GILZ EXPRESSION IN HUMAN DENDRITIC CELLS REDIRECTS THEIR MATURATION AND PREVENTS ANTIGEN-SPECIFIC T LYMPHOCYTE RESPONSE

Nicolas Cohen\textsuperscript{1*}, Enguerran Moully\textsuperscript{2*}, Haifa Hamdi\textsuperscript{1}, Marie-Christine Maillot\textsuperscript{1}, Marc Pallardy\textsuperscript{3}, Véronique Godot\textsuperscript{1}, Francis Capel\textsuperscript{1}, Axel Balian\textsuperscript{1,4}, Sylvie Naveau\textsuperscript{1,4}, Pierre Galanaud\textsuperscript{1}, François M.Lemoine\textsuperscript{2} & Dominique Emilie\textsuperscript{1,5}

\* : NC and EM contributed equally to this work.

\textsuperscript{1} INSERM UMR-S131, \textsuperscript{4} Service d’Hépato-Gastro-Entérologie and \textsuperscript{5} Service de Microbiologie - Immunologie Biologique, Hôpital Antoine Béclère, Assistance Publique-Hôpitaux de Paris, Institut Paris-Sud sur les Cytokines, Université Paris-Sud, 92140 Clamart, France
\textsuperscript{2} CNRS UMR 7087/UPMC, Hôpital Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Paris, 75013 France
\textsuperscript{3} INSERM UMR-S 461, Faculté de Pharmacie, Université Paris-Sud, 92286 Châtenay-Malabry, France

\textbf{Condensed title} : GILZ in human dendritic cells

\textbf{Correspondence} : Dominique EMILIE, INSERM U131, 32 rue des Carnets 92140 Clamart
phone: (33.1) 41 28 80 00 ; fax: (33.1) 46 32 79 93 ; \texttt{emilie@ipsc.u-psud.fr}
ABSTRACT

Interleukin (IL)-10 and glucocorticoids (GC) inhibit the ability of antigen-presenting dendritic cells (DC) to stimulate T lymphocytes. We show that induction of GILZ (Glucocorticoid-Induced Leucine Zipper) is involved in this phenomenon. IL-10, dexamethasone (DEX) and transforming growth factor (TGF)β stimulate GILZ production in human immature DC derived from monocytes and from CD34+ cells. GILZ is necessary and sufficient for DEX, IL-10 and TGFβ modulation of CD80, CD83, CD86, ILT3 and B7-H1 expression by DC and alteration of DC functions. GILZ stimulates the production of IL-10 by immature DC and it prevents the production of inflammatory chemokines by CD40L-activated DC. In contrast, GILZ does not prevent CD40L-mediated inhibition of phagocytosis, indicating that it affects some but not all aspects of DC maturation. GILZ prevents DC from activating antigen-specific T lymphocyte responses. Administration of GC to patients stimulates GILZ expression in their circulating antigen-presenting cells, and this contributes to the weak lymphocyte responses of GC-treated patients. Thus, regulation of GILZ expression is an important factor determining the decision of DC whether or not to stimulate T lymphocytes, and IL-10, GC and TGFβ share this mechanism for influencing DC functions and the balance between immune response and tolerance.
INTRODUCTION

Dendritic cells (DC) play a critical role in the activation of T lymphocytes through their ability to capture, process and present antigens in association with MHC molecules. Interaction between the co-stimulatory molecules CD80 and CD86, expressed by DC, and CD28, expressed by T lymphocytes, is required for optimal T lymphocyte activation. Immature DC express low levels of CD80 and CD86. They are activated by microbial-derived molecules through TLR, and this upregulates CD80/CD86 expression and ensures their maturation into fully efficient antigen-presenting cells. CD40 ligation also induces DC maturation and optimal T lymphocyte stimulation. Maturation of DC induced by TLR ligands and CD40L involves activation of the NF-κB and p38 MAP kinase pathways. DC may also generate immune tolerance by inducing T lymphocyte unresponsiveness or apoptosis, or by inducing T lymphocytes with regulatory functions. The mechanisms by which antigen presentation by DC leads to opposite patterns of T lymphocyte responses are not fully understood but they may depend on the stage of DC maturation. It was initially suggested that induction of tolerance resulted from a “default pathway”, in which antigen presentation in the presence of limited amounts of CD80/CD86 resulted in T lymphocyte anergy. This may account for tolerance induced by immature DC in tissues devoided of infectious agents or inflammation. More recent findings however suggest that the induction of tolerance is a more active process, requiring the production by DC of tolerance-inducing molecules such as IL-10, indoleamine 2,3-dioxygenase (IDO), B7-H1, immunoglobulin-like transcript (ILT)-3 and inducible costimulator ligand (ICOSL). Such molecules can be generated during the progression along an alternate pathway of DC maturation, leading to so-called “semi-mature” DC. This process may be induced by the local production of tolerance-inducing cytokines, including IL-10 and TGFβ, and by tolerance-inducing cells such as regulatory T lymphocytes. These mechanisms are not mutually exclusive as regulatory cells produce IL-10 and TGFβ.
Glucocorticoids (GC) are widely used to treat disorders involving uncontrolled immune activation, and especially autoimmune diseases, allergy and allograft rejection. Initial analyses focused on the effects of GC on T lymphocytes to explain their therapeutic properties, but recent reports show that the effects of GC are also due to silencing innate immune cells. In particular, GC alter the normal maturation of DC in response to TLR ligands and to CD40L. They down-regulate the expression of CD80, CD86 and MHC molecules and the production of IL-12, whereas they stimulate the production of IL-10. IL-10 has similar effects on DC, suggesting that the two agents share a common mechanism of action to interfere with DC maturation.

GILZ (Glucocorticoid-Induced Leucin Zipper) was identified in mice thymocytes treated with GC, protecting them from TCR/CD3-induced cell death. Both GC and IL-10 stimulate the production of GILZ in mice and human monocytes/macrophages and GILZ is involved in GC and IL-10 anti-inflammatory effects. GILZ blocks the NF-κB, MAPK and AP-1 signal transduction pathways in several types of cells. In view of the importance of these pathways in DC maturation, we addressed whether induction of GILZ in DC could explain why GC and IL-10 alter the maturation of DC and their ability to activate T lymphocytes.

MATERIALS AND METHODS

Antibodies and flow cytometry

The following mAbs FITC, PE or phycoerythrine-cyanin5 (PE-Cy5) were used: CD14-PE and CD80-PE from Becton Dickinson (Pont de Claix, France); CD3-PE, CD40-PE, CD34-PE-Cy5, CD83-PE and ILT-3-PE-Cy5 from Beckman-Coulter (Villepinte, France); CD1a-PE-Cy5, CD86-FITC or -PE, HLA-DR-PE-Cy5 and CD90-FITC or -PE from Pharmingen (San Diego, CA, USA) and B7-H1-PE from Cliniscience (Montrouge, France). Negative controls were irrelevant isotype-matched mAbs. GILZ protein was labeled by incubation with a rabbit polyclonal anti-GILZ Ab followed by a PE-conjugated anti-rabbit-F(ab')2 (Jackson
Immunoresearch, West Grove, PA). As controls, we used a rabbit polyclonal IgG (Santa Cruz, Santa Cruz, CA) and a PE-conjugated anti-IgG1 mAb (Immunotech, Marseilles, France). At least $10^6$ gated events were acquired with either a FACScalibur (Becton Dickinson) or an EPICS ELITE (Beckman Coulter) and analyzed with CellQuest (Becton Dickinson) or FlowJo (TreeStar, Can Carlos, CA) softwares.

**Human samples, cell purifications and DC differentiation**

Peripheral blood and leukapheresis products were collected with informed consent of the donors or patients and the study protocol was approved by the Institutional Review Board of the South Paris Medical School. To obtain Mo-DC, monocytes were isolated from mononuclear cells of healthy donors by negative selection (Dynal, Compiègne, France). They were plated in culture flasks at 6x$10^5$ cells/mL in RPMI 1640 medium (Invitrogen, Cergy-Pontoise, France) containing 10% human AB serum (hABS), 20 ng/mL Interleukin 4 (IL-4, a kind gift from Dr K. Thielemans) and 100 ng/mL GM-CSF (Schering Plough, Levallois-Perret, France) for 9 days, the culture medium being changed on day 3 as described, giving immature Mo-DC. To induce activation and maturation of DC, trimeric CD40 ligand (CD40L, 250 ng/mL, Immunex, Seattle, WA) was added on day 7 of culture. To obtain CD34-DC, leukapheresis samples from patients with solid tumors in remission were obtained after stem-cell mobilization with G-CSF/cyclophosphamide. CD34$^+$ cells were purified using immunomagnetic beads as described and frozen in FCS containing 10% dimethylsulfoxide until use. CD34$^+$ cells were thawed and seeded in 12-well tissue-culture plates (Costar, Cambridge, MA) at 5x$10^5$ cells/mL in 2mL of RPMI 1640 medium containing 10% hABs, 1% L-glutamine, 1% antibiotics, Stem Cell Factor (300 ng/mL, a gift from Amgen, Thousands Oaks), Flt3-Ligand (50 ng/mL, a gift from Immunex, Seattle, WA), 100 ng/mL GM-CSF and TNFα (5 ng/mL, Valbiotech, France). On day 5, cells were seeded at 2.5 to 5x$10^5$ cells/mL and incubated for an additional seven days in the same culture medium supplemented with 50 ng/mL IL-4, the medium being changed at day 8. In
some experiments, 250 ng/mL of CD40L was added on day 10. DEX (Sigma, L'isle d'Abeau, France) was used at 10^{-7}M, IL-10 (a gift from K. Moore, DNAX, Palo Alto, CA) at 100 ng/mL and TGFβ (R&D Systems Inc., Mineapolis, MN) at 1 ng/mL. The purity of freshly isolated monocytes and CD34^{+} cells was assessed by flow cytometry after staining of an aliquot of the preparation with CD14-PE mAb or CD34-PE-Cy5 mAb, respectively (purity >90%). Mo-DC were routinely >95% HLA-DR^{+} and >85% CD11c^{+}, and CD34-DC were >90% HLA-DR^{-}.

APC were obtained from patients undergoing treatment with oral glucocorticoids (prednisone or prednisolone, 0.5 mg/kg of body weight/day) for alcoholic hepatitis (n=7), symptomatic sinusitis (n=2) or acute cervico-brachial neuralgia (n=2). APC were isolated from mononuclear cells by negative selection (Dynal, Compiègne, France) before and after two days of GC treatment. After selection, 91.7% ± 2.9% of cells expressed CD14 (4 experiments). The fraction of DC (CD11c^{+} ILT3^{+} cells) was 0.26% ± 0.15% and 2.25% ± 0.20% before and after selection, respectively.

**RT-PCR studies**

Expression of the human GILZ gene was measured as mRNA by real time RT-PCR, and results were expressed as arbitrary units, as previously described^{17}.

**Production of GILZ lentiviral vectors**

The thy1-IRES-GILZ lentiviral vector construct was obtained in three steps using pGILZ, a GILZ-encoding vector^{17}, the intermediate plasmid pMLV-thy1-IRES-TK^{24}, the shuttle plasmid pTRE2 (Clontech, Palo Alto, CA, USA) and the pHIV-EF1L-thy1/GFP-W^{+} lentiviral vector^{25}. pGILZ was digested with BamHI and the ends blunted, and then digested with NotI. The resulting GILZ cDNA was ligated into pMLV-thy1-IRES-TK, replacing the TK gene, giving pMLV-thy1-IRES-GILZ. Then, the thy1-IRES-GILZ cassette was inserted between the AvrII and NotI sites in pTRE2 giving pTRE2-thy1-IRES-GILZ. The thy1-IRES-GILZ cassette was then ligated between the MluI and SalI sites in the pHIV-EF1L-thy1/GFP-W^{+} giving the pHIV-
EF1L-thy1/GILZ-W+ lentiviral vector (Lv GILZ). pHIV-EF1L-thy1/GILZ-W+ is a HIV1-derived vector composed of a bicistronic cassette coexpressing both the human thy1 reporter gene and GILZ by the mean of an IRES sequence from the encephalomyocarditis virus under the control of the full length Elongation Factor 1 alpha promoter (EF1L). pHIV-EF1L-thy1/GILZ-W+ also contains the cPPT (central polypurine tract) 26 and WPRE (woodduck hepatitis virus post-transcriptional regulatory element) sequences. Lv TK was constructed by replacing GILZ gene with HSV1-TK gene in Lv GILZ to obtain a control construct. pCMVΔR8.91 and pMD.G, both kindly provided by D. Trono, are HIV-derived packaging constructs which encode the HIV-1 Gag and Pol precursors and the regulatory proteins Tat and Rev and carries an envelope plasmid (env) encoding the vesicular stomatitis virus envelope G protein (VSV-G) used to pseudotype the vector particles. Lentiviral particles were produced as described elsewhere 25.

Aliquots of 2x107 293T cells were seeded in 162cm² tissue culture flasks and co-transfected using the calcium phosphate method with 9.9µg of pMD.G, 23µg of pCMVΔR8.91 and 29µg of the bicistronic pHIV-EF1L-thy1/GILZ-W+ or pHIV-EF1L-thy1/TK-W+. The next day, medium was replaced with medium without serum and lentiviral supernatants were collected 24 hrs later. The supernatants were spun at 800g and passed through a 0.45µm-pore-size filter (Costar, Cambridge, MA, USA), and concentrated by ultrafiltration using Centricon Plus-80 filter devices according to the manufacturer’s instructions (Centricon Plus-80, molecular weight cutoff 100kDa; Millipore, Bedford, MA). Concentrated supernatants were stored in aliquots at -80°C until further use. Viral titers were determined using 293T indicator cells as previously described 25, and were 2x10⁸ to 10⁹ infectious particles/mL.

**Transfer of GILZ gene**

Mo-DC were transduced with pGILZ or pcDNA3 17 by nucleofection after 7 days of culture, as described by the kit manufacturer (Amaxa System, Köln, Germany). Nucleofection efficiency, assessed using a GFP-reporter vector, was tested by flow cytometry 24 hours later: 59 ± 6 % (3 experiments) of the cells were GFP-positive. CD34-DC were infected with lentiviral vectors on
day 8 of culture. Briefly, 10^5 cells were suspended in 400µL of culture medium containing lentiviral supernatants at a multiplicity of infection of 100 and 8 µg/ml protamine sulfate and centrifuged at 20°C at 1000g for 3 hours (spinoculation) 25. The cells were then further cultured as described above. Transduction efficiency was assessed on day 12 of culture by flow cytometry using anti-CD90 mAbs, and was 21.9 ± 4.7% for Lv GILZ and 29.4 ± 6.6% for Lv TK (mean ± SEM of 5 separate experiments).

**RNA interference experiments**

The 21-nt-long interfering RNA duplexes with two 3'-end overhanging dT nucleotides on the antisense strand were synthesized. The sequences of the antisense strands of the siRNAs were for GILZ siRNA (siGILZ): 5'-AGU CCA GGA UUA UAG CCC CadTdT ; and for control siRNA (siC) with random nucleotides and no known specificity: 5'-ACG GGG GGC CCU UAA AAC AdTdT (MWG Biotec, Ebersberg, Germany or Dharmacon, Lafayette, CO).

Nucleofection of Mo-DC with siRNAs was conducted on day 6 of culture. Then, DEX, IL-10 or medium alone were added and the samples cultured for 24 hours. CD34-DC were transfected with siRNAs on day 8 using the JetSi endo transfection kit (Q-Biogene, Illkirch, France) as described by the manufacturer. Twenty-four hours later, the cells were washed and cultured as described above in the presence or absence of DEX or IL-10 until day 12. For APC, freshly isolated cells were transfected using the JetSi endo transfection kit.

**Lymphocyte responses**

Mo-DC or APC from BCG-vaccinated individuals were loaded with PPD (1 µg/mL, Statens Serum Institute, Copenhagen, Denmark) for 4 hours, washed and co-cultured (0.5x10^5 DC) with 1x10^5 freshly purified autologous CD4+ T-cells in round-bottomed 96-well culture plates in RPMI + 10% hABs. CD4+ T-cells were purified by negative selection using immunomagnetic beads (Dynal). In some experiments DC were transfected with siRNAs 24 hours before the end
of the Mo-DC culture. The response of CD4+ T lymphocytes was analyzed at the single cell level on day 7 of the co-culture using the dye Pkh26 (Sigma-Aldrich, Saint Quentin Fallavier, France), as previously described. For MLR, allogeneic T-cells were purified from PBMC using immunomagnetic beads as described and frozen until use. Thirty gray-irradiated CD34-DC were mixed in triplicate round-bottomed wells with 10^5 freshly thawed allogeneic T-cells/well and co-cultured in 200µL of RPMI + 10% hABs at 37°C for 5 days. Each CD34-DC preparation was tested against T lymphocytes from 3 unrelated donors. Cell proliferation was assayed by [³H]-TdR incorporation. Results are expressed as mean counts per minute (cpm).

ELISA assays

Production of cytokines and chemokines was assessed using ELISA kits from Diaclone (Besançon, France) for IFNγ and IL-10 and from R&D Systems for CCL3, CCL5 and CXCL8.

Endocytosis assay

The uptake ability of GILZ-expressing DC was measured using FITC-conjugated dextran particles (FITC-Dx, Sigma) diluted in PBS at 10mg/mL and used at a final concentration of 1mg/mL in culture medium. GILZ-expressing DC were seeded in culture medium at 1x10^6 cells/mL, kept at 37°C for 30min and then incubated with FITC-Dx at 37°C for 1 hour. Negative control of uptake was cells tested at 4°C. Then, cells were washed four times in cold PBS and analyzed immediately by FACS. When the endocytosis of FITC-Dx was studied on lentivirally-infected CD34-DC, cells were stained with CD90-PE mAbs prior to the uptake assay and analysis was performed on gated CD90+ CD34-DC, considered as transduced cells.
RESULTS

Expression of GILZ by DC

We monitored *gilz* gene expression by real time RT-PCR during the differentiation of monocyte-derived DC (Mo-DC). Freshly isolated monocytes expressed the *gilz* gene. This expression disappeared after 5 days of culture in the presence of GM-CSF + IL-4, and remained undetectable both in the presence and in the absence of CD40L-induced DC maturation. Addition of DEX led to an upregulation of *gilz* gene expression. This expression was maintained even in the presence of CD40L (Fig 1a). When DEX was added on day 5 and removed on day 7, *gilz* expression then declined to disappear by day 9, regardless of the addition of CD40L (Fig 1b). Therefore, *gilz* gene induction is reversible and its persistence depends on the continuous presence of DEX. IL-10 and TGFβ also stimulated *gilz* gene expression by DC, although to a lesser extent than DEX (Fig 1a). *gilz* gene expression was also studied during the differentiation of DC from CD34+ cells (CD34-DC). Freshly isolated CD34+ cells expressed the *gilz* gene strongly and this expression was progressively lost during their dendritic differentiation. Addition of DEX or IL-10 increased *gilz* gene expression and the increase persisted after CD40L addition (Fig 1c). Therefore, DEX and IL-10 stimulate *gilz* gene expression in immature DC, and this expression persists upon CD40L triggering. When DEX or IL-10 were added on day 7 to Mo-DC or on day 10 to CD34-DC, no induction of *gilz* gene expression was observed, regardless of the addition of CD40L (data not shown), indicating that sensitivity of DC to GC and IL-10 was restricted to a specific step of their differentiation. Expression of GILZ by Mo-DC was also studied by flow cytometry using an anti-GILZ polyclonal Ab. No GILZ was detected in immature Mo-DC cultured without DEX. Addition of DEX, IL-10 or TGFβ stimulated GILZ production (Fig 1d, upper panels and data not shown). Western blot studies gave similar findings (data not shown).
Fig 1: GILZ production by DC. *gilz* gene expression was determined by real time RT-PCR.

a) DEX (υ), IL-10 (θ), TGFβ (σ) or medium (⊙) were added to Mo-DC cultures on days 5 and 7, and CD40L was added on day 7; b) DEX was added to Mo-DC on day 5, cells were washed on day 7, and DEX, CD40L or medium were added; c) DEX (υ), IL-10 (θ), or medium (⊙) were added on day 9 of CD34-DC cultures, and CD40L was added on day 10; d) GILZ expression (bold curves) in untreated Mo-DC and in Mo-DC treated for 24 hours with DEX, DEX + control siRNA (siC) or DEX + GILZ siRNA (siGILZ) was determined by flow cytometry. Tinted histograms correspond to the control antibody.
Modulation of DC phenotype by GILZ

We determined whether GILZ is involved in the phenotypic changes of Mo-DC induced by DEX or IL-10. Inhibition of GILZ production was obtained by transfecting Mo-DC with GILZ small interfering (si)RNA. The GILZ siRNA prevented the DEX- or IL-10 induced production of GILZ mRNA, as assessed by real-time RT-PCR, and of GILZ protein, as assessed by flow cytometry and by western blotting. A control siRNA had no effect (Fig 1d, lower panels and data not shown).

DEX and IL-10 changed the phenotype of Mo-DC. When added on day 5 of culture to DC transduced with control siRNA, they inhibited the expression of CD80, CD83 and CD86, and they stimulated the expression of B7-H1. DEX and IL-10 inconsistently inhibited the expression of MHC class II molecules and stimulated that of ILT3 (Fig 2a and data not shown). In these experiments, expression of CD83 by control cells was higher than expected for DC cultured in the absence of differentiating agents, reflecting a partial activation of DC by nucleofection. Transduction of DC with GILZ siRNA prevented DEX- and IL-10-induced changes of phenotype: GILZ siRNA partially restored CD80 and CD83 expression and completely restored CD86 expression; it abolished induction of B7-H1 and ILT3 (Fig 2a). In experiments in which DEX or IL-10 inhibited MHC class II expression, this effect was reversed by GILZ siRNA (data not shown). Thus, GILZ is involved in the DEX- and IL-10-induced changes of Mo-DC phenotype. Next, a GILZ-encoding vector (pGILZ) was introduced into Mo-DC and cell phenotype was determined 24 hours later. We verified by flow cytometry and western blotting that nucleofection of Mo-DC with pGILZ resulted in production of the GILZ protein (data not shown). The effect of GILZ on CD86 expression was inconsistent. In contrast, GILZ-producing DC expressed lower levels of CD80 and CD83 and higher levels of B7-H1 and ILT3 than did DC transfected with a control vector (Fig 2b). Therefore, GILZ is sufficient to cause changes of DC phenotype.
Fig 2: Effects of GILZ on Mo-DC phenotype. a) Mo-DC were treated on day 6 with DEX, IL-10 or medium and with control (siC) or GILZ (siGILZ) siRNA. On day 7, DC phenotype was determined. One typical experiment of 3 is shown; b) Mo-DC were transduced with a control (pcDNA3) or a GILZ-encoding (pGILZ) vector on day 6 of culture, and they were stimulated with CD40L on day 7. DC phenotype was determined on day 9. One representative experiment of 2 is shown.
The effect of GILZ on the production of IL-10 and chemokines by DC

Treatment of immature Mo-DC with DEX stimulates their production of IL-10. We investigated whether GILZ was involved in this effect. DEX stimulated IL-10 production in Mo-DC transfected with control siRNA. In contrast, transfection with GILZ siRNA partially prevented DEX-induced upregulation of IL-10 production. We next tested the effect of gilz gene transfer into Mo-DC. Mo-DC nucleofected with pGILZ produced ~6 fold more IL-10 than control cells nucleofected with pcDNA3 (Table I). Therefore, induction of GILZ is at least partially responsible for the DEX-mediated stimulation of IL-10 production by DC.

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<td>pGILZ</td>
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Table 1. Role of GILZ in DEX-induced IL-10 production by Mo-DC

IL-10 concentration (mean ± sem) was measured in supernatants of immature Mo-DC treated with DEX alone, with DEX and control or GILZ siRNA, or in immature Mo-DC nucleofected with a control (pcDNA3) or a GILZ-encoding (pGILZ) vector.
Activation of Mo-DC with CD40L stimulated the production of the chemokines CCL3, CCL5 and CXCL8, but this was partially prevented by treatment with DEX or IL-10. Transfection of GILZ siRNA inhibited the effect of each DEX and IL-10 on chemokine production, whereas transfection with control siRNA had no effect (Fig 3).

Fig 3. Effects of GILZ on chemokine production by DC. Mo-DC were treated with control (□) or GILZ (■) siRNA on day 5, with DEX, IL-10 or medium on days 5 and 7, and with or without CD40L on day 7. Production of CCL3, CCL5 and CXCL8 was determined on day 9.
Results (mean ± SEM) are expressed as percentages of the values for controls, treated with control siRNA only (n = 3).

**GILZ effect on DC maturation**

The impact of GILZ expression on DC maturation was evaluated. Mo-DC were nucleofected with either pcDNA3 or pGILZ, stimulated or not with CD40L, and tested for dextran particles phagocytosis. As expected, the expression of CD80 and CD83 was lower in cells nucleofected with pGILZ than in controls (Fig 4a). In immature Mo-DC, phagocytosis of dextran was similar between GILZ-expressing and control cells. CD40L-induced activation of Mo-DC abolished phagocytosis to a similar extent in GILZ-expressing and control cells (Fig 4b). In these experiments, phagocytosis of dextran by immature Mo-DC nucleofected with either pcDNA3 or pGILZ was lower than in cells not nucleofected, whereas expression of CD80 and CD83 was stronger (data not shown). This indicates that nucleofection induced a partial maturation of Mo-DC. To avoid this, we generated GILZ-producing CD34-DC using lentiviral-mediated transfer of the *gilz* gene. A thymidine kinase-encoding lentivirus was used as control. Both lentiviruses also encoded for the CD90 reporter gene, and phenotypic changes were analyzed in CD90⁺ CD34-DC. In the absence of CD40L activation, CD34-DC expressed no CD83 and only low levels of CD80. GILZ expression partially inhibited the up-regulation of CD80 and CD83 induced by CD40L (Fig 4c). In immature CD34-DC, phagocytosis was slightly higher in GILZ-expressing cells than in controls (Fig 4d). Activation of CD34-DC with CD40L abolished phagocytosis in both GILZ-expressing and control cells. These findings confirm those obtained with Mo-DC, showing that GILZ has dissociated effects on the maturation of DC, preventing some but not all parameters of this process.
Fig 4: GILZ effect on DC maturation. a,b) Mo-DC were nucleofected with either pcDNA3 or pGILZ and stimulated or not with CD40L, giving mature (mDC) and immature (iDC) DC, respectively. a) CD80 and CD83 expression was analyzed in DC transfected with pcDNA3 (bold lines) or pGILZ (tinted histograms); b) phagocytosis of FITC-Dx was analyzed at 37°C (bold lines) or at 4°C (tinted histograms). c,d) CD34-DC were infected with LvGILZ or LvTK on day 8 of culture. CD40L was added or not on day 10. Results shown are gated on CD90+ (transduced) DC. c) DC phenotype was determined on day 12. Bold lines and tinted histograms correspond to LvTK- and LvGILZ-infected DC, respectively; d) Phagocytosis of FITC-Dx was determined at 37°C (bold lines) or 4°C (tinted histograms). Results are from one representative experiments of 3.

GILZ-expressing DC and T lymphocyte activation

To test the ability of GILZ-expressing DC to activate T lymphocytes, Mo-DC were transfected with control or GILZ siRNA, cultured with or without DEX or IL-10 and loaded with...
PPD. DC were then mixed with autologous Pkh26-labeled CD4\(^+\) T lymphocytes, and proliferation of lymphocytes was followed. Treatment of DC with DEX or IL-10 decreased their ability to stimulate CD4\(^+\) T lymphocytes (data not shown). The effect of DEX and IL-10 was preserved when DC were transfected with control siRNA. In contrast, GILZ siRNA abolished the effect of DEX or IL-10 on DC, restoring a strong proliferative response to PPD (Fig 5a). The effect of GILZ expression by DC on IFN\(\gamma\) production by CD4\(^+\) T lymphocytes was also tested. PPD-loaded Mo-DC stimulated IFN\(\gamma\) production by autologous CD4\(^+\) T lymphocytes. Treating DC with either DEX or IL-10 inhibited the production of IFN\(\gamma\) by CD4\(^+\) T lymphocytes. This inhibition was abolished when DC were transfected with GILZ siRNA, whereas control siRNA had no effect (Fig 5b).

The role of GILZ on CD34-DC-induced allogeneic response was also tested. Treatment of CD34-DC with DEX or IL-10 decreased their ability to stimulate the proliferation of allogeneic T lymphocytes. Transfection of CD34-DC with control siRNA had no effect, regardless of the addition of DEX or IL-10. Transfection with GILZ siRNA slightly enhanced proliferation in the absence of DEX or IL-10. It totally reversed the inhibitory effect of DEX and IL-10 (Fig 5c).

We tested whether GILZ expression in DC was sufficient to decrease CD4\(^+\) T lymphocyte stimulation. Mo-DC transfected with pGILZ or a control vector were loaded with PPD, washed and cocultured with autologous CD4\(^+\) T lymphocytes. The proliferation of CD4\(^+\) T lymphocytes was lower when GILZ-expressing DC were used as antigen-presenting cells (APC) (Fig 5d). Expression of GILZ in DC also decreased the production of IFN\(\gamma\) by CD4\(^+\) T lymphocytes (20.1 ± 11.3 % as compared to the production with control DC, p<0.05).
Fig 5: GILZ-expressing DC and antigen-specific response of CD4+ T lymphocytes.

Mo-DC were treated on day 5 with control (siC, □) or GILZ (siGILZ, ■) siRNA and with DEX, IL-10 or medium. They were loaded with PPD, washed and mixed with autologous CD4+ T lymphocytes on day 7. a) Lymphocyte proliferation, determined by Pkh26 fluorescence intensity (one representative experiment of 3). The mean (SEM) fraction of Pkh26low CD4+ T lymphocytes in the 3 experiments is shown in the upper right corner; b) IFNγ production (mean ± SEM of 2 experiments). In the absence of PPD loading there was no proliferation and <250 pg/mL IFNγ; c) CD34-DC were treated on day 8 with control (□) or GILZ (■) siRNA and with DEX, IL-10 or medium. On day 12, the DC were mixed with allogeneic T lymphocytes at a 1/2 ratio. Proliferation is expressed as percentages of values for controls, to which no DEX or IL-10 was added to DC. Results (mean ± SEM) are from one representative experiment of 2. Thymidine incorporation (mean ± SEM) by cells stimulated with control or GILZ siRNA-treated DC, without DEX or IL-10 addition, was 21,984 ± 5,206 cpm and 31,842 ± 9,023 cpm,
respectively; d) Mo-DC were nucleofected with pcDNA3 or pGILZ on day 6, loaded with PPD on day 7 and cocultured with Phk-26-labeled CD4⁺ T lymphocytes. T lymphocyte proliferation was determined 7 days later. Results are from one representative experiment of six, in which the inhibition of proliferation with GILZ-expressing DC was 50.3 ± 8.0 % (mean ± SEM) as compared to control DC (p<0.05).

We tested whether TGFβ has similar effects on DC than DEX. Treatment of DC with TGFβ inhibited their expression of CD80, CD83 and CD86, and it upregulated the expression of B7-H1. These effects were reversed when DC were nucleofected with GILZ siRNA (Fig 6A). When TGFβ-treated DC were used to present PPD, they stimulated CD4⁺ T lymphocytes to a lesser extent than control DC. This defect was reversed when DC where treated with GILZ siRNA (Fig 6B). Therefore, TGFβ-treated DC display phenotypic and functional alterations close to those of DEX-treated DC, and these changes also involve GILZ production by DC.
Fig 6: GILZ involvement in TGFβ-induced alterations of DC functions. a) Mo-DC were treated on day 6 with DEX, TGFβ or medium and with control (siC) or GILZ (siGILZ) siRNA.
On day 7, DC phenotype was determined; b) Mo-DC were treated on day 5 with control (siC) or GILZ (siGILZ) siRNA and with DEX, TGFβ or medium. They were loaded with PPD, washed and mixed with autologous CD4+ T lymphocytes on day 7. Lymphocyte proliferation was determined by Pkh26 fluorescence intensity. One typical experiment of 2 is shown.

**The effect of GILZ on the production of IL-10 and chemokines by DC**

Treatment of immature Mo-DC with DEX stimulates their production of IL-10 [14]. We investigated whether GILZ was involved in this effect. DEX induced IL-10 production in Mo-DC transfected with control siRNA. In contrast, transfection with GILZ siRNA prevented DEX-induced upregulation of IL-10 production. We next tested the effect of *gilz* gene transfer into Mo-DC. Mo-DC nucleofected with pGILZ produced ~6 fold more IL-10 than control cells nucleofected with pcDNA3 (Table I). Therefore, induction of GILZ is responsible for the DEX-mediated stimulation of IL-10 production by DC.

**In vivo induction of GILZ by GC treatment**

*gilz* gene expression was quantified by real-time PCR in circulating APC from patients treated with GC for various inflammatory disorders. Analyses were performed before and 48 hours after initiation of treatment. GC stimulated *gilz* gene expression by circulating APC (535 ± 43 AU of GILZ mRNA (mean ± SEM), vs 151 ± 35 AU before GC administration, p<0.05).

The ability of circulating APC from GC-treated patients to stimulate an antigen-specific CD4+ T lymphocyte response was tested. APC from GC-treated patients stimulated CD4+ T lymphocytes poorly, as assessed by proliferative and IFNγ production assays (data not shown). Findings were similar when APC were transfected with a control siRNA. In contrast, APC from GC-treated patients generated strong proliferation and IFNγ production when they were transfected with GILZ siRNA (Fig 7). Therefore, in GC-treated patients, GILZ expression by
circulating APC is increased, and this explains their poor effectiveness at triggering an antigen-specific CD4+ T lymphocyte response.

Fig 7: Effect of GILZ in GC-treated patients

Patients were tested before and after 48 hours of treatment with GC. Their APC were transfected with control (siC, □) or GILZ (siGILZ, ■) siRNA, loaded with PPD and cocultured with autologous CD4+ T lymphocytes. Proliferation (a,c) and IFNγ production (b) were determined on day 7 of culture. a,b) Results are expressed as means ± SEM for 4 patients; c) a typical experiment.
DISCUSSION

GC and IL-10 inhibit the expression of co-stimulatory and MHC molecules by DC and they prevent efficient antigen presentation to T lymphocytes. GC and IL-10 each stimulate DC expression of molecules directly inhibiting T lymphocyte responses, including B7-H1, IL-10 and ILT3. We show that these effects of GC and IL-10 involve a common mechanism, mediated by the induction of GILZ expression. DC derived in vitro from monocytes or CD34+ cells do not produce GILZ spontaneously, but GILZ production is induced by either DEX or IL-10. The continuous presence of DEX is required for the modulation of DC phenotype. GILZ production also requires the continuous presence of DEX or IL-10, and stops when these agents are removed. In contrast, GILZ production is not influenced by the addition of CD40L. In Mo-DC treated with DEX or IL-10, the decreased expression of co-stimulatory and MHC class II molecules and the increased expression of B7-H1 and ILT3 are prevented by GILZ siRNA. Moreover, gilz gene transfer to Mo-DC is sufficient to reproduce the phenotypic changes caused by DEX and IL-10.

Besides modulating the phenotype of DC, GILZ also affects their function. GILZ stimulated IL-10 production by DC to the same extent as GC. Both DEX and IL-10 inhibited the production of inflammatory chemokines by CD40L-stimulated DC. This effect was not found in DC transfected with GILZ siRNA. DEX and IL-10 inhibited DC stimulation of an antigen specific CD4+ T lymphocyte response and an allogeneic response. RNA interference experiments showed that GILZ expression is necessary for this effect and gene transfer experiments that it is sufficient. The effect of DEX, IL-10 and GILZ was stronger on DC phenotype changes than on T lymphocyte activation by DC. The inhibition of T lymphocyte stimulation by GILZ-expressing DC presumably results from a conjunction of effects: a decreased expression of co-stimulatory and MHC molecules, a decreased production of chemokines, which contribute to T lymphocyte activation, and an increased expression of IL-10, B7-H1 and ILT3. This redundancy of
mechanisms may ensure a strong and consistent inhibition of T lymphocyte activation by GILZ-expressing DC regardless of their origin and of their environmental conditions.

TGFβ also upregulated GILZ production by DC. TGFβ acts on T lymphocyte to prevent immune activation. However, TGFβ also affects DC functions directly \(^8,9\), and our findings show that GILZ induction is involved in this effect. GILZ production may thus be a common mechanism of immune control shared by several immunosuppressive cytokines. Interestingly, both IL-10 and TGFβ are produced by T lymphocytes with regulatory functions \(^12\), suggesting that these cells may control immune activation at least partly by inducing GILZ expression in DC. Because GILZ-expressing DC themselves produce IL-10, a self-amplifying loop may be induced locally to inhibit inflammation and immune activation.

GILZ inhibits cell activation via various molecular targets. It prevents NF-κB activation, at least partly by binding directly to its p65 subunit \(^17-19\). It also interferes with the MAPK pathway and the induction of AP-1 \(^19,20\). These signal transduction pathways are central to the maturation of DC, so a simple explanation of the effect of GILZ on DC function would be that it prevents DC maturation. The observation that GILZ inhibits several CD40L-induced modifications of DC, such as chemokine production and upregulation of co-stimulatory and MHC molecules, is consistent with this possibility. We also observed that GILZ prevents LPS-induced maturation of DC (data not shown). As immature DC are poor activators of T lymphocytes and may even promote tolerance, this would account for the inhibitory effect of GILZ on T lymphocyte responses. However, GILZ does not prevent all consequences of DC maturation, as it does not affect CD40L-induced inhibition of phagocytosis. Moreover, GILZ has specific effects, such as upregulation of IL-10 and B7-H1 expression. Expression of B7-H1 increases with DC maturation \(^5,7,30\), and therefore induction of IL-10 and B7-H1 by GILZ is not consistent with GILZ only preventing DC maturation. These observations rather suggest that GILZ drives DC to an alternative pathway of differentiation, leading to DC with many features of tolerogenic DC. The action of GILZ on DC maturation, regardless of its mechanism, presumably explains why IL-10 and TGFβ affect the function of immature DC but are not effective on fully mature DC.
The inability of DEX and IL-10 to induce GILZ when added to on fully differentiated DC may also explain this phenomenon.

Since their introduction as therapy, GC have become a cornerstone in most immunosuppressive treatments. Although their mechanisms of action remain incompletely understood, antigen-presenting cells are critical targets to their therapeutic effect. GC stimulate synthesis of IκBα and thus prevent NF-κB activation 31,32, suggesting a possible mechanism of GC action in DC. However, the intracellular effects of GC appear much more complex than initially thought, involving several molecular targets. We show here that GC administration to humans stimulates GILZ expression by circulating APC and that GILZ induction accounts for the poor efficacy of APC from GC-treated patients to stimulate T lymphocytes. Indeed, neutralization of GILZ by RNA interference restores fully potent antigen presentation by APC from GC-treated patients. By inducing GILZ in DC, GC may reproduce for therapeutic purposes a natural mechanism of immune regulation induced by immunosuppressive cytokines including IL-10 and TGFβ. Evidence from genetically-modified mice demonstrates that IL-10 and TGFβ are central to immune tolerance 33-37. In human and in mice, GILZ is constitutively produced by antigen-presenting cells (17 and this work). Possibly, this constitutive expression contributes to preventing unwarranted immune activation in healthy individuals. On the other hand, GILZ expression by DC may participate in immune defects in some disorders, and especially in cancers, which often produce IL-10, and in which IL-10-induced inhibition of CD80 8 and enhancement of B7-H1 6 expression by DC contribute to the failure of the immune system to control the tumor. Altogether, these results show that regulation of GILZ expression plays a critical role in the decision of DC to stimulate or not T lymphocytes, and IL-10, GC and TGFβ share this mechanism to affect DC functions and the balance between immune response and tolerance.
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REFERENCES


GILZ expression in human dendritic cells redirects their maturation and prevents antigen-specific T lymphocyte response

Nicolas Cohen, Enguerran Mouly, Haifa Hamdi, Marie-Christine Maillot, Marc Pallardy, Veronique Godot, Francis Capel, Axel Balian, Sylvie Naveau, Pierre Galanaud, Francois M Lemoine and Dominique Emilie