

# Glucocorticoid-Induced Leucine Zipper Governs the Therapeutic Potential of Mesenchymal Stem Cells by Inducing a Switch From Pathogenic to Regulatory Th17 Cells in a Mouse Model of Collagen-Induced Arthritis

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**Objective.** Mesenchymal stem cells (MSCs) are potent immunosuppressive cells that have shown promise in the treatment of rheumatoid arthritis (RA). Deciphering the intrinsic characteristics of MSCs that correlate with their biologic activity will facilitate their clinical use. Recently, the role of glucocorticoid-induced leucine zipper (GILZ) in the development of RA has been documented. The aim of this study was to evaluate whether GILZ expression by MSCs may contribute to their therapeutic effect.

**Methods.** MSCs were isolated from GILZ-deficient (GILZ<sup>-/-</sup>) mice and wild-type mice. MSCs (1 × 10<sup>6</sup> cells) were injected twice via the tail vein into mice with collagen-induced arthritis (CIA).

**Results.** In vitro, we showed that GILZ is a key factor involved in the immunosuppressive potential of MSCs. MSCs derived from GILZ<sup>-/-</sup> mice did not

suppress the proliferation of CD4+ T cells and were less efficient than MSCs derived from WT mice in altering Th17 cell polarization. Thus, we investigated the role of GILZ in an experimental model of arthritis and demonstrated that although WT MSCs significantly reduced paw swelling in arthritic mice, GILZ<sup>-/-</sup> MSCs did not. Moreover, the magnitude of the effects of GILZ<sup>-/-</sup> MSCs on Th17 cell frequency was significantly lower than that of WT MSCs. The therapeutic effect of MSCs correlated with the generation of Treg cells bearing the CD4 + RORγt + IL-17<sup>low</sup> IL-10+ signature, and Th17 cell polarization was GILZ dependent.

**Conclusion.** This study demonstrates that GILZ has an essential role in the therapeutic effectiveness of MSCs in arthritis by favoring Th17 cell polarization toward a regulatory phenotype. Therefore, potentiation of GILZ expression in MSCs could represent a means to enhance their therapeutic effect in autoimmune diseases.

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Currently, more than 50% of patients with rheumatoid arthritis (RA) are treated with systemic glucocorticoids (1–3). The effects of glucocorticoids are powerful, but their mechanisms of action are still not fully understood. Glucocorticoid-induced leucine zipper (GILZ), which is an important mediator of the action of glucocorticoids, has been suggested to exert a key role in the regulation of inflammation (4). GILZ acts as an early-response transcription factor that binds to the proinflammatory transcription factors NF-κB and activator protein 1 (AP-1). Despite the fact that GILZ is not a physiologic inhibitor of effector pathways in RA (5), GILZ silencing exacerbated the severity of murine collagen-induced arthritis (CIA; a commonly used animal model of human RA), while

GILZ overexpression inhibited chemokine and cytokine expression in human RA synovial fibroblasts (6) and in murine CIA (5). Because these observations suggested that GILZ mimics the antiinflammatory effects of glucocorticoids (4,7), we investigated whether the antiinflammatory effects of mesenchymal stem cells (MSCs) might be affected by GILZ expression.

RA is a systemic autoimmune disease characterized by synovial joint inflammation and infiltration of activated cells, cartilage destruction, and bone erosions (8,9). The murine CIA model is used extensively to investigate the mechanisms involved in the development of the disease and to test new treatment options, because it shares many immunologic and clinical features with human RA (10). Expression of interferon- $\gamma$  (IFN $\gamma$ ) is initiated early in the course of arthritis and is followed by interleukin-17 (IL-17) production after the clinical onset of disease (11). Therefore, the development of arthritis is characterized by a shift from a dominant Th1 cell response to dual Th1 cell/Th17 cell responses, suggesting that Th17 cells are key T<sub>H</sub> cells in the pathogenesis of arthritis (11). In addition to an increased frequency of Th1 cells and Th17 cells during early arthritis, a reduction in the number of Treg cells or a qualitative defect in Treg cell function has been suggested to mediate the development of arthritis (12,13). Taking these studies into account, inhibition of arthritis-associated inflammation using cell-based therapy has been of interest for several years as a potential addition to the biotherapies that are already widely used clinically (14).

In this context, MSCs have been extensively evaluated due to their capacity to suppress the host immune response (for review, see ref. 15). The potent immunosuppressive capacity of MSCs inhibits the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B lymphocytes, and natural killer cells and has been described both *in vitro* and *in vivo* in experimental models of disease (15–20). The mechanisms underlying immunomodulation induced by MSC treatment mediated by both soluble factors and cell–cell contact–dependent pathways are not fully understood. The possible mediators identified include factors specific to mouse MSCs, such as inducible nitric oxide synthase (21), or to human MSCs, including indoleamine dioxygenase (22) and HLA-G (23); other factors are overlapping molecules such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (16,24,25).

Several *in vitro* studies in which the immunomodulatory properties of MSCs were investigated revealed that MSCs might be of interest for the treatment of inflammatory arthritis (26–28). However, the therapeutic effect of MSCs in experimental animal models of

arthritis is controversial. The beneficial effect of MSCs in CIA occurs only if the cells are administered during a narrow window of time. MSC injection after disease onset did not prevent the occurrence of signs of arthritis (28). A previous study showed that a single injection of MSCs derived from mice of different genetic backgrounds, when administered at the time of antigen boost, exerts variable effects on CIA progression (29). The discrepancies between the reported therapeutic effects of MSCs might result from parameters including intrinsic molecular particularities of MSCs from different sources, genetic mismatch between the donor MSCs and the recipient, the time of MSC administration, and the number of MSCs injected (28,29).

The aim of the current study was to elucidate, *in vivo*, the role of GILZ expression in the therapeutic effect mediated by MSCs in an experimental model of arthritis. We investigated whether the beneficial effect of MSCs in arthritis was associated with GILZ-mediated restoration of the balance between Th17 cells and Treg cells that controls autoimmunity and inflammation. Our findings indicate a critical role of GILZ in the antiinflammatory effects of MSCs in arthritis and show that GILZ is required for the effects of MSCs on the generation of IL-10–producing regulatory Th17 cells. These data suggest the possibility that enhanced GILZ expression might improve the therapeutic effectiveness of MSCs in arthritis.

## MATERIALS AND METHODS

**Isolation, characterization, and transfection of murine MSCs.** MSCs were isolated and characterized as previously described (28), using bones from wild-type (WT) C57BL/6 mice and GILZ-deficient C57BL/6 mice (5). Transfection of WT mouse MSCs with the pCDNA3.1-GILZ plasmid (GILZ MSC plasmid, kindly provided by Carlo Riccardi) was performed using Lipofectamine according to the recommendations of the manufacturer (Invitrogen). Cells were transfected 48 hours before administration to the mice.

**Proliferation assay.** CD4<sup>+</sup> T cells were isolated from the spleens of DBA/1 mice by negative selection, using a Dynal CD4 Negative Isolation Kit (Invitrogen) according to the manufacturer's instructions, and activated with anti-mouse CD3/CD28 Dynabeads (Invitrogen). The activated CD4<sup>+</sup> T cells were then cultured alone or with MSCs at a 1:10 ratio (MSC:CD4<sup>+</sup> T cell) in complete Iscove's modified Dulbecco's medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 20 mM HEPES, and 50  $\mu$ M  $\beta$ -mercaptoethanol (Invitrogen). After 72 hours, CD4<sup>+</sup> T cells were quantified with a CellTiter-Glo Luminescent Cell Viability Assay (Promega). In order to test the suppressive effect of conditioned supernatants from either the splenocytes or lymph node cells (LNCs) of mice with CIA that were left untreated or were

treated with WT MSCs or GILZ<sup>-/-</sup> mouse MSCs (GILZ<sup>-/-</sup> MSCs), fresh splenocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and cultured with 5  $\mu$ g/ml of concanavalin A (Sigma-Aldrich). When neutralizing antibody to IL-10 was added, it was used at the concentration of 2.5  $\mu$ g/ml (BD Biosciences). After 72 hours, splenocyte proliferation was assessed by flow cytometry.

**Induction of Th17 cell differentiation.** Isolated CD4+ T cells were induced to differentiate into Th17 cells, as previously described (30). MSCs either were not added or were added at a 1:10 ratio (MSC:CD4+ T cell). After 72 hours, Th17 cell differentiation was assessed by flow cytometry.

**Real-time quantitative polymerase chain reaction (PCR) analysis.** Total RNA was extracted using an RNeasy Mini Kit (Qiagen). RNA was reverse transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems). Quantitative PCR was performed using a SYBR Green I Master kit and a LightCycler 480 system, according to the recommendations of the manufacturer (Roche Applied Science). Specific primers for neuropilin 1 and Helios were designed using Primer3 software (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>). Expression of messenger RNA (mRNA) for neuropilin 1 and Helios was normalized to that of the housekeeping gene ribosomal protein S9. The relative mRNA expression was then normalized to that of each specific gene, using the 2<sup>- $\Delta$ C<sub>t</sub></sup> method.

**Quantification of cytokines.** Enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems. Cytokines were quantified in culture supernatant (from T cell proliferation assays) or in sera. For analysis of cytokine production by splenocytes or LNCs, 2  $\times$  10<sup>6</sup> cells were seeded in proliferation medium and stimulated with 25  $\mu$ g/ml of bovine type II collagen. Supernatants were collected after 48 hours. Bovine type II collagen-specific immunoglobulins were quantified using alkaline phosphatase-labeled anti-mouse IgG1 and anti-mouse IgG2a antibodies (BD Biosciences), as previously described (31).

**Induction and scoring of arthritis.** Adult DBA/1 mice (9–10 weeks old) were obtained from our in-house animal breeding facility. Animal experiments were performed in accordance with the Committee on the Ethics of Animal Experiments in Languedoc-Roussillon (permit no. CEEA-LR-1042). Immunization was performed as previously described (32), and MSCs (1  $\times$  10<sup>6</sup>) were injected intravenously on days 18 and 24, as previously described (28). CIA was induced in 4 groups of mice: an (untreated) CIA group (n = 25), a group treated with WT MSCs (n = 25), a group treated with GILZ<sup>-/-</sup> MSCs (n = 25), and a group treated with the GILZ MSC plasmid (n = 10). Each experiment was performed 3 times. Signs of arthritis were assessed by measuring swelling of the hindpaws and by determining the arthritis score (using an extended scoring protocol with a maximum score of 40 per mouse). LNs and spleens were collected on the day the mice were killed.

**Flow cytometric analysis (fluorescence-activated cell sorting).** For intracellular cytokine detection, cells were stimulated with phorbol myristate acetate (50 ng/ml), ionomycin (1  $\mu$ g/ml), and brefeldin A (10  $\mu$ g/ml) for 4 hours. Cells were then stained with specific antibodies against mouse CD4 and CD25 before being fixed and permeabilized with Fixation/

Permeabilization Concentrate (eBioscience) and stained intracellularly with anti-IL-10, anti-IL-17, anti-IFN $\gamma$  (BD Biosciences), anti-FoxP3, and anti-retinoic acid receptor-related orphan nuclear receptor  $\gamma$ t (anti-ROR $\gamma$ t) antibodies (eBioscience). Samples were acquired using a FACSCanto II system and were analyzed using BD FACSDiva software (BD Pharmingen).

**Statistical analysis.** Results are expressed as the mean  $\pm$  SEM. All in vitro experiments were performed at least 3 times. First, the Kruskal-Wallis test was used to assess the statistical differences between all groups, and the Mann-Whitney test was performed to compare differences between 2 groups. Data were analyzed using GraphPad Prism software. *P* values less than 0.05 were considered significant.

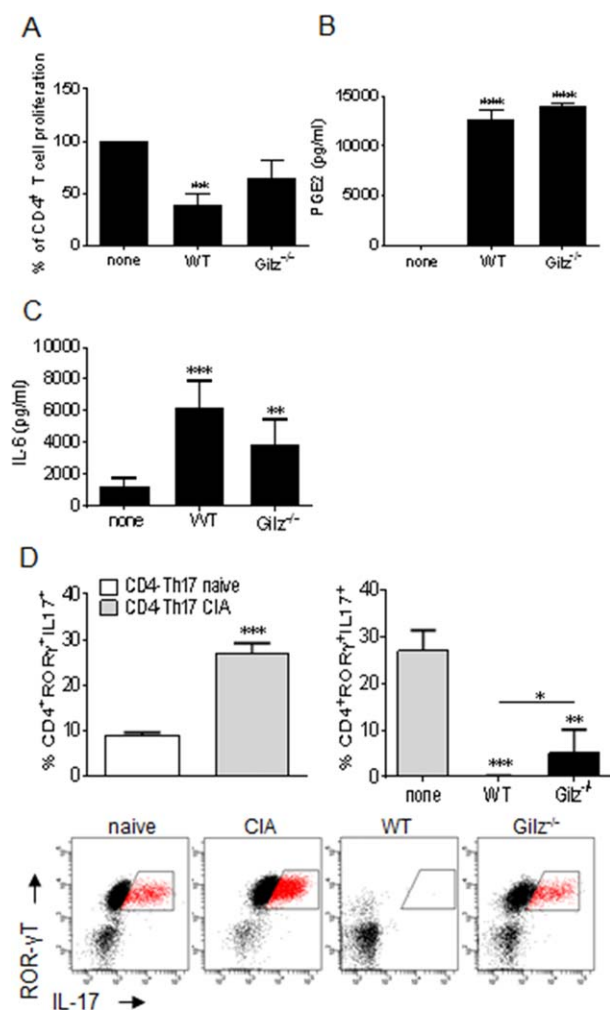
## RESULTS

**Reduced immunosuppressive properties of GILZ<sup>-/-</sup> MSCs in vitro.** First, we isolated and characterized MSCs from the bone marrow of GILZ-deficient C57BL/6 mice and showed that WT MSCs and GILZ<sup>-/-</sup> MSCs have a similar phenotype (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>). We then compared the ability of WT and GILZ<sup>-/-</sup> MSCs to inhibit CD4+ T cell proliferation. As shown in Figure 1A, WT MSCs induced significant suppression of CD4+ T cell proliferation, but GILZ<sup>-/-</sup> MSCs did not.

We next determined whether GILZ expression by MSCs affected PGE<sub>2</sub> and IL-6 secretion by MSCs, by quantifying these molecules in the supernatants of CD4+ T cells cultured with or without WT MSCs or GILZ<sup>-/-</sup> MSCs. The concentrations of PGE<sub>2</sub> and IL-6 were similarly increased in the supernatants of CD4+ T cells cultured with WT MSCs and GILZ<sup>-/-</sup> MSCs compared with the concentration in the supernatant of CD4+ T cells cultured alone (Figures 1B and C). These results revealed that GILZ plays a role in the suppressive function of MSCs on T cell proliferation and also demonstrated that this effect was not mediated via modulation of PGE<sub>2</sub> or IL-6 secretion.

**Role of GILZ in the suppressive effect of MSCs on Th17 cell differentiation.** Using activated CD4+ T cells isolated from naive mice and cultured under in vitro conditions of Th1 and Th17 lineage differentiation, we recently showed that MSCs inhibited proliferation of activated cells as well as cytokine production specific for Th1 and Th17 cells (30). We therefore addressed the question of whether MSCs could also affect the Th17 cell differentiation potential of CD4+ T cells derived from arthritic mice. For that purpose, we assessed the capacity of CD4+ cells isolated from the spleens of either naive or arthritic mice to differentiate into Th17 cells.



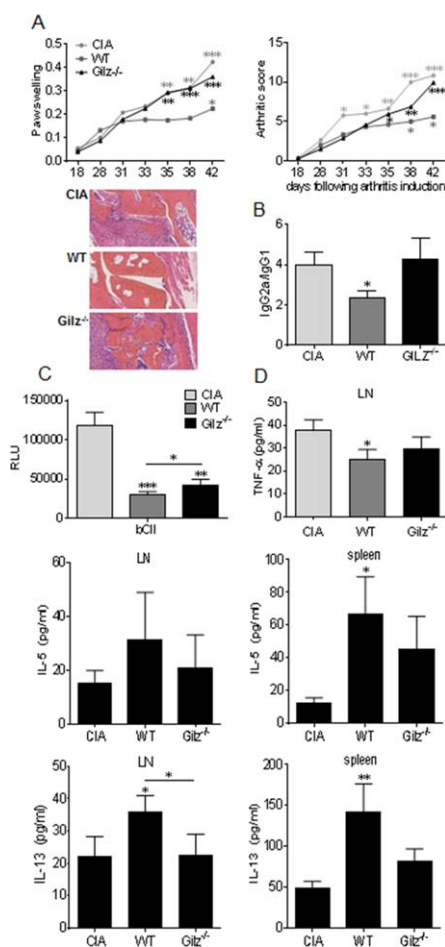


**Figure 1.** Mesenchymal stem cells (MSCs) derived from glucocorticoid-induced leucine zipper-deficient ( $GILZ^{-/-}$ ) mice are not immunosuppressive in vitro. **A**, Inhibition of  $CD4^{+}$  T cell proliferation by wild-type (WT) mouse MSCs or  $GILZ^{-/-}$  MSCs. Results are expressed as the percentage of  $CD3/CD28$ -induced proliferation, which was assigned a value of 100%. **B** and **C**, Concentrations of prostaglandin  $E_2$  ( $PGE_2$ ) (**B**) and interleukin-6 ( $IL-6$ ) (**C**) in the supernatants of  $CD4^{+}$  T cells cultured with WT or  $GILZ^{-/-}$  MSCs. **D**, Differentiation of  $CD4^{+}$  T cells toward the Th17 lineage. T cell differentiation was induced using  $CD4^{+}$  T cells from either naive mice or mice with collagen-induced arthritis (CIA). Top left, Detection of retinoic acid receptor-related orphan nuclear receptor  $\gamma t$  ( $ROR\gamma t$ ) and  $IL-17$  by flow cytometry defined the frequency of Th17 cells after  $CD4^{+}$  T cell differentiation. Top right, The frequency of Th17 cells after differentiation of  $CD4^{+}$  T cells from mice with CIA into Th17 cells, in the absence or presence of WT or  $GILZ^{-/-}$  MSCs, was assessed on day 4 of the differentiation process. Bottom, Representative dot plots of the histograms are shown. In **A–C** and **D** (top), values are the mean  $\pm$  SEM of 3 independent experiments. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$  (versus none, except where indicated otherwise). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>.

Compared with  $CD4^{+}$  T cells from naive mice,  $CD4^{+}$  T cells from arthritic mice displayed an enhanced capacity to differentiate into Th17 cells, as revealed by the significantly higher frequency of  $IL-17$ -secreting T cells (Figure 1D). As we previously demonstrated using  $CD4^{+}$  T cells isolated from naive mice (30), WT MSCs suppressed the differentiation of  $CD4^{+}$  T cells from arthritic mice toward the Th17 cell lineage (Figure 1D). However, the immunosuppressive potentials of WT MSCs and  $GILZ^{-/-}$  MSCs were significantly different (Figure 1D). Taken together, the data showed that  $CD4^{+}$  T cells from arthritic mice display a higher potential for differentiation into Th17 cells, and that  $GILZ$  is required for the suppressive effect of MSCs on Th17 polarization.

**Role of  $GILZ$  in the suppressive effect of MSCs in CIA.** Next, we used a murine CIA model to determine in vivo the role of  $GILZ$  in MSC-mediated therapeutic effects. Arthritis did not develop in mice treated with MSCs overexpressing  $GILZ$  ( $GILZ$  MSC plasmid), as revealed by the absence of arthritis symptoms (see Supplementary Figure 2A, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>). The severity of arthritis symptoms was significantly reduced in mice treated with WT MSCs compared with untreated control mice, while mice treated with  $GILZ^{-/-}$  MSCs developed arthritis of intermediate severity (Figure 2A). In addition, reduced infiltration of immune cells into the joints was observed only in mice treated with WT MSCs, as shown by histologic analysis (Figure 2A).

Concomitantly, the ratio of bovine type II collagen-specific IgG2a and IgG1 in serum, reflecting the Th1/Th2 cell balance in arthritic mice, was reduced by treatment with WT MSCs but not  $GILZ^{-/-}$  MSCs (Figure 2B). The ability of MSCs to regulate the proliferation of LNCs was assessed, using in vitro T cell proliferation assays in bovine type II collagen-sensitized mice on day 42. The proliferative responses of LNCs isolated from mice treated with either WT or  $GILZ^{-/-}$  MSCs were significantly lower than those observed in the control group of mice with CIA (Figure 2C). However, bovine type II collagen-specific responses were significantly lower in mice treated with WT MSCs compared with those treated with  $GILZ^{-/-}$  MSCs. The reduced proliferative response of LNCs from WT MSC-treated mice, but not mice treated with  $GILZ^{-/-}$  MSCs, was associated with significantly lower concentrations of  $TNF\alpha$  in supernatants (Figure 2D). In parallel, secretion of the Th2-associated cytokines  $IL-5$ ,  $IL-10$ , and  $IL-13$  was enhanced in the LNs and spleens of mice treated with WT MSCs but not in those of

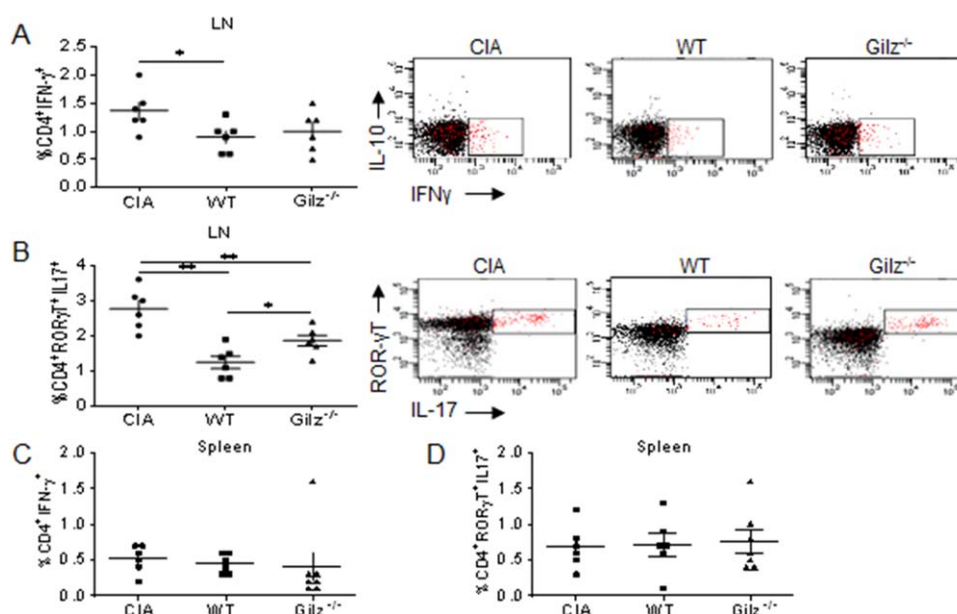


**Figure 2.** GILZ-dependent therapeutic effect of MSCs in mice with CIA. **A**, Evaluation of arthritis by measuring swelling of the hindpaw or the arthritis score (maximum possible score of 40 per mouse), in control mice, mice treated with WT MSCs, and mice treated with GILZ<sup>-/-</sup> MSCs. A score of 1 was assigned to each inflamed toe, and a score of 0 to 5 was assigned to each paw, according to its measured thickness. For the hind paws, this score was based on paw swelling increase (PSI) (score 0 if PSI < 0.1; score 1 if 0.1 < PSI < 0.15; score 2 if 0.15 < PSI < 0.25; score 3 if 0.25 < PSI < 0.45; score 4 if PSI > 0.45; score 5 if the paws are distorted and bent). For the fore-paws, a visual score was attributed (0 = noninflamed to 4 = highly inflamed and thick wrist; a score of 5 was assigned when the digits and front paw were distorted). Values are the mean  $\pm$  SEM swelling increase and arthritis score ( $n = 16$  mice per group). Representative photomicrographs of the joints are shown. Original magnification 11. **B**, Quantification of IgG1 and IgG2a in mouse sera, as determined by enzyme-linked immunosorbent assay (ELISA). **C**, Results of proliferation assay using lymph node (LN) cells incubated with 25  $\mu$ g/ml bovine type II collagen. **D**, Quantification of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), IL-5, and IL-13 secreted by splenocytes or LN cells activated with bovine type II collagen, as determined by ELISA. Values in **B–D** are the mean  $\pm$  SEM. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$  (versus day 18 [A] or CIA [B–D], except where indicated otherwise). RLU = relative luminescence units; bCII = bovine type II collagen (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>.

mice treated with GILZ<sup>-/-</sup> MSCs (Figure 2D). Taken together, these clinical and immunologic data suggest that GILZ plays a critical role in the capacity of MSCs to control the progression of CIA.

**Efficiency of GILZ<sup>-/-</sup> MSCs in reducing the numbers of Th1 and Th17 cells.** Because Th1 cells and Th17 cells have previously been considered to play a major role in the pathogenesis of arthritis (11), we examined the effect of MSCs on the number of pathogenic Th1 cells and Th17 cells in spleens and LNs on the day on which the mice were killed. Compared with untreated control mice, mice treated with WT MSCs exhibited a significantly reduced percentage of IFN $\gamma$ -producing Th1 cells in the LNs (Figure 3A). However, the percentages of IFN $\gamma$ -producing cells were not significantly different between mice treated with WT MSCs and mice treated with GILZ<sup>-/-</sup> MSCs (Figure 3A). In contrast, administration of both WT and GILZ<sup>-/-</sup> MSCs significantly reduced the percentage of IL-17-producing CD4<sup>+</sup> T cells in the LNs, but the effects of GILZ<sup>-/-</sup> MSCs were of significantly lower magnitude compared with the effects of WT MSCs (Figure 3B). These results indicated that GILZ is a critical mediator of the ability of MSCs to modulate Th1 and Th17 cell responses in vivo. Neither WT MSCs nor GILZ<sup>-/-</sup> MSCs reduced the frequency of Th1 cells or Th17 cells in the spleen (Figures 3C and D). Consistent with these observations, treatment of mice with the GILZ MSC plasmid resulted in a more noticeable suppressive effect on Th17 cell frequency in LNs compared with the suppressive effect of WT MSCs, while we did not observe a significant difference in spleens (see Supplementary Figures 2B and C, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>).

**Effect of GILZ deficiency on the capacity of MSCs to induce CD4+IL-10+ but not CD4+CD25+FoxP3+ Treg cells.** We next addressed the question of whether the reduced Th1 and Th17 cell responses observed following treatment with WT MSCs were associated with the generation of Treg cells. To characterize subsets of Treg cells, we used flow cytometry to analyze intranuclear FoxP3 staining in gated CD4<sup>+</sup>CD25<sup>+</sup> cells. On day 42, the percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells was significantly reduced in the LNs of mice treated with WT MSCs compared with untreated control mice (Figure 4A). A similar effect was observed in mice treated with GILZ<sup>-/-</sup> MSCs (Figure 4A). In contrast, we observed a significant increase in the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in the spleens of mice injected with WT or GILZ<sup>-/-</sup> MSCs compared with untreated control mice (Figure 4B).



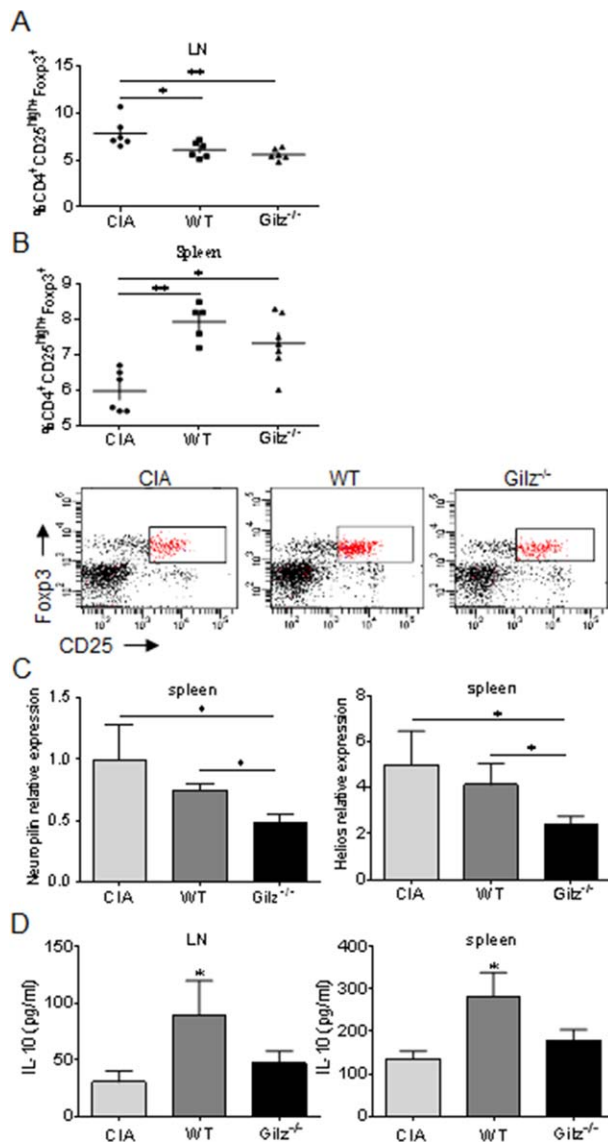
**Figure 3.** MSCs decrease the frequency of Th1 and Th17 cells in mice with CIA via GILZ expression. **A**, Left, Frequency of interferon- $\gamma$  (IFN $\gamma$ )-producing Th1 cells in the lymph nodes (LNs) of control mice, mice treated with WT MSCs, and mice treated with GILZ $^{-/-}$  MSCs. Right, Representative dot plots showing CD4 $^{+}$  cells positive for IFN $\gamma$  (Th1 cells). **B**, Left, Frequency of IL-17-producing cells in the LNs of control mice, mice treated with WT MSCs, and mice treated with GILZ $^{-/-}$  MSCs. Right, Representative dot plots showing CD4 $^{+}$  cells positive for ROR $\gamma$ t and IL-17 (Th17 cells). **C** and **D**, Frequency of Th1 cells (**C**) and Th17 cells (**D**) in the spleen of control mice, mice treated with WT MSCs, and mice treated with GILZ $^{-/-}$  MSCs. Each symbol represents an individual mouse; horizontal lines and error bars show the mean  $\pm$  SEM. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ . See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>.

To investigate whether these CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$  T cells were natural Treg cells or induced Treg cells, we assessed the expression levels of neuropilin 1 and Helios (33–35). While treatment with GILZ $^{-/-}$  MSCs decreased the expression of both neuropilin 1 and Helios, splenocytes isolated from control mice or mice treated with WT MSCs exhibited similar levels of neuropilin 1 and Helios (Figure 4C). These results suggested that the therapeutic effect of WT MSCs is not related to the generation of induced Treg cells. Further, the significant increase in the number of CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$  cells in the spleens of mice treated with either WT or GILZ $^{-/-}$  MSCs suggests that the therapeutic effects of MSCs are not mediated through the generation of CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$  Treg cells.

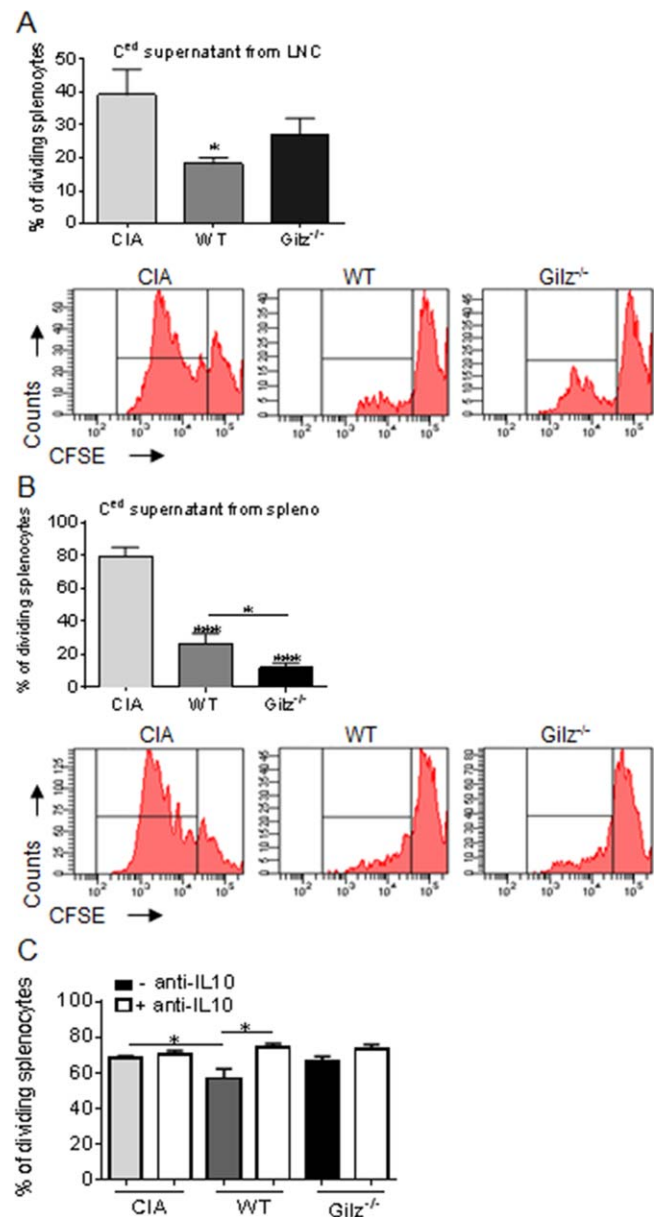
We therefore evaluated the possibility that MSCs could induce regulatory IL-10-producing Th cells (36,37). First, we performed ELISAs to quantify IL-10 production by bovine type II collagen-stimulated LN and spleen cells isolated from untreated control mice, WT MSC-treated mice, and GILZ $^{-/-}$  MSC-treated mice. IL-10 production by bovine type II collagen-stimulated LN and spleen cells was significantly higher in mice treated with WT MSCs compared

with that in untreated control mice and mice treated with GILZ $^{-/-}$  MSCs (Figure 4D). This secretion of IL-10 cannot be attributed to IFN $\gamma$ +IL-10 $^{+}$  regulatory cells, because T cells coexpressing IL-10 and IFN $\gamma$  were absent regardless of treatment (see Figure 3A). Taken together, these data suggest that the inability of GILZ $^{-/-}$  MSCs to protect against the development of CIA in mice paralleled their inability to generate IL-10-producing Treg cells.

**Role of Treg cell generation in the therapeutic effects of MSCs in CIA.** We next investigated whether inhibition of CIA induced by treatment with MSCs was related to the generation of T cells with regulatory function. For this purpose, we compared the immunomodulatory potential of the conditioned medium produced by bovine type II collagen-stimulated LNCs or splenocytes isolated from untreated control mice, mice treated with WT MSCs, and mice treated with GILZ $^{-/-}$  MSCs. A proliferation assay using CFSE-labeled splenocytes showed that the conditioned supernatant obtained from cultures of LNCs from mice treated with WT MSCs significantly reduced the proliferation of activated splenocytes compared with supernatant obtained from cultures of LNCs from untreated control mice (Figure 5A). In



**Figure 4.** MSCs induce classic Treg cell generation in the spleen independently of GILZ expression. **A** and **B**, Percentages of CD4<sup>+</sup>CD25<sup>high</sup>+FoxP3<sup>+</sup> Treg cells in individual lymph nodes (LNs) (**A**) and spleens (**B**, top) from control mice, mice treated with WT MSCs, and mice treated with GILZ<sup>-/-</sup> MSCs when the mice were killed. Each symbol represents an individual mouse; horizontal lines show the mean. **B**, Bottom, Dot plots of CD25<sup>high</sup>+FoxP3<sup>+</sup> cells gated for CD4<sup>+</sup> cells, representing 1 mouse per group. **C**, Levels of neuropilin 1 and Helios mRNA expression relative to expression of *RPS9* mRNA, as determined using the formula  $2^{-\Delta\Delta Ct}$ . **D**, Expression of IL-10 in the supernatants of LNs (left) and splenocytes (right) from control mice, mice treated with WT MSCs, and mice treated with GILZ<sup>-/-</sup> MSCs activated with 25  $\mu$ g/ml of bovine type II collagen for 72 hours, as determined by enzyme-linked immunosorbent assay. Values in **C** and **D** are the mean  $\pm$  SEM. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$  (versus CIA, except where indicated otherwise). See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>.



**Figure 5.** MSCs generate IL-10-producing Th17 cells with regulatory functions. **A** and **B**, Immunosuppressive activity of the conditioned (C<sup>cd</sup>) supernatant from cultures of lymph node cells (LNCs) (**A**) and splenocytes (spleno) (**B**) from control mice, mice treated with WT MSCs, and mice treated with GILZ<sup>-/-</sup> MSCs, as measured in a proliferative assay using carboxyfluorescein succinimidyl ester (CFSE)-labeled splenocytes after 3 days of coculture with freshly isolated splenocytes stimulated with concanavalin A. **C**, Effect of the addition of anti-IL-10-neutralizing antibody to the conditioned supernatant medium isolated from the cultured LNCs of each group of mice, as determined using a proliferative assay. Values are the mean  $\pm$  SEM. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$  (versus CIA, except where indicated otherwise). See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.39069/abstract>.



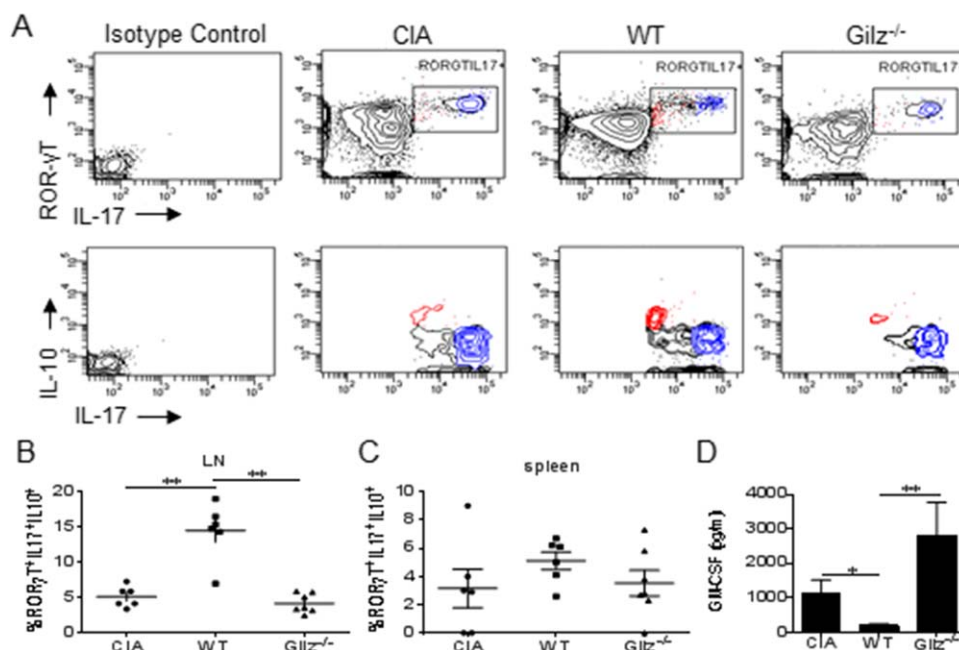
contrast, the reduction in the proliferation of splenocytes in the supernatant from cultures of LNCs from  $GILZ^{-/-}$  MSC-treated mice did not reach statistical significance (Figure 5A). This suggested a GILZ-dependent therapeutic effect of MSCs resulting in the production of immunosuppressive soluble factors by LNCs. Conditioned supernatant produced by splenocytes from both WT MSC-treated mice and mice treated with  $GILZ^{-/-}$  MSCs inhibited the proliferation of activated splenocytes (Figure 5B).

**Induction of CD4+ROR $\gamma$ t+IL-17+IL-10+ T cells by MSCs.** Given that Th17 cells and Treg cells are proposed to be reciprocally regulated during differentiation of naive T cells, and that Th17 cells generated in the presence of IL-6 and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) secrete IL-10, we investigated the possible generation of IL-10-producing nonpathogenic Th17 cells in mice treated with MSCs (38–40). The addition of anti-IL-10-neutralizing antibody to the conditioned medium isolated from the cultured LNCs of mice treated with WT MSCs resulted in significant restoration of the proliferation of activated splenocytes (Figure 5C).

We then investigated whether MSC treatment could influence the frequency of IL-10-producing

CD4+ T cells and did not observe any effect in LNs or spleens (data not shown). In our assessment of the Th17 cell populations present in the LNs or the spleens of mice, we observed a GILZ-dependent decrease in the percentage of ROR $\gamma$ t+IL-17+ Th17 cells in the LNs of MSC-treated mice (Figure 3B). A more-focused analysis revealed the presence of 2 populations of Th17 cells in the LNs: IL-17<sup>high</sup> and IL-17<sup>low</sup>. The IL-17<sup>low</sup> Th17 cells stained positive for IL-10, while the IL-17<sup>high</sup> Th17 cells did not (Figure 6A).

Compared with control mice or mice treated with  $GILZ^{-/-}$  MSCs, mice treated with WT MSCs showed a significant increase in the percentage of CD4+ROR $\gamma$ t+IL-17+IL-10+ T cells (Figure 6B). The increased frequency of CD4+ROR $\gamma$ t+IL-17+IL-10+ T cells was even more pronounced in mice treated with MSCs overexpressing GILZ compared with control mice (see Supplementary Figure 2B, available on the *Arthritis & Rheumatology* web site at <http://online.library.wiley.com/doi/10.1002/art.39069/abstract>). A similar tendency toward an increased frequency of CD4+ROR $\gamma$ t+IL-17+IL-10+ T cells was observed in the spleen cells of mice treated with WT MSCs, but not in mice treated with  $GILZ^{-/-}$  MSCs (Figure 6C).



**Figure 6.** Generation of IL-10-producing Th17 cells in the lymph nodes (LNs) of mice with CIA following MSC treatment depends on GILZ expression. **A**, Dot plots of ROR $\gamma$ t+IL-17+ cells (top) and ROR $\gamma$ t+IL-17+IL-10+ cells (bottom) in individual LNs of control mice, mice treated with WT MSCs, and mice treated with  $GILZ^{-/-}$  MSCs on the day the mice were killed, as determined by fluorescence-activated cell sorting analysis. Results are from 1 representative mouse per group. **C**, Frequency of CD4+ROR $\gamma$ t+IL-17+IL-10+ T cells in individual spleens from control mice, mice treated with WT MSCs, and mice treated with  $GILZ^{-/-}$  MSCs on the day the mice were killed. **D**, Quantification of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the conditioned supernatant obtained from LN cell (LNC) cultures of control mice, mice treated with WT MSCs, and mice treated with  $GILZ^{-/-}$  MSCs, as determined by enzyme-linked immunosorbent assay. Values are the mean  $\pm$  SEM and are representative of 2 independent experiments. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ . See Figure 1 for other definitions.



Because pathogenic Th17 cells are also characterized by the production of granulocyte-macrophage colony-stimulating factor (GM-CSF), which is inhibited by IL-10 (41), we quantified GM-CSF in the supernatant of bovine type II collagen-stimulated LNCs isolated from untreated control mice, mice treated with WT MSCs, or mice treated with GILZ<sup>-/-</sup> MSCs. Compared with the levels of GM-CSF in WT MSC-treated mice, the levels were significantly higher in the supernatant of cells isolated from the control mice or mice treated with GILZ<sup>-/-</sup> MSCs (Figure 6D). Taken together, these results suggested that MSC administration protects against the development of CIA in mice by inducing the generation of nonpathogenic IL-17<sup>low</sup>IL-10<sup>+</sup> T cells in a GILZ-dependent manner.

## DISCUSSION

This study is the first to provide evidence that GILZ plays a central role in the control of immune responses by MSCs. Using a murine CIA model, we showed that the therapeutic effect of MSCs is mediated by GILZ-dependent modulation of the Th17 cell response. Moreover, we demonstrated that the beneficial effect exerted by MSCs is related to a switch of the host response from a Th17 cell phenotype toward an IL-10-expressing Treg cell phenotype, which also depends on the expression of GILZ in MSCs. Taken together, our findings show that the therapeutic effect of MSCs depends on GILZ expression and is mediated by the induction of functional Treg cells bearing the CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup>IL-17<sup>low</sup>IL-10<sup>+</sup> signature.

Administration of MSCs during the course of arthritis induced a reduction in clinical signs, which was related to reduced LNC proliferation and proinflammatory cytokine production. This was associated with the release of Th2 cytokines by LNCs from mice treated with MSCs (28). The present study is the first to demonstrate that the systemic suppressive effect of MSCs on the host immune response is dependent on GILZ expression by MSCs. In mice with CIA, administration of MSCs generates a population of IL-17<sup>low</sup>IL-10<sup>+</sup> T cells that are likely to correspond to the nonpathogenic IL-10-producing Th17 cells induced in the presence of IL-6 and TGF $\beta$ 1, as previously characterized. This observation is consistent with the recently described regulatory or nonpathogenic T cells producing IL-17 (38–40,42). For example, Ghoreschi et al (38) demonstrated that Th17 cells generated in the presence of TGF $\beta$ 1 and IL-6, both of which are abundantly produced by MSCs, expressed higher levels of IL-9, IL-10, and CCL20 compared with Th17 cells generated in the

presence of IL-23, which expressed higher levels of IL-2, IL-33, and IL-18 receptor 1. The Th17 cells generated in the presence of TGF $\beta$ 1 and IL-6 were so-called nonpathogenic Th17 cells, while the Th17 cells generated in the presence of IL-23 displayed pathogenic potential.

IL-17-producing Th17 cells and IFN $\gamma$ -producing Th1 cells have previously been described to have critical roles in the pathogenesis of CIA (11,43–45). Here, we showed that treatment with MSCs prevented the progression of CIA by repressing both Th1 and Th17 cell responses in LNs but not spleens. The different responses observed in the 2 secondary lymphoid organs might be attributable to the differential distribution of MSCs after intravenous injection. Our results also revealed that the immunomodulatory effect on Th17 cells that is mediated by MSCs is GILZ dependent. These data are consistent with the known effects of GILZ on inflammatory signal transduction and cell activation. Indeed, GILZ may be an important regulator of the inflammatory responses in RA, because it exerts an inhibitory effect on proinflammatory cytokine and chemokine expression as well as antigen presentation and T cell function (7). This might be explained by its capacity to interact with many pathways that are relevant to inflammation, such as NF- $\kappa$ B and AP-1 (7).

In the present study, we addressed the possible involvement of GILZ in regulating the immune response in arthritis mediated by MSCs through the generation of classic Treg cells. We observed that the number of CD4<sup>+</sup>CD25<sup>high</sup>+FoxP3<sup>+</sup> cells in the spleen was similarly increased in mice treated with WT MSCs and mice treated with GILZ<sup>-/-</sup> MSCs, indicating that the reduced therapeutic effect of GILZ-deficient MSCs is not related to a defect in the generation of natural Treg cells. We also showed that both WT and GILZ<sup>-/-</sup> MSCs produced similar amounts of PGE<sub>2</sub>, a well-established mediator of the immunosuppressive properties of MSCs (16,24), that is also known to induce FoxP3-expressing Treg lymphocytes (46,47).

Next, in order to identify regulatory pathways specific for the therapeutic effects of MSCs, we investigated the generation of IL-10-producing nonclassic Treg cells. We observed that bovine type II collagen-activated LNCs from mice treated with MSCs produced higher levels of IL-10 than those from control mice with CIA, and this effect was abrogated when GILZ was silenced in MSCs. These results are consistent with those of a previous study showing that antigen-presenting cells treated with glucocorticoids express GILZ, which is required for generating IL-10-secreting Treg cells *ex vivo* (46). Although we observed that IL-10

production by the LNCs of mice treated with MSCs paralleled the generation of IL-17<sup>low</sup>IL-10<sup>+</sup> T cells, IL-10 can have several sources, including B cells and innate immune cells. This possibility has not yet been established.

Interestingly, we demonstrated the generation of a CD4<sup>+</sup>ROR $\gamma$ t+IL-17<sup>low</sup>IL-10<sup>+</sup> T cell population following therapeutic administration of MSCs to mice with CIA. Using GILZ-deficient MSCs, we show that GILZ displays a key role in the capacity of MSCs to induce IL-10-producing regulatory cells and to protect against arthritis progression in mice. The mechanisms responsible for the balance between CD4<sup>+</sup>ROR $\gamma$ t+IL-17<sup>low</sup>IL-10<sup>+</sup> and CD4<sup>+</sup>ROR $\gamma$ t+IL-17<sup>high</sup>IL-10-T cells in response to MSC treatment remain to be defined. However, the control of Th17 cell fate governed by MSCs in chronic inflammatory diseases such as RA is likely to be a major mechanism related to their immunomodulatory function.

In conclusion, this study is the first to show that GILZ plays a central role in the immunosuppressive properties of MSCs by enabling MSCs to induce the generation of Treg cells bearing the CD4<sup>+</sup>ROR $\gamma$ t+IL-17+IL-10<sup>+</sup> signature, which then inhibit T cell-mediated immune disease. Therapeutic administration of MSCs has the potential to favorably modify the balance between proinflammatory and antiinflammatory immune cells, and the potentiation of GILZ expression in MSCs could represent a means to enhance the therapeutic effect of these cells.

#### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Djouad had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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