vitro culture supernatants reflect cumulative effects of a given factor. The effects of ANXA1 on DEX regulation of TNF observed in vitro may not apply at the 1.5-h time point chosen for the in vivo studies reported here. In addition, the effects of GILZ on LPS and GC regulation of cytokines in vivo has not been reported. It is also possible that as yet unknown ANXA1-independent mechanisms are operative in vivo that do not apply in vitro. If a GILZ-deficient mouse becomes available, it will be of interest to observe the effects of ANXA1 on LPS and DEX regulation of cytokines in GILZ-deficient mice.

In conclusion, these data demonstrate that ANXA1 is a critical endogenous negative regulator of the expression of IL-6 and TNF by macrophages, via effects on MAPK phosphorylation, involving regulation of the expression of GILZ (Fig. 9). GILZ is a previously unrecognized target of ANXA1, and the functions of GILZ have the potential to explain many aspects of the proinflammatory phenotype of ANXA1-deficient mice and cells. Modulation of GILZ expression by manipulation of ANXA1 may afford a mechanism of inhibiting inflammation without GC, which would be an attractive modality for treatment of acute and chronic inflammatory diseases.

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Disclosures
The authors have no financial conflict of interest.


