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DC-SCRIPT Regulates IL-10 Production in Human Dendritic Cells by Modulating NF-κBp65 Activation

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Dendritic cells (DCs) are specialized immune cells uniquely capable of directing a naive T cell response toward immune activation or tolerance (1). Extracellular signals steer DC maturation either toward immunity against pathogens or tolerance to, for example, self-Ags, harmless food Ags, and commensal bacteria (1). The presence of tolerogenic DCs is crucial to maintain immune homeostasis, and they are characterized by expression of coinhibitory molecules and a high production of IL-10 (2, 3). In addition, IL-10 is also necessary to help resolve immune responses and avoid excessive immune activation leading to tissue pathology (4). Consequently, the regulation of IL-10 expression is under tight control in cells in general and in DCs in particular. Moreover, because the ability of DCs to direct the immune system is exploited in clinical vaccination settings to treat either autoimmune (5) or cancer (6), it is of crucial importance to thoroughly understand DC activation and tolerization pathways.

Several pathways are known to regulate DC activation, including the TLR and nuclear receptor (NR) pathways. TLRs recognize structurally conserved molecules derived from diverse pathogens such as LPS and viral RNA. TLR signaling results in the maturation of the DCs, displayed by a production of proinflammatory cytokines, upregulation of costimulatory molecules, and upregulation of Ag-presenting MHC molecules (7, 8). These maturation events create DCs that are able to present Ags to and activate naive T cells. NRs are ligand-inducible transcription factors that respond to ligands such as hormones (e.g., estrogen), vitamins (e.g., vitamin D3), and metabolites (e.g., fatty acids) (9). NRs can directly bind to DNA and thereby mediate gene transcription, which involves the recruitment of several corepressor or coactivator molecules to the promoters of their target genes (9). NRs such as the glucocorticoid receptor (GR) and the vitamin D3 receptor have been found to repress proinflammatory programs induced by TLRs (10, 11).

Common for NR and TLR signaling pathways is their ability to regulate transcription by NF-κB (12, 13). TLR signaling is known to activate the NF-κB complex (13), whereas GR has been found to inhibit NF-κB–mediated transcription (10, 14–16). NF-κB resides in the cytoplasm in an inactive form by binding to the NF-κB inhibitory protein IκBα (13). Upon IκBα phosphorylation, the NF-κB–IκBα complex disintegrates, allowing NF-κB to translocate to the nucleus and activate transcription of target genes. Five different subunits of NF-κB are known, which can form either homodimers or heterodimers (17). Three of the subunits (p65, RelB, and cRel) contain a transactivation domain, making them capable of initiating transcription. The other subunits (p50 and p52) only contain a Rel-homology domain, making them capable to bind other NF-κB family members. The most frequently studied complex in the TLR-signaling pathway is the p65-p50 heterodimer, but the p52-RelB heterodimer, and the p50-p50 homodimer also have been found to play a role in DCs (13). In addition to regulation of nuclear translocation, NF-κB activation also depends on posttranslational modifications (PTMs) such as phosphorylation, acetylation, ubiquitination, sumoylation, and nitrosylation (17). For instance, phosphorylation of Ser536 or Ser276 on p65 leads to activation of NF-κB–mediated transcription (18, 19), whereas phosphorylation of Ser547 or T505 on p65 renders NF-κB inactive, preventing transcription of its target genes (20, 21). Likewise, acetylation of K122 and K123 inhibits NF-κBp65 (22), whereas acetylation of K310 activates NF-κBp65–mediated transcription.
In the past, studies on NF-κB activation focused on the induction of proinflammatory cytokines (13). Recently, however, it was shown that the NF-κBp65 subunit can also bind upstream of the IL10 gene, suggesting it can also regulate the production of the anti-inflammatory cytokine IL-10 (24). The induction of IL-10 by NF-κBp65 was recently shown to be dependent on K310 acetylation of NF-κBp65 (25).

Recently, we have identified and characterized the transcription regulator DC-specific transcript (DC-SCRIPT) that is expressed by all DC subsets and is involved in regulating the activation status of DCs (26–28). In the absence of DC-SCRIPT, DCs secrete enhanced amounts of IL-10 in response to TLR stimulation, and less IL-12p70, whereas TNF-α and IL-6 remain unchanged (26). In correspondence with the increased IL-10 and decreased IL-12p70 production, DC-SCRIPT knockdown also led to dampened T cell proliferation and IFN-γ production (26). Interestingly, DC-SCRIPT has also been shown to regulate NR-mediated gene transcription both in DCs and in specialized epithelial cells (29–34). For DCs, we have shown that DC-SCRIPT is involved in inhibiting GR-mediated transcription, as well as expression of its target gene glucocorticoid-induced leucine zipper (GILZ). Together with IL-10, GR and GILZ are all factors known to be involved in tolerogenic DC function (35), suggesting that these pathways may be interconnected.

In this study, we have further investigated the molecular mechanism underlying IL-10 production in DC-SCRIPT knockdown DCs, with a focus on GR, GILZ, and NF-κB. Strikingly, we found that DC-SCRIPT does not control IL-10 production via the GR and GILZ pathway, but rather affects NF-κB activation.

### Materials and Methods

#### Generation of human monocyte-derived DCs

Human monocyte-derived DCs (moDCs) were generated from PBMCs as described previously (36). Buffy coats were obtained from healthy volunteers (Sanquin, Nijmegen, the Netherlands) after informed consent and according to institutional guidelines. Plastic-adhered monocytes were cultured for a total of 6 d in RPMI 1640 medium (Life Technologies) supplemented with 1% ultraglutamine (Cambrex), 0.5% antibiotic-antimycotic (Invitrogen), 10% (v/v) FCS (Greiner Bio-one), IL-4 (300 U/ml), and GM-CSF (450 U/ml) (both from Cellgenix). On day 3, moDCs were supplemented with new IL-4 (300 U/ml) and GM-CSF (450 U/ml).

#### Small interfering RNA-mediated knockdown

On days 3–4 of DC differentiation, cells were harvested and subjected to electroporation. For DC-SCRIPT silencing, a 23-nt custom ZNF366 small interfering RNA (siRNA) termed SC38 targeting the DC-SCRIPT gene at position 2349–2369 was used (Thermo Scientific). For GILZ silencing, the SMARTpool ON-TARGETplus TSC22D3 siRNA (Thermo Scientific) was used. siRNA ON-TARGETplus Non-Targeting siRNA#1 (Thermo Scientific) was used as control. Cells were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). A total of 10 μg siRNA (5 + 5 μg for double knockdown) was transferred to a 4-mm cuvette (Bio-Rad), and 10⁶ DCs were added in 200 μl OptiMEM and incubated for 3 min before being pulsed with an exponential decay pulse at 300 V, 150 mF, in a GenePulser Xcell (Bio-Rad), as previously described (37). Immediately after electroporation, the cells were transferred to preheated (37˚C) phenol red–free RPMI 1640 culture medium supplemented with 1% ultraglutamic acid, 10% (v/v) FCS, IL-4 (300 U/ml), and GM-CSF(450 U/ml).

#### Stimulations

Immature DCs were stimulated with 4 μg/ml R848 (Axxora), 100 ng/ml LPS from E. coli 0111:B4 (Sigma-Aldrich), or 100 nM prednisolone.
DC-SIGN Ab (clone DCN46; BD Biosciences), before addition of LPS.

calculated according to the cycle threshold method (38). The CFX Manager software (Bio-Rad) and checked for correct amplification by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen).

mRNA levels for the genes of interest were determined with a CFX96 sequence detection system (Bio-Rad) with SYBR Green (Roche) as the fluorophore and gene-specific oligonucleotide primers. Primers were used as follows (forward, reverse): DC-SCRIPT (ZenBio); (5'-AGCCTGAGTCATGGAG-3', 5'-TTCTGAGAGAGGTCAAAGG-3'), PBGD (5'-GCCAATTGCGGCTGCAA-3', 5'-GGGTACCCACGCGAATCAC-3'), GILZ (5'-AGAACCCTCAATACGGCAACAG-3', 5'-CATCAGATGATTCTTCACC-3'). Reaction mixtures and program conditions were used as recommended by the manufacturer (Bio-Rad). Quantitative PCR (qPCR) data were analyzed with the cycle threshold method (38).

**RNA isolation and quantitative PCR**

Total RNA was isolated from cells using an RNA isolation kit (Zymo Research). RNA quantity and purity were determined on a NanoDrop spectrophotometer. RNA was treated with DNase I (amplification grade; Invitrogen) and reverse transcribed into cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a CFX96 sequence detection system (Bio-Rad) with SYBR Green (Roche) as the fluorophore and gene-specific oligonucleotide primers. Primers used are as follows (forward, reverse): DC-SCRIPT (ZenBio); (5'-AAGGATGGGATGTCATGGAG-3', 5'-TTCTGAGAGAGGTCAAAGG-3'), PBGD (5'-GCCAATTGCGGCTGCAA-3', 5'-GGGTACCCACGCGAATCAC-3'), GILZ (5'-AGAACCCTCAATACGGCAACAG-3', 5'-CATCAGATGATTCTTCACC-3'). Reaction mixtures and program conditions were used as recommended by the manufacturer (Bio-Rad). Quantitative PCR (qPCR) data were analyzed with the cycle threshold method (38).

**ELISA**

Secreted cytokines were assayed in the supernatants of 24-h–stimulated moDCs. IL-10 was measured using the human IL-10 ready-set-go kit (Sanquin), and TNF-α was measured using the human TNF-α ELISA kit (eBioscience), IL-6 was measured using the PeliPair human IL-6 ELISA kit (Sanquin), and TNF-α was measured using the human TNF-α ELISA set (BD Biosciences). All cytokines were measured according to the manufacturers’ instructions.

**NF-κB DNA binding assay**

Immature moDCs were stimulated for 1–8 h with R848, followed by two quick washes in 4°C cold PBS. Next, nuclear extracts were prepared with the NucBuster protein extraction kit (Novagen) according to the manufacturer’s protocol. Protein amount in isolated nuclei was measured using the Coomassie Plus Protein Assay (Thermo Scientific), and 7 µg protein was used as input for the TransAM NF-κB family kit (Active Motif). Binding of the NF-κB subunits to an immobilized consensus sequence for NF-κB was detected by specific mAbs for NF-κB p50, p52, p65, RelB, and cRel and secondary HRP-conjugated Abs using a colorimetric readout. The kit was used according to the manufacturer’s protocol.

**Phosphorylation of NF-κBp65**

Immature moDCs were plated at a cell concentration of 5 × 10^5/ml and allowed to rest for a minimum of 1 h before stimulation. After stimulation, cells were lysed in a concentration of 10^6/100 µl 4°C cold lysis buffer consisting of 62.5 mM Tris (pH 6.8, Sigma-Aldrich), 1% SDS (Invitrogen), and freshly added complete protease inhibitor mixture (Roche), 1 mM PMSE (Sigma-Aldrich), as well as the phosphatase inhibitors 1 mM Na3VO4 (Sigma-Aldrich) and 10 mM NaF (Merck). Cell lysates were mixed 1:4 with sample buffer containing 5% glycerol (Invitrogen), 6% SDS, 125 mM Tris-HCl (pH 6.8), 0.1 mg/ml bromophenol blue (Gebr. Schmid), and 10% 2-ME (Sigma-Aldrich). Heated at 95°C for 5 min; and then cooled on ice. The proteins were resolved by electrophoresis on a 10% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) and transferred to Protran nitrocellulose transfer membranes (Amersham) at 4°C. Membranes were blocked and stained in 1% skimmed milk powder (Campina) and 3% BSA (Roche) in PBS with 0.1% Tween 20 for assays using the NF-κBp65 (1:100 dilution of clone sc-8008; Santa Cruz Biotechnology), or 2% skimmed milk powder and 2% BSA in TBST for assays using the phospho-specific NF-κBp65 (1:1000 dilution of clone 93H1; Cell Signaling Technology). Membranes were blocked for 1 h at room temperature, stained overnight with primary Abs (including mouse [AC-40] or rabbit [20–33] anti-actin, 1:10,000 dilution; Sigma-Aldrich), and stained for 1 h at room temperature with corresponding secondary Abs (diluted 1:5000: goat anti-mouse IgG IRDye 800CW, goat anti-rabbit IgG IRDye 800CW, goat anti-mouse IRDye 680RD (all three from LI-COR Biosciences), or goat anti-rabbit IgG Alexa Fluor 640 (Invitrogen). Membranes were scanned by using an Odyssey Infrared Imaging System (LI-COR Biosciences).
Chromatin immunoprecipitation-qPCR

Chromatin immunoprecipitation (ChIP) was performed as previously described (39), with some modifications in the buffer compositions. Five million human moDCs were stimulated for 1 h with R848, followed by cross-linking with 1% formaldehyde for 15 min at room temperature. The reaction was quenched with 0.125 M glycine and washed at 4 °C with three buffers: 1) PBS; 2) buffer consisting of 0.2% Triton X-100, 0.2% NP-40, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 10 mM Tris-HCl pH 7.5, 10 mM NaCl; and 3) buffer consisting of 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES pH 7.6. Cells were then resuspended in ChIP incubation buffer (0.15% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES, 1/10 protease inhibitor mixture [Roche], 0.1% BSA) and sheared using a Bioruptor Next Generation sonicator (Diagenode). Sonicated chromatin was centrifuged for 5 min at 4 °C and supernatant was snap-frozen. Subsequently, the chromatin was incubated overnight with 1 μg rabbit anti-human NF-κBp65 Ab (clone D14E12; Cell Signaling Technology) or isotype control (rabbit IgG; Jackson Immunoresearch) and protein A/G beads (Santa Cruz). Beads were washed, eluted, and de-cross-linked for 5 h as previously described (39). De-cross-linked DNA was purified using the QIAquick PCR purification kit (Qiagen) and quantified using Qubit (Invitrogen). qPCR was performed on input and purified chromatin immunoprecipitated (ChIPed) chromatin using the primers for the IL10 enhancer: forward 5’-GAGGACAGTGTGCCATCCCG-3’, reverse 5’-AAACCGGATTAAGCGCCC-3’, as previously described (40).

Detection of K310 acetylation of NF-κBp65

Immature moDCs were plated at a cell concentration of 5 × 10^7/ml and allowed to rest for a minimum of 1 h before stimulation. After stimulation, paraformaldehyde (Merck) was added directly to the culture medium at a final concentration of 1.6%. Fixation was allowed to take place for 10 min at room temperature protected from light. Subsequently, culture medium was aspirated, and cells were resuspended in remaining medium (to avoid clumps when MeOH is added). For permeabilization, cells were vigorously resuspended before FACS staining. Cells were blocked and stained in PBS with 1% BSA, 0.05% NaN3 (Merck), and 2% human serum (Sanquin) (4 ×) to remove residual MeOH. Cells that remained adherent were scraped loose before FACS staining. Cells were blocked and stained in PBS with 1% BSA, 0.05% NaN3, and 2% human serum, using 1:100 diluted acetyl-K310 NF-κBp65 Ab (catalog no. 3045; Cell Signaling Technology) or isotype control (rabbit IgG; Jackson Immunoresearch). Cells were stained with primary Ab/isotype control for 45 min on ice, followed by 30 min on ice with a secondary PE-Cy5.5 goat anti-rabbit IgG Ab (catalog no. L42018; Invitrogen). Data were acquired on a FACSCyan (Beckman Coulter). Isotype controls gave a staining intensity similar to unstimulated moDCs, indicating that blocking conditions were sufficient to avoid unspecific staining (data not shown). Data were analyzed using FlowJo (Tree Star).

Statistics

Statistical analysis was done using Excel (Microsoft) or Prism 5 (GraphPad Software). The specific method has been described in each figure legend.

Results

IL-10 production mediated by DC-SCRIPT knockdown is GILZ and GR independent

Previously we have shown that knockdown of the transcription regulator DC-SCRIPT leads to GILZ upregulation and IL-10 production in human moDCs, most effectively after TLR stimulation (26, 33). Because GILZ has been shown to mediate IL-10 production in moDCs (25), we hypothesized that DC-SCRIPT may affect NF-κB signaling and thereby regulate IL-10 production. Interestingly, blocking NF-κB signaling with the NF-κB inhibitor BAY11-7082 partially inhibited IL-10 production in SCRIPT-KD-DCs (Fig. 2A).

Fig. 3. DC-SCRIPT knockdown increases phosphorylation of NF-κBp65. SCRIPT-KD-DCs or Ctrl-DCs were stimulated with R848 for 0–120 min. Protein lysates were assayed by Western blot for phosphorylated NF-κBp65. (A) Representative donor displaying that DC-SCRIPT knockdown induces a higher phosphorylation of NF-κBp65. (B and C) p-NF-κBp65 and total NF-κBp65 were quantified for a range of donors. Statistics: Ctrl-DCs were compared with SCRIPT-KD-DCs at each time point, using the Student two-tailed t test (n = 3–4). Error bars = SEM. *p < 0.05.
Because activation of the NF-κB pathway is dependent on translocation and subsequent DNA binding of its subunits, we investigated whether the absence of DC-SCRIPT expression affects DNA binding of the different NF-κB subunits in moDCs (Fig. 2B). To this end, nuclear lysates were isolated from SCRIPT-KD-DCs and Ctrl-DCs before and 1 h after R848 treatment. As expected, and in line with previous reports from LPS-stimulated DCs (25, 41), our data show that R848 stimulation of moDCs leads to a significant increase in DNA binding of p65, p50, and cRel ($p < 0.01$ for Ctrl-DCs, $p < 0.001$ for SCRIPT-KD-DCs), whereas no significant increase was observed for p52 and RelB. Interestingly, SCRIPT-KD-DCs showed a significantly enhanced DNA binding of p65 when compared with Ctrl-DCs upon stimulation with R848. Collectively, these data link DC-SCRIPT knockdown to NF-κB activation and enhanced nuclear DNA binding of the p65 subunit of NF-κB.

**DC-SCRIPT knockdown leads to more S536 phosphorylation of NF-κB**

NF-κBp65 has been demonstrated to regulate production of a range of different cytokines in DCs, dependent on the specific PTMs it acquires upon stimulation (13, 25). One of the most important phosphorylation sites in NF-κBp65 activation is Ser$^{536}$ (17). Interestingly, the NF-κB inhibitor BAY11-7082 inhibits the IκB kinase (42), which phosphorylates NF-κBp65 exactly at this Ser$^{536}$ position (18, 43). Because BAY11-7082 decreased IL-10 production in SCRIPT-KD-DCs (Fig. 2A), we determined whether NF-κBp65 is differentially phosphorylated at Ser$^{536}$ in SCRIPT-KD-DCs. Intriguingly, already without stimulation (0 min) increased NF-κBp65 Ser$^{536}$ phosphorylation was discerned in SCRIPT-KD-DCs compared with Ctrl-DCs (Fig. 3A, 3B). Upon stimulation with R848, both Ctrl-DCs and SCRIPT-KD-DCs increased Ser$^{536}$ phosphorylation. Time-course analysis showed that levels of Ser$^{536}$ phosphorylation in SCRIPT-KD-DCs remained significantly higher than Ctrl-DCs after 30-min stimulation, which leveled out at later time points. Notably, total NF-κBp65 protein expression did not change and could therefore not account for the observed effect (Fig. 3A, 3C).

**DC-SCRIPT knockdown leads to more K310 acetylation of NF-κBp65**

In addition to phosphorylation, NF-κBp65 activity is also regulated by acetylation. Specifically, acetylation of K310 in NF-κBp65 has been linked to regulation of IL-10 production (25). Therefore, we investigated whether K310 is acetylated in SCRIPT-KD-DCs (Fig. 4). Fig. 4A and 4B shows that Ctrl-DCs do not acetylate NF-κBp65 at K310 upon R848 stimulation as expected. In contrast, SCRIPT-KD-DCs do acetylate K310 upon R848 stimulation for 30 min. Interestingly, even nonstimulated SCRIPT-KD-DCs exhibited a higher acetylation level relative to Ctrl-DCs, similar to that observed for NF-κBp65 Ser$^{536}$ phosphorylation. Moreover, the baseline acetylation status of NF-κBp65 K310 in unstimulated SCRIPT-KD-DCs shows equal intensity as moDCs stimulated with the previously reported positive control compounds (Fig. 4C, 4D).

**FIGURE 4.** DC-SCRIPT knockdown increases acetylation of NF-κBp65. Ctrl-DCs or SCRIPT-KD-DCs (SC) were stimulated for 0–60 min with R848, followed by intracellular acetyl NF-κBp65 (K310) FACS staining. (A and B) Overlays between unstimulated and 30-min R848-stimulated moDCs. (C) Positive control: LPS + anti–DC-SIGN Ab (DCN46). (D and E) moDCs were pretreated for 1 h with the HATi AA or vehicle control before R848 stimulation to assay the effect of HATs on the observed acetylation. The line going through (A)--(E) indicates the peak of unstimulated SCRIPT-KD-DCs. (F) Summary of the relative fluorescence intensity (FI) for siRNA-treated moDCs stimulated with R848. Values are normalized to the mean FI for unstimulated Ctrl-DCs. Statistics: ANOVA with a Bonferroni posttest, $n = 3–4$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017**
Supplemental Fig. 1A) (25). Further quantification of the data confirmed the occurrence of a significant increase in NF-κBp65 K310 acetylation peaking at 30 min after stimulation (Fig. 4F). Because histone acetyltransferases (HATs) have previously been reported to acetylate NF-κB (23), we next determined whether blocking HATs with the HAT inhibitor (HATi) AA (44–46) could diminish K310 acetylation in SCRIPT-KD-DCs. As shown in Fig. 4D, 4E, and Supplemental Fig. 1B, HATi pretreatment significantly decreased NF-κBp65 K310 acetylation in SCRIPT-KD-DCs. In contrast, the level of acetylation in Ctrl-DCs was so low or absent that the HATi treatment did not mediate any change. These data indicate that DC-SCRIPT knockdown affects the HAT-dependent acetylation of NF-κBp65 at K310.

**IL-10 production from SCRIPT-KD-DCs depends on p300/CBP**

To link the increase in acetylation in SCRIPT-KD-DCs to the increase in IL-10 production, we stimulated SCRIPT-KD-DCs and Ctrl-DCs in the presence or absence of HATi. In addition to the broad specificity HATi AA (44–46), we also included the p300/CBP-specific HATi’s curcumin (47, 48) and C646 (49) because p300/CBP has previously been implicated in NF-κB acetylation (23). As shown in Fig. 5, all three HATis significantly decreased IL-10 production in SCRIPT-KD-DCs to a similar extent. These data indicate that DC-SCRIPT knockdown affects the HATi-dependent acetylation of NF-κBp65 at K310.

**DC-SCRIPT knockdown increases NF-κBp65 binding to the IL10 enhancer**

Until recently, NF-κBp65 was mainly considered to induce inflammatory cytokine expression (13), but recently it was found that specific signaling through Raf-1 leads to both p300/CBP-mediated acetylation of NF-κBp65 and increased binding of NF-κBp65 to a \( IL10 \) cis-regulatory enhancer element (24, 25, 40). To determine whether DC-SCRIPT would also affect the binding of NF-κBp65 to this specific \( IL10 \) enhancer, we stimulated SCRIPT-KD-DCs and Ctrl-DCs for 1 h with R848 and ChIPed with a NF-κBp65 Ab or isotype control. Subsequent qPCR for the \( IL10 \) enhancer showed that SCRIPT-KD-DCs had a significantly higher binding of NF-κBp65 to the \( IL10 \) enhancer compared with Ctrl-DCs and isotype control (Fig. 6). These data suggest that DC-SCRIPT decreases NF-κBp65 binding to the \( IL10 \) enhancer, and thereby limits IL-10 production in human DCs.

**Discussion**

In this study, we sought to increase our understanding of the molecular pathways of the NR coregulator DC-SCRIPT uses to regulate TLR-mediated IL-10 production by DCs. We report that the IL-10 production induced in SCRIPT-KD-DCs was neither dependent on GILZ nor on GR. Instead, IL-10 production in the absence of DC-SCRIPT is at least in part dependent on NF-κB signal transduction and can be blocked by HATis. Moreover, the NF-κB subunit p65 exhibits increased PTMs and an enhanced \( IL10 \) enhancer-binding capacity in SCRIPT-KD-DCs.

The transcription regulator DC-SCRIPT not only an important NR coregulator important in DC and cancer biology (30–34, 50), it also affects TLR-mediated cytokine production by DCs (26). Our current findings now show that the pleiotropic protein DC-SCRIPT modulates NF-κBp65 activity in DCs. DC-SCRIPT has previously been shown to bind the transcription corepressors receptor-interacting protein 140 (RIP140) and C-terminal binding protein 1, as well as the histone deacetylase 1 (HDAC1), HDAC3, and HDAC6 (27, 29). We now show that the IL-10 production observed after DC-SCRIPT knockdown is dependent on NF-κB and p300/CBP. Interestingly, both HDAC3 and RIP140 have been demonstrated to interact with NF-κBp65 (51–53) and HDAC3 has been found to mediate deacetylation of NF-κBp65 (53). Our finding that DC-SCRIPT knockdown leads to more K310 acetylation and subsequent IL-10 production is in line with a previous report that shows that concomitant triggering of DC-SIGN and TLR4 leads to HAT p300/CBP-dependent K310 acetylation of NF-κBp65 with subsequent IL-10 induction (25). In addition to enhanced acetylation of K310, we also show that DC-SCRIPT knockdown leads to enhanced phosphorylation of Ser536 NF-κBp65. Interestingly, it has been shown that enhanced phosphorylation of this specific site is linked to enhanced interaction between NF-κBp65 and p300/CBP (54). Therefore, it is tempting to...
speculate that DC-SCRIPT modulates p300/CBP-dependent acetylation of NF-κBp65 K310 by reducing the level of Ser536 NF-κBp65 phosphorylation. Subsequent recruitment of the co-pressors such as HDAC3 and RIP140 would then result in decreased DNA binding of NF-κBp65, thereby controlling IL-10 production in DCs. However, further studies are required to define the exact molecular mechanism.

NF-κBp65 also regulates other cytokines besides from IL-10, such as IL-6 and TNF-α. However, previously we have demonstrated that neither IL-6 nor TNF-α is affected by DC-SCRIPT knockdown (26). It is therefore likely that the combination of the specific Ser56 acetylation and K310 acetylation on NF-κBp65 is specific for only a subset of NF-κBp65-regulated genes like the observed increase in IL-10 production. Interestingly, under basal conditions, SCRIPT-KD-DCs readily displayed enhanced phosphorylation of Ser56 and acetylation of K310 NF-κBp65 compared with Ctrl-DCs. This suggests that already in immature DCs, DC-SCRIPT controls activation of NF-κBp65 and subsequent IL-10 production. After TLR7/8 triggering, the effect of DC-SCRIPT on IL-10 production was even more pronounced, as can be expected in mature DCs where NF-κB activity is strongly increased (25). Possibly, DC-SCRIPT increases the threshold for IL-10 induction in immature professional Ag-presenting DCs to prevent a premature tolerogenic phenotype and allows for the full immune potentiation function of mature DCs, by limiting IL-10-mediated feedback control. In the absence of DC-SCRIPT, NF-κBp65 thus appears to be more preactivated. Intriguingly, NF-κBp65 has been found to be constitutively active in prostate carcinoma (55), a cancer found to correlate with low DC-SCRIPT expression (30). This may suggest that, both in the immune system and in cancer, DC-SCRIPT is involved in controlling signaling cascades that lead to enhanced activation of NF-κBp65.

Inhibiting the NF-κB pathway or blocking p300/CBP showed only a partial reduction in IL-10 secretion after TLR ligation of SCRIPT-KD-DCs. This suggests that DC-SCRIPT not only dampens the NF-κB pathway but may also regulate other pathways that control IL-10 production in TLR-stimulated DCs. Therefore, it will be very interesting in future experiments to perform genome-wide analyses in mDCs to further investigate the pathways that are affected by DC-SCRIPT.

In conclusion, our current data provide novel insight into how DCs regulate expression of the important immune-dampening cytokine IL-10, and indicate that DC-SCRIPT may play a key role in the immunogenic potential of DCs. Understanding the immunogenic potential of DCs is crucial for exploiting these cells in immunotherapy to the benefit of patients with cancer, autoimmunity, or infectious diseases.

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Disclosures
The authors have no financial conflicts of interest.

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