Glucocorticoid-Induced Leucine Zipper Enhanced Expression in Dendritic Cells Is Sufficient To Drive Regulatory T Cells Expansion In Vivo

Joseph Calmette, Mehdi Ellouze, Thi Tran, Soumaya Karaki, Emilie Ronin, Francis Capel, Marc Pallardy, Françoise Bachelerie, Roman Krzysiek, Dominique Émilie, Géraldine Schlecht-Louf and Véronique Godot

*J Immunol* published online 31 October 2014
http://www.jimmunol.org/content/early/2014/10/31/jimmunol.1400758
Glucocorticoid-Induced Leucine Zipper Enhanced Expression in Dendritic Cells Is Sufficient To Drive Regulatory T Cells Expansion In Vivo

Joseph Calmette,*‡,† Mehdi Ellouze,*‡,† Thi Tran,*‡,† Soumaya Karaki,*‡,† Emilie Ronin,*‡,† Francis Capel,*‡ Marc Pallardy,*‡ Françoise Bachelerie,* Roman Krzysiek,*‡ Dominique Emilie,*‡ Géraldine Schlecht-Louf,*‡ and Véronique Godot§,*,¶

Tolerance induction by dendritic cells (DCs) is, in part, mediated by the activation of regulatory T cells (Tregs). We have previously shown in vitro that human DCs treated with glucocorticoids (GCs), IL-10, or TGF-β upregulate the GC-Induced Leucine Zipper protein (GILZ). GILZ overexpression promotes DC differentiation into regulatory cells that generate IL-10–producing Ag-specific Tregs. To investigate whether these observations extend in vivo, we have generated CD11c-GILZhi transgenic mice. DCs from these mice constitutively overexpress GILZ to levels observed in GC-treated wild-type DCs. In this article, we show that GILZhi DCs strongly increase the Treg pool in central and peripheral lymphoid organs of aged animals. Upon adoptive transfer to wild-type recipient mice, OVA-loaded GILZhi bone marrow–derived DCs induce a reduced activation and proliferation of OVA-specific T cells as compared with control bone marrow–derived DCs, associated with an expansion of thymus-derived CD4+Foxp3+ CD4 T cells. Transferred OVA-loaded GILZhi DCs produce significantly higher levels of IL-10 proliferation of OVA-specific T cells as compared with control bone marrow–derived DCs, associated with an expansion of peripheral Treg; SDLN, skin draining lymph node; Tconv, conventional CD4 T cell; Treg, regulatory T cell; Treg, thymically derived CD4+Foxp3+CD25+ Treg.

The online version of this article contains supplemental material.

immune homeostasis requires the equilibrium between responses against pathogens and tolerance toward self-Ags and innocuous environmental Ags. Regulatory T cells (Tregs) are recognized as essential contributors to this balance. Two main Treg subsets have been described: the thymically derived CD4+Foxp3+CD25+ Tregs (tTreg) that differentiate in the thymus, and the peripheral Tregs (pTregs) that differentiate from naive conventional CD4 T cells (Tconvs) and acquire Foxp3 expression in the periphery. Tregs depend on dendritic cells (DCs) for both their differentiation and activation. Indeed, DCs are required to activate tTreg in periphery and to convert Tconvs into pTregs (2). The exact nature of the regulatory DCs (DCregs) that are endowed with Treg induction and activation capacity is not entirely understood. Convergent studies show that this activation is not a default pathway but rather an active process that requires the expression of a regulatory genetic program by specialized DC subsets (3). Both developmental characteristics and environmental signals are reported to impact tolerogenic potential of DCs. IL-10, TGF-β, glucocorticoids (GCs), vasoactive intestinal peptide, vitamin D3, antioxidative vitamins, and retinoic acid, used alone or in combination, skew DC maturation toward DCregs (4, 5). However, the molecular mechanisms controlling the maturation of DCs into DCregs remain elusive.

The GC-Induced Leucine Zipper protein (GILZ) is a GC-induced protein initially reported in dexamethasone (Dex)-treated thymocytes (6). We have previously shown ex vivo that the level of GILZ expression in human DCs controls their tolerance-inducing function (7–9) and extended to other hematopoietic cells these observations (10, 11), which were confirmed by others (12–15). GILZ expression is partly dependent on endogenous steroids in vivo (12), and its expression in DCs is enhanced in vitro by GC, IL-10, TGF-β (7, 8), mitomycin C (16), rapamycin (15), vitamin D3 (15), and tumor environment (17). Human GILZhi-DCs secrete large amounts of IL-10 compared with GILZlow-DCs and by this way induce Ag-specific CTLA-4-IL-10+ CD4+ Tregs, some of which coexpress Foxp3 (7, 8). Furthermore, we have shown in humans that GILZ mediates the immunoregulatory effects of GC

Address correspondence and reprint requests to Prof. Véronique Godot or Dr. Gérardine Schlecht-Louf, INSERM U955, 51 Avenue du Maréchal de Lattre de Tassigny, Créteil, 94 010 Cedex, France (V.G.) or geraldine.schlecht-louf@u-psud.fr (G.S.-L.).

© 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

Received for publication March 24, 2014. Accepted for publication October 6, 2014.

This work was supported by the Agence Nationale pour la Recherche (Grant 09-GENO-030-01), the Fondation pour la Recherche Médicale, the Université Paris-Sud (Pôle de Recherche et d’Enseignement Supérieur UniverSud and Appel à Projet Attractivité 2012), a doctoral fellowship from the Ecole Doctorale Innovation Thérapeutique (Grant ED 425 to J.C.), a doctoral fellowship from INSERM (to M.E.), a doctoral fellowship from the Région Ile de France (to S.K.), and a one-year postdoctoral fellowship from the Agence Nationale pour la Recherche (Grant 09-GENO/030/01 to S.K.).

Abbreviations used in this article: BM-DC, bone marrow–derived DC; cDC, conventional DC; ctrl, control littermate; DC, dendritic cell; DCreg, regulatory DC; Dex, dexamethasone; eGFP, enhanced GFP; GC, glucocorticoid; GILZ, GC-induced leucine zipper protein; MFI, mean of fluorescence intensity; MLN, mesenteric lymph node; pDC, plasmacytoid DC; polyIC, polyinosinic-polycytidylic acid; pTreg, peripheral Treg; SDLN, skin draining lymph node; Tconv, conventional CD4 T cell; Treg, regulatory T cell; Treg, thymically derived CD4+Foxp3+CD25+ Treg.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1400758
on DCs (8, 9). Downregulation of GILZ in DCs promotes the activation of effector CD4+ T cells (10, 11, 12, 17).

To gain insight into the impact of GILZ on the regulatory functions of DCs in vivo, we generated CD11c–GILZhi transgenic mice. DCs from these mice constitutively overexpress GILZ to the same extent as GC-treated wild type DCs. In in vitro studies, GILZhi DC phenotype and function is not different from that observed in DCs from control littersmates (ctl). However, CD11c–GILZhi mice display an age-dependent accumulation of Foxp3+ Tregs in vivo. In adoptive transfer models, we further demonstrate that the GILZhi DCs acquire a regulatory phenotype after cognate interaction with T cells. They express reduced levels of MHC class II molecules, secrete higher amounts of IL-10, poorly induce Tconv proliferation and activation, but promote Treg expansion.

In conclusion, our work formally identifies GILZ as a factor that contributes to DC tolerogenic function in vivo and validates the therapeutic potential of GILZ-expressing DCregs in immunopathologies relying on Treg defects.

Materials and Methods

Mice

CD11c–GILZhi mice were generated by a single targeted insertion (at the hprt docking site on the X chromosome) of GILZ coding sequences driven by the CD11c promoter (Nucleus, Lyon, France; Supplemental Fig. 1). Genetic uniformity was reached by a 10-generation backcross to the C57BL/6J background (c>0.98%). The resulting CD11c–GILZtransgenic mice are viable and fertile, with litters of normal size and sex ratio. Mice were maintained by heterozygous crossing with C57BL/6 males and CD11c–GILZhi mice were compared with controls from the same litter. Unless specified, hemizygous male mice were used between 8 and 14 wk of age.

OT-II TCR transgenic mice were obtained from Charles River (L’Arbresle, France) and from CDTA (Orleans, France). Foxp3enhanced GFP (eGFP) and IL-10–GFP mice were kind gifts from Dr. B. Malissen (Centre d’Immunologie de Marseille-Luminy, Marseille, France) (18) and Dr. R. Flavell (Yale University School of Medicine, New Haven, CT) (19), respectively.

Mice were bred in the Animex animal facility under specific pathogen-free facilities. Experimentation was approved by the local Ethics Committee for Animals (C2EA-126; Animal Care and Use Committee, Villejuif, France) and complied with French and European guidelines for the use of laboratory animals.

Proteins, peptides, and activators

The synthetic OVA233-349 (IQSVAVHAAHINEAGPR) peptide corresponding to the immunodominant I-Aβ-restricted T cell epitope from OVA was purchased from AnaSpec (Fremont, CA). The OVA protein was obtained from Sigma-Aldrich. TLR agonists were purchased from Invivogen (Cayla SAS).

RNA extraction and quantitative RT-PCR

RNAs were extracted from 2 × 10⁶ bone marrow-derived DCs (BM-DCs) according to manufacturer’s instructions (RNeasy Plus Mini kit; Qiagen, France). CDNA were obtained by reverse transcription with the MuLV retrotranscriptase (Applied Biosystems). Quantitative RT-PCR was performed using SYBR Green I Master Mix kit (Roche Applied Science) and LightCycler 480 (Roche). Normalization was made by standard curve method based on β-actin expression. The primers sequences are available upon request.

Cell preparations

BM-DCs were generated as previously described (20), using DC culture medium (CM; Life Technologies; RPMI 1640, 5% FCS, 50 μg/ml gentamicin, 5 × 10⁻⁵ M 2-ME, and 20 ng/ml murine rGM-CSF; PreproTech). Cells (containing >90% of CD11c+ cells) were used at days 9 and 10. Splenic DCs were recovered from CD11c–GILZlo/low littermate mice as described by Schlecht et al. (21). Magnetic cell sorting was done using autoMACS (Miltenyi Biotech). Cell purity ranged from 90 to 99%.

Splenic DCs were recovered from CD11c–GILZhi or littermate mice as described in Schlecht et al. (27). Cells were either injected i.v. into CD45.1 C57BL/6 mice (5 × 10⁶ cells/mouse) or used in vitro.

Western blots

Protein extracts from 2 × 10⁶ BM-DCs or splenic DCs were lysed with 20 μl lysis buffer (Roche Applied Science) as previously described (8). Fifty micrograms of proteins was loaded on a 4-12% gradient polyacrylamide gel (NuPAGE; Invitrogen). GILZ staining was performed with a rabbit anti-GILZ Ab (FL-134 clone; Santa Cruz) and a donkey anti-rabbit secondary Ab conjugated to peroxidase (Amersham, GE-Healthcare) as described in Hamdi et al. (8). β-Actin (eBioscience SAS) expression was used as loading control.

Ag capture and processing

A total of 2 × 10⁵ DCs was distributed in wells of a 96-well plate. Ag capture was measured using Alexa 488–labeled OVA (Molecular Probes), dextran-FTTC, and zymosan-FTTC (both from Sigma-Aldrich). Dextran polymers are preferentially taken up by mannose receptor (CD206)–dependent endocytosis (22). OVA by both CD206-dependent endocytosis and macropinocytosis (23, 24), and zymosan by dectin–1–dependent endocytosis (25). OVA DQ (Molecular Probes) was used for Ag processing assay (26). After 90 min or 5 h of incubation at 4˚C or 37˚C, DCs were labeled with anti-CD11c Abs and analyzed on FACS Calibur or LSR Fortessa (BD Biosciences, San Diego, CA). ∆ means of fluorescence intensity (MFIs) was calculated by subtracting the MFI at 4˚C (control) from the MFI at 37˚C.

DC activation for phenotype and cytokine/chemokine detection

The phenotype and cytokine/chemokine secretion profiles of DCs were assessed by incubating 2 × 10⁵ BM-DCs in 96-well plates with 1.5 μM Cpg (TLR9 agonist), 1 μg/ml LPS (TLR4 agonist), 10 μg/ml polyinosinic-polycytidylic acid (polyIC; TLR3 agonist), or 10 μg/ml zymosan (TLR2 agonist). After 15 h, culture supernatants were recovered for cytokine measure, and DC phenotype was analyzed by flow cytometry. Cytokines and chemokines were assessed by ELISA using IL-10 and IL-12p70 BD OptEIA Set Mouse (BD Biosciences) or by Lumixen assay using the Procarna mouse 26-plex from eBioscience.

T cell stimulation in vitro and in vivo

BM-DCs were loaded with OVA or OVA233-349 peptide for 5 h and 90 min, respectively, at 37˚C. In in vitro assays, 2.5 × 10⁵, 5 × 10⁵, or 10 × 10⁵ DCs per well were used in triplicate to stimulate 5 × 10⁵ CFSE–labeled OT-II CD4 T cells. After 72 h of incubation, T cell proliferation was analyzed by flow cytometry.

Splenic DCs were loaded with OVA for 4 h and cocultured with OT-II CD4 T cells at a 1:10 ratio. After 72 h, [3H]thymidine (5 μCi/well) was added to cultures. Cells were harvested 6 h later to evaluate incorporated [3H]thymidine by scintillation counting.

In vivo assays, 5 × 10⁵ BM-DCs were transferred i.v. into CD45.1 mice 1 d after OT-II T cell injection. Sixty-six hours later, CD45.1 mice were sacrificed and spleens recovered as described in Schlecht et al. (27). T cell phenotype and proliferation were analyzed by flow cytometry.

Flow cytometry

Cells suspended at 10⁶ cells/ml in PBS-FCS 2% were first incubated with anti-CD16/32 (2.4G2 clone) Fc-Block, then labeled for 15–30 min in 4˚C in the dark with combinations of mAbs (clone): anti-CD11c (HL-3), -CD20 (RA3-6B2), -CD45RA (14.8), -CD11b (M1/70), -CD86 (53-6.7), -CD40 (HM40-3), -CD86 (GL1), -CD3 (17A2), -CD4 (RM4-5), -CD8 (53-6.7), -B220 (RA3-6B2), -CD45.2 (104), -CD27 (LG.7F9), -CD44 (IM7), -CD11b (M1/70), -CD25(PIC61), -MHCII (M114.15.2), -PD-L1 (MIHS), -PD-L2 (TY-25), -ICOSL (H5K.3), -OX40 (OX86), -H2Kb (CTKb), -DC25 (104.2), -CD27 (LD.7G9), -ICOS (7E.17G9), and -NK1.1 (PK136). mAbs and isotype controls were purchased from BD Biosciences (San Diego, CA), eBioscience (San Diego, CA), Biolegend (San Diego, CA), or Caltag (Buckingham, U.K.).

For intracellular Foxp3 staining, Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA) was used. For anti-IL-10 and anti-IFN-γ staining, 10⁶ splenocytes were stimulated with 5 h with 1 μg/ml OVA233-349 peptide at 37˚C in the presence of GolgiPlug (BD Biosciences) were permeabilized using the Fix/Perm and Perm/Wash kit (BD Biosciences). Fluorescence was acquired on a FACSAria or a LSR Fortessa (BD Biosciences) flow cytometer and analyzed using the FlowJo Software (Tree Star).

Statistical analysis

Statistical analysis of results was performed with GraphPad Prism software. Depending on experimental settings, (un)paired t test, one- or two-way ANOVA, or Wilcoxon signed rank test was used. The p values obtained
from multiple comparisons were adjusted by Bonferroni correction. The 5% threshold was considered significant. \( *p < 0.05, **p < 0.01. \)

**Results**

Mice with constitutive overexpression of GILZ in DCs display normal lymphocyte and DC distribution in lymphoid tissues

In the CD11c-GILZ\textsuperscript{hi} transgenic mice, we used the CD11c promoter to drive enforced expression of GILZ in DCs (28). We confirmed an overexpression of GILZ mRNA and protein in splenic DCs and BM-DCs from CD11c-GILZ\textsuperscript{hi} mice as compared with their ctrl (Fig. 1A, 1B). Therefore, DCs from CD11c-GILZ\textsuperscript{hi} mice will be referred to as GILZ\textsuperscript{hi} DCs throughout the text. The enforced expression of GILZ driven by the CD11c promoter in transgenic mice and the expression of GILZ induced by Dex in ctrl mice were comparable, thereby confirming a regular level of GILZ in CD11c-GILZ\textsuperscript{hi} mice (Fig. 1A, 1B). Likewise, the level of GILZ expression was slightly increased in GILZ\textsuperscript{hi} BM-DCs after Dex treatment (Fig. 1A, 1B), pointing out a suboptimal level of GILZ in these transgenic cells.

GILZ overexpression was restricted to DCs because no alteration in GILZ mRNA levels was observed in other hematopoietic cells such as CD4 T cells, CD8 T cells, splenic NK cells, or liver NK cells, a population known to be enriched in CD11c-expressing cells (29) (Fig. 1C–E).

No change in the architecture (data not shown) and total cell numbers in spleen, skin draining lymph nodes (SDLNs), and mesenteric lymph nodes (MLNs) was observed in CD11c-GILZ\textsuperscript{hi} mice as compared with ctrl (Table I). Furthermore, the frequencies and absolute numbers of B cells, T cells, conventional DCs (cDCs), and plasmacytoid DCs (pDCs) were similar in secondary lymphoid organs of CD11c-GILZ\textsuperscript{hi} and ctrl littermates (Fig. 1F and Table I).

Increased Treg pool in CD11c-GILZ\textsuperscript{hi} mice

Because human monocyte-derived DCs overexpressing GILZ induce Treg differentiation in vitro (7–9), we investigated whether GILZ overexpression in DCs modified the Treg compartment in vivo. To address this issue, we first traced Foxp3-eGFP CD4\textsuperscript{+} Tregs in several lymphoid organs of young (8–12 wk old) and aged (18 mo) Foxp3-eGFP \( \times \) CD11c-GILZ\textsuperscript{hi} female mice obtained by crossing CD11c-GILZ\textsuperscript{hi} mice with Foxp3-eGFP reporter mice. We observed an increase of Foxp3\textsuperscript{+}CD25\textsuperscript{hi} Treg frequencyrestricted to the spleen in young Foxp3-eGFP \( \times \) CD11c-GILZ\textsuperscript{hi} mice and generalized to the thymus and the secondary lymphoid organs for both CD25\textsuperscript{+} and CD25\textsuperscript{−} Tregs in aged Foxp3-eGFP \( \times \) CD11c-GILZ\textsuperscript{hi} mice as compared with Foxp3-eGFP ctrl littermates (Fig. 2A, 2B). Because both the CD11c-GILZ transgene and the Foxp3 gene are carried on X chromosome, subjected to X-inactivation, we checked GILZ overexpression in DCs from Foxp3-eGFP \( \times \) CD11c-GILZ\textsuperscript{hi} females (Fig. 2C) and verified that the proportion of Foxp3-eGFP Tregs corresponded to half the Tregs detected by intracellular labeling as expected (Fig. 2D). Likewise, we confirmed the expansion of Tregs in lymphoid organs of young and 24-mo-old CD11c-GILZ\textsuperscript{hi} male by Foxp3 intracellular staining (Fig. 2E, 2F). However, no difference in the percentages and numbers of CD4\textsuperscript{+} Foxp3\textsuperscript{−} Tconv was observed between ctrl and CD11c-GILZ\textsuperscript{hi} mice, whatever their age (Fig. 2E, 2F).

The Treg phenotype analyzed in aged CD11c-GILZ\textsuperscript{hi} mice was similar to that of ctrl littermates for CD69, CTLA4, GITR, CD103, PD1, and CCR7 (data not shown). The thymus and SDLN of aged CD11c-GILZ\textsuperscript{hi} mice showed an increase of the ICOS\textsuperscript{hi} Treg subset (Fig. 2H), a subset reported to be highly suppressive and biased toward IL-10 production (30–32). This increase of ICOS\textsuperscript{hi} Tregs was also confirmed in young CD11c-GILZ\textsuperscript{hi} mice (Fig. 2G). Thus, the sole GILZ overexpression in DCs clearly promotes Treg accumulation in vivo, and this accumulation is associated with an increase in the ICOS\textsuperscript{hi} Treg subset.

GILZ\textsuperscript{hi} DC phenotype in vitro

The basal phenotype of DCs was investigated on either freshly isolated splenic DCs or BM-DCs. In both cases, the expression levels of CD80, CD86, CD40, MHC class I, or MHC class II molecules were similar between CD11c-GILZ\textsuperscript{hi} and ctrl mice (Fig. 3A, 3B). We further looked at the expression of several...
membrane markers for tolerogenic DCs. The results showed no increased expression of PD-L1, PD-L2, ICOSL, and OX40L on GILZ hi splenic DCs or GILZ hi BM-DCs as compared with their controls (Fig. 3A, 3B). Thus, the sole GILZ overexpression in freshly isolated DCs or BM-DCs does not significantly modify their basal phenotype ex vivo and in vitro.

We next addressed the impact of GILZ overexpression on DC maturation in vitro. Analysis of the expression of CD86, CD40, and

<table>
<thead>
<tr>
<th>Table I. DC, B cell, and T cell frequency in secondary lymphoid organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Ctrl</td>
</tr>
<tr>
<td>Cell number ($\times 10^6$)</td>
</tr>
<tr>
<td>cDCs, %</td>
</tr>
<tr>
<td>pDCs, %</td>
</tr>
<tr>
<td>B cells, %</td>
</tr>
<tr>
<td>CD4 T cells, %</td>
</tr>
<tr>
<td>CD8 T cells, %</td>
</tr>
</tbody>
</table>

Spleens, SDLNs, and MLNs were harvested and single-cell suspensions prepared. The cells were counted, and the proportions of cDCs (CD11c hi, pDCs (CD11c lo PDCA-1+), B cells (CD19+B220+), CD4 T cells (CD3+CD4+), and CD8 T cells (CD3+CD8+) were determined by flow cytometry. Results are from six to eight mice per group.

**FIGURE 2.** Treg accumulation in CD11c-GILZ hi mice. (A and B) Foxp3-eGFP × CD11c-GILZ hi female mice were obtained by crossing transgenic CD11c-GILZ hi female mice with Foxp3-eGFP male mice. Thymuses, spleens, and SDLNs from young (8–12 wk old) (A; n = 6–8 mice/group) and aged (18-mo-old) (B; n = 5–6 mice/group) Foxp3-eGFP × CD11c-GILZ hi or Foxp3-eGFP littermate control mice were analyzed by flow cytometry. T cells were gated as single CD3 CD4 T cells and assessed for Foxp3 and CD25 expression. (C) GILZ overexpression in splenic DCs from Foxp3-eGFP × CD11c-GILZ hi females was confirmed by quantitative RT-PCR (n = 5–6 mice/group). (D) Treg detection in the spleen of Foxp3-eGFP × CD11c-GILZ hi and littermate control Foxp3-eGFP mice using eGFP signal (left) or intracellular Foxp3 staining (right) was compared to confirm the proportionality between the two detection systems (n = 4–5 mice/group). (E and F) Thymuses and spleens from young (E) or 24-mo-old (F) CD11c-GILZ hi and control male mice were recovered. Frequencies and numbers of CD3+CD4+ Foxp3+ CD25+ or CD25- Tregs (left) and CD3+CD4+Foxp3- Tconv (right) are shown (n = 3–6 mice/group). (G and H) Frequency of ICOS hiFoxp3+ T cells in lymphoid organs of young (G; n = 6–8 mice) and aged (H; n = 5–6 mice) Foxp3-eGFP × CD11c-GILZ hi or Foxp3-eGFP littermate female mice are shown. Mean ± SEM, two-tailed unpaired t test. *p < 0.05, **p < 0.01.
MHC class II molecules on GILZhi and ctrl BM-DCs after stimulation with TLR2 (zymosan), TLR3 (polyI:C), TLR4 (LPS), and TLR9 (CpG) ligands revealed no statistically significant differences. Because Dex downregulated costimulatory molecules on human DCs through GILZ induction (7, 8), we evaluated the expression of MHC class II molecules, CD40, and CD86 on Dex-treated-GILZhi and ctrl DCs. An equivalent downregulation of MHC and costimulatory molecules was observed in both DC subsets (Fig. 3C). This might be explained by the higher expression of GILZ reached in vitro after DC treatment with Dex (see Fig. 1A, 1B) or by GILZ-independent effects of Dex on DC phenotype in mice.

No differences in IL-10 or IL-12p70 production could be observed between GILZhi and control BM-DCs either in the steady-state or after activation (Fig. 3D). Other cytokines and chemokines were not differently expressed either (Supplemental Fig. 2 and data not shown). Taken together, these data indicate that GILZhi BM-DCs display no alteration of surface phenotype and cytokine production in vitro.

Ag capture and presentation by GILZhi DCs in vitro

The absence of a tolerant phenotype for GILZhi DCs in vitro prompted us to verify whether these DCs loaded with OVA were able to activate OVA-specific OT-II Tregs after in vivo adoptive transfer. Because murine DCs overexpressing GILZ upon lentiviral transduction display deficient OVA uptake (17), we first tested the capacity of GILZhi splenic DCs and BM-DCs to internalize and degrade soluble (OVA, dextran) and particulate (zymosan) Ags. No differences in dextran or zymosan capture were observed (Fig. 3A). Consistently with the results published by Lebson et al. (17), GILZhi BM-DCs displayed a reduced OVA uptake as compared with control BM-DCs after a short incubation period with this Ag, and OVA degradation was similarly reduced (Fig. 4A). However, these capture and degradation defects were transient and disappeared completely at later times (i.e., 5 h of incubation; Fig. 4B), and were not observed for splenic DCs (Fig. 4C). To test Ag presentation capacity, we loaded GILZhi and ctrl BM-DCs with OVA for 5 h to avoid differences in Ag access, and incubated them with CFSE-labeled OT-II CD4 T cells. As shown in Fig. 4D, GILZhi DCs induced OT-II cell proliferation as efficiently as ctrl BM-DCs. Similar results were obtained with splenic DCs (Fig. 4E). Overall, these data demonstrated that GILZhi DCs display no major alteration in their capacity to take up and present Ags in vitro.

GILZhi DCs poorly activate effector T cells but promote iTreg expansion in vivo

We next investigated the ability of OVA-loaded GILZhi DCs to prime naive Ag-specific T cells in vivo, and determined whether they favor the expansion of Ag-specific Tregs. To this end, CFSE-labeled DCs were harvested from the spleens (A) or derived from the bone marrow (B) of CD11c-GILZhi (GILZhi) and littermate control (ctrl) mice and analyzed by flow cytometry. DCs were gated as CD11c+ cells, and their expression of MHC class II molecules, costimulatory molecules, or inhibitory molecules was assessed. MFI and percents of positive cells as compared with isotype controls are indicated in regular and bold font, respectively. Results shown are representative of three experiments. (C) GILZhi and ctrl BM-DCs phenotype after stimulation for 15 h with TLR2 (zymosan), TLR3 (polyI:C), TLR4 (LPS) or TLR9 (CpG ODN) agonists, or Dex. MHC II molecules, CD40, and CD86 expression were measured by FACS on CD11c+ cells. Means ± SEM of MFI fold increase for five to eight mice in each group are shown. (D) IL-10 and IL-12p70 concentrations were measured by ELISA in the culture supernatants from GILZhi and ctrl BM-DCs prepared in (C). Wilcoxon signed rank test corrected by Bonferroni for multiple comparison was used to compare treated ctrl or GILZhi DCs with unstimulated ctrl or GILZhi DCs, respectively. ***p < 0.005, **p < 0.01, *p < 0.05.
OT-II T cells (CD45.2) and GILZ hi or control DCs (CD45.2) loaded with OV A were i.v. injected to CD45.1 congenic recipient mice (Fig. 5A). Three days after DC injection, spleens were recovered and CD45.2 OT-II T cells were analyzed by flow cytometry. CD4 T cells triggered by OV A-loaded GILZ hi DCs proliferated less than those stimulated with control DCs (Fig. 5B, 5D). This decreased proliferation capacity was associated with a lower OT-II cell activation state as revealed by CD44 and CD62L staining (Fig. 5E, 5F). Similar results were obtained when DCs were loaded with the OVA323–339 peptide, further demonstrating that the difference in T cell activation between GILZ hi and ctrl DCs is not due to a defect in Ag processing (Fig. 5B, 5D–F). The analysis of cytokine production by splenic OT-II T cells after a 5-h in vitro restimulation with OV A323–339 peptide revealed that OV A-loaded GILZ hi DCs induced less IFN-\(\gamma\)–producing T cells and more IL-10–producing T cells than ctrl DCs (Fig. 5G). Thus, although GILZ hi DCs were as efficient as control DCs in inducing T cell proliferation in vitro, they were significantly less efficient in inducing T cell stimulation and proliferation in vivo.

To test whether this reduced stimulation capacity was associated with the proliferation of Tregs, we performed a Foxp3 intracellular staining on the OT-II T cells recovered after the adoptive transfer. This experiment revealed a significantly higher proportion of CD4+Foxp3+CD25+ Tregs after T cell priming with GILZ hi DCs (Fig. 5H, 5J). These Tregs were mainly found among dividing cells (Fig. 5I), suggesting that the increase of Tregs observed after OV A-loaded GILZ hi DC injection results from Treg proliferation.

To determine whether these proliferating Tregs derived from tTregs or were differentiated from Foxp3+ Tconv, we reproduced this experiment using OT-II T cells depleted from CD25+ T cells, which correspond essentially to Tregs in naive OT-II mice. Although CD25-depleted OT-II T cells primed by OV A-loaded GILZ hi DCs still proliferate far less than those activated by OV A-loaded ctrl DCs (Fig. 5C, 5D) and display a less activated phenotype (Fig. 5E, 5F), no Treg accumulation was observed in mice that had received OV A-loaded GILZ hi DCs (Fig. 5J). These adoptive transfers formally demonstrated that GILZ hi DCs are poor inducers of CD4 T cell proliferation and activation, but that they preferentially support tTreg expansion in vivo.

GILZ hi DCs display a regulatory phenotype in vivo

To shed light on the apparent paradoxical phenotype displayed by GILZ hi DCs in vitro and in vivo, where they show no specific phenotype or cytokine secretion, respectively, (Fig. 3) and tolerogenic properties, we analyzed GILZ hi DC phenotype in vivo after an encounter with T cells. We were particularly interested in their production of IL-10 because we previously demonstrated that one characteristic of tolerant human GILZ hi DCs was a higher IL-10 production. Therefore, we crossed the CD11c-GILZ hi and ctrl mice with IL-10–GFP reporter mice (19) and reproduced the earlier-described adoptive transfer in CD45.1 congenic recipient mice, using GILZ hi or ctrl DCs coexpressing GFP with IL-10 (Fig. 6A). Equivalent numbers of DCs were recovered in each condition (data not shown), showing that the differences in T cell priming by GILZ hi DCs cannot be assigned to deficient homing to the spleen. Although no difference was observed for CD86 expression on GILZ hi and ctrl DCs (Fig. 6B), the analysis of MHC class II molecules expression revealed a significant decrease on OV A-loaded GILZ hi DCs (Fig. 6C). OV A-loaded GILZ hi DCs also produced significantly more IL-10 than ctrl OV A-loaded DCs.
Using DCs from CD11c-GILZhi IL-10–GFP and ctrl IL-10–GFP mice, we confirmed that the DCs used for the adoptive transfer displayed the same capacity to produce IL-10 in vitro upon TLR2 engagement (Fig. 6E), results consistent with those obtained in Fig. 3D. The significant increase in IL-10 production in vivo was observed only for OVA-loaded GILZhi DCs, and not for nonloaded GILZhi DCs, suggesting a role for DC–T cell interactions in DC phenotype modification. However, the sole engagement of CD40 on GILZhi DCs by anti-CD40 Abs was not sufficient to increase their IL-10 production in vitro (data not shown). The analysis of the CD45.2+CD4+ OT-II T cells recovered from the spleens 15 h after DC transfer revealed a similar activation of T cells that had interacted with OVA-loaded GILZhi and ctrl DCs, as assessed by CD25 and CD62L staining (Fig. 6F). Overall, these data show that upon in vivo transfer, GILZhi DCs acquire a regulatory phenotype, characterized by a reduced MHC class II expression at the cell surface and an increased IL-10 production.

Discussion

We set up a new mouse strain that constitutively expresses enhanced GILZ levels in DCs to investigate in vivo the contribution of GILZ to the tolerogenic function of DCs. In this study, we show that the sole GILZ overexpression in DCs is sufficient to promote thymus-derived Foxp3+ Treg expansion in vivo.

We previously reported that high levels of GILZ expression in human DCs endow these cells with a regulatory potential, stimulating the proliferation of IL-10–secreting Ag-specific Tregs in vitro (7–9). In this article, we show that young CD11c-GILZhi mice have significantly increased frequencies of Tregs in the spleen. Noteworthy, this increase in Treg frequency is exacerbated and generalized to all the lymphoid organs in 18- to 24-mo-old animals (33, 34), pointing to an age-dependent accumulation of Tregs driven by GILZ-expressing DCs. In addition, the pool of ICOS-expressing Tregs is significantly increased in CD11c-GILZhi mice. ICOS contributes to the homeostatic survival of Tregs and to the expansion of Ag-specific Tregs (31). The ICOShi Tregs were reported to be prone to produce IL-10 (30, 31, 35). The increase of the ICOShi Tregs in the CD11c-GILZhi mice may therefore result from a constitutively enhanced activation of Tregs that could participate in Treg accumulation over time.

To exclude the possibility that the increase in Treg frequency observed in the CD11c-GILZhi mice results from a CD11c-driven expression of GILZ in a subset of T cells (36) that could promote their survival (37) or alter their differentiation (38), we used an
adoptive transfer model in which only the transferred DCs over-express GILZ. When OVA-loaded GILZhi DCs and OT-II T-cells were cotransferred to wild-type recipient mice, OVA-specific T cell proliferation and activation were reduced as compared with that induced by OVA-loaded control DCs. This was associated with an increase in the percentages of positive cells as compared with endogenous DCs are indicated in regular and bold fonts, respectively. Right histograms represent the MFI ± SEM for the three experiments. (E) IL-10 production by GILZhi × IL-10–GFP and ctrl IL-10–GFP DCs in the resting state (medium) and upon in vitro stimulation with zymosan (n = 3). Two-way ANOVA followed by Bonferroni posttests were used for statistical analysis. **p < 0.01, *p < 0.05. (F) CD45.2+CD3+CD4+ OT-II T cells activation was revealed by the percentages of cells expressing CD25 (left panel) and CD69 (right panel) (n = 3).

FIGURE 6. GILZhi DCs acquire a regulatory phenotype upon in vivo transfer. (A) Adoptive transfer approach to analyze DC phenotype (B and C) and their IL-10 production (D) after T cell encounter in vivo. Transferred DCs were gated as CD45.2+CD11c+MHCII+ cells. (B) CD86 expression at the surface of the CD45.2 DCs (C), MHC class II expression, and (D) IL-10 production were assessed by FACS analysis. Left histograms show the results for one representative experiment out of three. MHC class II and IL-10 expression by CD45.1+ endogenous DCs (gray lines) and CD45.2+ transferred DCs (black lines) are presented. MFI and percents of positive cells as compared with endogenous DCs are indicated in regular and bold fonts, respectively. Right histograms represent the MFI ± SEM for the three experiments. (E) IL-10 production by GILZhi × IL-10–GFP and ctrl IL-10–GFP DCs in the resting state (medium) and upon in vitro stimulation with zymosan (n = 3). Two-way ANOVA followed by Bonferroni posttests were used for statistical analysis. **p < 0.01, *p < 0.05. (F) CD45.2+CD3+CD4+ OT-II T cells activation was revealed by the percentages of cells expressing CD25 (left panel) and CD69 (right panel) (n = 3).

a state of anergy as classically observed when a T cell meets its Ag in the absence of costimulation (39) or alternatively when it is activated in the presence of IL-10 (40). At this stage of their differentiation, it is not possible to distinguish anergic T cells induced by TCR ligation in the absence of costimulation from IL-10–anergized T cells, because both populations fail to produce cytokines (40, 41). IL-10 and TGF-β production by IL-10–anergized T cells require several rounds of restimulation (42). In this article, we report that OVA-loaded GILZhi DCs produce significantly higher levels of IL-10 and do not harbor reduced CD86 expression as compared with OVA-loaded control DCs. Thus, GILZhi DCs could contribute to the induction of IL-10–anergized T cells, a hypothesis supported by our previous observation in humans (8).

Remarkably, the regulatory phenotype of GILZhi DCs was only observed in vivo and restricted to Ag-loaded DCs, as opposed to the results we observed with human monocyte-derived DCs (8). This discrepancy may result from the
experimental procedures used to upregulate GILZ in human DCs (Dex treatment and GILZ gene transfection) or intrinsic differences, which may exist between human and murine DCs. The latter hypothesis is supported by the fact that Dex treatment of murine DCs did not induce IL-10 production in contrast with human DCs, pointing to interspecies differences in responses to GC exposure.

Collectively, our work demonstrates that DCs overexpressing GILZ acquire a tolerogenic function, associated with their ability to produce IL-10, and expand Foxp3+ Tregs in vivo. These results support the hypothesis that GILZ acquire a tolerogenic function, associated with their ability to limit the efficacy of dendritic cell vaccines, Cancer Gene Ther. 18: 563–570.


