



UWA Research Publication

Ngo, D., Beaulieu, E., Gu, R., Leaney, A., Santos, L., Fan, H., Yang, Y., Kao, W., Xu, J., Escriou, V., Loiler, S.A., Vervoordeldonk, M.J.B.M. & Morand, E.F. (2013). Divergent effects of endogenous and exogenous glucocorticoid-induced leucine zipper in animal models of inflammation and arthritis. *Arthritis and Rheumatism*, 65(5), 1203-1212.

Copyright © 2013 by the American College of Rheumatology

This is pre-copy-editing, author-produced version of an article accepted for publication in *Arthritis & Rheumatism* following peer review. The definitive published version (see citation above) is located on the article abstract page of the publisher, Wiley.

This version was made available in the UWA Research Repository on 1 May 2014, in compliance with the publisher's policies on archiving in institutional repositories.

The definitive version is available at www3.interscience.wiley.com

Use of the article is subject to copyright law.

Divergent effects of endogenous and exogenous GILZ in models of inflammation and arthritis

Devi Ngo, BSc(Hons)¹, Elaine Beaulieu PhD¹, Ran Gu BSc(Hons)¹, Alexandra Leaney BMedSci¹, Huapeng Fan PhD¹, Yuanhang Yang PhD¹, Wenping Kao PhD¹, Leilani Santos, PhD¹, Jiake Xu, PhD², Virginie Escriou, PhD³, Scott Loiler, PhD⁴ Margriet J. Vervoordeldonk, PhD^{4,5}, and Eric F. Morand MBBS(Hons) FRACP PhD¹

¹Centre for Inflammatory Diseases, Monash University, Melbourne Australia

²School of Pathology and Laboratory Medicine, The University of Western Australia, Nedlands, Australia.

³Inserm, U 640, F-75006 Paris, France

⁴ArthroGen BV, Amsterdam, The Netherlands

⁵Div Of Clinical Immunology and Rheumatology, AMC/University of Amsterdam, Amsterdam, The Netherlands

Address correspondence and reprint requests to:

Eric Morand, Southern Clinical School, Monash University Monash Medical Centre, 246 Clayton Road, Clayton, Australia, 3168.

T: +61 3 9594 5525 F: +61 3 9594 6437

E-mail: eric.morand@monash.edu

This work was supported by a Project grant from the National Health and Medical Research Council of Australia. SL and MV are employees of ArthroGen BV, Amsterdam. The authors have no conflict of interest to declare.

Abstract

Objectives: Glucocorticoid-induced leucine zipper (GILZ) has effects on inflammatory pathways that suggest it as a key inhibitory regulator of the immune system, and its expression is exquisitely sensitive to induction by glucocorticoids. We therefore hypothesized that GILZ deficiency would exacerbate models of immune-mediated inflammation, and impair the effects of glucocorticoids on inflammation, and correspondingly that exogenous GILZ would inhibit these events.

Methods: GILZ^{-/-} mice were generated using the LoxP/Cre system and responses were studied in delayed-type hypersensitivity (DTH), antigen-induced arthritis (AIA), K/BxN serum transfer arthritis, and LPS-induced cytokinemia. Therapeutic expression of GILZ via injection of recombinant adeno-associated virus expressing GILZ gene was compared to the effects of glucocorticoid in collagen-induced arthritis (CIA).

Results: Increased T cell proliferation and DTH were observed in GILZ^{-/-} mice but neither AIA or K/BxN arthritis were affected, and GILZ deficiency did not affect LPS-induced cytokinemia. Deletion of GILZ did not impair the effects of exogenous glucocorticoids on CIA or cytokinemia. In contrast, overexpression of GILZ in joints significantly inhibited CIA, with an effect similar to that of dexamethasone.

Conclusion: Despite effects on T cell activation, GILZ deficiency was without impact on effector pathways of arthritis, and was unexpectedly redundant in glucocorticoid effects. These findings do not support GILZ being central to the actions of glucocorticoids. Despite this, effectiveness of exogenous GILZ in inhibiting CIA suggests further evaluation of GILZ in inflammatory disease.

Profound anti-inflammatory actions of glucocorticoids underpins their widespread use in RA, despite adverse effects reflecting their metabolic actions (1-3). Greater mechanistic understanding may enable the development of therapies mimicking the anti-inflammatory but not metabolic effects of glucocorticoids. Glucocorticoid actions are integrated at the molecular level by the effects of the glucocorticoid-glucocorticoid receptor (GR) complex on gene transcription (4). The glucocorticoid/GR complex binds as a dimer to glucocorticoid response elements in target gene DNA, or inhibits ('transrepresses') gene expression by tethering as a monomer to other transcription factors such as NF- κ B and AP-1 (5). Among the genes most sensitive to transcriptional activation by glucocorticoids is glucocorticoid-induced leucine zipper (*GILZ* (*TSC22D3*)) (6). *GILZ* inhibits NF- κ B through a physical interaction with the NF- κ B p65 subunit which impedes nuclear translocation (7). *GILZ* also binds to the AP-1 components c-Jun and c-Fos (8), and also inhibits Raf-1 (9) and Ras (10). *GILZ* silencing increased the severity of murine collagen-induced arthritis (CIA), and *GILZ* overexpression inhibited chemokine and cytokine expression in human RA synovial fibroblasts (RASf) (11). These observations have led to the concept that *GILZ* is a critical mediator of glucocorticoid anti-inflammatory effects (12, 13).

The majority of the effects of *GILZ* have been reported in forced overexpression studies. Although male infertility was recently reported in a *GILZ*^{-/-} mouse strain (14), the physiological effects of *GILZ* on the immune system, and whether *GILZ* is required for the therapeutic effects of glucocorticoids, remain unknown. In order to investigate these issues, and probe the potential for *GILZ*-based RA therapies, we created *GILZ*^{-/-} mice and the means to locally overexpress *GILZ* *in vivo*. We report that although *GILZ* deficiency resulted in increased T cell activation, there was no effect on effector pathways of inflammation, or on the expression of two distinct models of RA. Moreover, and unexpectedly, glucocorticoid effects on inflammation were retained in the absence of *GILZ*. In contrast, therapeutic

overexpression of GILZ mimicked the inhibitory effects of glucocorticoids in a model of RA. These findings revise understanding of the role of GILZ in immunity and inflammation, and in the actions of glucocorticoids, yet support the possibility of GILZ manipulation being explored as a therapy for RA.

Methods

Generation of GILZ knockout mice

GILZ knockout mice were generated in conjunction with Ozgene (Perth, Australia), by disruption of the GILZ gene in C57BL/6 mouse embryonic stem (ES) cells via homologous recombination using the LoxP/Cre system (15). The targeting construct of the GILZ gene (**Supplementary Figure 1A**) was built by using the in house targeting vector backbone (FLSniper, Ozgene) with homologous sequences spanning approximately 6Kb upstream and 6Kb downstream of GILZ. The floxed genomic area was approximately 2.2Kb with two LoxP sites flanking the last exon of GILZ. The neomycin cassette was removed by crossing with FLP C57BL/6 deleter mice. The resulting heterozygous floxed animals (GILZ^{floxed}ΔNeo/wt) were crossed with a general Cre C57BL/6 deleter, and the resulting GILZ^{flox}/wt, cre⁺ mice were backcrossed to WT C57BL/6 mice to remove the Cre gene. Genotypes of the resulting mice were determined by PCR using genomic DNA isolated from mice tails (**Supplementary Figure 1B**). The reported infertility of male GILZ^{-/-} mice (14) was confirmed; consequently, GILZ^{wt/KO} females and GILZ^{wt/Y} males were bred and male offspring genotyped to select GILZ-deficient (GILZ^{-/-}) mice (**Supplementary Figure 1B**). GILZ^{-/-} mice developed normally in relation to size and general development, had no difference in spleen neutrophil, monocyte or total T cell numbers, but had a modest but statistically significant increase in spleen CD4:CD8⁺T cell ratio (data not shown).

Induction of delayed type hypersensitivity (DTH), antigen-induced arthritis (AIA), collagen induced arthritis (CIA), and K/BxN serum transfer arthritis

Ovalbumin (OVA)-induced DTH was induced in WT and GILZ^{-/-} mice and paw swelling assessed at 24hours using calipers as previously described (16). Lymph node T cells were stimulated with OVA (10-00µg/mL) for 24hours, and IFN-γ and IL-17A ELISPOT assays (BD Biosciences) performed according to the manufacturer's protocol. Methylated bovine serum albumin (mBSA)-induced DTH and AIA were induced in WT and GILZ^{-/-} mice, and assessed by measurement of joint swelling using calipers and histological analysis, as described (17). CIA was induced in DBA/1 mice, as described (11). Joints were scored daily for arthritis development as described, with a maximum score of 12 per mouse (11). Antigen-specific T cell proliferation was analysed as previously described (17) using single cell suspensions of lymph node or spleen cells stimulated with Concanavalin A (Con A) (Sigma-Aldrich), or mBSA. DNA synthesis (incorporation of ³Hthymidine (0.5µCi, GE Healthcare)) was measured using a liquid scintillation β-counter (Cambridge Scientific). K/BxN serum transfer arthritis was induced as previously described, and each limb was scored daily from 0 – 5 allowing for the greater degree of swelling and erythema observed in this model (18). Histological severity was evaluated on 4µm sections stained with Safranin–O and counterstained with fast green/iron hematoxylin (11). DBA/1 mice developing CIA were treated from day 21 with liposome-encapsulated control and GILZ siRNA sequences as previously described (11). Mice were injected intravenously (i.v.) with 200µl of cationic liposome/nucleic acid formulation thrice weekly. All animal experiments were performed in accordance with the regulations of the Monash University Animal Ethics Committee.

LPS-induced cytokine production

WT and GILZ^{-/-} mice were injected i.p with LPS 10mg/kg. Mice were bled via tail vein at 0, 30 minutes, 1, 2, 4 and 24hours and serum collected. In some experiments Dex 1mg/kg (or

vehicle) was injected i.p 1hour prior to LPS. Murine dermal fibroblasts (MDF) were isolated as previously described (19) and maintained in complete RPMI. Cells were seeded overnight, and serum starved (0.5% FBS RPMI) prior to 24hours treatment with 10^{-7} M - 10^{-10} M Dex +/- LPS (200ng/mL).

RA synovial fibroblasts

RA synovial fibroblasts (RASf) were cultured as previously described (20). RA patients fulfilled the ACR criteria for the classification of RA (21). All experiments were approved by the Human Research Ethics Committee, Monash Medical Centre. RASf were transfected with 100nM control siRNA or GILZ siRNA for 24hours using RNAiMAX (Invitrogen) as previously described (11). Cells were treated with 1ng/ml TNF (BioSource, Camarillo, CA) with or without Dex (10^{-7} - 10^{-10} M) and supernatants collected 7hours later.

ELISA

Anti-CII IgG, IgG1, IgG2a and cytokine concentrations were measured by enzyme-linked immunosorbent assays (ELISA) as previously described (11, 22). Murine tumor necrosis factor (TNF) and interleukin-6 (IL-6) (R&D systems, Minneapolis, U.S.A), IL-1 β (Endogen, Rockford, U.S.A), interferon gamma (IFN- γ) and monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences, Australia) were detected following recommendations of the manufacturer. RASf supernatant cytokine concentrations were analyzed using cytometric bead array (BD Biosciences, Australia) as recommended by the manufacturer.

Construction, production and administration of GILZ-recombinant adeno-associated virus

5 (rAAV5)

GILZ-recombinant adeno-associated virus 5 (GILZ-rAAV) was constructed as described (23). Briefly, the mouse gene coding for GILZ flanked by AAV2 inverted terminal repeats and under control of a CMV promoter was packaged into the capsid from AAV5 resulting in the

GILZ-rAAV vector. Vectors were produced with a adenovirus-free system in HEK-293 cells using a triple transfection method (24) and purified by density gradient centrifugation. AAV titers were measured by QPCR, and final titers were 7×10^{13} (mock (empty vector)) and 3.85×10^{13} vector genomes/mL (GILZ-rAAV). DBA1 mice induced with CIA (as above) were treated with an intra-articular injection of PBS, mock rAAV or GILZ-rAAV in both knee joints ($5\mu\text{l}$, 2×10^{11} vg) and both ankle joints ($2,5\mu\text{l}$, 1×10^{11} vg), administered in each mouse on the day of onset of arthritis (n=10 per group).

Western blotting and quantitative polymerase chain reaction (QPCR)

Cell lysates were collected and Western blotting performed as previously described (11). Primary and secondary antibodies were rabbit anti-GILZ (FL134) (Santa Cruz), mouse anti- β -actin (Sigma), anti-rabbit Alexa680 for GILZ and anti-mouse Alexa750 for β -actin (Biolabs). Bands were detected using Odyssey Infrared Imaging System (LI-COR Biosciences). GILZ mRNA expression was measured by quantitative PCR using methods and primers previously described (11).

RAW264.7 macrophage cell lines

The P3K (25) cell line derived from RAW264.7 macrophages expresses a NF- κ B luciferase reporter construct. Cells were maintained in minimal essential medium-alpha (MEM) (Gibco BRL, Gaithersburg, MD) containing 10% FCS, 2 mM L-glutamine, 1% penicillin-streptomycin-fungizone. GILZ expression was induced using GILZ-rAAV. After 72hours cells were stimulated with 100ng/mL RANKL for 6hours and luciferase activity detected as described (25).

Statistical analysis

Student's t-tests or Mann-Whitney tests were used for continuous or discontinuous variables, respectively. *P* values less than 0.05 were considered significant.

Results

Effect of GILZ deficiency on T cell activation and inflammatory arthritis

As inhibitory effects of GILZ overexpression on Th1 activation in response to OVA have been reported (26), we examined these responses in WT and GILZ^{-/-} mice. Cutaneous DTH responses to OVA were significantly increased in GILZ^{-/-} compared to WT mice (**Figure 1A**). This was accompanied by increased lymph node T cell IFN- γ production as measured by ELISPOT (**Figure 1B**). Lymph node T cell IL-17A production was also increased in GILZ^{-/-} compared to WT mice (**Figure 1C**). To establish whether these effects would impact on inflammatory arthritis, we next examined the Th1-dependent mBSA-AIA model, which also allows examination of DTH (17). Cutaneous DTH responses to mBSA were significantly increased in GILZ^{-/-} compared to WT mice (**Figure 1D**), and antigen-induced T cell activation, as measured by proliferation and IFN- γ release, were significantly increased in lymph node cells from GILZ^{-/-} mice (**Figure 1E-F**). In contrast, no difference in arthritis severity, measured by knee thickness or histological examination, was observed between WT and GILZ^{-/-} mice (**Figure 1G-H**). These findings suggest that endogenous GILZ is a physiological inhibitor of T cell activation, but that the loss of this inhibition in GILZ^{-/-} mice is not associated with activation of effector pathways that mediate joint inflammation. To further explore this, we next examined the effect of physiological GILZ on the K/BxN serum transfer arthritis model, which is T cell-independent but depends on chemokine-mediated recruitment of myeloid effector cells (27). No significant difference in clinical or histological arthritis severity was observed between WT and GILZ^{-/-} mice (**Figure 2A-B**). Lastly, we investigated the effect of physiological GILZ on LPS induction of TNF, IL-1 β , and IL-6. LPS increased serum cytokines in both WT and GILZ^{-/-} mice, and no significant differences were observed (**Figure 2C-E**). These observations were confirmed *in vitro* using thioglycollate-induced peritoneal macrophages (22) from WT and GILZ^{-/-} mice (data not

shown). Together, these data suggest that despite its effects on T cell activation, physiological expression of GILZ may not exert dramatic inhibitory effects on B cell activation, myeloid effector cell recruitment, or the expression of pro-inflammatory effector cytokines, and thus on models of RA that depend on these pathways.

Effects of glucocorticoids in GILZ^{-/-} mice

The lack of effect of physiological GILZ on effector pathways suggested that GILZ may not be required for the actions of glucocorticoids on these events, and hence on arthritis. We therefore examined Dex effects on LPS-induced cytokines in WT and GILZ^{-/-} mice. Dex inhibited LPS-induced TNF, IL-1 β , and IL-6 in both WT and GILZ^{-/-} mice, and no significant difference between WT and GILZ^{-/-} mice was detected (**Figure 3A-C**). We next examined the effect of GILZ deficiency on glucocorticoid sensitivity *in vitro*. Dex 10⁻⁷M robustly induced the expression of GILZ in MDF over 4-8 hours (**Figure 3D**). The chemokine MCP-1 is abundantly produced by MDF (19). Dex dose-dependently inhibited basal and LPS-induced MCP-1 in both WT and GILZ^{-/-} cells, and no significant difference between WT and GILZ^{-/-} cells was observed (**Figure 3E-F**).

As CIA in C57Bl/6 mice is insufficiently severe to permit the examination of glucocorticoid effects, we examined the effects of glucocorticoids on CIA in DBA/1 mice depleted of GILZ using liposome-encapsulated siRNA that we have shown effectively silences GILZ expression in this model(11). Exacerbation of CIA severity by GILZ siRNA treatment in the absence of Dex was confirmed (data not shown and **Figure 4B**). In contrast, GILZ siRNA treatment had no significant impact on Dex inhibition of CIA (**Figure 4A-B**). Inhibition of Dex-induced GILZ expression in human RASF by siRNA was demonstrated using Western blotting (**Figure 4C**). GILZ siRNA had no effect on Dex inhibition of RASF TNF, IL-8, and IL-6, either basally (data not shown) or in response to TNF (**Figure 4D-F**). These data do not

support an essential role of GILZ in the anti-inflammatory effects of glucocorticoids on these phenomena.

Effect of therapeutic GILZ-rAAV on CIA

The preceding experiments suggest that endogenous GILZ is not a significant inhibitor of effector pathways or the effects of glucocorticoids in arthritis. To test the hypothesis that therapeutic induction of GILZ could nonetheless suppress established disease, we induced CIA in DBA/1 mice, and induced GILZ local expression with GILZ-rAAV. Western blot analysis indicated that infection of RAW246.7 macrophages with GILZ-rAAV resulted in GILZ protein expression (**Supplementary Figure 2A**) and inhibition of NF- κ B luciferase activity (**Supplementary Figure 2B**). Compared to mock-rAAV or PBS injected joints, intra-articular injection of GILZ-rAAV significantly increased GILZ mRNA in distal paw and knee (**Figure 5A**). In contrast, there was no increase in GILZ mRNA in the spleen (**Figure 5A**). A single GILZ-rAAV injection on the day of onset of clinical disease significantly reduced arthritis severity over the subsequent 15 days (**Figure 5B**). In separate experiments, mice developing CIA were treated from the same timepoint with daily s.c injections of Dex. The requirement for daily injections limited to the duration of this experiment to 7 days.

Dexamethasone resulted in a significant reduction in arthritis severity that was similar in magnitude to the effect of GILZ-rAAV treatment (**Figure 5C**). Clinical scores calculated as the area under the curve (AUC) adjusted for the number of days observed were significantly reduced by both GILZ-rAAV and Dex treatment, and the effects of these treatments were not significantly different (GILZ rAAV 1.5 ± 0.3 , Dex 1.0 ± 0.2 , $P = 0.26$) (**Figure 5D**). Separate experiments in which mice were observed for up to 21 days after GILZ-rAAV injection demonstrated that the inhibition of arthritis persisted (data not shown). Histological severity was also markedly attenuated in GILZ-rAAV treated mice (**Figure 5E**). In contrast, GILZ-rAAV treatment did not affect serum levels of anti-CII IgG, IgG1 and IgG2a antibodies

(**Figure 5F**), or CII-induced T cell proliferation in cells from draining lymph nodes (**Figure 5G**). These data indicate an inhibitory effect on joint inflammation during CIA of therapeutically-induced local GILZ expression.

Discussion

Since the discovery that dimerisation of the GR is not required for inhibition of the NF- κ B and AP-1 pathways (28), transrepression has been considered the dominant means through which glucocorticoids regulate inflammation. Recently, however, it has been shown that many effects of glucocorticoids on inflammation require homodimeric GR effects (29), and that selectivity for transrepression reduces effectiveness of GR ligands (30). This suggests that genes transcriptionally activated by glucocorticoids are essential for the control of immune-inflammatory responses. GILZ is among the most sensitively glucocorticoid-induced proteins. Together with its ability to directly inhibit NF- κ B and AP-1, this suggests GILZ as a potential mediator of the immune-inflammatory effects of glucocorticoids. Whether endogenous GILZ is required for the effects of glucocorticoids, or acts as a constitutive inhibitory regulator of the immune response like other glucocorticoid-induced anti-inflammatory proteins(31, 32), has not previously been established.

Our first experiments demonstrated increased OVA-induced DTH responses and IFN- γ production in GILZ^{-/-} mice, consistent with previous findings in mice transgenic for GILZ in T cells (33). In addition, a previously unsuspected effect of GILZ on Th17 activation was suggested by increased IL-17A production in the setting of GILZ deficiency. The mechanism of these effects may depend on the reported effects of GILZ on Ras (10) or on other pathways such as NFATc1, as both impact on T cell IL-17A expression (34). Unexpectedly, increased T cell activation in GILZ^{-/-} mice was not accompanied by increased AIA severity. Although AIA requires the activation of T cells (31, 32), activation of effector cells resulting in the production of pro-inflammatory cytokines is also required, and such effects may vary between

tissues. Our observations suggest either that physiological GILZ is not a major regulator of effector responses to T cell activation, that increased T cell activation in the absence of GILZ was insufficient to exacerbate joint disease, or that GILZ deficiency resulted in a paradoxical insensitivity of local effector cells to T cell-driven activation.

To exclude the possibility that the lack of effect of endogenous GILZ on arthritis severity was specific to the relatively mild AIA model, we studied the more robust K/BxN serum transfer arthritis model, which is mediated by chemokine-dependent recruitment of myeloid cells (27). Here we also found no significant difference between WT and GILZ^{-/-} mice in clinical or histological arthritis severity. Finally, LPS-induced production of the cytokines TNF, IL-6 and IL-1 β was unaffected by the absence of GILZ. Although multiple pathways are involved in the pathogenesis of arthritis models, one explanation for our findings is that the effects of physiological GILZ on T cells are not accompanied by effects on effector events such as antibody production, leukocyte recruitment, or cytokine expression that are essential for the development of arthritis.

Glucocorticoids act therapeutically via effects on both T cell and effector leukocyte function. The lack of effect of GILZ deficiency in these experiments called into question the hypothesized requirement for GILZ in the effects of glucocorticoids, suggested by previous *in vitro* studies using GILZ silencing (35, 36). Therefore, we examined the requirement for GILZ in the effects of glucocorticoids using GILZ^{-/-} mice and cells, and *in vivo* siRNA silencing. In contrast to expectations, LPS-induced serum cytokines were inhibited *in vivo* by Dex to an equivalent degree in WT and GILZ^{-/-} mice, and dose-dependent effects of Dex on cytokine release *in vitro* were equivalent in WT and GILZ^{-/-} cells. Moreover, the attenuation of CIA by Dex was unaffected by treatment with a GILZ-siRNA regimen that we have previously shown to inhibit GILZ expression *in vivo* (11). Finally, GILZ silencing in RASF did not impair Dex inhibition of cytokine production by these cells. Together, these data do

not support the hypothesis that GILZ is required for glucocorticoids to exert their therapeutic effects on these aspects of the immune response.

Assessment of a potential therapeutic molecule based on results observed in its absence may be incompletely informative. Previous studies have demonstrated that T cell overexpression of GILZ leads to reductions in severity of models of colitis and spinal cord injury *in vivo* (26, 37). Therefore, to determine if GILZ could exert therapeutic effects in CIA despite the redundancy demonstrated by its deletion, we used rAAV as a means to induce the synthesis of GILZ in the joints (38). AAV serotype 5 has been shown to have tropism for dendritic cells, synovial fibroblasts and endothelial cells *in vitro*, and to successfully deliver genes to the inflamed joint *in vivo* (23, 39). Here, GILZ-rAAV injection at the onset of disease in CIA successfully induced local GILZ expression, accompanied by significant attenuation of clinical and histological joint inflammation. The reduction of joint disease in response to GILZ-rAAV, despite the lack of effect on T cell proliferation and anti-CII antibody production, is consistent with the restriction of GILZ overexpression to the joint, and to the fact that GILZ-rAAV treatment was given during established arthritis, after the time at which T and B cell responses against CII are established. These effects also differ from those observed in AIA in the context of GILZ-deficiency, in which increased T cell activation was observed without increased joint disease. These two observations, that systemic deletion of GILZ during the development of immune responses resulted in increased T cell activation but not arthritis, while local GILZ overexpression inhibited joint inflammation without affecting T cell responses, suggest differences between endogenous and supra-physiological, and local and systemic, effects of GILZ.

Despite the effects of exogenous GILZ, we demonstrated redundancy of endogenous GILZ to glucocorticoid anti-inflammatory effects. No published studies inform as to the potential redundancy of GILZ in glucocorticoid metabolic effects. However, GILZ has been described

to promote osteoblast differentiation (40), suggesting the possibility that therapeutic GILZ induction may not transduce pro-resorptive signals of glucocorticoids. A wide array of studies is needed to probe the participation of GILZ in the metabolic effects of glucocorticoids, and the availability of GILZ-deficient mice such as those we have generated here will enable such studies for the first time.

Several caveats apply to the interpretation of the results presented here. The mechanisms through which physiological GILZ impacts on T cell activation, and the functional consequences of these effects, requires detailed examination. The lack of a phenotype in AIA in GILZ^{-/-} mice is at odds with the previously reported exacerbation of CIA in DBA/1 mice by GILZ silencing (11), that our current results with GILZ siRNA confirm. Our GILZ^{-/-} mice are on the C57Bl/6 background, a strain relatively resistant to the induction of CIA (41).

Backcrossing the GILZ^{-/-} mouse strain onto the DBA/1 background would assist in addressing the effects of GILZ on CIA, however the infertility of GILZ^{-/-} male mice renders this infeasible. Moreover, it remains possible that a GILZ-based therapy would be insufficiently broad in its effects to be an effective therapy for inflammatory disease. For example, the ability of GILZ to mimic the recently highlighted direct GR-mediated repression of gene expression (42) is unknown.

In conclusion, we have demonstrated the unexpected finding that despite attenuation of CIA in response to therapeutic GILZ overexpression, and increased T cell activation in GILZ^{-/-} mice, GILZ is not a significant physiological inhibitor of effector pathways of arthritis. Moreover, our results indicate that GILZ is redundant for the inhibitory effects of glucocorticoids on CIA and on LPS-induced cytokine production. These findings suggest that, rather than being essential to the actions of glucocorticoids, the expression of GILZ may represent a 'backup' pathway, wherein the effects of GILZ on transcription factors such as NF- κ B and AP-1 operate in parallel to direct GR transrepression of these pathways. Given the

lethal effects of inflammation in the absence of endogenous glucocorticoids (43), the existence of such redundancy in glucocorticoid effects could confer a survival advantage. Nonetheless, the inhibitory effect observed in response to local GILZ overexpression in CIA suggests that further exploration of potential glucocorticoid-mimicking therapeutic effects of GILZ is warranted. Understanding of the contribution of GILZ to metabolic effects of glucocorticoids would be required prior to advancing such an application to the clinic.

Acknowledgements

The authors wish to thank Dr. Qiang Cheng for his helpful ideas throughout this work, Steven Lim for his assistance in tissue culture and Dr. Camden Lo for his assistance with imaging.

Figure Legends

Figure 1 Effect of GILZ deficiency on DTH responses to OVA and T cell responses in AIA.

(A-C) OVA/CFA was injected into WT (n=6) and GILZ^{-/-} (n=6) mice and footpad DTH induced on day 10. (A) DTH responses (mm) were measured after 24hours. (B-C) WT and GILZ^{-/-} lymph node T cells re-stimulated with OVA or non-treated (NT) for 24hours and IFN- γ (B) and IL-17A (C) measured by ELISPOT in response to OVA. (D-H) AIA was induced in WT (n=16) and GILZ^{-/-} (n=14) mice with mBSA/CFA and DTH initiated on day 27 into the footpads as described. (D) DTH responses were measured after 24hours. (E) Lymph node T cells were re-stimulated with mBSA (0.1, 1, 10 μ g/mL), Con A or non-treated (NT) for 48hours prior to measurement of thymidine incorporation. (F) IFN- γ was measured by ELISA in T cell supernatants. (G) AIA severity was measured by change in knee thickness (mm) measured on day 28. (H) Representative images of knee joints stained with Safranin-O from WT and GILZ^{-/-} mice with AIA. Data represent the mean \pm SEM. *P < 0.05, WT vs GILZ^{-/-}.

Figure 2 Effect of GILZ deficiency on K/BxN serum transfer arthritis and LPS induced cytokinemia.

(A-B) K/BxN serum transfer arthritis was induced in WT (n=5) and GILZ^{-/-} (n=4) mice. Clinical score (A) and histological score (B) were measured as described. (C-E) WT and GILZ^{-/-} mice were injected i.p with LPS (10mg/kg). Serum was collected at indicated timepoints and concentrations of (C) TNF, (D) IL-1 β and (E) IL-6 in WT and GILZ^{-/-} mice measured by ELISA. Data represents the mean \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001, WT vs GILZ^{-/-}.

Figure 3 Effect of glucocorticoid treatment in GILZ deficient mice.

(A-C) WT (n=11) and GILZ^{-/-} (n=13) mice were injected with vehicle or Dex (1mg/kg) 1 hour prior to i.p injection of LPS (10mg/kg). Serum was collected at indicated timepoints after LPS injection and concentrations of (A) TNF, (B) IL-1 β and (C) IL-6 in WT and GILZ^{-/-} mice measured by ELISA. Data is represented as the mean \pm SEM. †P < 0.05, †††P < 0.001, WT+Dex vs WT; *P < 0.05, ***P < 0.001 GILZ^{-/-} + Dex vs WT. (D) WT and GILZ^{-/-} mouse dermal fibroblasts (MDF) were treated with Dex at 10⁻⁷M for 4 and 8 hours and GILZ expression detected by Western blot. (E) WT and GILZ^{-/-} MDF were treated with Dex (10⁻⁷M – 10⁻¹⁰M) for 24 hours and MCP-1 concentration measured in supernatants by ELISA. Values are expressed relative to no treatment (NT) controls. (F) WT and GILZ^{-/-} MDFs were pre-treated with Dex (10⁻⁷M – 10⁻¹⁰M) for 1 hour prior to LPS (200ng/mL) stimulation for 24 hours and MCP-1 concentration measured in supernatants by ELISA. Values are expressed relative to LPS-treated controls (n=4 independent experiments). Data is represented as the mean \pm SEM. *P < 0.05, **P < 0.01, Dex-treated vs control.

Figure 4 Effect of glucocorticoid treatment in the absence of GILZ

(A-B) DBA/1 mice were induced with CIA and treated thrice weekly from the onset of disease with control (ct) (n=10) or GILZ siRNA (n=10) as described, and daily with vehicle or Dex (0.25mg/kg s.c) for 10 days. (A) Arthritic scores were observed in control and GILZ siRNA-treated mice undergoing Dex treatment. *P < 0.05, **P < 0.01 Dex vs vehicle. (B) Scores were expressed as area under the curve (AUC)/days observed. *P < 0.05. No significant (NS) difference was observed between control- and GILZ siRNA-treated mice undergoing Dex treatment. (C-F) GILZ expression was silenced in RA synovial fibroblasts (RASf) by treatment with control or GILZ siRNA using RNAiMAX. (C) Cells were treated with Dex (10⁻⁷M) for 5 hours and protein lysates isolated for GILZ expression measured by Western blot. (D-F) Cells were co-treated with Dex (10⁻⁷M – 10⁻¹⁰M) and TNF (1ng/mL) for 7 hours and supernatant concentrations of (D) TNF, (E) IL-8 and (F) IL-6 measured using

Cytometric Bead Array kit (n=3 independent experiments). Data are represented as the mean \pm SEM.

Figure 5 Effect of GILZ-rAAV on CIA

CIA was induced in DBA/1 mice as described. Mice were treated with an intra-articular injection in knees and ankles with either PBS, mock-rAAV or GILZ-rAAV from the onset of arthritis (n=10 per group). (A) GILZ mRNA was measured in distal paw, knee synovium and spleen from mice treated with PBS, mock-rAAV or GILZ-rAAV. (B) CIA clinical score was recorded in mice treated with PBS, mock-rAAV or GILZ-rAAV. *P < 0.05, **P < 0.01 GILZ-rAAV vs mock-rAAV. (C) Arthritic scores were measured in mice with CIA treated with vehicle (control) or Dex (0.05mg/kg/day). *P < 0.05, Dex vs control (D) GILZ-rAAV and Dex-treated sCIA clinical scores were expressed as AUC/days observed. *P < 0.05, **P < 0.01 GILZ-rAAV or Dex vs mock-rAAV treatment. No significant difference (NS, P=0.26) was observed between mice treated with GILZ-rAAV and Dex. The following results are from PBS, mock-rAAV or GILZ-rAAV treated mice with CIA. (E) Representative images of ankle joints stained with Safranin-O. (F) Serum anti-CII IgG, IgG1 and IgG2a antibody titres measured by ELISA. (G) Lymph node T cells were re-stimulated with and CII (10, 50 μ g/mL) or no treatment (NT) for 72hours prior to measurement of proliferation by thymidine incorporation. Data are represented as the mean \pm SEM.

Supplementary Figure Legends

Supplementary Figure 1. Strategy for the generation of GILZ^{-/-} mice.

(A) The targeting vector was constructed to insert LoxP sites on either side of the last exon and 3'UTR of the *Gilz* gene. A neomycin resistance cassette was also added to the targeting vector within the floxed sequence, in order to select positive transformants. This neomycin cassette was excised by crossing the floxed mice with an Ozgene FLP deleter to create the floxed Δ Neo conditional GILZ KO strain. Mating the floxed Δ Neo mice with the Ozgene general Cre-deleter mice resulted in the recombination of the floxed sequence and generation of the GILZ KO. (B) Quantitative PCR primers pairs were designed to genotype GILZ KO mice. Two primers pairs were used to confirm WT, floxed or KO alleles. Primer pair 1 (red) amplified a 346bp DNA fragment in WT alleles, a 444bp fragment in floxed alleles and will not amplify any fragment in KO alleles. The second primer pair (blue) amplified a 2261bp fragment in wt and floxed alleles and a 538bp fragment in KO alleles. (C) PCR amplified fragments were separated by electrophoresis and band sizes indicated mouse genotype as indicated. (D) DNA was isolated from tail samples of mice and processed for genotyping. Example of genotyping by PCR from heterozygous female mating with a WT male resulting in pups, which are female heterozygous (GILZ^{+/-}), female or male WT (GILZ^{+/+}) or male knockout (GILZ^{-/-}). PCR fragments were separated by electrophoresis and the band size indicated genotypes.

Supplementary Figure 2. *In vitro* effects of GILZ-rAAV on GILZ protein expression and NF- κ B luciferase activity

Mock-rAAV or GILZ-rAAV was infected into P3K RAW264.7 macrophages at 400,000 MOI for 72hours. (A) GILZ protein was measured using Western blot. β -actin was blotted as the

loading control. (B) P3K RAW264.7 macrophages infected with mock-rAAV or GILZ-rAAV were treated with 100ng/mL RANKL for 6hours and cell lysates measured for NF- κ B luciferase activity. Results are expressed as fold change luciferase activity compared to non-RANKL-treated (NT) cells. Data is represented as the mean \pm SEM of n=5 experiments. Statistical analysis was performed using Student's t-test. * $P < 0.05$.

References

1. Saag KG, Koehnke R, Caldwell JR, Brasington R, Burmeister LF, Zimmerman B, et al. Low dose long-term corticosteroid therapy in rheumatoid arthritis: An analysis of serious adverse events. *The American Journal of Medicine*. 1994;96(2):115-23.
2. Huscher D, Thiele K, Gromnica-Ihle E, Hein G, Demary W, Dreher R, et al. Dose-related patterns of glucocorticoid-induced side effects. *Ann Rheum Dis*. 2009;68(7):1119-24.
3. Davis JM, 3rd, Maradit Kremers H, Crowson CS, Nicola PJ, Ballman KV, Thorneau TM, et al. Glucocorticoids and cardiovascular events in rheumatoid arthritis: a population-based cohort study. *Arthritis Rheum*. 2007;56(3):820-30.
4. Chinenov Y, Rogatsky I. Glucocorticoids and the innate immune system: Crosstalk with the Toll-like receptor signaling network. *Molecular and Cellular Endocrinology*. 2007;275(1-2):30-42.
5. Barnes PJ. Glucocorticosteroids: current and future directions. *Brit J Pharmacol*. 2011;163(1):29-43.
6. D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A, et al. A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity*. 1997;7(6):803-12.
7. Ayroldi E, Migliorati G, Bruscoli S, Marchetti C, Zollo O, Cannarile L, et al. Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood*. 2001;98(3):743-53.
8. Mittelstadt PR, Ashwell JD. Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J Biol Chem*. 2001;276(31):29603-10.
9. Ayroldi E, Zollo O, Macchiarulo A, Di Marco B, Marchetti C, Riccardi C. Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Mol Cell Biol*. 2002;22(22):7929-41.
10. Ayroldi E, Zollo O, Bastianelli A, Marchetti C, Agostini M, Di Virgilio R, et al. GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling. *J Clin Invest*. 2007;117(6):1605-15.
11. Beaulieu E, Ngo D, Santos L, Yang YH, Smith M, Jorgensen C, et al. Glucocorticoid-induced leucine zipper is an endogenous antiinflammatory mediator in arthritis. *Arthritis Rheum*. 2010;62(9):2651-61.
12. Ayroldi E, Riccardi C. Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. *FASEB J*. 2009;23(11):3649-58.
13. Beaulieu E, Morand EF. Role of GILZ in immune regulation, glucocorticoid actions and rheumatoid arthritis. *Nature Reviews Rheumatology*. 2011;7(6):340-8.
14. Bruscoli S, Velardi E, Di Sante M, Bereshchenko O, Venanzi A, Coppo M, et al. Long glucocorticoid-induced leucine zipper (L-GILZ) protein interacts with ras protein pathway and contributes to spermatogenesis control. *J Biol Chem*. 2011;287(2):1242-51.
15. Kontgen F, Suss G, Stewart C, Steinmetz M, Bluethmann H. Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *Int Immunol*. 1993;5(8):957-64.
16. Ooi JD, Phoon RK, Holdsworth SR, Kitching AR. IL-23, not IL-12, directs autoimmunity to the Goodpasture antigen. *J Am Soc Nephrol*. 2009;20(5):980-9.
17. Santos LL, Milenkovski GP, Hall PH, Leech M, Sharma L, Takeda K, et al. IL-18 is redundant in T-cell responses and in joint inflammation in antigen-induced arthritis. *Immunol Cell Biol*. 2006;84(2):166-73.
18. Santos LL, Fan H, Hall P, Ngo D, Mackay CR, Fingerle-Rowson G, et al. Macrophage migration inhibitory factor regulates neutrophil chemotactic responses in inflammatory arthritis in mice. *Arthritis Rheum*. 2011;63(4):960-70.

19. Yamana J, Santos L, Morand E. Enhanced induction of LPS-induced fibroblast MCP-1 by interferon- γ : Involvement of JNK and MAPK phosphatase-1. *Cellular Immunology*. 2009;255(1-2):26-32.
20. Toh ML, Yang Y, Leech M, Santos L, Morand EF. Expression of mitogen-activated protein kinase phosphatase 1, a negative regulator of the mitogen-activated protein kinases, in rheumatoid arthritis: up-regulation by interleukin-1 β and glucocorticoids. *Arthritis Rheum*. 2004;50(10):3118-28.
21. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*. 1988;31(3):315-24.
22. Yang YH, Aeberli D, Dacumos A, Xue JR, Morand EF. Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *J Immunol*. 2009;183(2):1435-45.
23. Adriaansen J, Tas SW, Klarenbeek PL, Bakker AC, Apparailly F, Firestein GS, et al. Enhanced gene transfer to arthritic joints using adeno-associated virus type 5: implications for intra-articular gene therapy. *Ann Rheum Dis*. 2005;64(12):1677-84.
24. Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, et al. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther*. 1998;5(7):938-45.
25. Cheng T, Pavlos NJ, Wang C, Tan JW, Lin JM, Cornish J, et al. Mutations within the TNF-like core domain of RANKL impair osteoclast differentiation and activation. *Mol Endocrinol*. 2009;23(1):35-46.
26. Cannarile L, Cuzzocrea S, Santucci L, Agostini M, Mazzon E, Esposito E, et al. Glucocorticoid-induced leucine zipper is protective in Th1-mediated models of colitis. *Gastroenterology*. 2009;136(2):530-41.
27. Jacobs JP, Ortiz-Lopez A, Campbell JJ, Gerard CJ, Mathis D, Benoist C. Deficiency of CXCR2, but not other chemokine receptors, attenuates a murine model of autoantibody-mediated arthritis. *Arthritis and rheumatism*. 2010.
28. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, et al. DNA binding of the glucocorticoid receptor is not essential for survival. *Cell*. 1998;93(4):531-41.
29. Kleiman A, Hübner S, Rodriguez Parkitna JM, Neumann A, Hofer S, Weigand MA, et al. Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. *FASEB J*. 2012;26(2):722-9.
30. Weinstein DS, Gong H, Doweiko AM, Cunningham M, Habte S, Wang JH, et al. Azaxanthene based selective glucocorticoid receptor modulators: design, synthesis, and pharmacological evaluation. *J Med Chem*. 2011;54(20):7318-33.
31. Hammer M, Mages J, Dietrich H, Servatius A, Howells N, Cato AC, et al. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *J Exp Med*. 2006;203(1):15-20.
32. Yang YH, Morand EF, Getting SJ, Paul-Clark M, Liu DL, Yona S, et al. Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis Rheum*. 2004;50(3):976-84.
33. Cannarile L, Fallarino F, Agostini M, Cuzzocrea S, Mazzon E, Vacca C, et al. Increased GILZ expression in transgenic mice up-regulates Th-2 lymphokines. *Blood*. 2006;107(3):1039-47.
34. Liu XK, Lin X, Gaffen SL. Crucial role for nuclear factor of activated T cells in T cell receptor-mediated regulation of human interleukin-17. *J Biol Chem*. 2004;279(50):52762-71.
35. Hamdi H, Bigorgne AI, Naveau S, Balian A, Bouchet-Delbos L, Cassard-Doulcier A-M, et al. Glucocorticoid-induced leucine zipper: A key protein in the sensitization of monocytes to lipopolysaccharide in alcoholic hepatitis. *Hepatology*. 2007;46(6):1986-92.

36. Yang N, Zhang W, Shi XM. Glucocorticoid-induced leucine zipper (GILZ) mediates glucocorticoid action and inhibits inflammatory cytokine-induced COX-2 expression. *J Cell Biochem.* 2008;103(6):1760-71.
37. Esposito E, Bruscoli S, Mazzon E, Paterniti I, Coppo M, Velardi E, et al. Glucocorticoid-induced leucine zipper (GILZ) over-expression in T lymphocytes inhibits inflammation and tissue damage in spinal cord injury. *Neurotherapeutics.* 2011;9(1):210-25.
38. Adriaansen J, Vervoordeldonk MJ, Tak PP. Gene therapy as a therapeutic approach for the treatment of rheumatoid arthritis: innovative vectors and therapeutic genes. *Rheumatology (Oxford).* 2006;45(6):656-68.
39. Xin KQ, Mizukami H, Urabe M, Toda Y, Shinoda K, Yoshida A, et al. Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. *J Virol.* 2006;80(24):11899-910.
40. He L, Yang N, Isales CM, Shi XM. Glucocorticoid-induced leucine zipper (GILZ) antagonizes TNF-alpha inhibition of mesenchymal stem cell osteogenic differentiation. *PloS one.* 2012;7(3):e31717.
41. Campbell IK, Hamilton JA, Wicks IP. Collagen-induced arthritis in C57BL/6 (H-2b) mice: new insights into an important disease model of rheumatoid arthritis. *Eur J Immunol.* 2000;30(6):1568-75.
42. Surjit M, Ganti KP, Mukherji A, Ye T, Hua G, Metzger D, et al. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell.* 2011;145(2):224-41.
43. Leech M, Metz C, Bucala R, Morand EF. Regulation of macrophage migration inhibitory factor by endogenous glucocorticoids in rat adjuvant-induced arthritis. *Arthritis Rheum.* 2000;43(4):827-33.