Glucocorticoid-Induced Leucine Zipper (GILZ) Mediates Glucocorticoid Action and Inhibits Inflammatory Cytokine-Induced COX-2 Expression

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Abstract   Cyclooxygenase-2 (COX-2) plays an important role in rheumatoid arthritis and therefore, has been a major target for anti-arthritis therapies. The expression of COX-2 is induced by inflammatory cytokines such as TNF-α and IL-1β, and inhibited by glucocorticoids. However, the molecular mechanisms underlying the anti-inflammatory and immune suppressive actions of glucocorticoids are not well defined. Here we report that glucocorticoid-induced leucine zipper (GILZ) mimics glucocorticoid action and inhibits inflammatory cytokine-induced COX-2 expression in bone marrow mesenchymal stem cells, the cells that have been recently implicated in the pathogenesis and progression of rheumatoid arthritis. Using a retrovirus-mediated gene expression approach we demonstrate that overexpression of GILZ inhibits TNF-α and IL-1β-induced COX-2 mRNA and protein expression, and knockdown of GILZ by shRNA reduces glucocorticoid inhibition of cytokine-induced COX-2 expression. Consistent to these results, overexpression of GILZ also inhibits NF-κB-mediated COX-2 promoter activity. Finally, we show that GILZ inhibits COX-2 expression by blocking NF-κB nuclear translocation. Our results suggest that GILZ is a key glucocorticoid effect mediator and that GILZ may have therapeutic value for novel anti-inflammation therapies. J. Cell. Biochem. 103: 1760–1771, 2008.   © 2007 Wiley-Liss, Inc.

Key words: arthritis; COX-2; GILZ; glucocorticoid; mesenchymal stem cell

Rheumatoid arthritis is a chronic inflammatory disease characterized by pain, swelling, and joint destruction [Firestein, 2003]. The exact causes of rheumatoid arthritis are not clear but an elevated cyclooxygenase-2 (COX-2) expression seems tightly associated with this disease [Crofford, 2000; Fitzpatrick, 2004]. Therefore, COX-2 has been a major target for anti-arthritis therapies [Hinz and Brune, 2002; Buttgereit et al., 2004]. The expression of COX-2 is induced by many stimuli, including pro-inflammatory cytokines such as IL-1β and TNF-α, which are abundant in the arthritic joints [Dayer, 2002;

Wadleigh and Herschman, 1999], and is inhibited effectively by glucocorticoids (GCs) [Newton et al., 1997; Morisset et al., 1998]. However, the molecular mechanisms by which GCs exert their anti-inflammatory and immune suppressive actions are not clear despite the fact that GCs have been widely used for the treatment of rheumatoid arthritis and other chronic inflammatory and autoimmune diseases [Conn, 2001; Buttgereit et al., 2004]. Importantly, long-term GC therapy causes, among other adverse effects, rapid bone loss resulting in osteoporosis [Lukert and Raisz, 1990; Kirwan, 2000]; therefore, their use is limited.

Recently, a new transcription factor, glucocorticoid-induced leucine zipper (GILZ), was identified [D'Adamio et al., 1997]. GILZ, which is also induced by anti-inflammatory IL-10 [Berrebi et al., 2003], is a member of the leucine zipper protein family [Cannarile et al., 2001] and belongs to the transforming growth factor-beta (TGF-β)-stimulated clone-22 (TSC-22) family of transcription factors [Shibanuma

et al., 1992]. GILZ has been shown to interact with and inhibit the activities of the key inflammatory signaling mediators NF-κB and AP-1 [Ayroldi et al., 2001; Mittelstadt and Ashwell, 2001]. GILZ can also interact with the mitogen-activated protein kinase (MAPK) family member, Raf1, resulting in inhibition of Raf-1 phosphorylation and, subsequently, inhibition of MEK/ERK-1/2 phosphorylation and AP-1-dependent transcription [Ayroldi et al., 2002]. Moreover, GILZ can inhibit IL-2 and IL-5 expression [Berrebi et al., 2003; Arthaningtyas et al., 2005] and stimulates IL-10 production by immature dendritic cells (DCs), therefore preventing the production of inflammatory chemokines by activated DCs [Cohen et al., 2005]. These lines of evidence suggest that GILZ is capable of transducing the anti-inflammatory and immune suppressive actions of GCs.

Mesenchymal cells or fibroblast-like synovio-cytes (FLS) are one of the two major populations of resident synoviocytes that form the pannus, a structure that causes invasive joint destruction and acute inflammation in rheumatoid arthritis [Firestein, 2003]. Recent evidence shows that the onset of rheumatoid arthritis is associated with a massive influx of mesenchymal cells into the joint [Jorgensen et al., 2002; Jones et al., 2004]. These mesenchymal cells or FLS are now identified as bona fide bone marrow mesenchymal stem cells (MSCs). These MSCs are recruited to the arthritic joints but, due to the inflammation, their normal differentiation is arrested and they acquire a "tumor-like" phenotype and are thought to play a key role in the pathogenesis of rheumatoid arthritis [Li and Makarov, 2006]. In this report we investigated the molecular mechanisms by which GILZ mediates GC effects and inhibits COX-2 expression in mouse bone marrow-derived MSCs.

MATERIALS AND METHODS

Reagents

Recombinant murine IL-1β and TNF-α (Cat # 401-ML-005 and 401-MT-010, respectively) were purchased from R&D Systems; COX-2 antibody (Cat # 160126) was from Cayman Chemical; p65 monoclonal antibody (Cat # sc-8008) was from Santa Cruz Biotech, Inc.; anti-rabbit, anti-mouse IgG-Cy3 conjugate and anti-rabbit IgG-FITC conjugate (Cat # 81-6115, 81-6515, and 81-6111, respectively) were from Zymed Laboratories, Inc.; IRDye 800 anti-rabbit IgG (Cat # 611-132-122) was from Rockland Immuno-chemicals, Inc. GILZ antibody was custom-made and described previously [Shi et al., 2003]. All other reagents were purchased from Sigma-Aldrich except where specified.

Mouse Bone Marrow MSCs

Bone marrow-derived MSCs used in this study were isolated from 18-month-old C57BL/6 mice (National Institute on Aging, Bethesda, MD). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Georgia. In brief, the MSCs were isolated from long bones (from 6 mice) using negative-immuno-depletion followed by positive-immuno-selection approaches. These MSCs, which are characterized as CD-11b⁻, CD-11c⁻, CD45R/B220⁻, PDCA-1⁻, and Sca-1⁺, are capable of undergoing osteogenic, adipo-genic, and myogenic differentiation (Zhang et al., unpublished work). The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone) and penicillin/streptomycin. MC3T3-E1 cells (ATCC) were maintained in α-MEM supplemented with 10% FBS and antibiotics. C3H10T1/2 cells (ATCC) were maintained in DMEM supplemented with 10% FBS and antibiotics.

Production of Recombinant Retroviruses and Infection

The retroviral vectors expressing GILZ (ΔU3-GILZ) or green fluorescent protein (GFP) (ΔU3-GFP) were constructed in a replication-defective ΔU3nlsLacZ vector [Ory et al., 1996]. In brief, the LacZ sequence in the parental ΔU3nlsLacZ vector was excised by *Xba*I and *Bam*HI and replaced with the full-length coding region of mouse GILZ (Accession #: AF024519) or GFP cDNA. *Xba*I and *Bam*HI restriction sites were incorporated into the 5′ and the 3′ ends of the PCR products, respectively, when GILZ and GFP cDNA were amplified by PCR. The retroviral particles (Ret-GILZ and Ret-GFP) were produced by transfecting ΔU3-GILZ or ΔU3-GFP plasmid DNA into the retroviral packaging cell line 293GPG and harvested as described [Ory et al., 1996].

For infection, 2 ml of viruses prepared above was added to the cells (seeded in a 60-mm dish the previous day at ∼70% confluence) and incubated at 37°C in the presence of 8 μg/ml

polybrene. The medium containing viruses was removed from the cells 6 h after infection and replaced with fresh media.

For in vitro experiments, the infected cells were seeded at a density of 1×10^6 cells/cm^2 and stimulated as indicated.

Western Blot Analysis

Western blot analyses were performed as previously described [Shi et al., 2003]. In brief, the cells were treated as indicated and then harvested in lysis buffer. Equal amounts of total protein were separated on 12% SDS–PAGE, transferred onto nitrocellulose membrane, and blocked in 5% non-fat dry milk for 2 h at RT. The membranes were then incubated with the indicated primary antibodies for at least 1 h at RT. After several washes the membranes were incubated with IRDye 800 secondary antibody and scanned using Odyssey Infrared Imaging System (LI-COR Biotechnology).

GILZ Knockdown Constructs and Lentivirus Production

Knockdown constructs expressing small hairpin interfering RNA (shRNA) targeting endogenous GILZ mRNA (shGILZ) were generated in a lentivirus-based shRNA vector pLL3.7 [Rubinson et al., 2003]. This vector contains an EGFP cassette downstream of the shRNA sequence and is driven by CMV promoter. In brief, the GILZ targeting sequences were designed using shRNA Target Finder tool (www.genscript.com). Three candidate sequences with different GC content were selected and inserted into the *Hpa*I and *Xho*I sites of the pLL3.7 vector. Two constructs, shGILZ-1, which blocks Dex-induced endogenous GILZ protein expression, and shGILZ-2, which does not block Dex-induced GILZ expression (as a control), were used in the study. The targeting sequences are as follows: shGILZ-1: 5′-T<u>CCAGGATTTGGATTTGGAT</u>**TT-CAAGAGA**<u>ATCCAAATCCAAATCTGG</u>*TTTTT-T*C-3′ (top strand), and 5′-TCGAG*AAAAAA*<u>CCAG-GAT TTGGATTTGGAT</u>**TCTCTTGAA**<u>ATCCAA-ATCCAAATCCTGG</u>A-3′ (bottom strand); shGILZ-2: 5′-T<u>GCAGAAGCAACCTCTC TCT</u>**TTCA-AGAGA** <u>AGAGAGAGGTT GCTTCTGC</u>*TT-TTTT*C-3′ (top strand), and 5′-TCGAG*AAA-AAAA*<u>GCAGAAGCA ACCTCTCTCT</u>**TCTCTT-GAA**<u>A-GAGAGAGGTTG CTTCTGCA</u>-3′ (bottom strand). Underlined: hairpin sequence; bolded: loop sequence; italicized: terminator

sequence (6 Ts in top strand or 6 As in bottom strand). *Hpa*I and *Xho*I restriction sites are included at the 5′ and 3′ ends, respectively. To produce lentivirus, shGILZ-1 or shGILZ-2 vector was cotransfected with packaging vectors pCMV-VSV-G (envelope) and pHR'8.9 ΔVPR (core protein) into 293T cells using Fugene 6 Reagent (Roche Diagnostic Corp.). The lentiviral particles were harvested 48 h post-transfection as described [Rubinson et al., 2003].

RNA Extraction and Real-Time RT-PCR

RNA isolation, reverse transcription, and PCR analysis were performed as previously described [Shi et al., 2003]. In brief, total cellular RNA was isolated using TRIzol reagent (Invitrogen Corp.), 2 µg of RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems), and the mRNA levels of the indicated genes were analyzed in triplicate using SYBR Green Mater Mix (Applied Biosystems) and a Chromo-4 real-time RT-PCR instrument (MJ Research). The mRNA levels were normalized to β-actin (internal control) and gene expression was presented as fold changes (ΔΔCt method) [Pfaffl, 2001]. The primer sequences used in the PCR reactions were: 5′-ACTCACTCAGTTTGTTGAGTCAT T-C-3′ (forward) and 5′-TTTGATTAGTACTG-TAGGGTTAATG-3′ (reverse) for COX-2 (Gene Bank Accession #: M64291), and 5′-CTGGCAC-CACACACCTTCTACA-3′ (forward) and 5′-GGTA-CGACCAGAGGCATACA-3′ (reverse) for β-actin (GeneBank Accession #: NM_007393).

Transient Transfection and Luciferase-Reporter Assays

Transient transfection and promoter luciferase reporter assays were performed as previously described [Shi et al., 2003]. Two luciferase reporters WT724-Luc and pNF-κB-Luc were used. WT724-Luc is a well-characterized mouse COX-2 promoter fragment linked to luciferase reporter gene. This 724-bp COX-2 promoter fragment contains well-characterized NF-κB binding sites within the first 400-nucleotide relative to the transcription start site [Wadleigh et al., 2000]. The pNF-κB-Luc consists of three κB concatamers from the HIV-long terminal repeat inserted upstream of a concanavalin-A minimal promoter driving the expression of luciferase. In brief, WT724-Luc or pNF-κB-Luc was cotransfected with the

indicated amounts of expression vectors and an internal control plasmid (pRL-TK) into C3H10T1/2 mouse mesenchymal cells using Lipofectamine Plus reagents (Invitrogen Corp.). Total amounts of DNA in each transfection were kept constant by adding empty vector (pcDNA3). Twenty-four hours after transfection, the cell lysates were harvested and luciferase activity measured using Dual-Luciferase Reporter Assay Kit (Promega Incorp.) and a Vector3V Multilabel Counter (Perkin-Elmer).

Immunofluoresence Labeling and Imaging

Cells grown in chamber slides were fixed with freshly prepared 4% paraformaldehyde containing 0.2% Triton X-100 for 15 min and blocked in 2% BSA for 1 h at RT before incubating with GILZ, or p65 primary antibodies (1:200 dilution) for at least 1 h at RT. After several washes, the slides were incubated with goat anti-rabbit or anti-mouse IgG-Cy3 or IgG-FITC secondary antibody (1:600 dilution) for 1 h at RT in dark. The slides were washed three times in PBS for 5 min each and stained with DAPI (300 nM) to visualize the nucleus.

Finally, the slides were washed, mounted with Vectorshield mounting media (Vector Laboratories), and analyzed using a Nikon TE2000 fluorescence microscope equipped with COOLSNAP Monochrome Camera. Images were acquired and processed with Metamorph Imaging System.

RESULTS

Induction of COX-2 Expression by Pro-Inflammatory Cytokines in MSCs

TNF-α and IL-1β are major inflammatory cytokines that induce COX-2 expression and orchestrate inflammation in rheumatoid arthritis. GCs inhibit this cytokine-induced COX-2 expression. To investigate whether TNF-α and IL-1β can induce COX-2 expression in MSCs, we treated the cells with either different concentrations of TNF-α and IL-1β, or a mixture of TNF-α and IL-1β (10 ng/ml each) for the indicated times. The cells were lysed and equal amounts of total protein were separated on SDS–PAGE and analyzed by Western blot using COX-2-specific polyclonal antibody. The
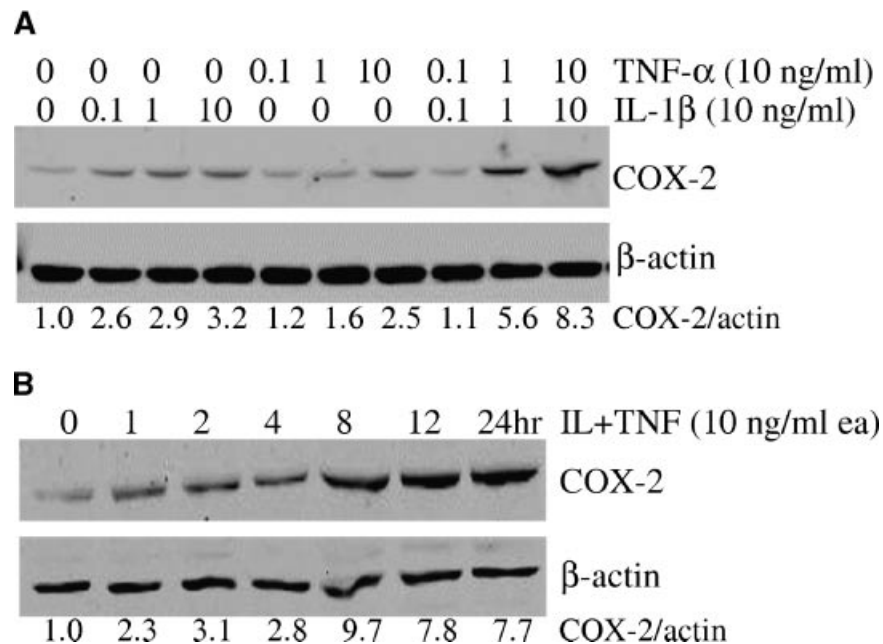

Fig. 1. Cytokine dose- and time-course induction of COX-2 in MSCs. Mouse MSCs were serum-starved (0.2% FBS) for 16 h and then treated with different concentrations of IL-1β, TNF-α, or both IL-1β and TNF-α for 8 h (**A**), or treated with IL-1β and TNF-α (10 ng/ml each) for the indicated times (**B**). The cells were lysed and equal amounts of total protein were separated on SDS–PAGE, transferred onto membranes, and probed with COX-2-specific antibody. Levels of COX-2 protein were detected by incubating the membrane with IRDye 800 anti-rabbit IgG and imaging with Odyssey Infrared Imaging System. Equal loading of the lanes is demonstrated by stripping and re-probing the same membranes with β-actin antibody. The intensity of the bands was measured densitometrically, and the levels of COX-2 expression were expressed as a ratio of COX-2/β-actin. The value from vehicle treated Ret-GFP retrovirus-infected cells is arbitrarily set as 1. The experiment was performed independently a minimum of three times and a representative results of these experiments is shown.

results show that COX-2 expression is induced by TNF-α or IL-1β in dose- and time-dependent manners (Fig. 1), indicating that bone marrow MSCs are responsive to cytokine stimulation and that they may play a role in the pathogenesis of arthritis.

Overexpression of GILZ Inhibits Inflammatory Cytokine-Induced COX-2 Expression

To test the hypothesis that GC inhibition of COX-2 is mediated by GILZ, we first examined whether GCs can induce GILZ expression in MSCs. MSCs were treated with dexamethasone (Dex, 100 nM), and whole cell lysates were analyzed by Western blot using GILZ polyclonal antibodies. The results show that Dex induces GILZ expression rapidly (within 1 h) and the expression reaches a peak level between 3 and 6 h (Fig. 2A).

Next, we generated GILZ-expressing retroviruses (Ret-GILZ) and infected MC3T3-E1 cells, a cell line that has been shown to express COX-2 in response to cytokine stimulation [Wadleigh and Herschman, 1999; Okada et al., 2000]. As a control, the cells were also infected with GFP-expressing retroviruses (Ret-GFP)
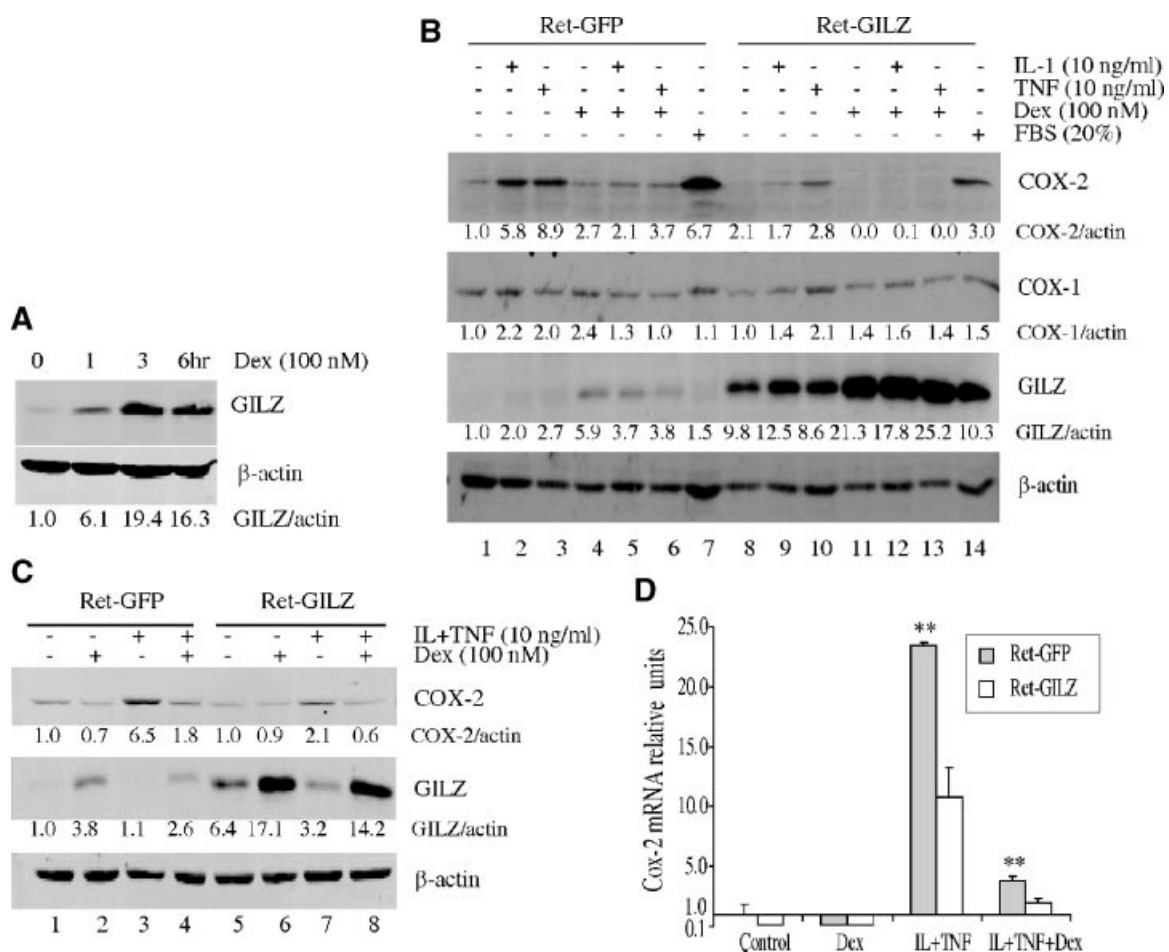

Fig. 2. Effect of GILZ Overexpression on COX-2 Induction by Inflammatory Cytokines. **A:** Dex induces GILZ expression in MSCs. MSCs were treated with Dex (100 nM) for 1, 3, or 6 h and total cell lysates (40 μg/lane) were analyzed by Western blot using GILZ antibody. **B:** MC3T3-E1 cells were infected with Ret-GILZ or Ret-GFP retroviruses, serum-starved, and treated with vehicle (EtOH), IL-1β, TNF-α, Dex, FBS, or in combinations of IL-1β + Dex and TNF-α + Dex for 8 h. Total cell lysates (30 μg/lane) were analyzed by Western blot using antibodies against proteins indicated on the right. This experiment was repeated a minimum of three times. **C:** MSCs were infected as in B and treated as indicated. Western blot was performed as in B. The intensity of bands in each blot was measured densitometrically. The changes of COX-2 (or GILZ) expression level are presented as a ratio of COX-2 (or GILZ)/β-actin and are shown at the bottom of the panel. The value of COX-2 (or GILZ)/β-actin from control (**panel A**) or Ret-GFP retrovirus-infected cells (**panels B** and **C**) is arbitrarily set as 1. **D:** GILZ inhibits COX-2 mRNA expression. MSC-GFP and MSC-GILZ cells were treated as in C, and levels of COX-2 mRNA were analyzed by real-time RT-PCR. The relative amounts of COX-2 mRNA are presented as arbitrary units after normalization to β-actin (fold change relative to Ret-GFP control group). Student's t-test was used to determine the statistical differences between each group; **$P < 0.01$.

and used in parallel. The retrovirus-infected cells were serum-starved and then stimulated with IL-1β (10 ng/ml), TNF-α (10 ng/ml), Dex (100 nM), 20% FBS (positive control), vehicle (ethanol, negative control), IL-1β plus Dex, or TNF-α plus Dex for 8 h before they were harvested and subjected to Western blot analysis. The results show that IL-1β and TNF-α induce COX-2 expression in Ret-GFP retrovirus-infected cells (Fig. 2B, compare lanes 2 and 3 to lane 1), and as expected, Dex represses it (lanes 5 and 6). However, this cytokine-induced COX-2 expression is significantly inhibited in Ret-GILZ retrovirus-infected cells (compare lanes 9 and 10 to lanes 2 and 3). It is noted that while the 20% FBS-induced COX-2 is attenuated by GILZ overexpression, it is not reduced to the degree as is seen in TNF-α and IL-1β treated cells (lane 14). This suggests that alternative mechanisms of COX-2 induction are involved since FBS contains multiple stimuli (cytokines, hormones, and growth factors) that can induce COX-2 expression. Further investigation is required to address this issue. The expression of another COX isoform, COX-1, is not affected. Since the combination of TNF-α and IL-1β shows an additive effect, and both cytokines share a common signaling pathway, we used the combination of these two cytokines (10 ng/ml each) in rest of the experiments to highlight GILZ anti-inflammatory effect. A similar experiment was then performed using GILZ- or GFP-expressing retrovirus-infected MSCs, with the same results, that is, overexpression of GILZ inhibits IL-1β- and TNF-α-induced COX-2 expression (Fig. 2C). It is noted that in the presence of Dex, the inhibition appears to be much stronger in cells overexpressing GILZ (B, lanes 12 and 13; C, lane 8). This, most likely, is due to the induction of endogenous GILZ (middle panel) and the activation of GC receptors (GRs) by Dex, an event that is known to inhibit NF-κB and AP-1 transcriptional activities [Yang-Yen et al., 1990; Ray and Prefontaine, 1994]. In both experiments, equivalent loading of lanes is demonstrated by immuno-labeling for β-actin (bottom panels). The intensity of bands was measured densitometrically, and the changes of COX-2 and GILZ expression levels are presented as a ratio of COX-2/β-actin or GILZ/β-actin. The value from vehicle treated Ret-GFP retrovirus-infected cells is arbitrarily set as 1.

To determine whether COX-2 mRNA is also inhibited by GILZ, we treated retrovirus-infected MSCs as in C except that total RNA was isolated 4 h post-treatment, and performed real-time RT-PCR analysis. As expected, and consistent with the Western blot results, the expression of basal and cytokine-induced COX-2 mRNA is inhibited significantly in Ret-GILZ retrovirus-infected MSCs (Fig. 2D). In contrast, COX-2 mRNA is induced significantly (∼25-fold) by IL-1β and TNF-α in Ret-GFP retrovirus-infected control MSCs. Together, these results demonstrate that overexpression of GILZ inhibits inflammatory cytokine-induced COX-2 mRNA and protein expression, an effect that is equivalent to that produced with GC treatment.

Knockdown of GILZ Reduces GC Inhibition of Inflammatory Cytokine-Induced COX-2 Expression

To confirm that GC inhibition of cytokine-induced COX-2 expression is mediated by GILZ, we performed loss-of-function studies. Two lentivirus-based small hairpin RNAi constructs (shGILZ-1 and -2) were generated and viral particles were produced. The knockdown efficiency of these constructs was tested by infecting MSC cells. After induction with Dex (100 nM for 6 h), the lentivirus-infected cells were fixed and immuno-labeled with GILZ antibody. As shown in Figure 3A, shGILZ-1 significantly reduced GC-induced GILZ expression (top panels), but shGILZ-2 had no effect (middle panels) and used as negative controls in the subsequent experiments. Induction of GILZ by Dex in uninfected MSCs was also shown (bottom panels). These lentivirus-infected MSCs were treated with Dex (100 nM for 2 h to induce GILZ expression), washed and replaced with fresh media before they were treated with IL-1β and TNF-α (10 ng/ml each for 8 h to induce COX-2 expression). As a control, MSCs were also infected with lentiviruses produced from empty vector pLL3.7, which does not contain any shRNA sequence. Western analysis results show (Fig. 3B) that shGILZ-1, which reduced Dex-induced GILZ expression (middle panel, lane 6), failed to inhibit IL-1β and TNF-α-induced COX-2 expression in the presence of Dex (top panel, compare lane 7 to lane 8). In contrast, IL-1β and TNF-α-induced COX-2 expression was reduced significantly by Dex in cells that were infected with pLL3.7
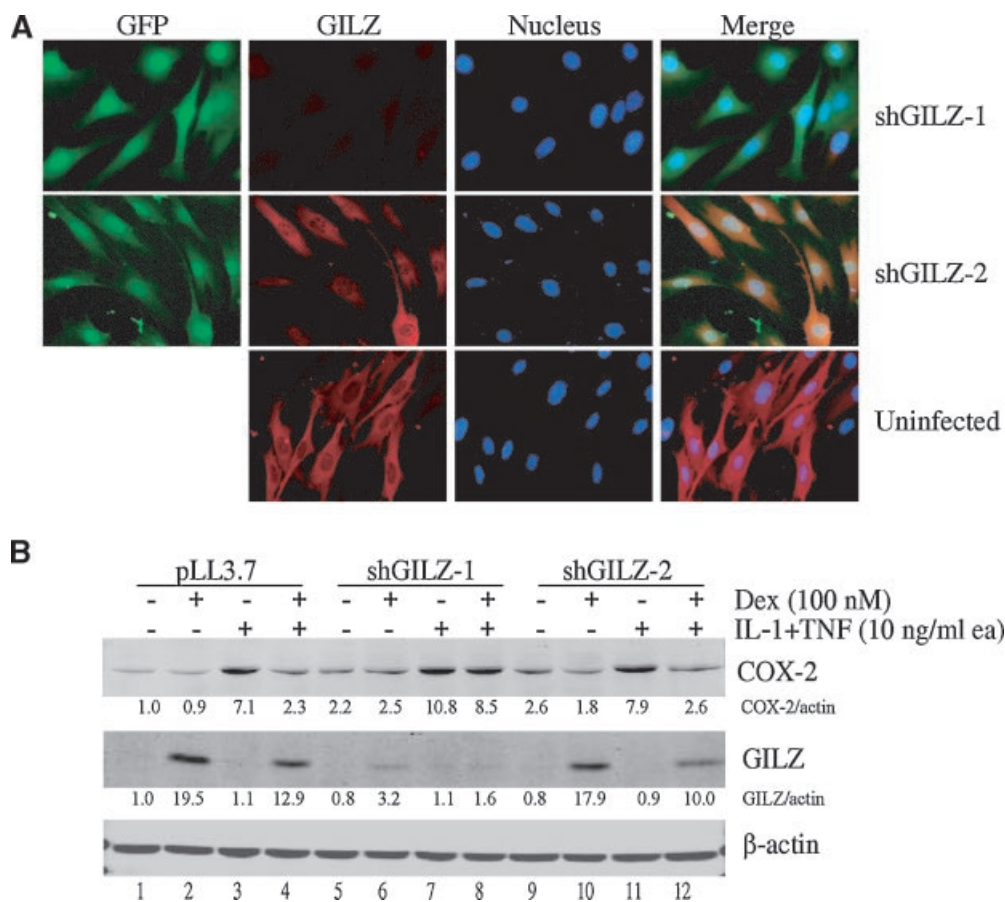
Fig. 3. Effect of GILZ knockdown on GC inhibition of inflammatory cytokine-induced COX-2 expression. **A**: MSCs were infected with lentiviruses expressing shRNA (shGILZ-1 and -2) targeting GILZ. Forty-eight hours after infection the cells were treated with Dex (100 nM for 6 h), immuno-labeled with GILZ antibody and detected by anti-rabbit-Cy3 using a fluorescence microscope. The infected cells are visualized by the expression of GFP, an expression cassette included in the viral vector. Note: shGILZ-1 significantly reduced Dex-induced GILZ expression (**upper panel**) but shGILZ-2 had no effect (**middle panel**) and was used in B as a control. Dex-induced GILZ expression in uninfected MSCs is also shown (**bottom panel**). This experiment was repeated at least three times. **B**: MSCs were infected with a control (pLL3.7); shGILZ-1; or shGILZ-2 lentiviruses. The cells were pretreated with

Dex (100 nM for 2 h) and then treated with IL-1β and TNF-α (10 ng/ml each) for 8 h before harvest. Equal amounts of total protein were analyzed by Western blot for COX-2 (top) and GILZ (middle) expression. Equal loading of lanes is shown by labeling of β-actin (bottom). Note: the shGILZ-2 virus, which does not knockdown Dex-induced GILZ expression (compare lane 10 to lane 6), has no effect on Dex inhibition of cytokine-induced COX-2 expression (compare lane 12 to lane 10). The intensity of bands in each blot was measured densitometrically. The changes of COX-2 (or GILZ) expression level are presented as a ratio of COX-2 (or GILZ)/β-actin and are shown at the bottom of the panel. The value of COX-2 (or GILZ)/β-actin from vehicle treated pLL3.7 lentivirus infected cells (lane 1) is arbitrarily set as 1. This experiment was repeated at least three times.

or shGILZ-2 control lentiviruses (top panel, compare lanes 3 to 4 and 11 to 12). It should be emphasized that these control viruses did not inhibit Dex-induced GILZ expression (middle panel, lanes 2 and 10). Equal loading of lanes is shown by labeling for β-actin (bottom panel). The intensity of bands was measured densitometrically, and the expression levels of COX-2 or GILZ were expressed as a ratio of COX-2 or GILZ to β-actin. These results confirm that inhibition of cytokine-induced COX-2 by Dex is dependent on GILZ expression.

GILZ Inhibits NF-κB-Mediated COX-2 Transcription

The transcription of COX-2 is activated by direct binding of NF-κB to the COX-2 gene promoter [Mestre et al., 2001]. To determine whether GILZ inhibits NF-κB-mediated COX-2 transcription, we performed transient transfection and luciferase-reporter assays using a well-characterized mouse COX-2 promoter-luciferase reporter construct WT724-Luc and an artificial NF-κB-responsive luciferase reporter construct

(see Materials and Methods Section for details). C3H10T1/2 cells were cotransfected with WT724-Luc, NF-κB (p65 and p50) and increasing amounts of GILZ expression plasmids, and the luciferase activity was measured 24 h after transfection. Results show that overexpression of NF-κB activated COX-2 promoter activity (sevenfold), but this activation was inhibited dose-dependently by increasing levels of GILZ expression (Fig. 4A). The specific inhibition of NF-κB activity by GILZ was further confirmed in a similar experiment by GILZ dose-dependent inhibition of pNF-κB-Luc, an artificial NF-κB-dependent luciferase reporter (Fig. 4B). In the absence of NF-κB overexpression, GILZ also showed some inhibition effects on promoter activity, most likely due to the inhibition of endogenous NF-κB activity. Together, these and real-time RT-PCR results demonstrate that GILZ inhibits NF-κB-mediated COX-2 gene transcription.

GILZ Inhibits Cytokine-Induced NF-κB Nuclear Translocation

Inactive NF-κB (p65 subunit) is localized in the cytoplasm. Upon activation (i.e., cytokine stimulation), p65 translocates into the nucleus and activates target gene transcription. Studies by Ayroldi et al. [2001] showed that GILZ interacts with p65 and blocks its nuclear translocation in T cells. Because p65 nuclear translocation is a key step for NF-κB to function in the nucleus, we also examined whether GILZ can

block cytokine-induced p65 nuclear translocation in MSCs. MSCs were infected with GILZ (Ret-GILZ)- or GFP-expressing (Ret-GFP) retroviruses and treated with or without TNF-α (10 ng/ml for 30 min) as indicated. The Ret-GILZ virus-infected cells were immuno-labeled with GILZ (green) polyclonal and p65 (red) monoclonal antibodies and detected by FITC- or Cy3-conjugated secondary antibodies using fluorescence microscopy. The Ret-GFP virus-infected cells were labeled with p65 and detected by Cy3-conjugated secondary antibody. As shown in Figure 5, treatment of the cells with TNF-α induced p65 nuclear translocation of Ret-GFP virus-infected or uninfected control cells. In contrast, this cytokine-induced p65 nuclear translocation was largely blocked by GILZ overexpression. The p50 subunit is located in the nucleus with or without GILZ overexpression or cytokine stimulation (data not shown). These results suggest that inhibition of cytokine-induced NF-κB nuclear translocation is a key mechanism by which GILZ inhibits COX-2 and other cytokine-induced inflammatory genes.

DISCUSSION

We present evidence in this report that GILZ transduces the anti-inflammatory effect of GCs and inhibits inflammatory cytokine-induced COX-2 expression in bone marrow MSCs. We used GILZ gain-of-function and loss-of-function approaches and showed that overexpression of
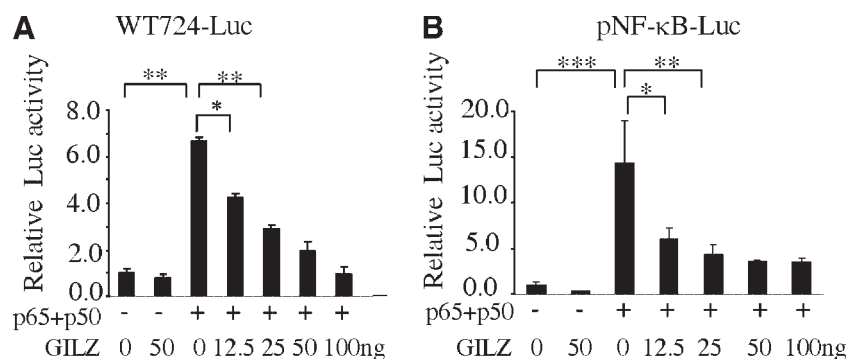

Fig. 4. Effect of GILZ overexpression on COX-2 promoter activity. **A**: C3H10T1/2 cells were cotransfected with WT724-Luc, NF-κB (p65 and p50), and increasing amounts of pcDNA-GILZ expression plasmids to show GILZ dose-dependent inhibition of NF-κB transactivational activity. **B**: C3H10T1/2 cells were cotransfected with pNF-κB-Luc, NF-κB, and increasing amounts of GILZ expression plasmids to show GILZ dose-dependent inhibition of NF-κB-dependent trans-criptional activity. Luciferase activity was measured 24 h after transfection using a dual-luciferase assay kit. The experiments were performed in triplicate a minimum of three times. Results are shown as fold changes and are presented as mean ± SEM. The luciferase activity from reporter (WT724-Luc or pNF-κB-Luc) and empty vector (pcDNA3) transfected cells was arbitrarily set as 1. Student's t-test was used to determine the statistical differences between each group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.
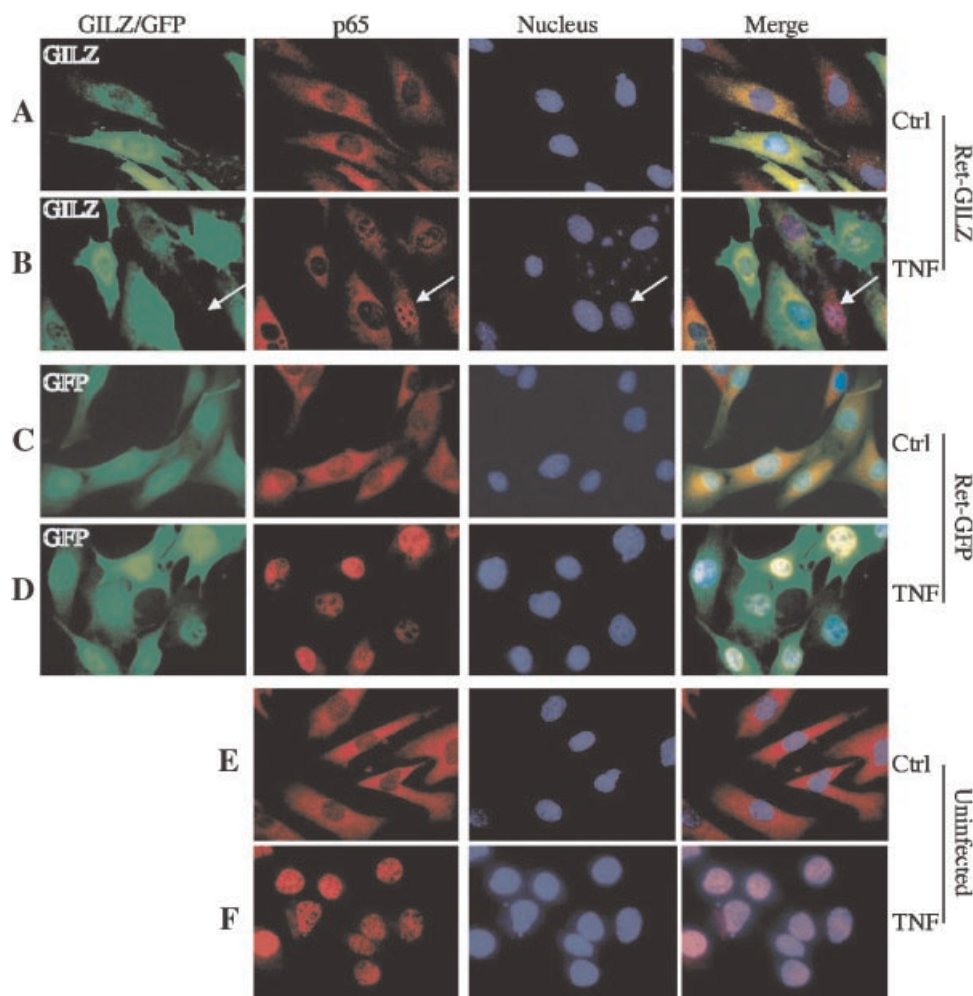
Fig. 5. Effect of GILZ overexpression on inflammatory cytokine-induced p65 nuclear translocation. MSCs were infected with Ret-GILZ (**A, B**), Ret-GFP (**C, D**) retroviruses, or left uninfected (**E, F**). After overnight incubation, the cells were stimulated with or without TNF-α (10 ng/ml for 30 min) as indicated and immuno-labeled with GILZ (**A, B, first column**) and p65 (**A–F, second column**) antibodies. Subcellular localization of GILZ and p65 was visualized by FITC- and Cy3-conjugated secondary antibodies, respectively, using a fluorescence microscope. Nuclei were visualized by counter-staining of the cells with DAPI (**third column**). Note: in the presence of TNF-α, p65 is concentrated in the nucleus of Ret-GFP virus-infected cells (**D**), uninfected control cells (**F**), and the cells that were not infected by Ret-GILZ virus (indicated by an arrow in **B**). This TNF-α-induced p65 nuclear translocation is largely inhibited in Ret-GILZ retrovirus-infected cells (**B**). This experiment was repeated at least four times. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

GILZ inhibits TNF-α and IL-1β-induced COX-2 expression, and knockdown of GILZ reduces GC inhibitory effect on cytokine-induced COX-2 expression.

GCs are widely used for the treatment of many chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis [Conn, 2001; van Everdingen et al., 2002] and asthma [Bazzy-Asaad, 2001]. GCs are known to counteract the key inflammatory signaling mediators, NF-κB and AP-1, through a mechanism involving the GC receptors (GRs) [Auphan et al., 1995; De Bosscher et al., 2003]. Current understanding of the action of GCs focuses on the interactions between these transcription factors and is summarized in three models [De Bosscher et al., 2000; Almawi and Melemedjian, 2002]: (1) the IκB-α up-regulatory model, which proposes that GCs induce the expression of IκB-α and that the newly synthesized IκB-α sequesters the p65 subunit of the NF-κB in the cytoplasm and thereby inhibits NF-κB nuclear functions; (2) the protein-protein

interaction model, which proposes that activated GRs physically interact with c-Jun/AP-1 and with the p65 subunit of NF-κB, resulting in inhibition of genes activated by AP-1 or NF-κB; and (3) the competition model, which proposes that the activated GRs compete with NF-κB and/or AP-1 for transcription coactivators such as CBP/p300 and SRC-1, and thereby modulate the transcription of the target genes. There is controversy, however, regarding these models. For example, the effect of GCs on IκB-α synthesis and subsequently on NF-κB nuclear translocation is cell-type specific [Scheinman et al., 1995; Brostjan et al., 1996]. In addition, a GR mutant that does not enhance IκB-α expression was still able to repress NF-κB activity [Heck et al., 1997]. Importantly, most of, if not all, the published studies were carried out in the presence of GCs, and it is now known that these also induce GILZ [D'Adamio et al., 1997; Cannarile et al., 2001], which had not been identified at that time, and GILZ also interacts with NF-κB and AP-1.

In this study, we tested the hypothesis that GILZ is a GC-effect mediator using COX-2 and bone marrow MSCs as a model system. The reasons for choosing this system include: (1) COX-2 is a major player in rheumatoid arthritis and its expression is up-regulated by inflammatory cytokines such as TNF-α and IL-1β which are abundant in the arthritic joint, and is down-regulated by GCs; (2) The COX-2 promoter has been well-characterized; it contains functional NF-κB binding sites and is known to be activated by NF-κB through this site in response to cytokines [Wadleigh and Herschman, 1999; Tak and Firestein, 2001]; and (3) MSCs have been recently implicated in the pathogenesis of rheumatoid arthritis [Li and Makarov, 2006], and manipulation of these cells (i.e., overexpression of GILZ) has potential for cell-based therapy of joint diseases [Jorgensen et al., 2004]. Our data show that overexpression of GILZ can inhibit pro-inflammatory cytokines TNF-α- and IL-1β-induced COX-2 expression, and knockdown of GILZ reduces GC inhibition of cytokine-induced COX-2 expression. Further, we demonstrate that GILZ inhibits COX-2 expression by preventing NF-κB (p65 subunit) nuclear translocation. Together, these results demonstrate that GILZ is a prominent candidate for new anti-inflammatory therapies. It is noted that while this manuscript was in preparation, a similar study by Eddleston et al.

[2007] also found that GILZ mediates GC anti-inflammatory effect in epithelial cells by attenuating cytokine-induced NF-κB activity, which is consistent with our conclusion.

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