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DC-SCRIPT Regulates Glucocorticoid Receptor Function and Expression of Its Target GILZ in Dendritic Cells

Saartje Hontelez, Nina Karthaus, Maaike W. Looman, Marleen Ansems, and Gosse J. Adema

Dendritic cells (DCs) play a central role in the immune system; they can induce immunity or tolerance depending on diverse factors in the DC environment. Pathogens, but also tissue damage, hormones, and vitamins, affect DC activation and maturation. In particular, glucocorticoids (GCs) are known for their immunosuppressive effect on DCs, creating tolerogenic DCs. GCs activate the type I nuclear receptor (NR) glucocorticoid receptor (GR), followed by induced expression of the transcription factor glucocorticoid-inducible leucine zipper (GILZ). GILZ has been shown to be necessary and sufficient for GC-induced tolerogenic DC generation. Recently, we have identified the DC-specific transcript (DC-SCRIPT) as an NR coregulator, suppressing type I steroid NRs estrogen receptor and progesterone receptor. In this study, we analyzed the effect of DC-SCRIPT on GR activity.

We demonstrate that DC-SCRIPT coexists with GR in protein complexes and functions as a corepressor of GR-mediated transcription. Coexpression of DC-SCRIPT and GR is shown in human monocyte–derived DCs, and DC-SCRIPT knockdown enhances GR-dependent upregulation of GILZ mRNA expression in DCs. This demonstrates that DC-SCRIPT serves an important role in regulating GR function in DCs, corepressing GR-dependent upregulation of the tolerance-inducing transcription factor GILZ. These data imply that by controlling GR function and GILZ expression DC-SCRIPT is potentially involved in the balance between tolerance and immunity. 

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they initiate target gene transcription. In contrast, GR-D isoforms are, independent of ligand binding, confined to the nucleus and have markedly lower transcriptional activity (16).

Recently, we have identified DC-specific transcript (DC-SCRIPT) as a transcription factor and an NR coregulator in human breast and prostate carcinoma tissue. DC-SCRIPT was found to be present in type I and type II NR protein complexes, repressing type I NRs estrogen receptor–, progesterone receptor–, or androgen receptor–mediated transcription while enhancing type II NRs retinoic acid receptor α/retinoid X receptor (RXR), vitamin D receptor/RXR, or peroxisome proliferator activated receptor γ/RXR function (19–22).

In the immune system, DC-SCRIPT is specifically expressed in DCs and, in contrast to other DC markers, identifies all DC subsets tested to date (23, 24). Interestingly, we have demonstrated an important role for DC-SCRIPT in TLR4- and TLR7/8-mediated DC maturation. DC-SCRIPT knockdown in moDCs increased secretion of the anti-inflammatory cytokine IL-12, which subsequently impaired both the production of the proinflammatory cytokine IL-12 as well as T cell proliferation (25). In this study, we investigated the putative role of DC-SCRIPT in controlling the anti-inflammatory function of GR in moDCs. Our data show the presence of DC-SCRIPT in GR protein complexes and demonstrate altered GR-mediated transcription in the presence and absence of DC-SCRIPT. Our findings therefore suggest active regulation of GR function by DC-SCRIPT in DCs.

Materials and Methods

Cell lines

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing GlutaMAX (Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated FCS (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), 1% nonessential amino acids (Invitrogen), and 0.5% antibiotic–antimycotic (Invitrogen). Human hepatocellular carcinoma Hep3B cells were cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated FCS and 0.5% antibiotic–antimycotic (Invitrogen).

Generation of human DCs

Human moDCs were generated from PBMCs as described previously (26). Monocytes were derived from buffy coats from healthy donors (Sanquin, Nijmegen, The Netherlands). Buffy coats were obtained from healthy volunteers after informed consent and according to institutional guidelines. Plastic-adherent monocytes were cultured for 6 d in DCs culture medium (phenol red-free RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 1% ultra-glutamine, 0.5% nonessential amino acids (Invitrogen), 10% (v/v) FCS (Greiner Bio-one), IL-4 (300 U/ml), and GM-CSF (450 U/ml) (both from cellgenix). During day 3 moDCs were supplemented with new IL-4 (300 U/ml) and GM-CSF (450 U/ml). Mature moDCs were generated from day 6 immature moDCs through 24-h stimulation with vehicle (0.1% EtOH), followed by 24-h stimulation with 200 ng/ml LPS (InvivoGen, Toulouse, France). Tolerogenic moDCs were generated from day 6 immature moDCs through 24-h stimulation with 100 nM dexmethasone (dex) and subsequent 24 h with 200 ng/ml LPS. DC maturation was ensured by FACS staining.

Confocal laser scanning microscopy

Round ø 12-mm cover slides (Thermo Scientific, Braunschweig, Germany) were coated with poly-l-lysine (Sigma-Aldrich, Zwijndrecht, The Netherlands). Immature, mature and tolerogenic moDCs were seeded on coverslides (50,000 cell/slide) and adhered for 2 h in serum-free, phenol red-free RPMI 1640 medium supplemented with 1% normal donkey serum (Sigma–Aldrich) in PBS, stained 1 h with 2.5 μg/ml (moDCs) goat–anti-human DC-SCRIPT (R&D Systems, Abingdon, U.K.) and 2.5 μg/ml (moDCs) mouse–anti-human GR (Abcam, Cambridge, U.K.) and 1 h with 1/400 Alexa Fluor 488 donkey–anti-goat IgG and 1/400 Alexa Fluor 647 rabbit–anti-mouse IgG (Invitrogen). The nucleus was stained 5 min with 0.3 μg/ml DAPI (Sigma-Aldrich), washed with PBS, and mounted on 76 × 26-mm microscope slide (Thermo Scientific) with mowiol + 2.5% azide (Calbiochem, San Diego, CA). Confocal laser scanning microscopy (CLSM) was carried out with an Olympus FV1000 Confocal Laser Scanning Microscope with an argon (457, 488, 515 nm) and 405, 559, and 635 diode lasers at the Microscopic Imaging Facility of the Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands).

Plasmids

The NR isoforms GR, GR-C3, and GR-D3 (National Center for Biotechnology Information accession number NM_001018077) were isolated and cloned into plasmids p3α-3gluc (described previously in Ref. 20 generating p3α-3gluc-GR, p3α-3gluc-GR-C3, and p3α-3gluc-GR-D3). The vectors pCATCH and pCATCH-DCSCRIPT were described previously (24). pCATCH-DCSCRIPT was used to generate pCATCH-DCSCRIPT-tGFP/pdm and pCATCH-DCSCRIPT-tGLu. The transcription reporter plasmid pMMTV-luc containing the mouse mammary tumor virus (MMTV) promoter was provided by Dr. H. Stunnenberg (Department of Molecular Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University). pMMTV-luc was used to generate pMMTV-luc and pMMTV-NF-I/Oct1-luc and pMMTV-GR-luc. pGRE3-luc, pGRE2-luc, pHRE3-luc, and pHRE4-luc.

Coimmunoprecipitation assay

HEK293 cells were seeded in 10-cm culture dish (6 × 106 cells/dish) 24 h prior to transfection. Cells were cotransfected with 5 μg pCATCH-DCSCRIPT or pCATCH (control) and 5 μg p3α-3gluc-GRα or pα-3gluc (control) by using Metafectene transfection reagent (Biontex, Martinsried/Planegg, Germany), according to the manufacturer’s protocol. Cells were lysed 24 h after transfection in immunoprecipitation (IP) assay buffer (50 mM NaCl, 1% Triton X-100, 0.1% NaDodSO4, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.5% sodium deoxycholate) containing the protease inhibitors 2 μg/ml leupeptin (Sigma–Aldrich), 2 μg/ml aprotinin (Roche), and 1 mM PMSF (Sigma–Aldrich). Cell lysates were used for IP of DC-SCRIPT using anti–DC-SCRIPT–coupled dynabeads (GE Healthcare, Hoekvelden, The Netherlands), according to the manufacturer’s protocol. Isotype–coupled Dyna-Beads were used as a control.

Western blotting

Protein samples were subjected to electrophoresis on an 8% 37.5:1 acrylamide gel and transferred onto Protran nitrocellulose transfer membrane (Schleicher and Schuell, Dassel, Germany) and blocked in 1% Elb/3% BSA in PBS with 0.01% Tween. DC-SCRIPT was detected with anti–DC-SCRIPT (1.2 μg/ml; R&D Systems) and IRDye 680CW donkey–anti-goat IgG (1:5000; Li-cor Biosciences) as secondary Ab. GR was detected with a rat anti-HA (1:1000, 3F10; Roche) and IRDye 800CW goat–anti-rat IgG (1:5000; Li-cor Biosciences) or anti-GR Ab (3D5) (200 μg/ml, 1:30, Abcam) and IRDye 680CW goat–anti-mouse IgG (1:5000 Li-cor Biosciences) as secondary Ab. Blots were probed with a mouse–anti-histone H4 (Nanogen, Germany), β-actin (1:20,000; Roche Applied Science) or rabbit–anti-actin (Sigma–Aldrich) and IRDye 680CW donkey–anti-mouse IgG or goat–anti-rabbit (1:5000; Li-cor Biosciences) as secondary Ab, loading control. Both actin and DC-SCRIPT or actin and GR were visualized on the same blot using two different secondary detection Abs conjugated with fluorescent molecules detected at 800 and 680 nm. After staining, the membranes were scanned using the Odyssey Infrared Imaging system to visualize the labeled proteins.

Luciferase transcription assay

Hep3b cells were plated (6 × 103) in 24-wells plates 8 h before transfection and transfected using the Calcium Phosphate precipitation method (Invitrogen). HEK293 cells were plated at (1 × 105) in 24-well plates 24 h before transfection and transfected using metatexcene. Transfected cells were stimulated with 100 nM dex, 100 nM prednisolone (pred), or vehicle (0.1% EtOH) for 24 h. Cells were harvested 24 h after stimulation, and cell lysates were analyzed for luminescence according to manufacturer’s protocol (Dual Luciferase Reporter assay; Promega) using a Victor3 luminometer (PerkinElmer). Relative light units were calculated after correction for transfection efficiency based on the activity of the cotransfected pRL-SV40 (Promega). The data are expressed as the mean activity of at least four independent experiments ± SE.

DC-SCRIPT knockdown

Human moDCs day 4 were electroporated with a 23-nt Custom ZNF366 by guest on April 15, 2017 http://www.jimmunol.org/ Downloaded from https://www.jimmunol.org/ DOI: 10.4049/jimmunol.200108077
DC-SCRIPT REGULATES GR FUNCTION

Luciferase expression (MMTV-luc). In the MMTV promoter, four hormone-responsive elements (HRE) have been identified that bind ligand-activated GR, thereby inducing transcription of the luciferase gene (29). Eighteen hours after transfection, the cells were treated with vehicle or the GR ligands dex or pred. Luciferase production was analyzed 24 h after stimulation.

Hep3B cells lack endogenous GR expression; therefore, only background luciferase activity was detected after GR–ligand stimulation in the absence of ectopically expressed GR. Cells that were cotransfected with the GR expression vector did show luciferase activity in a GR ligand–dependent manner. Both dex and pred were able to induce luciferase production (Fig. 2A). Interestingly, increasing expression levels of DC-SCRIPT resulted in a dose- and ligand-dependent repression of GR activity. Previously, DC-SCRIPT has been shown to directly interact with the global transcription corepressor C-terminal binding protein 1 (CtBP1) via its CtBP1 interaction motif (24). In addition, DC-SCRIPT harbors an LxxLL motif, a motif known to facilitate interactions with NRs (5). Next, we examined whether the CtBP1 and the NR binding (LxxLL) motifs within DC-SCRIPT were important for its repressive function on GR. We therefore mutated both CtBP1 domains or deleted the LxxLL motif. Both mutants were tested for protein expression levels (data not shown), which resembled the expression levels of wild-type DC-SCRIPT. Cellular localization was also expected to be similar, because deletion of the complete acidic domain does not alter its nuclear location (24). Fig. 2B shows that both mutants are still able to strongly repress GR-dependent luciferase production. Thus, DC-SCRIPT functions as a corepressor of GR-induced transcription on the MMTV promoter, independent of its CtBP1 binding and LxxLL motif.

Efficient transcription initiation via the MMTV promoter requires the presence of the most distal HRE site (HRE1), the regulatory element AA upstream of HRE1 (30) and binding of the transcription factors NF-I and octamer-binding transcription factor-1 (Oct1) (29, 31, 32). To assess the requirement of these regulatory elements for DC-SCRIPT–mediated corepression, we deleted these sequence elements from the MMTV-luc reporter. In the MMTV.A1-luc and MMTV.A2-luc reporters, the AA element alone or both the HRE1 and the AA element are deleted, respectively, whereas the MMTV.AF-I/Oct1-luc reporter lacks the NF-I and Oct1 binding sites (Fig. 2C) (33). The wild-type MMTV-luc reporter was used as positive control. Deletion of the AA element or the NF-I/Oct1 binding sites reduced GR-dependent luciferase expression compared with the wild-type reporter, which was even
further decreased upon deletion of both AA and HRE1 (Fig. 2D). On all MMTV reporters/mutants, DC-SCRIPT expression effectively repressed luciferase production. These data imply that the repressive function of DC-SCRIPT does not depend on the HRE1 and the AA element nor does the NF-I and Oct1 binding sites.

DC-SCRIPT represses transcriptional activity of GR isoforms

GR has been shown to be translated into eight different isoforms using alternative translational start sites (Fig. 3A) (17). The GR expression vector includes all translational start sites, thus, could express all GR isoforms. Indeed transfection of GR in HEK293 cells demonstrates expression of the full-length GR-A, as well as the GR-B, -C, and, albeit at markedly lower levels, GR-D isoforms (Fig. 3A). To examine DC-SCRIPT function on different GR isoforms, we also cloned GR-C3 and GR-D3. Of note, the GR-C3 vector includes downstream ATGs, hence, in addition to GR-C3, this vector could theoretically also express GR-D1, -D2, and -D3. However, only GR-C3 and GR-D1 could be detected (data not shown).

The effect of DC-SCRIPT on transcription initiation of the reporter construct MMTV-luc by these isoforms was tested using luciferase transcription assays. GR isoforms expressed by both GR and GR-C3 expression vectors equally induced luciferase production in a ligand-dependent manner. Cells expressing the GR-D3 isoform, however, were less potent in inducing luciferase expression, displaying a 4-fold reduction in luciferase production.

Increasing expression levels of DC-SCRIPT resulted in a dose- and ligand-dependent repression of the transcriptional activity of all GR isoforms (Fig. 3B). This indicates that the 1–336 aa N-terminal part of GR that was deleted in the GR-D3 expression vector is not required for the effect of DC-SCRIPT on GR-dependent transcription.

DC-SCRIPT and GR expression in immature, mature, and tolerogenic DCs

Next, we investigated DC-SCRIPT and GR protein expression by western blotting and CLSM in immature (iDCs), mature (mDCs) and tolerogenic DCs (tolDCs). iDCs and mDCs were obtained by...
stimulating immature moDCs with vehicle or 24 h of LPS, respectively. tolDCs were generated from immature moDCs through 24-h stimulation with dexamethasone and subsequent 24 h with LPS. Western blot analysis of DC-SCRIPT and GR protein levels showed equal expression of both proteins in iDCs, mDCs and tolDCs (Fig. 4A). The translational isoforms GR-A, -B, -C, and -D isoforms visualized with rat-anti-HA in GR-HA–transfected HEK293 cells. (B) Luciferase reporter assay for GR-A-, GR-C-, and GR-D–mediated transcription. Hep3B cells were cotransfected with the firefly luciferase reporter plasmid MMTV-luc, expression plasmids for GR-A, GR-C, or GR-D and increasing amounts of the expression plasmid for DC-SCRIPT. Cells were stimulated with vehicle (○) or 100 nM dex (■) for 24 h. Luciferase activity is displayed relative to luciferase production upon dex stimulation in the presence of GR-A and in the absence of DC-SCRIPT. Data from at least three independent experiments. Error bars correspond to ± SEM.

 Knockdown of DC-SCRIPT enhances GILZ expression in moDCs

To determine the physiological relevance of the interaction between DC-SCRIPT and GR, DC-SCRIPT expression was silenced in moDCs using siRNA electroporation. Because DC-SCRIPT was demonstrated to repress GR-mediated transcription on the MMTV promoter, we hypothesized a similar effect on the endogenous GR target GILZ in moDCs. DC-SCRIPT knockdown was therefore expected to enhance GR-dependent transcription, which could be detected by an increase in GILZ expression with vehicle or the GR agonist prednisolone for 24 h. After stimulation, GILZ mRNA levels were measured. As expected, little or no GILZ mRNA expression was detected in vehicle-treated control DCs. Stimulation with pred resulted in an upregulation of GILZ.
expression levels in control DCs (Fig. 5C), which could be effectively blocked by inhibiting GR activation with the GR antagonist RU-486. Strikingly, vehicle-treated siSC DCs already demonstrated a significant (p = 0.05) increase in GILZ expression compared with control DCs. This upregulation appears to be GR independent, because it is also detected in the presence of the GR antagonist RU-486. In the absence of the GR antagonist, treatment with pred further increased GILZ expression in DC-SCRIPT knockdown DCs, with relative GILZ mRNA levels being significantly higher than in control siRNA-treated DCs (p = 0.01). These data thus indicate DC-SCRIPT to function as corepressor of GR-mediated transcription of GILZ in DCs. Blocking GR activation with the GR antagonist abolished GILZ upregulation in DC-SCRIPT knockdown DCs, confirming that this was indeed dependent on GR activation. These results indicate that DC-SCRIPT affects GR activity in DCs and represses expression of the endogenous GR target gene GILZ, a transcription factor that has been associated with the generation of tolDCs.

**Discussion**

The present study demonstrates that the NR coregulator DC-SCRIPT modulates GR function in human moDCs. In cell lines, DC-SCRIPT was present in GR-containing protein complexes, and GR-mediated transcription was found to be repressed by DC-SCRIPT. Human moDCs coexpress DC-SCRIPT and GR in the nucleus and cytoplasm, and DC-SCRIPT silencing resulted in enhanced expression of the GR target gene GILZ, a transcription factor that is instrumental for the generation of tolDCs.

Copresence of DC-SCRIPT and GR in protein complexes could be demonstrated in transfected HEK293 cells; however, we could not co-IP the endogenous complex from DCs. This could have several reasons. Human monocyte derived DCs express at least 20 different NRs of which at least 6 (GR, progesterone receptor, estrogen receptor, retinoic acid receptor α, vitamin D receptor, and peroxisome proliferator activated receptor γ) possibly contain DC-SCRIPT in their repressor or activator complexes (19–22). Therefore, enrichment of a specific NR via DC-SCRIPT IP may be extremely difficult. Furthermore, endogenous DC-SCRIPT not present in protein complexes may possibly bind more efficiently to the beads, and is therefore more likely to be purified. In addition, DC-SCRIPT and GR likely interact indirectly. Hence, lysis buffers need to be mild to keep the complex intact but at the same time sufficiently stringent to properly lyse the nucleus. Nonetheless, our co-IP findings in HEK293 cells are further supported by functional data that demonstrate the effect of DC-SCRIPT expression on GR–ligand-mediated GR target gene expression, not only in HEK293 cells, but also in dendritic cells.

The present data describing the interaction between DC-SCRIPT and GR in cell lines is in line with our previous findings, suggesting that DC-SCRIPT interacts with NRs via other proteins present in NR protein complexes. DC-SCRIPT has been shown to bind the transcription corepressors receptor-interacting protein 140 and CtBP1, as well as histone deacetylase-1, -3, and -6, all known to be present in NR protein complexes (5, 33, 34). The absence of a direct contact between DC-SCRIPT and GR is also consistent with the finding that deletion of the NR interaction motif LxxLL and the CtBP1 interaction motif in the acidic domain of DC-SCRIPT did not affect its repressive function on GR transcription. This implies that interaction with CtBP1 and the presence of the LxxLL motif is not required for the repressive effect of DC-SCRIPT. Interaction with NR complexes could, however, be facilitated via other, less defined NR interaction motifs (35–39). In addition to the LxxLL motif, DC-SCRIPT contains multiple putative NR interaction motifs that are possibly involved in the interaction with various NRs (data not shown).

Coexpression of DC-SCRIPT and GR in DCs was demonstrated with CLSM, albeit areas containing either DC-SCRIPT or GR were equally abundant. Ligand-dependent GR translocation was observed in iDCs. However, in the absence of its ligand, GR was also detected in the nucleus of iDCs. The nuclear GR expression most likely reflects the expression of GRβ isoforms, known to have restricted nuclear localization (18). DC-SCRIPT expression was
Because we have recently identified DC-SCRIPT as a NR coregulator, GR activation was only detected for FKBP5 (51 kDa FK506-binding protein 5) and PTX3 (pentraxin 3); however, induction levels were low and DC-SCRIPT silencing did not affect expression of these genes (data not shown). One major reason could be the CRE sequence or the context of this sequence in the promoter of these genes. Meijising et al. (43) recently demonstrated that a single nucleotide change in the GRE sequence influences GR binding affinity and conformation, affecting cofactor recruitment and transcription activation. Similarly, the binding properties of DC-SCRIPT might differ depending on GR conformation and affect the binding of other cofactors. Furthermore, adjacent binding sites of other transcription factors can also affect GR-mediated transcription and possibly DC-SCRIPT function (19). In line with this, we have preliminary data that suggest that DC-SCRIPT can also activate GR-dependent transcription, depending on the promoter context (data not shown). As soon as a suitable DC-SCRIPT Abs for chromatin IP become available, it would be extremely interesting to perform chromatin IP–sequencing studies and expression arrays to further investigate gene regulation specifically in DCs with respect to DC-SCRIPT and GR activation and expression.

Finally, besides GR, also other NRs are known to function in DC biology, affecting either differentiation, maturation, or both. The human NR superfamily contains 48 members, of which 20 NRs have been described to be expressed in monocyte-derived DCs (44). Because we have recently indentified DC-SCRIPT as a NR coregulator in breast (20) and prostate carcinoma (22), affecting both type I and type II NR function, it is tempting to speculate about a role for DC-SCRIPT as NR coregulator in DC biology. It would therefore be interesting to study target gene expression of various NRs after DC-SCRIPT knockdown in DCs.

Disclosures

The authors have no financial interests of conflict.

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