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Micro article

A focused Real Time PCR strategy to determine GILZ expression in mouse tissues

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ABSTRACT

Glucocorticoid-Induced Leucine Zipper (GILZ) is a glucocorticoid-inducible gene that mediates glucocorticoid anti-inflammatory effects. GILZ and the isoform L-GILZ are expressed in a variety of cell types, especially of hematopoietic origin, including macrophages, lymphocytes and epithelial cells, and strongly upregulated upon glucocorticoid treatment.

A quantitative analysis of GILZ expression in mouse tissues is technically difficult to perform because of the presence of a pseudogene and the high homology of *GILZ* gene with other genes of TSC22 family. We here propose specific primer pairs to be used in Real Time PCR to avoid unwanted amplification of GILZ pseudogene and TSC-22 family member d1iso3. These primer pairs were used to determine GILZ and L-GILZ expression, in either untreated or *in vivo* and *in vitro* dexamethasone-treated tissues. Results indicate that GILZ and L-GILZ are upregulated by glucocorticoids, being GILZ more sensitive to glucocorticoid induction than L-GILZ, but they are differently expressed in all examined tissues, confirming a different role in specific cells. An inappropriate primer pair amplified also GILZ pseudogene and TSC22d1iso3, thus producing misleading results. This quantitative evaluation may be used to better characterize the role of GILZ and L-GILZ in mice and may be translated to humans.

1. Introduction

Glucocorticoid-Induced Leucine Zipper (GILZ) is a glucocorticoid-inducible gene belonging to the TSC22 family, whose members share TSC box and leucine zipper domains. GILZ is one of the earliest and rapidly induced gene by glucocorticoids (GCs), mainly in lymphoid cells, which regulates activation and apoptosis. Evidence is accumulating that GILZ is an important mediator of GC anti-inflammatory effects in cell lineages and in inflammatory mouse models, through inhibition of the expression of pro-inflammatory genes [1,10,13,16,3,5]. This effect depends on the ability of GILZ to homo- and hetero-dimerize, thereby acting as a regulator of gene transcription.

L-GILZ is an isoform of GILZ capable to bind Ras. It is particularly expressed in testis, where its absence causes failure of spermatogenesis [23,26,8]. In addition, L-GILZ has been found to mediate the anti-myogenic effect of GCs [7] and, interestingly, can bind p53, thus leading to tumor growth suppression [2].

Although GILZ and L-GILZ have been studied in diverse cell types, especially in the immune system, a systematic analysis of their tissue distribution has never been done. Furthermore, the high homology of GILZ with TSC22d1 and the existence of a GILZ pseudogene may lead to false results of GILZ expression.

We here show the data that prompted us to find a specific strategy to amplify undoubtedly GILZ through quantitative Real Time PCR. Finally, by using this method, we performed a deep analysis of GILZ and L-GILZ expression in immune and non-immune tissues and in the same tissues after exposure to glucocorticoids (GC), both *in vitro* and *in vivo*.

2. Materials and methods

2.1. In vivo and in vitro dexamethasone treatment

Six–eight weeks old C57Bl/6 mice were treated with dexamethasone (DEX) 10 mg/kg by intraperitoneal administration, 3 h before sacrifice and removing organs. *In vitro* experiments were performed by reducing organs to single cell suspension through cell strainer separation and treating cells (2×10^6/ml) by either DEX 10^{-6} and 10^{-7} M for 3 h.

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2.2. Reverse transcriptase PCR

The RNA of tissues or single cells was isolated using the Trizol reagent (Ambion, Life Technologies), and conversion of total RNA to cDNA was performed with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. PCR was performed with 20 ng of cDNA, and all primers are listed in supplementary Table 1. Taq polymerase was purchased from Life Technologies. For primer couples A-B and C-D reactions were incubated in a 96-well Eppendorf Mastercycler ep gradient S at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s, 34 cycles. For primer couple E-F reactions were incubated in a 96-well Eppendorf Mastercycler ep gradient S at at 95 °C for 30 s, 58 °C for 20 s and 72 °C for 30 s, 36 cycles. For primer couple W-Z reactions were incubated in a 96-well Eppendorf Mastercycler ep gradient S at 95 °C for 30 s, 57 °C for 20 s and 72 °C for 30 s, 36 cycles.

2.3. Real Time PCR

Real Time PCR reactions were performed using the ABI 7300 Real Time Cycler (Applied Biosystems), and amplification was achieved using the Sybr select master mix (Applied Biosystems) with primers listed in supplementary Table 1. Time and temperature conditions are the same as described above. GAPDH was used as housekeeping gene and its expression was evaluated by Taqman assay using standard conditions (4352339E, Life Technologies). All experiments were carried out in triplicate, and the ΔΔCt method was used to determine expression of GILZ and L-GILZ.

3. Results

3.1. Non-specific PCR amplification with some primers for GILZ

When we tried to amplify GILZ by reverse transcriptase PCR (RT-PCR), we found that several couples of primers located along GILZ mRNA (for instance, Forward primer in exon 3-W- and Reverse primer in exon 6-Z) amplified a product even in GILZ knock-out (KO) cells, demonstrating that they amplify other sequences homologous to GILZ (Fig. 1a and b).

In an attempt to find genes sharing high homology with murine GILZ, in chromosome 1 we found a pseudogene (NC_000067.6, 66062746-66064858, NCBI, February 2015) that shows 95% overall homology to GILZ. The major portion of the small difference between GILZ and the pseudogene is due to non-contiguous point mutations (see, for example, supplementary Fig. 1). Moreover, the pseudogene shows a 191 bp sequence in a region located in exon 6, which is missing in GILZ (dotted in Fig. 1a). To demonstrate that GILZ pseudogene can be amplified, we used primers A and B located in exon 6 (Fig. 1a), which can theoretically amplify both GILZ (177 bp) and its pseudogene (361 bp). Fig. 1c shows that GILZ pseudogene can be amplified and derived from genomic DNA contamination of cDNA samples, as demonstrated by the fact that amplification is observed in RNA samples in which RT has not been performed (not shown).

TSC22 family member TSC22d1iso3 (NM_001177751.2) is another gene that shows a high homology with GILZ. In particular, the homology is very high between the exon 5 of GILZ and the exon 2 of TSC22d1iso3, and the exon 6 of GILZ and the exon 3 of Tsc22d1iso3 (supplementary Fig. 1). There is even some homology between the exon 3 of GILZ and the exon 1 of TSC22d1iso3. Reasonably, some couples of primers annealing on regions of high similarity between GILZ and TSC22d1iso3 can amplify GILZ together with TSC22d1iso3. To test whether this was the case, we designed a strategy of digestion of PCR products with 2 restriction enzymes (supplementary Fig. 1 and Table 2). The digestion releases 3 different fragments, which are TSC22d1iso3, GILZ pseudogene and GILZ. Digestion of the WT PCR products shown in Fig. 1d clearly demonstrates that this primer pair amplifies not only GILZ, but also TSC22D1 and the pseudogene (Fig. 1d). This result suggests that quantification of GILZ is not accurate, if inappropriate primers are used, especially when GILZ is expressed at low levels or not at all. As a consequence, quantitative analysis by Real Time PCR could be biased.

3.2. Specific PCR amplification of GILZ

In order to circumvent the above problems, we identified a specific region in exon 6 (hatched in Fig. 1a), spanning 7 bp, which is unique in GILZ and missing in the pseudogene (supplementary

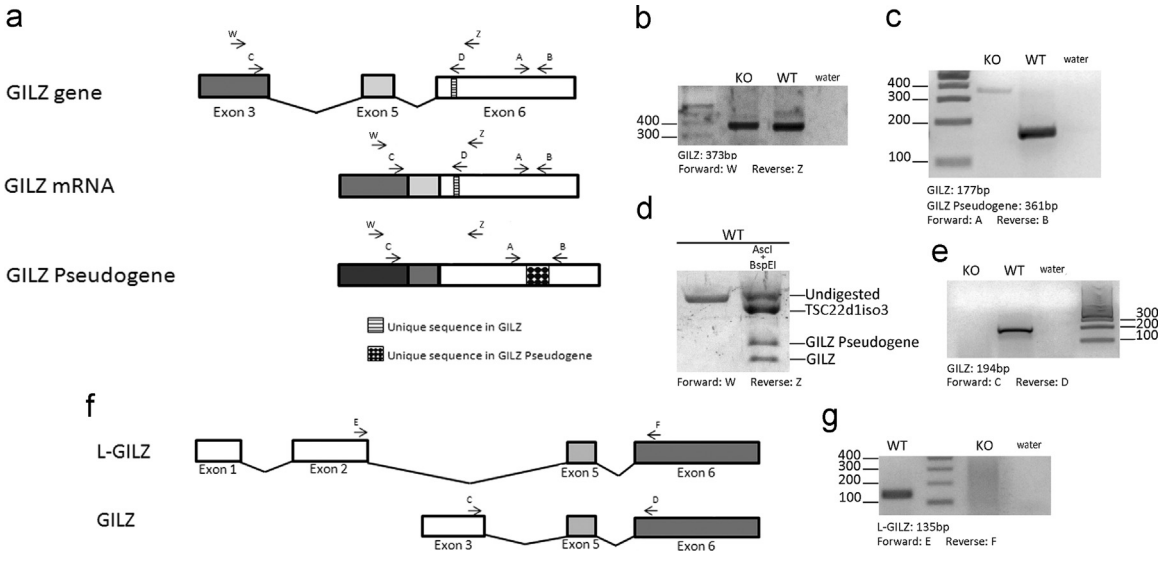

Fig. 1. PCR strategy to amplify GILZ and L-GILZ: (a) structure of GILZ gene, GILZ mRNA and GILZ pseudogene. W and Z represent the primers used in RT-PCR shown in (b) and (d), A and B represent the primers used in the RT-PCR shown in (c), whereas C and D are the primers used in (e). (b) RT-PCR performed with inappropriate primers W–Z. (c) RT-PCR performed with A and B primers. (d) Restriction enzyme digestion performed in the WT PCR product shown in (b). (e) PCR to amplify GILZ. All PCRs used thymus-derived cDNA. The expected lengths of digested fragments are indicated for each amplified gene: (f) gene structure of L-GILZ compared with GILZ. E and F are specific primers to amplify L-GILZ. (g) RT-PCR to amplify L-GILZ in testis.

Fig. 2), so that the GILZ reverse primer (D) could be designed overlapping this region. The same primer presents four mismatches with TSC22d1iso3, two of them towards the 3′ end of the primer (-2 and -5, see supplementary Fig. 2). Forward primer (C) in exon 3 has a perfect match with both GILZ and GILZ pseudogene, and 11 mismatches with TSC22d1iso3, three of them towards the 3′ end of the primer (-2, -3 and -5, see supplementary Fig. 2). Therefore, it is reasonable to hypothesize that the primer pair C–D uniquely amplifies GILZ, as demonstrated by the absence of GILZ in KO mice (Fig. 1e). A further proof is the digestion of the PCR product from WT mice with BspEI restriction enzyme (not shown), whose site is present in GILZ but not in TSC22d1iso3 mRNA.

3.3. Specific amplification of L-GILZ

The GILZ pseudogene does not include exon 1 and 2 of L-GILZ and, to our knowledge, the other genes belonging to TSC22 do not present similar isoforms. Therefore, it can be hypothesized that L-GILZ specific primers amplify uniquely L-GILZ. Indeed, when we tested forward primer E and reverse primer F (annealing to exon 2 and exon 6, respectively), amplification of L-GILZ was obtained in WT and none in KO mice (Fig. 1f and g).

3.4. GILZ and L-GILZ tissue distribution and induction by GCs

Once designed the appropriate PCR strategy through the use of specific primers (C and D for GILZ, E and F for L-GILZ), a quantitative analysis of GILZ and L-GILZ expression in tissues were performed by Real Time PCR. Fig. 2a and b show GILZ and L-GILZ expression in a variety of fresh mouse tissues. Fig. 2c summarizes and compares the expression of both transcripts: GILZ and L-GILZ are differently expressed in almost all tissues.

Because GILZ is a GC-induced gene, we treated mice with the synthetic glucocorticoid dexamethasone (DEX) for 3 h, and recovered tissues from diverse organs. Data in Fig. 3 clearly show how both GILZ (Fig. 3a) and L-GILZ (Fig. 3b) are upregulated after GC exposure in almost all tissues. GILZ is more sensitive to GC induction than L-GILZ, reaching high levels of expression especially in thymus and in lung.

Lymphoid tissues from untreated mice were further characterized *in vitro* by 3 h DEX treatment. Two doses of DEX were used and results shown in Fig. 4 demonstrate that there is always upregulation of GILZ and L-GILZ after DEX exposure. L-GILZ and GILZ upregulation is dose-dependent only in thymus and bone marrow, while is dose-independent in spleen and lymph nodes, suggesting that a low DEX dose is enough to induce both genes.

3.5. Reliable results with the use of specific primers

To test whether the use of inappropriate primer couples would produce biased results in a Real Time PCR assay, we tested the expression of GILZ and its induction by DEX in some tissues, using the couple of primer W–Z. Results shown in supplementary Fig. 3 demonstrate that the expression of GILZ investigated by using inappropriate primers W and Z is completely different from the one obtained with the specific primers C and D (left panel) and that the effect of DEX is underestimated when GILZ expression is evaluated by inappropriate primers (right panel). This is a consequence of overestimation of GILZ expression in untreated specimens.

In conclusion, in our opinion the use of C–D primer couple allows a correct quantification of GILZ and the use of E–F primer couple allows a correct quantification of L-GILZ.

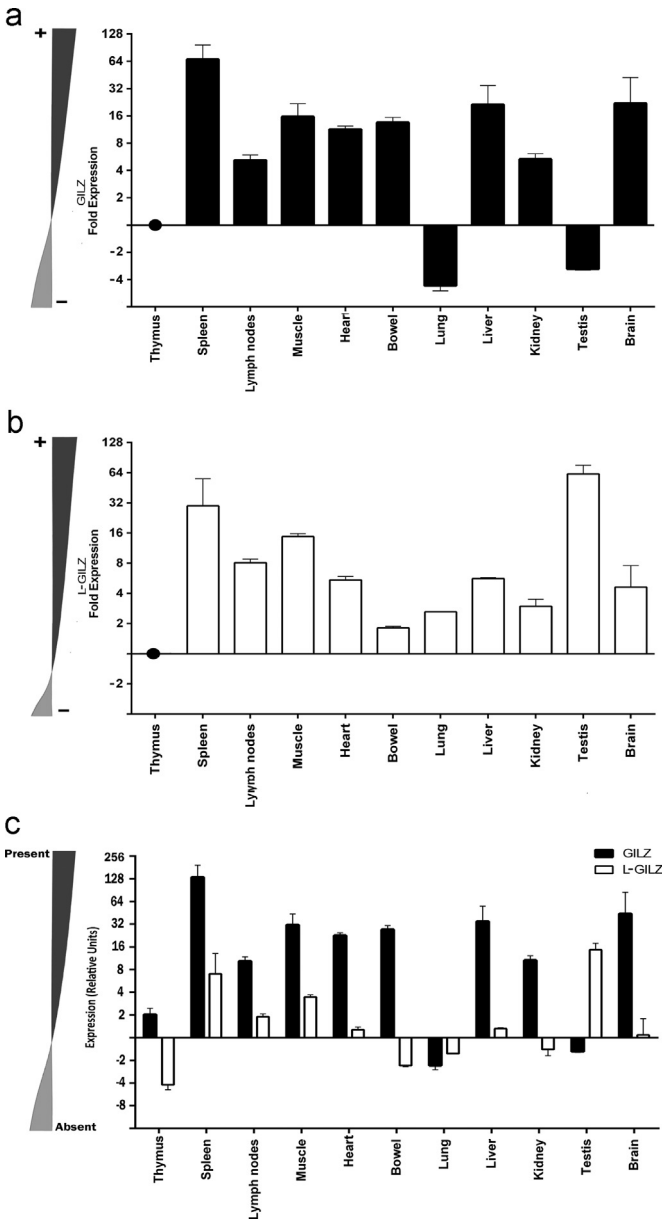

Fig. 2. Real Time PCR quantification in fresh mouse tissues: (a) and (b) GILZ and L-GILZ expression was normalized using the housekeeping gene GAPDH. All values were calculated with respect of thymus, set equal to 1 and expressed in a base 2 log scale. (c) Comparison of GILZ and L-GILZ in the same tissues as in (a) and (b) in which values were considered relatively to the housekeeping gene expression, setting arbitrarily a basal level (*X*-axis). Results are mean of triplicates ± SD of two experiments, and shown on a base 2 log scale.

4. Discussion

The evaluation of gene expression is currently performed mostly by Real Time PCR. Gene expression is correctly evaluated if only one gene product is amplified, by using specific primers. Moreover, the amplification of contaminating DNA can be simply avoided by using a forward and a reverse primer annealing in separate exons. Evaluation of GILZ expression is complicated by two problems: the presence of a GILZ pseudogene and the high homology of GILZ with TSC22d1iso3. GILZ pseudogene comprises all GILZ exons, without any introns. Therefore, GILZ pseudogene can be amplified even when using primer annealing on separate exons, in the presence of contaminating cellular DNA, which is usually present even in technically good RNA samples. Our data show that GILZ pseudogene was amplified even in the presence of

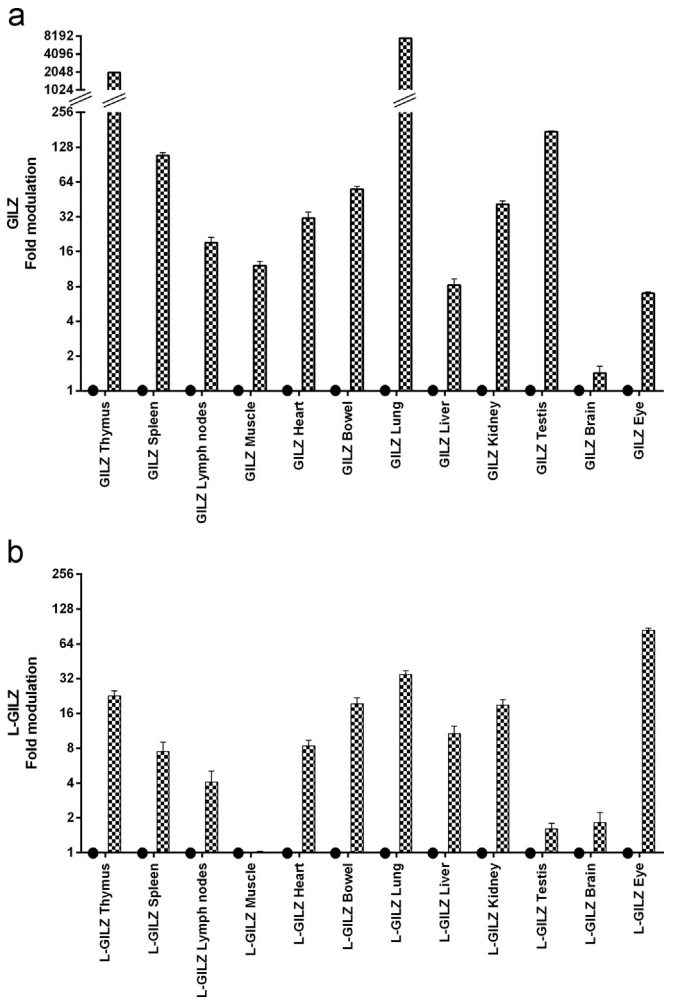

Fig. 3. Modulation of GILZ and L-GILZ investigated by Real Time PCR in fresh mouse tissues 3 h after *in vivo* DEX treatment (10 mg/kg). The fold values in each group were calculated *versus* the same tissues from untreated mice, set equal to 1, on a base 2 log scale. Results are mean of triplicates ± SD of two experiments.

GILZ, which competes with the pseudogene (Fig. 1d). The homology between the exon 5 of GILZ and the exon 2 of TSC22d1iso3, and the exon 6 of GILZ and the exon 3 of TSC22d1iso3 is very high (supplementary Fig. 1). Therefore, the possibility that a reverse primer designed on exons 5 and 6 of GILZ anneals also on Tsc22d1iso3 sequence is quite high. Some homology is also present between the exon 3 of GILZ and the exon 1 of TSC22d1iso3 and it is possible that a forward primer designed on exon 3 of GILZ has some identity with TSC22d1iso3 gene. If this similarity is quite good in the 3′ end of the primer, the possibility that it anneals and generates a PCR product is good. We show that W–Z primer pair (Fig. 1a and supplementary Table 1) generated a PCR product including TSC22d1iso3 isoform, not only in KO tissues but also in WT cells, despite the presence of GILZ and the pseudogene as competing templates. The PCR products deriving from GILZ pseudogene and Tsc22d1iso3 isoform have a very similar length as GILZ (supplementary Table 1). As a consequence, they cannot be separated by a gel run. Therefore, the possibility that GILZ expression is wrongly evaluated using inappropriate primers is high, as shown in the supplementary Fig. 3.

To avoid the above mentioned problems, we designed primers C and D having a lower tendency to anneal to GILZ pseudogene and to TSC22d1iso3 isoform. In particular, the reverse primer D cannot anneal at the 3′ end in the pseudogene sequence, and the 3′ end of both forward and reverse primers are suboptimal for the annealing to TSC22d1iso3 isoform. The lack of any PCR products in KO cells (Fig. 1g) confirmed that these primers specifically amplify GILZ. Importantly, the primer pair C–D has been optimally designed to work properly at 60 °C, as, for example, in a Taqman assay.

Anti-inflammatory effects of GCs encompass multiple mechanisms, among which induction of specific genes in immune cells represents one of the most important. GILZ is one of the earliest induced genes and has been widely studied in diverse cell types, including T cells [11,14,22,24,6], macrophages [18], dendritic [9] and epithelial cells [19]. However, a systematic screening of GILZ expression in mouse tissues and its regulation after DEX treatment has never been done so far.

Our results demonstrate an almost ubiquitous presence of GILZ in all tissues with low levels in testis, as already known [8], and in lungs, where a surge in its expression occurs after DEX treatment. We exactly do not know which is the cell type expressing GILZ in the lung, but it stands to reason that macrophages could be the candidate cells. This could be suggested by the observation of a higher expression of GILZ in spleen than in lymph nodes, maintained after *in vivo* DEX administration, and by previous work [12,17,18,28]. Such results partially overlap with a very recent work done in rat tissues [4].

L-GILZ was found to be expressed in all the examined tissues, and, most importantly, was found to be upregulated by DEX at different extent. Of note is the more exquisite sensitivity of GILZ than L-GILZ to DEX induction, as can be observed in Fig. 2c, and this feature represents a further difference between GILZ and L-GILZ. Reasonably, this difference is due to the diverse promoter regions of GILZ and L-GILZ, which are separated by about 54 kb, thus being differently regulated. Furthermore, GILZ promoter contains 6 putative Glucocorticoid Responsive Elements (GRE) while L-GILZ only 4, as assessed by *in silico* analysis through Matinspector software (Genomatix). GILZ and L-GILZ even differ in the intra-cellular localization (L-GILZ is predominantly cytoplasmic while GILZ is both in the cytoplasm and in the nucleus) and function [13,18,1,27,2]. Of note, GILZ and L-GILZ are differently expressed in untreated tissues as demonstrated here, further suggesting diverse roles according to the cell type.

The difference in GC sensitivity between GILZ and L-GILZ *in vivo* is not reproduced in the *in vitro* studies from a quantitative point of view. This is not surprising since different experimental conditions were used. *In vivo*, distribution of GCs varies according to the tissues and their metabolism can influence the response of cells in tissues and change local GC concentration as well. Furthermore, *in vivo* GC can modulate activity of immune cells and their migration, resulting in a different cell type organization in each lymphoid organ, while untreated organs may be slightly different. The microenvironment in organs *in vivo* can influence the response to GCs, which does not occur in isolated organs. The *in vivo* GC treatment and consequent analysis of GILZ and L-GILZ induction is very close to what happens under pharmacological treatment during a disease. However, the common feature is the raise in expression of GILZ and L-GILZ after GC exposure, which is maintained in both experimental conditions (*in vivo* and *in vitro*).

The application of the strategy proposed here will add new information about the role played by GILZ in inflammation and cancer and could help find a correlation between GILZ/L-GILZ expression and diseases [15,20,21,25], especially those involving treatment with GCs. Finally, such a quantitative strategy could be applied to human cells, since a pseudogene does exist in human genome (TSC22 domain family member 3 pseudogene LOC 100287033).

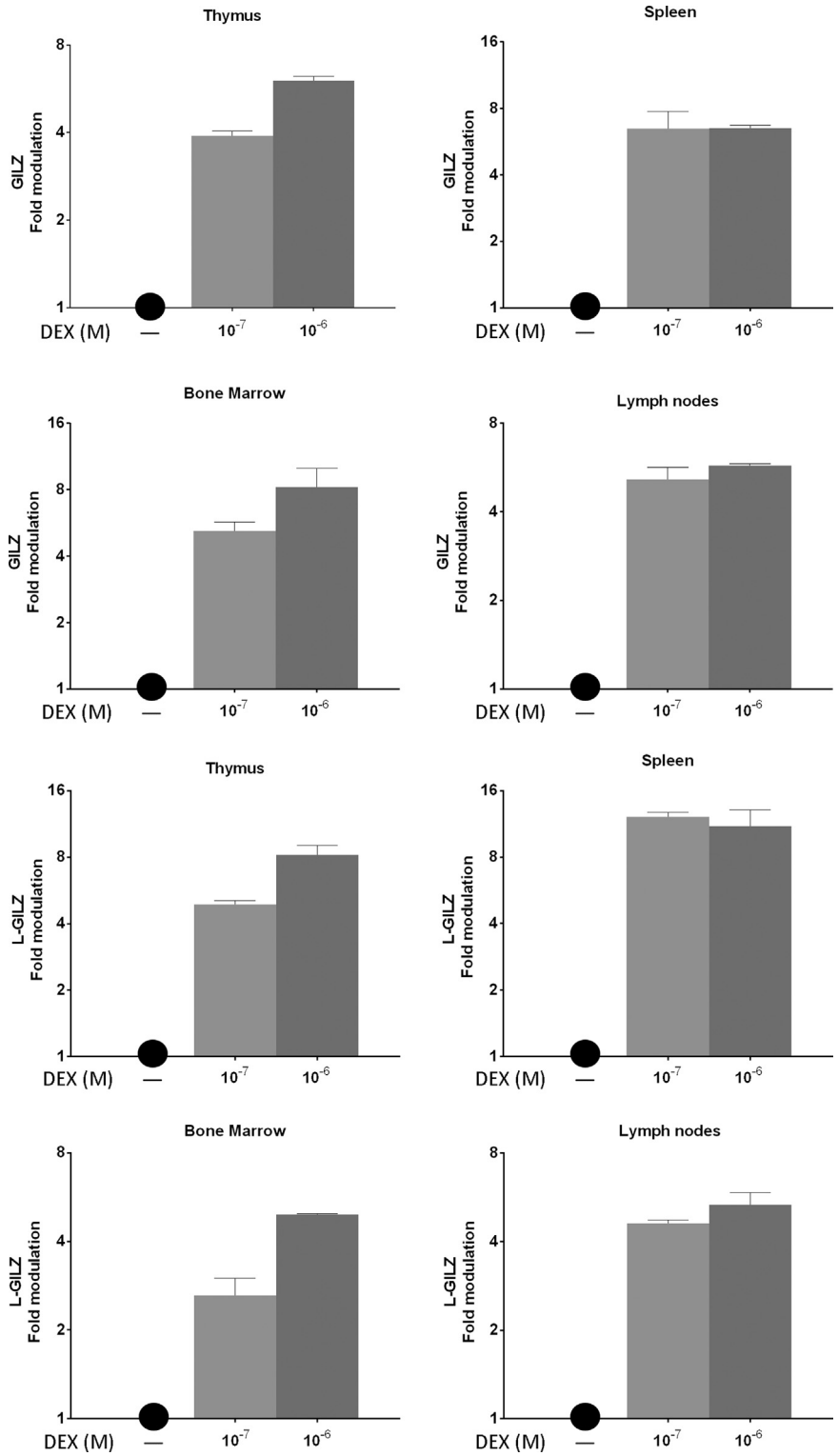

Fig. 4. GILZ and L-GILZ expression investigated by Real Time PCR in *in vitro* DEX-treated lymphoid tissues. Cells from thymus, spleen, bone marrow and lymph nodes were treated with two doses of DEX, for 3 h. The fold values in each group were calculated *versus* the same untreated cells, set equal to 1, on a base 2 log scale. Results are mean of triplicates ± SD of two experiments.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.rinim.2015.10.003.

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