Dendritic Cell-Specific Transcript: Dendritic Cell Marker and Regulator of TLR-Induced Cytokine Production

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Dendritic cells (DCs) are the professional APCs of the immune system that dictate the type and course of an immune response. Molecular understanding of DC biology is important for the design of DC-based immunotherapies and optimal clinical applications in vaccination settings. Previously, we isolated and characterized the cDNA-encoding dendritic cell-specific transcript (DC-SCRIPT; also known as ZNF366). DC-SCRIPT mRNA expression in the immune system was confined to DCs and was reported to be an early hallmark of DC differentiation. In this study, we demonstrate IL-4 to be the dominant factor for DC-SCRIPT expression in human monocyte-derived DCs. In addition, to our knowledge, we show for the first time endogenous DC-SCRIPT protein expression in human DCs both in vitro and in situ. DC-SCRIPT protein is detected early upon differentiation of monocytes into DCs and is also present in multiple freshly isolated DC subsets. Maturation of DCs with TLR ligands further increased DC-SCRIPT mRNA expression, suggesting a role in DC maturation. Indeed, small interfering RNA-mediated knockdown of DC-SCRIPT affected the cytokine response upon TLR stimulation. These DCs displayed enhanced IL-10 and decreased IL-12 production, compared with wild-type DCs. Silencing of IL-10 in DC-SCRIPT knockdown DCs rescued IL-12 expression, suggesting a primary role for DC-SCRIPT in the regulation of IL-10 production. The Journal of Immunology, 2012, 189: 138–145.

Dendritic cell-specific transcript: dendritic cell marker and regulator of TLR-induced cytokine production

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Dendritic cells (DCs) are the professional APCs of the immune system and play an essential role in the initiation and modulation of immune responses. DCs reside in the tissue in an immature state, and are capable of recognizing and capturing microbial Ags through specific receptors. Upon infection or inflammation, they undergo a complex process of maturation, where they change from Ag-capturing cells into APCs (1). With the expression of costimulatory or co-inhibitory molecules and the secretion of pro- or anti-inflammatory cytokines, DCs generate either immunity or tolerance through T lymphocyte stimulation (2). The type of molecules that are expressed greatly depend on the activation status of the DC, and is affected by environmental stimuli (3–5).

A broad range of DC subsets has been described, including the in vitro monocyte-derived DCs (moDCs) and the in vivo blood-derived myeloid and plasmacytoid DCs (mDCs and pDCs, respectively). mDCs act as sentinels in the periphery and have a specialized function depending on their location and pattern recognition receptor expression profile. The cellular cues present at different locations, for example, gut, skin, or other organs, inflicted by local invading pathogens direct the mDCs toward a specific response. pDCs are considered the front line of defense in antiviral immunity, as they rapidly produce massive amounts of type I IFN in response to viral infection and prime T cells against viral Ags (6–8). In vitro, DCs can be generated from monocytes through stimulation with IL-4 and GM-CSF (9). These cytokines trigger DC differentiation while inhibiting macrophage and osteoclast differentiation (10–12).

The differentiation of the DC subsets from their precursors is a highly complex process. Genetic analyses have identified different transcription factors, including IRF4, RelB, and PU.1, to be crucial in the development of specific DC subsets in lymphoid organs (13–17). DC differentiation and maturation require a complete change in the DC gene expression profile, mediated by the combinatorial effect of a few key transcription factors and chromatin reorganization (18).

In 2006, we identified and characterized a new DC-expressed transcription factor, termed DC-specific transcript (DC-SCRIPT; also known as ZNF366). DC-SCRIPT mRNA is present in all DC subsets tested to date, including moDCs, mDCs, pDCs, and Langerhans cells. Interestingly, expression was not detected among other leukocyte populations (19), suggesting an essential role of DC-SCRIPT in DC biology. Outside the immune system, DC-SCRIPT has also been detected in epithelial cells in the breast and in tumors derived thereof (20, 21). DC-SCRIPT is located on human chromosomes 5q13.2 (22) and is encoded by an 8-kb mRNA. It is well conserved in evolution, with the human and mouse genes both located in syntenic chromosomal regions, sharing 80% amino acid sequence homology (23). The protein consists of a proline-rich region, 11 C2H2-type zinc fingers, and an acidic region. In addition, it bears a functional CbP1 motif and a LxxLL nuclear receptor (NR) interaction motif (19, 24). NRs are ligand-inducible transcription factors that bind specific DNA-regulatory response elements. NRs and their coregulators have been described...
to play an important role in a wide variety of biological processes, including immunobiology and cancer biology (25–30). Moreover, we demonstrated that DC-SCRIPT is a unique modulator of NR function and a strong and independent prognostic marker in breast carcinoma (20).

To date, the expression and function of DC-SCRIPT in DCs remain largely unknown. In this study, we characterized the endogenous DC-SCRIPT protein expression dynamics in the in vitro moDCs as well as in primary blood-derived DCs and studied its functional role in DC maturation.

Materials and Methods

Generation of human DCs

Human moDCs were generated from PBMCs, as described previously (31). Monocytes were derived from buffy coats. Plastic-adherent monocytes were cultured for 6 d in phenol red-free RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 1% ultraglutamine (Cambrex, Wiesbaden, Germany), 0.5% antibiotic-antimycotic (Invitrogen, Breda, The Netherlands), 10% (v/v) FCS (Greiner, Kremsmuenster, Austria), IL-4 (300 U/ml), and GM-CSF (450 U/ml) (both from Cellgenix). Differentiation of moDCs were supported with new IL-4 (300 U/ml) and GM-CSF (450 U/ml). Mature moDCs were generated from day 6 immature moDCs through 48-h stimulation with 200 ng/ml LPS (InvivoGen, Toulouse, France). Human myeloid dendritic cells (mDCs) were isolated from PBMCs using the CD1c (BDCA-1)+ Dendritic Cell Isolation Kit (Miltenyi Biotec, Leiden, The Netherlands). Human pDCs were cultured for 6 d in phenol red-free RPMI 1640 medium without AA and supplemented with 1% ultraglutamine, 0.5% antibiotic-antimycotic, IL-4 (300 U/ml), and GM-CSF (450 U/ml). DCS were fixed using 1% paraformaldehyde extra pure DAC 1 (Merck, Haarlem, The Netherlands) in PBS for 15 min at room temperature. DCs were permeabilized with 100% isopropanol (Merck, Haarlem, The Netherlands) for 1 h at −20°C, washed with PBS, blocked for 1 h with 3% BSA (Roche) and 1% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA) and stained with mouse anti-human glucocorticoid receptor (GR; Abcam, Cambridge, U.K.) or mouse anti-human glucocorticoid receptor (GR; Abcam, Cambridge, U.K.) and 10 μM DAPI (Sigma-Aldrich) or 1 μM 1,028 software (Bio-Rad) and checked for correct amplification and dissociation of the products. mRNA levels of the genes of interest were normalized to mRNA levels of the housekeeping gene porphobilinogen deaminase (PBGD) and were calculated according to the cycle threshold method (32).

Immunohistochemistry

Snap-frozen tonsil specimens were obtained from the Department of Pathology, Radboud University Nijmegen Medical Centre, St. Radboud, and approved by the institutional ethics committee of the Radboud University Nijmegen Medical Centre. The specimens were embedded in OCT embedding matrix (CellPath, Newtown, U.K.) and sectioned in 5-μM-thick tissue sections. The sections were placed on Superfrost slides (Thermo Scientific, Ettensis-Leur, The Netherlands), fixed with acetone, and incubated with 4 μg/ml mouse anti-human DC-SCRIPT Ab (R&D Systems, Abingdon, U.K.), 4 μg/ml mouse anti-human DC-specific intercellular adhesion molecule-3–grabbing nonintegrin (DC-SIGN; AZN-D1), followed by incubation with a biotinylated horse anti-goat IgG or horse anti-mouse (Vector Laboratories, Burlingame, CA), and signal development was performed using a Vectastain ABC-HPR Kit (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands). Isotype-matched mouse IgG1 (BD Bioscience) and mouse IgG1 (BD Bioscience) were used as controls. Sections were counterstained with hematoxylin to visualize the cell nuclei and analyzed by using a Leica DM LB microscope (Leica Microsystems B.V., Rijswijk, The Netherlands).

Western blotting

Cells were lysed in 1% SDS and 62.5 mM Tris (pH 6.8) and the protease inhibitors 2 μg/ml leupeptin (Sigma-Aldrich), 2 μg/ml aprotonin (Roche), and 1 mM PMSF (Sigma-Aldrich). Cell lysates were mixed with sample buffer containing 5% glycerol, 6% NaDodSO4, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.05% bromophenol blue (Gebr. Schmidbaur, Frankfurt, Germany), 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 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) and transferred overnight to Protran nitrocellulose transfer membranes (Schleicher & Schuell,’s-Hertogenbosch, The Netherlands) at 30 mA and 4°C. To block nonspecific protein binding, the membranes were incubated in 1% skimmed milk powder and 3% BSA in PBST. The membranes were incubated for 1 h with 2.5 μg/ml goat anti-human DC-SCRIPT Ab (R&D Systems, Abingdon, U.K.), washed three times in PBST, and subsequently incubated for 1 h with the secondary Ab IRDye 800CW donkey anti-goat IgG (1:5000 dilution; Li-cor Biosciences, Bad Homburg, Germany) to detect DC-SCRIPT. To detect actin, the membranes were incubated with a mouse anti-actin (1:20,000 dilution; Sigma-Aldrich clone AC140), washed three times in PBST, and incubated for 1 h with the secondary Ab Alexa Fluor 680-conjugated donkey anti-mouse IgG (1:5000 dilution; Invitrogen).

All membranes were then washed three times in PBST. After staining, the membranes were scanned by using an Odyssey Infrared Imaging System (Li-cor Biosciences) to visualize the proteins.

Confocal laser-scanning microscopy

Round 12-mm cover slides (Thermo Scientific, Braunschweig, Germany) were coated with poly-L-lysine (Sigma-Aldrich). Immature and mature moDCs were cultured for day 6, mDCs were cultured for 5 d, and pDCs were cultured for 6 d, and were seeded on cover slides (300 cells/slide) and adhered for 2 h in serum-free, phenol red-free RPMI 1640 supplemented with 1% ultraglutamine, 0.5% antibiotic-antimycotic, IL-4 (300 U/ml), and GM-CSF (450 U/ml). DCs were fixed using 1% paraformaldehyde extra pure DAC 1 (Merck, Haarlem, The Netherlands) in PBS for 15 min at room temperature. DCs were permeabilized with 100% isopropanol (Merck, Haarlem, The Netherlands) for 1 h at −20°C, washed with PBS, blocked for 1 h with 3% BSA (Roche) and 1% normal donkey serum (Sigma-Aldrich) in PBS, and stained 1 h with 2.5 μg/ml goat anti-human DC-SCRIPT (R&D Systems) and 1 h with 1/400 Alexa Fluor 488 donkey anti-goat IgG (Invitrogen). The nucleus was stained for 5 min with 0.3 μg/ml DAPI (Sigma-Aldrich) or 1 μg/ml propidium iodide (ITK, Uithoorn, The Netherlands), washed with PBS, and mounted on 70% 20-μm microscope slide (Thermo Scientific) with nuclei stain plus 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). The nucleus/cytoplasm (N/C) ratio (mean nuclear values/mean cytoplasmic values) of DC-SCRIPT expression was calculated using a custom written quantitative image analysis algorithm in Fiji/ImageJ software (http://fiji.sc).

Goat anti–DC-SCRIPT validation

Immature moDCs were stained with 2.5 μg/ml goat anti-human DC-SCRIPT or mouse anti-human glucocorticoid receptor (GR, Abcam, Cambridge, U.K.) for 40 min and 1 h with 1/400 Alexa Fluor 488 donkey anti-goat or goat anti-mouse. Prior to staining, the primary Abs were supplemented with vehicle (2.9 μl 10 mM HCl + 2.9 μl 10 mM NaOH) or 9.96 μg/ml recombinant human DC-SCRIPT (R&D Systems).

Small interfering RNA-mediated knockdown

For DC-SCRIPT silencing, a 23-nt Custom ZNF363 small interfering RNA (siRNA) termed 5CS8 targeting the DC-SCRIPT gene at position 2349–2369 was used (Dharmacon, Lafayette, CO). For IL-10 silencing, the ON-TARGETplus SMARTPool was used (Dharmacon). Western blotting for IL-10 targeting siRNA oligos each 21 nt was long. The irrelevant siRNA ON-TARGETplus Non-Targeting siRNA#1 (Dharmacon) was used as control. Cells were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). A total of 10 μg siRNA was transferred to a 4-mm cuvette (Bio-Rad), and 10 × 10^6 DCs were added in 200 μl OptiMEM and incubated for 3 min before being pulsed with an exponential decay pulse at 300 V, 150 μA, and 60 Hz (Apcb, Almere, The Netherlands) as described previously (33). Immediately after electroporation, the cells were transferred to warm (37°C) DC culture medium without AA and supplemented with 1% ultraglutamine, 0.5% antibiotic-antimycotic, 10% (v/v) FCS, IL-4 (300 U/ml),
and GM-CSF (450 U/ml). Day 6 (72 h after transfection) DCs were stimulated with vehicle, 200 ng/ml LPS, 4 μg/ml R848 (Axxora, Raamsdonkveer, The Netherlands), or 20 μg/ml poly(I:C) (Sigma-Aldrich) for 24 h. RNA was isolated with the Quick-RNA MiniPrep Kit (Zymo Research). Total lysates were prepared 72 h after transfection, lysing 50,000 cells in 50 μl 1% SDS lysis buffer containing 1% SDS and 62.5 mM Tris (pH 6.8) plus the protease inhibitors 2 μg/ml leupeptin, 2 μg/ml aprogin, and 1 mM PMSF.

ELISA

Cytokines were measured in the supernatants 24 h after induction of maturation. IL-12p70 production was measured using a standard sandwich ELISA (Pierce Biotechnology, Etten-Leur, The Netherlands). IL-6 was measured using PeliPair human IL-6 ELISA kit (Sanquin, Amsterdam, The Netherlands). TNF was measured using Human TNF ELISA Set (BD Biosciences, Breda, The Netherlands), and IL-10 was measured using Human IL-10 Module Set (Bender MedSystems, Vienna, Austria). Differences in cytokine production were assessed using t tests. Two-sided p values <0.05 were a priori considered to be statistically significant.

Mixed leukocyte reaction

DCs were electroporated at day 4 of differentiation with DC-SCRIPT targeting siRNA (siSC) or control siRNA and seeded in a 96-well plate (50,000 cells/well). At day 7, DCs were stimulated with vehicle or 4 μg/ml R848 for 8 h, after which the medium was replaced with fresh DC medium. At day 8, 24 h after R848 stimulation, PBLs were added to the DCs, in a ratio of 1:1, and cocultured for 120 h. After 4 d of coculture, cells were pulsed with [3H]thymidine for 15 h and harvested, and [3H]thymidine incorporation was determined as a measure for T cell proliferation. To analyze the Th cell profile, supernatants were collected after 2 d of DC-PBL coculture. Cytokine production in the supernatant was analyzed with a human Th1/Th2 Multiplex Kit (eBioscience, Vienna, Austria), according to manufacturer’s instructions.

Results

IL-4 induces DC-SCRIPT mRNA expression in monocytes

Within the immune system, human DC-SCRIPT mRNA has been found to be preferentially expressed by DCs (19). To obtain more insight into the expression characteristics of DC-SCRIPT, we investigated DC-SCRIPT mRNA expression during differentiation of monocytes into DCs. Hereeto, adherent monocytes were cultured in the presence of IL-4 (34, 35), GM-CSF, or the combination of both cytokines. Cells were analyzed at different time points after start of differentiation. In the absence of cytokines (vehicle), essentially no DC-SCRIPT mRNA expression could be detected. In the presence of GM-CSF alone, only small amounts of DC-SCRIPT mRNA were discerned. Incubation with IL-4 and GM-CSF or IL-4 alone resulted in DC-SCRIPT mRNA expression within 2 h after the start of stimulation, indicating IL-4 as the dominant factor for DC-SCRIPT induction. An increase in DC-SCRIPT mRNA levels was observed up to 8 h after stimulation (Fig. 1A). At later time points, DC-SCRIPT mRNA levels decreased somewhat, but remained stable from day 3 to day 8. DCs matured with LPS demonstrated an increase in mRNA expression levels (Fig. 1B), suggesting a role for DC-SCRIPT in DC maturation.

DC-SCRIPT protein is expressed in different DC subsets

To confirm endogenous DC-SCRIPT protein expression, cell lysates were prepared from monocytes at different time points after the onset of differentiation toward DCs. Within 4 h, DC-SCRIPT protein expression could be observed. Protein levels steadily increased during differentiation to DCs and remained constant from day 6 onward (Fig. 2A). In line with its mRNA expression, DC-SCRIPT protein expression is also dependent on IL-4 (data not shown).

To prove that DC-SCRIPT protein is also present in freshly isolated blood mDCs and pDCs, cell lysates were prepared from purified mDCs and compared with total PBMCs. DC-SCRIPT protein could not be detected in total PBMCs (Fig. 2B, lane 1), nor could it be detected in the mDC-negative fraction (Fig. 2B, lane 2). However, in the mDC fraction, DC-SCRIPT protein expression was readily observed (Fig. 2B, lane 3). Similarly, DC-SCRIPT protein expression could be observed in isolated mDCs and pDCs. Protein levels of cell lysates of the indicated cell fractions were subjected to immunoblotting with anti-DC-SCRIPT Abs and anti-actin as loading control. (A) Cell lysates of mDCs harvested at the indicated time points. Day 6 DCs were stimulated with vehicle or LPS for 48 h to obtain immature and mature day 8 DCs, respectively. Representative data from one of three donors.
SCRIPT protein was present in pDCs, albeit at lower levels compared with moDCs and mDCs (Fig. 2C, lane 3). No DC-SCRIPT expression could be detected in the total PBL fraction (Fig. 2C, lane 1) and the pDC negative fraction (Fig. 2C, lane 2). To our knowledge, these data show for the first time that DC-SCRIPT is endogenously expressed at protein level in freshly isolated mDCs and pDCs.

**DC-SCRIPT subcellular distribution varies among DC subsets**

The localization of endogenous DC-SCRIPT protein in the different subsets of DCs was investigated with CLSM. Hereito, DCs were stained with anti–DC-SCRIPT Abs recognizing the C-terminal part of DC-SCRIPT. The specificity of the Ab was validated by DC-SCRIPT peptide-blocking experiments (Supplemental Fig. 1). Our data show that DC-SCRIPT is predominantly localized in the nucleus of moDCs, and reveal that localization does not change upon maturation of the cells with LPS (Fig. 3A). Some DC-SCRIPT expression could be discerned in the cytoplasm. In fresh mDCs, DC-SCRIPT localization is also most pronounced in the nucleus of the cells. In pDCs, expression levels of DC-SCRIPT were apparently lower compared with mDC and moDC subsets. DC-SCRIPT staining could be found in both the cytoplasm and nucleus of pDCs, depending on the donor. Quantification of DC-SCRIPT expression in the nucleus and the cytoplasm was used to confirm localization differences between pDC donors, and between moDC and pDCs. Between pDC donors, the N/C ratio varied between 1.1 and 2.4 (mean: 1.56 ± 0.59). In contrast, mDCs displayed a N/C ratio of 2.5, whereas moDCs showed an average ratio of 3. The variation in N/C ratio within pDCs and mDCs from a single donor was minimal. These data show that the DC-SCRIPT localization is predominantly nuclear in moDCs and mDCs, whereas in pDCs a more pronounced cytoplasmic DC-SCRIPT staining is observed that varies between different pDC donors.

To further confirm DC-SCRIPT protein expression in DCs in immunological tissue, frozen tonsil sections were analyzed for DC-SCRIPT expression. The presence of DCs was confirmed by staining the consecutive section with the DC marker DC-SIGN (Fig. 3B). As expected, DCs with myeloid appearance were mainly present in the T cell area in between the germinal centers, as shown by the DC-SIGN staining. The area in which DC-SCRIPT-positive cells were found overlapped with the area containing DC-SIGN, a previously defined marker for mDCs (36). Furthermore, DC-SCRIPT expression was also observed in cells with mDC morphology located in the T cell area, further substantiating its protein expression in mDCs. In situ, DC-SCRIPT expression in the observed mDCs appeared to be mostly confined to the nucleus. The low expression levels of DC-SCRIPT in pDCs, and their low abundance in lymph nodes, did not allow proper assessment of pDCs with this approach.

**DC-SCRIPT knockdown affects IL-10 secretion by moDCs**

DC-SCRIPT mRNA and protein are expressed during the complete life cycle of moDCs, including in mature DCs. To investigate its function in DC maturation, DC-SCRIPT was silenced using a siRNA oligo (siSC) targeting the acidic region of the DC-SCRIPT gene at position 2349–2369. DCs treated with nontargeting siRNA oligos as well as nontreated DCs were used for comparison. Subsequently, immature DCs were stimulated for 24 h with ligands for TLR4, TLR7/8, and TLR3, respectively; LPS; R848; and poly(I:C). Our data demonstrate an efficient knockdown of DC-SCRIPT protein expression in siSC-treated DCs at day 6 of differentiation prior to stimulation, but not control siRNA-treated and nontreated DCs (Fig. 4A).

The effect of DC-SCRIPT knockdown on DC maturation was examined by investigating cell surface maturation marker expression and cytokine secretion in the supernatant, at, respectively, 48 and 24 h after stimulation. As expected, nonelectroporated DCs markedly increased expression of both maturation markers CD80 and CD83 upon TLR stimulation (Fig. 4B). DCs electroporated with siSC or irrelevant siRNA (control) also enhanced CD80 and CD83 expression upon activation, at equal intensities. Relative to untreated DCs, electroporated DCs showed some disparity in CD83 expression upon LPS stimulation, possibly due to the variable maturation effects by LPS.

DC maturation was also qualified by analyzing cytokine secretion (Fig. 4C). Secretion of the proinflammatory cytokines IL-6 and TNF by nonstimulated DCs, electroporated or not, could not be detected, in agreement with their immature status. Stimulation with TLR ligands differentially induced IL-6 and TNF secretion, with highest levels found upon R848 treatment, and lowest levels after poly(I:C) stimulation. Both electroporated and not electroporated DCs secreted equal amounts of IL-6 and TNF. No significant effect of DC-SCRIPT silencing was detected relative to control siRNA-treated and nontreated DCs. Variation after LPS treatment was again detected between donors. In line with the absence of proinflammatory cytokine expression, secretion of the
anti-inflammatory cytokine IL-10 was also minimal in all conditions in immature DCs. As expected, TLR-mediated maturation did induce only a minimal amount of IL-10 expression in both siRNA control DCs and nontreated DCs. Surprisingly, DCs electroporated with siSC displayed a significant increase in IL-10 secretion after treatment with LPS or R848. Little or no effect of DC-SCRIPT silencing could be detected upon polyI:C stimulation. Hence, these data demonstrate that DC-SCRIPT expression in DCs is important for repression of IL-10 secretion during TLR4- and TLR7/8-induced maturation.

Increased IL-10 secretion in siSC DCs impairs IL-12 secretion

The anti-inflammatory cytokine IL-10 is known to impair DC maturation, including IL-12 production (2). To gain more insight into the kinetics of cytokine production in siRNA control and DC-SCRIPT knockdown DCs, cytokine mRNA and protein levels were monitored in time. To this end, IL-6, TNF, IL-12, and IL-10 mRNA and protein expression of control siRNA or siSC electroporated DCs was measured at 0, 2, 4, 8, 16, and 24 h after R848 stimulation (Fig. 5). Both siSC- and control siRNA-treated cells demonstrated maximum mRNA expression between 2 and 16 h for all cytokines, which decreased at later time points. Maximum levels of IL-6 and TNF expression were detected 4 h after stimulation, whereas IL-12p35 and IL-10 mRNA expression peaked at 8 h. The protein expression of IL-6, TNF, and IL-10 followed the mRNA expression kinetics, reaching maximum levels at later time points, after which expression remained relatively stable. When comparing control siRNA- and siSC-treated DCs, no effect was found for IL-6 or TNF secretion. In contrast, siSC and control siRNA DCs differed greatly in the expression of IL-10 and IL-12. In addition to the increase in IL-10 production, IL-12 production was significantly impaired at both the mRNA and the protein level in siSC DCs. The impaired IL-12 expression observed in siSC DCs was preceded by the increased IL-10 secretion, suggesting a role for IL-10 in reducing IL-12 levels in siSC DCs.

IL-10 silencing rescues IL-12 secretion in DC-SCRIPT knockdown DCs

To confirm the role of enhanced IL-10 secretion on the expression of IL-12, both DC-SCRIPT and IL-10 expression were silenced. To this end, DCs were electroporated with either control siRNA, siSC with control siRNA, or siSC with IL-10 targeting siRNA (siIL-10). Day 6 DCs were stimulated with R848 for 24 h, after which cytokine production was measured in the supernatant. As an additional control, untreated DCs stimulated with R848 received 10^6 U/ml IL-10 or vehicle 4 h after R848 stimulation, to mimic the enhanced IL-10 secretion in siSC DCs.

Again, efficient knockdown of DC-SCRIPT protein expression was detected in all conditions electroporated with siSC prior to stimulation (Fig. 6A). IL-10 protein expression in the supernatant was measured at both 8 h (data not shown) and 24 h after stimulation and demonstrated efficient IL-10 silencing at both time points. DC-SCRIPT knockdown DC again showed decreased IL-12 secretion upon R848 stimulation. Likewise, addition of rIL-10 to untreated DCs stimulated with R848 resulted in a significant (p = 0.011) reduction in IL-12 secretion (Fig. 6B). Silencing of both DC-SCRIPT and IL-10, however, resulted in normal IL-12 protein levels, confirming that IL-10 signaling mediates downregulation of IL-12 secretion in siSC DCs (Fig. 6C). In line with previous results, no effect was detected on IL-6 and TNF secretion, suggesting that the enhanced IL-10 production specifically affects IL-12 expression in these cells.

DC-SCRIPT knockdown impairs T cell responses

Next, we investigated the biological consequences of DC-SCRIPT silencing on DC-mediated T cell responses in an allogeneic MLR. SiSC or control siRNA electroporated DCs were stimulated at day 7 with vehicle or R848 for 8 h, after which the medium was replaced with fresh DC medium. Twenty-four hours after stimulation, PBLs were added and both T cell proliferation and cytokine secretion were determined as a measure of T cell activation. As shown (Fig.
T cell proliferation was readily detected upon stimulation with control siRNA-treated DCs and was significantly impaired after stimulation with siSC-treated DCs. The effect of R848 stimulation on T cell proliferation was limited, possibly reflecting the overall immune activation at these allogeneic conditions. In contrast to T cell proliferation, IFN-γ secretion by T cells in these cocultures was largely dependent on the presence of R848 (Fig. 7B). Strikingly, DC-SCRIPT–silenced DCs showed an impaired capacity to induce IFN-γ secretion by T cells relative to control siRNA-silenced DCs. No significant differences were detected for the proinflammatory cytokine levels of IL-6 and TNF between control- and siSC-treated DCs in these cocultures (Supplemental Fig. 2). Collectively, these data strengthen the finding that DC-SCRIPT plays an important role during DC maturation and the induction of T cell responses.

**Discussion**

Previously, we have isolated and characterized the cDNA encoding the transcription regulator DC-SCRIPT (19, 23) that is preferentially expressed in DCs within the immune system. In the current study, DC-SCRIPT mRNA and protein expression were found to be induced early in DC differentiation and were dependent on IL-
4. Silencing of DC-SCRIPT expression affected DC maturation and induced IL-10 secretion in mature DCs, which consequently impaired IL-12 secretion by these cells. Furthermore, DC-SCRIPT-silenced DCs were shown to have a significantly impaired capacity to induce T cell proliferation and IFN-γ responses. Hence, DC-SCRIPT appears to be an important factor in regulating DC maturation.

In human moDCs, DC-SCRIPT expression was dependent on the presence of IL-4. Control experiments demonstrated no DC-SCRIPT expression in PBLs upon IL-4 stimulation (data not shown). In addition, although in vivo studies previously demonstrated DC-SCRIPT expression in breast epithelial cells (20), IL-4 stimulation of the DC-SCRIPT-negative MCF-7 breast carcinoma cells did not induce DC-SCRIPT expression (data not shown). These data therefore suggest that the IL-4-mediated induction of DC-SCRIPT is related to the differentiation of monocytes to DCs. It is therefore important to further deduce the expression and function of DC-SCRIPT in the DC differentiation process itself.

Next to moDCs, DC-SCRIPT expression was readily detected in mDCs and pDCs. Previous reports demonstrated DC-SCRIPT mRNA expression in all DC subsets tested, including Langerhans cells, mDCs, and pDCs (19). In this study, to our knowledge, we show for the first time endogenous DC-SCRIPT protein expression in both mDCs and pDCs. Moreover, in vivo DC-SCRIPT expression was observed in DCs present in T cell areas of tonsil tissue. In immunohistochemistry and CLSM slides, DC-SCRIPT is predominantly localized in the nucleus of moDCs and mDCs, concomitant with the presence of a nuclear localization motif. Interestingly, some expression was also detected in the cytoplasm of moDCs and mDCs. This was even more pronounced in pDCs; however, it must be noted that expression is lower and that variations between donors were observed for this subset. In addition, our recent work demonstrated a predominant cytoplasmic expression of DC-SCRIPT in breast epithelial cells (20). This suggests that DC-SCRIPT might also have important functions outside the nucleus.

Further insight into the cytoplasmic expression of DC-SCRIPT might be gained from the NR biology. In a previous publication, we have characterized DC-SCRIPT as a NR coregulator (20). NRs and many of their coregulators are found both in the nucleus as well as in the cytoplasm. The type I NRs are classically sequestered in the cytoplasm, and translocate to the nucleus upon ligand binding, where they bind to specific DNA sequences (26, 37–40). NR function is controlled by NR coregulators, such as N-CoR and SMRT. Recent evidence suggests shuttling of these coregulators between the nucleus and cytoplasm, triggered by changes in signaling at the cell surface (41). Further research is necessary to fully elucidate the pattern and dynamics of DC-SCRIPT expression in different DC subsets. Investigating the effects of various extracellular signals, such as NR ligands, will provide more insight into the DC-SCRIPT localization characteristics in DCs.

In addition, our data uncovered an important role for DC-SCRIPT in DC maturation. DCs with silenced DC-SCRIPT expression displayed enhanced IL-10 and decreased IL-12 cytokine production upon maturation. Silencing IL-10 expression rescued the IL-12 secretion in DC-SCRIPT knockdown DCs, suggesting that DC-SCRIPT primarily affects the expression of IL-10. No effect was detected on IL-6 and TNF secretion, or after poly(I:C) maturation. Interestingly, TLR4 and TLR7/8 signal via the adaptor protein MyD88, whereas TLR3 activates TRIF-mediated signaling (42), suggesting that DC-SCRIPT primarily functions in the MyD88 pathway. Functionally, DC-SCRIPT-silenced DCs are less capable of inducing T cell proliferation and IFN-γ secretion in a coculture with allogeneic PBLs.

Unraveling the underlying molecular mechanisms by which DC-SCRIPT can regulate IL-10 expression in DCs will be an important next step. In DCs, IL-10 transcription is induced through NF-κB activation (43). One way of enhanced and prolonged IL-10 transcription is NF-κB acetylation (44). It would therefore be of great interest to see whether and how DC-SCRIPT is involved in NF-κB acetylation. Furthermore, besides immunostimulatory DCs, future studies on DC-SCRIPT expression and function should also include tolerogenic DCs (tolDCs). These immunosuppressive DCs are known to secrete elevated levels of IL-10, while having diminished IL-12 secretion, thereby preventing T cell proliferation (45). Studying tolDCs is even more interesting, as we have recently found that DC-SCRIPT also affects the function of the GR (S. Hontelez, N. Karthaus, M.W. Looman, M. Ansems, and G.J Adema, submitted for publication). GR is well known for its central role in the generation of tolDCs, and known to induce IL-10 production. Moreover, the IL-10 promoter contains a glucocorticoid-responsive element, which could serve as a binding site for GR to stimulate IL-10 transcription (46, 47).

DCs are in the center of the immune system, controlling the type and course of an immune response. They regulate both innate and adaptive immunity and serve as a bridge between both systems. Therefore, DCs are regularly used in immunotherapy. Detailed understanding of DC differentiation and maturation will allow for the generation of the best suitable DC for these therapies. Collectively, our data provide important insight in the DC biology, highlighting DC-SCRIPT as an essential factor in DC maturation.

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Disclosures
The authors have no financial interests of conflict.

References
Supplemental data

**Figure S1. Validation of the goat-anti-DC-SCRIPT antibody**

CLSM validation of the anti-DC-SCRIPT antibody using a recombinant human DC-SCRIPT peptide (rh-DC-SCRIPT). This peptide represents the epitope for the anti-DC-SCRIPT antibody. Mouse-anti-GR antibody was used as control. Cells were stained with goat-anti-DC-SCRIPT or mouse-anti-GR pre-incubated with vehicle or rh-DC-SCRIPT.

**Figure S2. DC-SCRIPT knock-down does not affect IL-6 and TNF secretion in a MLR**

Effect of DC-SCRIPT silencing on IL-6 and TNF secretion in an allogeneic mixed leukocyte reaction (MLR). Day 4 moDCs were electroporated with control siRNA or siSC oligos. At day 7 DCs were stimulated with vehicle (not shown) or R848 for 8 hours. Day 8 DCs were co-cultured with PBLs in a ratio of 1:1. Data from 3 donors. Error bars correspond to +/- SEM. Data indicated with *N.S.* (not significant) did not meet the criteria in the *t* test to be considered statistically significant.