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Chapter 8

The Role of GILZ in Anti-Inflammatory and Immunosuppressive Actions of Glucocorticoids

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1. Introduction

Chronic inflammatory diseases place a social and financial burden on society. Glucocorticoids, a class of steroid hormones existing in almost every vertebrate, have been exploited for more than 60 years as a therapeutic option for the inflammatory diseases. For example, the therapeutic effects of synthetic glucocorticoids, first observed in rheumatoid arthritis (RA), led to the awarding of a Nobel prize in 1950 (Slocumb *et al.*, 1950). Based on their rapid, profound and wide-ranging effects, glucocorticoids are a mainstay of treatment for virtually all inflammatory diseases besides RA, and now are among the most frequently prescribed of all medications (Hillier, 2007). Indeed, a community practice survey in 2000 indicated that up to 1% of the entire adult population is taking systemic glucocorticoids at any given time (van Staa *et al.*, 2000). Glucocorticoids have widespread systemic effects, particularly on the inflammation and immune response (Barnes, 2006; Chrousos, 1995). However, glucocorticoids are associated with dose dependent side effects, including diabetes mellitus, osteoporosis, weight gain, and hypertension (Huscher *et al.*, 2009), as well as increased risk of cardiovascular events (Davis *et al.*, 2007). Much effort has been expended identifying glucocorticoid anti-inflammatory mechanisms of action (Barnes, 2006; Scha¨cke *et al.*, 2002). Understanding of the mechanism of action of glucocorticoids is essential in order to devise better ways to treat inflammatory disease, ideally retaining the beneficial effects of glucocorticoids but not their adverse effects.

The discovery of a glucocorticoid-induced protein that could emulate the beneficial, but not harmful, effects of glucocorticoids, would represent a landmark in inflammation translational research on a glucocorticoid alternative therapy. Glucocorticoid induced leucine zipper (GILZ) may be such a candidate molecule. GILZ was first identified in 1997 in a gene extraction library, where it was found to be dramatically induced by dexamethasone (D'Adamio *et al.*, 1997). Subsequent studies, mostly utilizing forced over expression of GILZ have ascertained that GILZ has anti-inflammatory functions that include interactions with

the NF-κB and AP-1 pathways (Di Marco *et al.*, 2007; Mittelstadt & Ashwell, 2001), which closely mimics the anti-inflammatory effects of glucocorticoids. Moreover, it has recently been shown that GILZ is also expressed in rheumatoid arthritis (RA) synovial tissues, where it exerts inhibitory effects on cytokine expression, and inhibiting the expression of GILZ results in exacerbation of disease in a mouse model of RA. As we will summarise in this Chapter, GILZ is a pivotal endogenous regulator of inflammation and immune responses, which could represent a potential new therapeutic alternate to glucocorticoids.

2. GILZ structure and expression

2.1. Molecular structure of GILZ

As shown in **Fig 1**, GILZ, also named TSC22 domain family protein 3 (TSC22D3), is a 137-amino acid protein, consisting of three major domains: the N-terminal (1-75 aa), leucine zipper (76-97 aa), and C-terminal domains (98-137 aa) (Beaulieu & Morand, 2011). To date, four isoforms of GILZ have been characterized as splice variants from the *Tsc22d* gene and named GILZ1-4 (Soundararajan *et al.*, 2007). The leucine zipper motif of GILZ is located in the central part of the protein and mainly mediates the homodimerization of GILZ required for many of its functions (Di Marco *et al.*, 2007), while the other two domains are responsible for protein-protein interactions between GILZ and transcription factors and signaling molecules. For example, the C-terminal of GILZ is a proline-rich region necessary for direct binding of GILZ to the p65 subunit of NF-κB (Di Marco *et al.*, 2007; Riccardi *et al.*, 2001). In 2001, Aryoldi and colleagues showed that the over expression of GILZ in T cells inhibits the activation of NF-κB by binding the p65 subunit of NF-κB and preventing its nuclear translocation (Ayroldi *et al.*, 2001). GILZ was co-precipitated with the p65 subunit of NF-κB in macrophages stimulated with glucocorticoids, and expression of GILZ with an NF-κB reporter inhibits reporter activity (Berrebi *et al.*, 2003). The N-terminal domain of GILZ directly binds with the upstream MAP kinase pathway activating molecule Raf-1, to inhibit its function. The interaction between GILZ and c-Fos and c-Jun (two constituents of AP-1) also occurs via the N-terminal domain of GILZ. Moreover, GILZ also binds to Ras via its tuberous sclerosis complex (TSC) box (61-75 aa), or even interacts with Ras and Raf together to form a trimer.

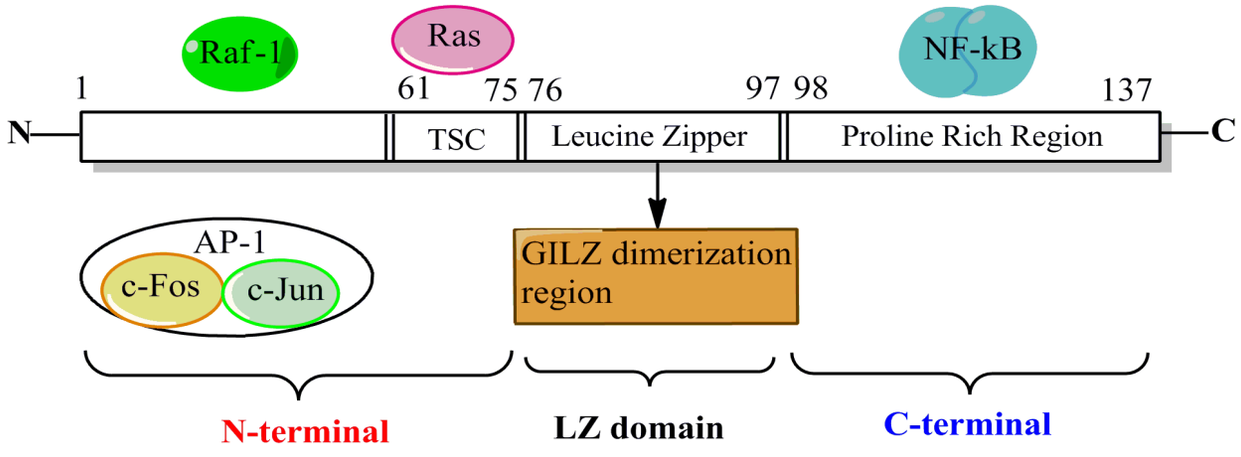

Figure 1. Functional domains of GILZ

2.2. Expression of GILZ

GILZ gene expression is exquisitely sensitive to induction by glucocorticoids. For example, in human RA synovial fibroblasts, dexamethasone (Dex), a synthetic drug derived from glucocorticoid class of steroid, induced a more than 10-fold increase in GILZ transcripts at a concentration of only 1 nM, while 100 nM dexamethasone increased GILZ mRNA by over 100-fold (Beaulieu *et al.*, 2010a). *In vivo*, exogenous glucocorticoids induce GILZ expression, while blockade of endogenous glucocorticoids inhibits GILZ expression in mouse, and GILZ expression is reduced in response to reductions in circulating cortisol in humans (Beaulieu *et al.*, 2010a; Lekva *et al.*, 2009). The dramatic effect of glucocorticoids on GILZ is mediated via the direct binding of the glucocorticoid/glucocorticoid receptor (GR) complex to six glucocorticoid-responsive elements (GREs) located in the promoter region of the *GILZ* gene. The *GILZ* promoter also contains two functional forkhead-responsive elements (FHREs), which when bound to the transcription factor forkhead box O 3 (FoxO3) facilitate maximal GILZ expression induced by glucocorticoid receptor binding (Asselin-Labat *et al.*, 2005b).

Although GILZ expression is mainly controlled by glucocorticoids (Beaulieu *et al.*, 2010b; Berrebi *et al.*, 2003; Eddleston *et al.*, 2007a), it is also modulated by a variety of cytokines (Eddleston *et al.*, 2007a). For example, GILZ is up-regulated by IL-10 (Berrebi *et al.*, 2003), IL-15 and TGF-β (Ayroldi & Riccardi, 2009; Cohen *et al.*, 2006a), whereas GILZ is down-regulated by IL-2 in some cell types. IL-2 can inhibit FoxO3 transcriptional activity, thus inhibiting glucocorticoid induced GILZ expression (Asselin-Labat *et al.*, 2005a). Some inflammatory stimuli, such as tumor necrosis factor (TNF) and lipopolysaccharide (LPS), were also found to reduce GILZ mRNA expression in fibroblast like synoviocytes (Beaulieu *et al.*, 2010b). GILZ expression can also be regulated by other anti-inflammatory molecule such as Annexin A1. Yang et al have reported that in Annexin A1 deficient cells, dexamethasone failed to significantly induce GILZ, in contrast to wild type cells (Yang *et al.*, 2009), which indicates a regulatory role of Annexin A1 on GILZ expression.

Interestingly, GILZ expression is also modulated by the oxygen environment. Wang and colleagues recently found that hypoxia not only remarkably upregulated the expression of GILZ, but also significantly enhanced Dex-induced expression of GILZ in macrophages and the spleen of rats (Wang *et al.*, 2011). They also reported ERK MAP kinase activity is involved in the upregulation of GILZ induced by hypoxia.

To date, GILZ has been discovered to be expressed in a variety of tissues (**Table 1**). This information indicates a widespread distribution of GILZ in the human body and suggests it is well placed for a role as a pivotal regulator of inflammation. Moreover, growing evidence showed that GILZ is present in a wide range of cell types that are sensitive to glucocorticoids *in vitro.* In 1997, GILZ was first identified in T cells, in which GILZ inhibited T cell receptor (TCR)-mediated T cell activation (D'Adamio *et al.*, 1997). Since then, GILZ has been shown to be expressed in other immune cells, including monocytes/macrophages, mast cells, and dendritic cells (Berrebi *et al.*, 2003; Cohen *et al.*, 2006b; Godot *et al.*, 2006; Hamdi *et al.*, 2007a), and to have numerous anti-inflammatory functions in these cells. These functions

are outlined in the following sections. Besides immune cells, GILZ has also been shown to express in other cell types such as epithelial cells and bone-marrow-derived mesenchymal stem cells (MSCs). For example, GILZ was reported to inhibit NF-κB activation in epithelial cells and MSCs (Eddleston *et al.*, 2007b; Yang *et al.*, 2008). The studies also reported the expression of GILZ is necessary for dexamethasone-mediated inhibition of IL-8 production in respiratory epithelial cells and similarly for the dexamethasone-dependent inhibition of cyclo-oxygenase 2 expression in MSCs.

Tissue types	Expression of GILZ	Reference(s)
Lymphoid tissue	Lymphocytes mainly from thymus, spleen, and lymph nodes	(Asselin-Labat *et al.*, 2004; D'Adamio *et al.*, 1997; Riccardi *et al.*, 2001)
Brain	Ubiquitously expressed in rat brain	(van der Laan, 2008)
Renal epithelium	mammalian kidney epithelial cells	(Soundararajan *et al.*, 2009)
Collecting duct	the cortical collecting duct of the mouse kidney	(Robert-Nicoud *et al.*, 2001)
Ovaries	normal ovary and epithelial ovary cancer	(Redjimi *et al.*, 2009)
Bone tissue	fetal osteoblasts, mesenchymal stem cells, and osteoclasts	(Lekva *et al.*, 2010)
Skeletal muscle and cardiac tissue	skeletal muscle tissue and myoblasts	(Bruscoli *et al.*, 2010)

Table 1. Expression of GILZ in different tissues

3. Effects of GILZ on the immune response

3.1. Innate immunity

A variety of studies suggest that GILZ has critical inhibitory effects on the activity of the innate immune system. In a monocytic cell line (THP-1), RANTES (also known as CCL5) and MIP-1α (also known as CCL3), antigen presenting MHC class II molecules, B7 co-stimulatory molecules CD80 and CD86, and the the pathogen-associated molecular pattern (PAMP) receptor TLR2, are all modulated by GILZ, with an expected effect on reducing recruitment and activation of inflammatory cells (Berrebi *et al.*, 2003; Cohen *et al.*, 2006b). Furthermore, in liver disease, GILZ expression in Kupffer macrophages due to glucocorticoids treatment reduces the production of pro-inflammatory mediators in response to LPS (Hamdi *et al.*, 2007a). Besides macrophages and monocytes, GILZ is also expressed in human airway epithelial cells and inhibited by IL-1β, TNF and interferon (IFN)-γ, and overexpression of GILZ inhibited the activation of NF-κB by IL-1β and TLR ligands (Eddleston *et al.*, 2007b). Currently, it is known that GILZ expression is inhibited by different pro-inflammatory mediators. For example, GILZ was not produced in granulomas in Crohn disease and tuberculosis since the macrophages in the granulomas were activated

with the strong expression of the RANTES gene (Berrebi *et al.*, 2003). By contrast, GILZ expression is retained in macrophages in Burkitt lymphomas, potentially contributing to the failure of the immune system to reject the tumor (Berrebi *et al.*, 2003). Taken together, these results above indicate a wide range of inhibitory effects of GILZ in a variety of innate immune responses. Clearly, immune responses, such as the expression of cytokines, chemokines and TLRs, are highly pertinent to the known pathology of inflammatory diseases such as RA. Of note, no *in vivo* studies of the role of GILZ in regulating classic innate immune responses, such as responses to endotoxin or other TLR ligands, have been reported.

3.2. Adaptive immunity

Parallel to innate immune responses, multiple critical functions of GILZ have been found which regulate the activity of antigen-presenting and effector cells of the adaptive immune response. For example, GILZ can mediate the effects of glucocorticoids on dendritic cells (DCs), whose maturation and antigen presentation are impaired in the presence of increased GILZ. Cohen *et al* demonstrated that GILZ over expression altered MHC and co-stimulatory molecule expression, resulting in reduced antigen presentation (Cohen *et al.*, 2006a), and subsequent decreased T lymphocyte activation. Moreover, the expression of GILZ is induced by glucocorticoids and transforming growth factor β (TGF-β) in immature DCs, and oral administration of glucocorticoids to patients increased the expression of GILZ in antigen-presenting cells (Cohen *et al.*, 2006b). The overexpression of GILZ is also able to drive the development of regulatory DCs, which secrete IL-10, and prevent the production of pro-inflammatory cytokines induced by CD40L. Furthermore, these GILZ-expressing regulatory DCs were found to induced $CD25^{hi}FoxP3^{+}CTLA\text{-}4^{+}$, IL-10 secreting T-regulatory cells from $CD4^{+}$ T-lymphocytes (Hamdi *et al.*, 2007b), resulting in inhibition of subsequent immune responses to specific antigens (Suffia *et al.*, 2006). Regulatory T cells (Tregs) that inhibit activation of other T lymphocytes are generated in response to GILZ over expressing dendritic cells (Hamdi *et al.*, 2007b), providing a further immunomodulatory effect of GILZ. This immunosuppressive effect of GILZ on DCs is extremely relevant in the context of inflammatory pathology since DCs determine whether antigen presentation will lead to an immune response or a tolerogenic response.

Additional knowledge of the effects of GILZ in adaptive immunity arises from studies of modified GILZ expression in T lymphocyte cells. For example, Cannarile and colleagues have demonstrated increased secretion of cytokines associated with a T_H2 response, such as IL-4, IL-10, IL-5, and IL-13, and reduced expression of cytokines associated with a T_H1 response such as IFN-γ in GILZ overexpressing cells compared with wild type T cells (Cannarile *et al.*, 2006a). In their study on GILZ transgenic T lymphocytes, they found there was decreased expression of T-box protein 21 (T-bet) (Cannarile *et al.*, 2006a), a transcription factor specifically associated with a T_H1 response, and increased expression of the transcription factors GATA-3 and STAT6. As STAT6 modulates GATA3 which is important for polarization towards a T_H2 phenotype (Wurster *et al.*, 2000; Zheng & Flavell, 1997), these studies indicate that the expression of GILZ promotes T lymphocyte development towards a

T_H2 instead of T_H1 phenotype. Moreover, mice transgenic for GILZ under the control of the CD2 promoter, that overexpresse GILZ in T cells, display a T_H2-skewed phenotype, and are protected from the T_H1-dependent model of dinitrobenzene sulfonic acid (DNBS)-induced colitis but exhibit an increase in the 'allergenic' T_H2 Oxazolone-induced colitis (Cannarile *et al.*, 2009). Another study investigated the levels of inflammatory mediators in wild type and GILZ transgenic mice induced with DNBS, and were able to demonstrate a decrease in pro-inflammatory cytokines and NF-κB activation in GILZ transgenic mice compared to wild type. Furthermore, in T-cell specific GILZ transgenic mice, young animals do not exhibit a significant difference in thymic weight. However, there was a significant decrease in $CD4^+CD8^+$ double positive thymocytes. There was also a parallel increase in $CD4^-CD8^-$ double negative and $CD8^+$ T-cells, but no change in the $CD4^+$ population (Delfino *et al.*, 2004). In the aged mice, the $CD4^+$ population also increased in a significant manner, although not as dramatically as the $CD8^+$ or the $CD4^-CD8^-$ populations, signifying a disturbance in thymic maturation. The observation above is interesting since GILZ might mediate some of the glucocorticoid-triggered apoptotic effects during thymic development. Microarray studies also show that GILZ is expressed in resting B cells, and it is presumed that GILZ down-regulation facilitates B-cell activation (Glynne *et al.*, 2000).

4. Effect of GILZ on signalling pathways

4.1. NF-κB pathway

To date, much research has focused on the function of the NF-κB pathway in the anti-inflammatory effects of glucocorticoids (Auphan *et al.*, 1995; DeBosscher & Haegeman, 2009; Gossye *et al.*, 2009). Glucocorticoids are known as effective inhibitors of the NF-κB pathway, and considerable research directed at understanding the antagonistic effects of glucocorticoids on this pathway has been undertaken (De Bosscher *et al.*, 2003). As shown in **Fig 2**, GILZ, significantly up regulated in the presence of glucocorticoids, participates in the inhibition of NF-κB by glucocorticoids through a physical interaction with the NF-κB p65 subunit (Ayroldi *et al.*, 2001), preventing its nuclear translocation. The inhibition of GILZ is independent from other IκB- or Rel-related proteins, since GILZ was found to co-immunoprecipitate with NF-κB p65 subunit in the presence or absence of IκB (Ayroldi *et al.*, 2001). Subsequently, Yang *et a*l demonstrated the role of GILZ in the inhibition of the inflammatory mediator COX-2 in MCSs in response to IL-1α and TNF-α, by preventing NF-κB p65 subunit nuclear transport (Yang *et al.*, 2008). Similarly, the mechanism of GILZ-NF-κB mediated inhibition of COX-2 transcription has been shown in epithelial cells (Eddleston *et al.*, 2007b). The inhibition of NF-κB by GILZ has also been demonstrated *in vivo*, in a transgenic mouse model in which GILZ expression is driven by the CD2 promoter, resulting in the overexpression of GILZ in thymocytes (Delfino *et al.*, 2006). When subjected to T-cell receptor-triggered apoptosis, the nuclear translocation and DNA binding of NF-κB were impaired in T cells from GILZ transgenic mice, whereas the translocation of transcription factors belonging to the NFAT family was not affected. All the findings above arouse interest in GILZ and its

function in the pathogenesis of NF-κB–related inflammatory diseases. For example, GILZ transgenic mice demonstrated reduced NF-κB activation in spinal cord injury in comparison to wild type mice, and overexpression of GILZ protects TH1 inflammatory responses in colitis associated with inhibition of nuclear and phosphorylated p65 (Cannarile *et al.*, 2009; Esposito *et al.* 2011). Moreover, Srinivasan and colleagues have described a novel NF-κB p65-binding GILZ-derived peptide which exhibited therapeutic potential as a small molecule NF-κB inhibitor in experimental autoimmune encephalomyelitis (EAE), a model of human multiple sclerosis (Srinivasan & Janardhanam, 2011a).

4.2. AP-1 pathway

The transcription factor AP-1 is another major participant in inflammatory and immune responses (Adcock & Caramori, 2001). AP-1, as a heterodimer of the c-Fos and c-Jun proteins, can be phosphorylated to significantly increase its transcriptional activity. The direct inhibitory effects of the glucocorticoid-glucocorticoid receptor complex on AP-1 signaling are well documented (De Bosscher *et al.*, 2003). It is also known that glucocorticoids lead to the repression of mitogen-activated protein kinase (MAPK) activity and hence AP-1 activation through the expression of phosphatases that exert inhibitory interactions with various MAPK members (Aeberli *et al.*, 2006). Of interest, Mittelstadt and colleagues showed direct binding of GILZ to the AP-1 components c-Jun and c-Fos (**Fig 2**) in Jurkat cells (an immortalized line of T cells) (Mittelstadt & Ashwell, 2001). The paper also showed GILZ is critical for the regulation of FasL expression in response to glucocorticoids. FasL is a promoter containing NFAT binding elements and under regulation of the NFAT/AP-1 complex signalling. FasL promoter and its enhancer elements, early growth response factor (Erg) -2 and Erg-3 were inhibited by transient transfection of GILZ in Jurkat cells. The authors demonstrated that c-Fos and c-Jun do in fact interact with N-terminal, but not the LZ or PER domains, of GILZ. Furthermore, Ayroldi et al. confirmed the interaction of GILZ with c-Fos and c-Jun, and further showed that GILZ expression interferes with c-Fos transcription in response to anti-CD3 stimulation of IL-2, but not c-Jun, and also by negatively interfering with upstream signalling of Raf-1-ERK pathway (Ayroldi *et al.*, 2002).

4.3. MAP kinase pathways

The MAP kinase family consists of extracellular signal-regulated kinase (ERK), p38 kinase, and JNK, all of which can be activated by upstream molecules such as Ras and Raf-1 (Rincon, 2001) and all of which phosphorylate downstream proteins in the respective cascades to regulate expression of a variety of genes related to inflammation, cell proliferation, differentiation and apoptosis. GILZ has also been shown to bind to both Raf-1 (Ayroldi *et al.*, 2002) and Ras (Ayroldi *et al.*, 2007) and thereby to modulate downstream signalling (**Fig 2)**. Ayroldi and colleagues reported that GILZ overexpression in anti-CD3-stimulated T cells can bind to Raf-1, which inhibits phosphorylation of Raf-1 and results in

suppression of MEK and ERK1/2 phosphorylation (Ayroldi *et al.*, 2002). They also found that GILZ can bind to Raf-1 via the NH_2-terminal region of GILZ and Ras via the TSC box of GILZ. The interaction of GILZ with Ras or Raf-1 has been shown to be dependent on the activation of Ras, where GILZ will bind predominately to Raf-1 in the absence of active Ras. However, as Ras is activated, Raf-1 will bind to Ras to a stronger degree than it will bind to GILZ. Furthermore, the affinity of GILZ to Ras will also increase, leading to predominately GILZ-Ras complexes (Ayroldi, 2007). GILZ may also form a trimer with both Ras and Raf-1, and this is also dependent on Ras activation. All the results above suggest that GILZ inhibits cell activation and inflammation via regulation of MAPK signaling molecules.

4.4. PI3K/Akt and apoptotic signalling pathways

The inhibition by GILZ of Ras and Raf-1 also decreases the activation of another downstream signalling pathway, the PI3 kinase (PI3K)/Akt pathway, which is involved in cell survival as well as activation. Recent studies have uncovered a crucial role for FoxO3 in mediation of PI3K/Akt pathway. In the cell, non-phosphorylated FoxO3 migrates into the nucleus and up-regulates several mediators of cell cycle progression, such as G1/S-specific cyclin-D1, $p27^{KIP1}$ (also known as cyclin-dependent kinase inhibitor 1B), Fas ligand (also known as tumor necrosis factor ligand superfamily member 6) and Bim (also known as Bcl-2-like protein 11), to inhibit cell proliferation (Schmidt *et al.*, 2002). Activation of Akt leads to the phosphorylation of FoxO3, which results in the nuclear exclusion of FoxO3 and thus leads to the inhibition of their cognate transcriptional targets. Interestingly, the gene encoding GILZ has been identified as a transcriptional target of FoxO3 (Asselin-Labat *et al.*, 2005b). Other studies have demonstrated that GILZ can inhibit its own expression through a negative feedback effect to promote nuclear exclusion of FoxO3 shown in **Fig 2** (Latre de Late et al., 2010). However, until now, the net effect of the PI3K/Akt-FoxO3–GILZ regulatory loop on cell proliferation has not yet been clearly defined.

GILZ has also been shown to modulate the expression of a variety of apoptosis pathway proteins in accordance with the observations that GILZ can prevent anti-CD3 mediated apoptosis (D'Adamio *et al.*, 1997). Asselin-Labat and colleagues showed that an increase in GILZ expression down-regulates the expression of Bim, a pro-apoptotic member of the Bcl-2 family, but has no effect on Bcl-x_L protein, an anti-apoptotic Bcl-2 family protein, to prevent apoptosis. In the same study, they demonstrated that knockdown of GILZ accelerates IL-2-deprivation-mediated apoptosis in the IL-2-dependent, CTLL-2 cell line, through increased levels of Bim (Asselin-Labat *et al.*, 2004). Furthermore, they showed that GILZ acts on Bim through the transcriptional factor FoxO3. Using a Bim-promoter-luciferase construct, GILZ expression was shown to repress Bim transcription, and these effects were abrogated with the co-expression of FoxO3 (Asselin-Labat *et al.*, 2004). However, it had been previously shown in $CD4^+CD8^+$ double positive T cells that overexpression of GILZ leads to an increase in the spontaneous apoptosis, and an interaction with, and reduction of, Bcl-x_L. Furthermore, GILZ expression was associated with an increase in the activation of extrinsic apoptotic caspases -3 and -8, but not caspase-9, involved in the mitochondrial/cytochrome C

pathway (Delfino *et al.*, 2004). The authors suggested that NF-κB inhibition is the mechanism by which TCR mediated apoptosis is inhibited by GILZ in over expression models, and that NF-κB may be involved in control of Bcl-x_L (Delfino *et al.*, 2006). Whilst the mechanism of GILZ in regulation of cell apoptosis has yet to be well understood, it follows the general trend of GILZ to display a dual activity in regards to apoptosis and cell survival.

4.5. Other non-inflammatory signalling pathways

In addition to mediating glucocorticoid effects in inflammation and immunity, GILZ may also play a critical role in non-immune function such as adipogenesis and osteogenesis. For example, Shi *et al* reported that GILZ directly binds to CCAAT/enhancer-binding protein (C/EBP) DNA binding sites in the *PPAR-γ2* promoter, with consequent inhibition of mesenchymal cell adipogenesis (Shi *et al.*, 2003). Previously, glucocorticoids had been shown to activate C/EBP directly, and therefore promote PPAR-γ2 expression and adipocyte differentiation (Shi *et al.*, 2003). This observation thus suggests a potential role of GILZ as a direct transcriptional repressor of gene expression in a direction opposite to the effects of Glucocorticoids. Of note, this is in contrast to the effects of GILZ binding to pro-inflammatory transcription factors where it mimics the effects of glucocorticoids. Moreover, as PPAR-γ2 is a key regulator of adipogenesis, GILZ's prevention of C/EBP action inhibits adipogenesis, and thereby promotes osteogenesis (Zhang *et al.*, 2008b), again an effect opposite to those of glucocorticoids which promote osteoporosis. The findings offer the suggestion of a possible GILZ-based therapy wherein GILZ exhibits beneficial glucocorticoid anti-inflammatory actions without the negative side effects of adiposity and osteoporosis.

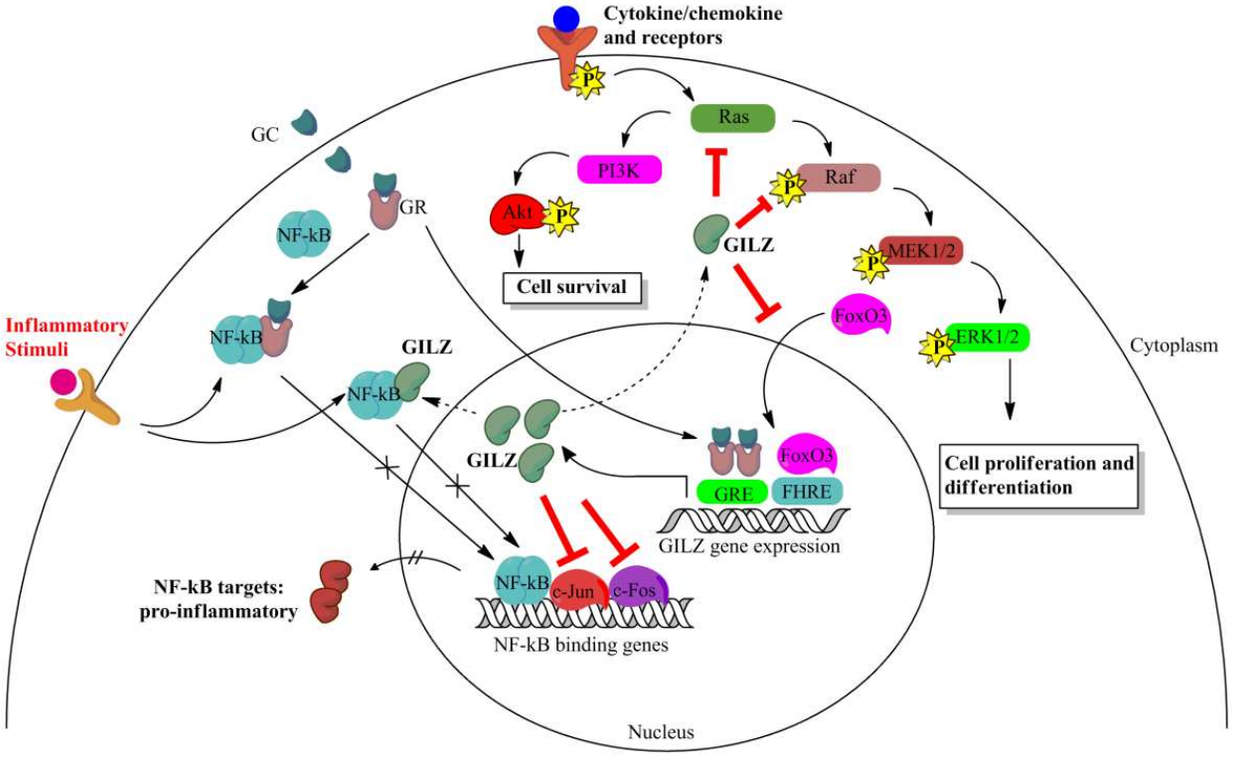

Figure 2. Roles of GILZ as a mediator in immune signaling pathways

NF-κB, activated by a variety of inflammatory stimulation, translocates into the nucleus and binds to target genes encoding pro-inflammatory factors. Glucocorticoid bound to the receptor GR can directly interact with NF-κB to prevent its nuclear translocation. In addition, the GC/GR complex can translocate into the nucleus and bind to glucocorticoid response elements on the *GILZ* gene to induce GILZ expression. GILZ in turn binds to NF-κB and prevents its nuclear translocation. GILZ can also directly bind to c-Jun and c-Fos, two constituents of AP-1, to inhibit their transcriptional activity and gene expression of pro-inflammatory molecules. The location, in the cytoplasm or nucleus, where GILZ binds to AP-1 subunits is still unknown. In the cytoplasm, GILZ also modulates cell survival by blocking Ras activation and the downstream PI3K/Akt signaling pathway. GILZ binds and inhibits Ras and Raf phosphorylation and thus inhibits downstream MEK-1/2 and ERK-1/2 activation. As part of a negative feedback loop, GILZ prevents nuclear translocation of FoxO3, which is in turn a key transcriptional factor to upregulate *GILZ* gene expression.

5. Effect of GILZ on inflammatory and autoimmune diseases

The reported actions of GILZ suggest GILZ may exert anti-inflammatory effects in immune and inflammatory diseases. Studies in animal disease models, or in human pathology, remain limited, but favour a role for GILZ as a modulator of immune-inflammatory responses. For example, Cannarile and colleagues reported GILZ effects on delayed-type hypersensitivity (DTH) responses in the GILZ transgenic mouse (Cannarile *et al.*, 2006b). In response to ovalbumin (OVA) immunization, GILZ overexpression mice exhibited significantly less swelling than wild type control, which indicates the essential role of GILZ in T cells in inhibiting T_H1 dependent DTH responses. The authors also investigated a murine model of colitis, in which it was shown that significant inhibition was observed in mice overexpressing GILZ in T cells (Cannarile *et al.*, 2009). In addition, studies of these T cells showed reduction of the T_H1 cytokine IFN-γ. Moreover, colon lysates from GILZ overexpressing mice have lower total and phosphorylated Ser536 NF-κB p65, which indicates that GILZ overexpression in T cells protects mice from T_H1-mediated colitis disease by inhibition of NF-κB activity.

Recently, Beaulieu and colleagues investigated the role of endogenous GILZ in RA (Beaulieu *et al.*, 2010a). GILZ was potently induced by glucocorticoids in cultured human RA synovial cells *in vitro*, and in murine arthritis *in vivo*. GILZ silencing by *in vivo* siRNA administration resulted in increased severity of the collagen-induced model of RA in mice, and in parallel GILZ overexpression inhibited chemokine and cytokine expression in human synovial cells. These results suggest GILZ as a key endogenous regulatory molecule in RA. Another study showed that GILZ was noticeably absent in granulomas in Crohn disease and tuberculosis (Berrebi *et al.*, 2003), which suggests inhibitor regulation of GILZ in the presence of chronic inflammatory disease, while human asthma patients demonstrated increased GILZ expression in response to glucocorticoid therapy (Kelly *et al.*, 2011).

GILZ is also reported to attenuate experimental autoimmune encephalomyelitis (EAE), a disease model of human multiple sclerosis. Srinivasan and colleagues demonstrated that

delivery of a GILZ-derived peptide is protective against EAE in mice (Srinivasan & Janardhanam, 2011b). The GILZ fragment they isolated, containing a proline rich domain, can directly interact with p65 NF-κB, thereby inhibiting p65 translocation from activated human $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PBMCs) (Srinivasan & Janardhanam, 2011a). As T cells are a major target of glucocorticoids in EAE (Wust *et al.*, 2008), these data provide further evidence that exogenous GILZ could exert therapeutically useful anti-inflammatory properties.

6. Perspective and expectations: GILZ as a glucocorticoid sparing target

GILZ, a molecule mainly modulated by glucocorticoids, play a pivotal role in the regulation of inflammation and immune responses. Expressed in multiple cells and tissues, GILZ inhibits the expression of a variety of inflammatory mediators and modulates the immune response. In this chapter, we have summarised GILZ structure and function, the effects of GILZ in immune responses, and its interaction with a number of key transduction pathways pivotal to the pathogenesis of inflammatory diseases. The more recent observations that GILZ exerts immunomodulatory and anti-inflammatory effects *in vivo* that mimic the inhibitory actions of glucocorticoids strongly suggests GILZ is a potential substitute for glucocorticoids in the therapy of inflammatory diseases.

As we have noted, currently a number of important anti-inflammatory molecules, such as Annexin A1 and MKP-1, are induced by glucocorticoids, and evidence that synthetic glucocorticoids lose their effectiveness in the absence of these molecules has been adduced (Furst *et al.*, 2007; Ralph & Morand, 2008; Yang *et al.*, 2009; Yang *et al.*, 2004; Yang *et al.*, 2006). Attention to the molecules that glucocorticoids amplify the expression of will permit discovery of the means to develop a surrogate for glucocorticoids' beneficial impact on immune activation without their toxicity. Importantly, the presence of GILZ exerts immune and inflammation modulatory effects in the absence of glucocorticoids. A GILZ-based therapeutic approach, therefore, could potentially offer profound glucocorticoid-like regulatory effects in autoimmune disease. Investigation of the metabolic effects of any GILZ-based therapy is required in order to ensure that the undesirable effects of glucocorticoids are not recapitulated. Early results are encouraging in this regard. In mesenchymal stem cells, differentiation towards osteogenic precursors is enhanced by GILZ, whereas silencing of GILZ reduced osteogenic differentiation (Zhang *et al.*, 2008a), suggesting that a GILZ therapy might have protective rather than harmful effects on bone. GILZ expression was also associated with osteoblast development, and GILZ silencing increased osteoblast expression of OPG and RANKL in favour of osteoclastogenesis (Lekva *et al.*, 2010), further suggesting that GILZ-based therapy might have a bone-protective effect. Studies of the role of GILZ in glucocorticoid-induced osteoporosis *in vivo* are eagerly awaited.

GILZ-based therapies could be based around the administration of recombinant protein or NF-κB binding peptides. As we have introduced above, Srinivasan and colleagues have described a novel NF-κB p65 binding GILZ peptide which exhibited therapeutic potential as a NF-κB inhibitor in EAE (Srinivasan & Janardhanam, 2011b). Alternatively, a gene therapy

approach, which has already been successfully used *in vivo* to suppress arthritis via delivery of the anti-inflammatory cytokine IL-10 (Apparailly *et al.*, 2002), could be applied to GILZ. Inducing GILZ expression other than through the use of glucocorticoids, for example by modifying activity of the transcription factor FoxO3, could represent a further means to increase available GILZ protein, as could inhibition of the as-yet unidentified mechanisms of GILZ protein turnover. Finally, structure-function analysis of the molecules with which GILZ interacts in order to achieve its immune modifying effects could reveal targets for synthetic GILZ mimetics. Although considerable work remains, the first proof of concept studies of an *in vivo* GILZ-based therapeutic approach is under development in the authors' laboratory (unpublished observations).

In conclusion, glucocorticoids remain among the most widely used drugs in human diseases, and in particular in autoimmune disease. Their effectiveness is increasingly well understood, based on their effects on inflammatory signal transduction, but their use is constrained by toxicity, which also relates to their specific physiological actions. GILZ is a key molecule in glucocorticoid biology, which now represents a candidate mediator of glucocorticoid regulation of immune and inflammatory responses, and deserves further investigation.

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