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1,25-Dihydroxyvitamin D₃ Promotes Tolerogenic Dendritic Cells with Functional Migratory Properties in NOD Mice

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The biologically active form of vitamin D, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], is able to promote the generation of tolerogenic mature dendritic cells (mDCs) with an impaired ability to activate autoreactive T cells. These cells could represent a reliable tool for the promotion or restoration of Ag-specific tolerance through vaccination strategies, for example in type 1 diabetes patients. However, successful transfer of 1,25(OH)₂D₃-treated mDCs (1,25D₃-mDCs) depends on the capacity of 1,25(OH)₂D₃ to imprint a similar tolerogenic profile in cells derived from diabetes-prone donors as from diabetes-resistant donors. In this study, we examined the impact of 1,25(OH)₂D₃ on the function and phenotype of mDCs originating from healthy (C57BL/6) and diabetes-prone (NOD) mice. We show that 1,25(OH)₂D₃ is able to imprint a phenotypic tolerogenic profile on DCs derived from both mouse strains. Both NOD- and C57BL/6-derived 1,25D₃-mDCs decreased the proliferation and activation of autoreactive T cells in vitro, despite strain differences in the regulation of cytokine/chemokine expression. In addition, 1,25D₃-mDCs from diabetes-prone mice expanded CD25⁺Foxp3⁺ regulatory T cells and induced intracellular IL-10 production by T cells in vitro. Furthermore, 1,25D₃-mDCs exhibited an intact functional migratory capacity in vivo that favors homing to the liver and pancreas of adult NOD mice. More importantly, when cotransferred with activated CD4⁺ T cells into NOD.SCID recipients, 1,25D₃-mDCs potently dampened the proliferation of autoreactive donor T cells in the pancreatic draining lymph nodes. Altogether, these results argue for the potential of 1,25D₃-mDCs to restore Ag-specific immune tolerance and arrest autoimmune disease progression in vivo. The Journal of Immunology, 2014, 192: 000–000.
donors as in cells derived from diabetes-resistant donors. This concern arises from observations on the abnormal behavior and, in particular, defective maturation of the myelocytic lineage in NOD mice compared with C57BL/6 mice (16), an attribute that is mirrored by abnormalities within this lineage in humans with T1D compared with healthy individuals (17). This introduces a potential flaw in studies presenting drug effects on DCs, especially when using only nonautoimmune donors. In this regard, we (18–20) showed that modulation of the DC phenotype is possible in bone marrow (BM)-derived DCs from C57BL/6 or NOD mice, but data on the functionality of these cells are lacking or incomplete.

In the current study, we aimed to establish whether 1,25(OH)2 D3 could induce tolerogenic DCs from diabetes-prone NOD mice and whether these 1,25D3-mDCs would home to the pancreas and pancreatic draining lymph nodes (pLNs) when administered to NOD mice. Despite inherent disease-related abnormalities, we demonstrated that 1,25(OH)2 D3 imprints a tolerogenic profile on murine DCs derived from either C57BL/6 or NOD mice, which was reflected in their efficient ability to blunt autoaggressive T cell proliferation and activation in vitro. Furthermore, NOD-derived 1,25D3-mDCs were able to expand Tregs and to promote intracellular IL-10 production by T cells in vitro. Finally, NOD-derived 1,25D3-mDCs efficiently restrained T cell proliferation in vivo and promptly migrated to the pancreas and liver of adult NOD mice. Taken together, these data open the avenue for the use of 1,25D3-mDCs as promising tools to restore the balance between immunogenicity and tolerogenicity in an autoimmune disease context.

Materials and Methods

Animals

C57BL/6 (C57BL/6/NHsd; H-2b) mice were purchased from Harlan (Horst, The Netherlands), and NOD mice were obtained originally from Prof. C. Y. Wu (Department of Endocrinology, Peking Union Medical College Hospital, Beijing, China). They were bred and housed under semibarrier conditions in our animal facility at Katholieke Universiteit Leuven. OT-II-transgenic (Tg) mice, which carry the MHC class II (MHCIId)-restricted Tg TCR for OVA323–339, were kindly provided by Prof. M. Moser (Université Libre de Bruxelles, Brussels, Belgium) and bred in our animal facility. BDC2.5 TCR-Tg NOD mice and NOD.SCID mice were bred from stocks purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained according to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals, and all experimental procedures were approved and performed in accordance with the Ethics Committee of the Katholieke Universiteit Leuven.

In vitro generation of BM-derived murine DCs

BM-derived DCs were obtained in vitro, as previously described (18). Briefly, BM was isolated from long bones of C57BL/6 or NOD mice aged 4–5 wk. BM cells were differentiated in vitro toward immature DCs (iDCs) for 8 d in the presence of 20 ng/ml murine rGM-CSF and 20 ng/ml murine rIL-4 (both from PeproTech, Rocky Hill, NJ) in RPMI 1640 medium (Invitrogen, Meerbeke, Belgium) containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. The medium was refreshed on days 3 and 6. At day 8, nonadherent cells were harvested, and iDCs were purified after a CD11c+ magnetic bead selection (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s specifications. After CD11c+ magnetic selection, iDCs were cultured in vitro toward immature DCs (iDCs) or 1,25D3-mDCs were loaded for 2 h at 37°C with OVA257–264 (Invogen, Lund, Sweden) or BDC2.5 mIg (AnaSpec, Freemont, CA) at various concentrations (0.1, 1, and 10 μg/ml) prior to the addition of T cells or transfer in vivo.

Real-time quantitative PCR

Total RNA was extracted from iDCs, Ctrl-mDCs, and 1,25D3-mDCs using the commercially available RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands). A constant amount of RNA was reverse transcribed using 100 U Superscript II Reverse Transcriptase (Invitrogen) and 5 mM oligo (dT)16 at 42°C for 30 min. The quantitative PCR amplification reaction was performed on a StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA) using Fast SYBR Green Master Mix or TaqMan Fast Universal Master Mix (both from Applied Biosystems), as described previously (21, 22). Cytokine primers and probe sequence were as follows: FW 5′-GGCAAGACGTGGAGAATGGC-3′, probe 5′-AGACGCTTCTTTGATTTG-3′, and probe sequence were as follows: FW 5′-CAGGTTGACCTTCTCGTTAAG-3′, probe 5′-GGCCGCCGCTTGTTGATC-3′ (Eurogentec, Liege, Belgium); isotype primers and probe sequence were as follows: FW 5′-CATTTGAAGTGAAGCTGTTTG-3′, probe 5′-GGGCGGCCGCTTGTTGATC-3′ (Eurogentec); ido primers were as follows: FW 5′-TGACTACGGTGAGCCTGCCT-3′, and probe sequence were as follows: FW 5′-TGAGCTCAGAGTGTCGGCTG-3′, probe 5′-TTACGGCCTACTGCTGTCTGAC-3′ (Eurogentec). All samples were normalized to the geometrical mean of ribosomal protein L27 (22) (primers: FW 5′-GTCGAGATTTGGGAACTTCA-3′, probe 5′-TTCTCTGATGACCCGTTCT-3′), hydroxymethylbilane synthase (hmbs) (primers: FW 5′-GAAACTGCCTGTCGCTGATCT-3′, probe 5′-TGCTTCCGCTCTTCTTGCAT-3′), and lypRT (primers: FW 5′-TGTCGCACCTCAGCTGAAAGC-3′, probe 5′-GGCTCACATTGCAATCAAAGTG-3′). All data were analyzed using the comparative Ct method, as previously described (21).

Flow cytometry

After 9 d of culture, nonadherent DCs were analyzed for their surface marker expression by flow cytometry (FACS). All staining procedures were performed in PBS containing 2 mM EDTA and 0.1% BSA. Cells were preincubated with anti-FcγR (clone 32-1.8) for 30 min at 4°C to minimize nonspecific binding. Thereafter, 2.5 × 10^5 cells were labeled directly with the following conjugated Abs: CD11c, I-Ad (clone 39-10-8 for NOD), I-Ab (clone AF6-120.1 for C57BL/6), H-2kd (clone SF1-1.1 for NOD), H-2kb (clone AF6-88.5 for C57BL/6), CD80, CD86, PD-L1, and matching isotype controls. Signal height and widths were used to exclude doublets. Dead cells were excluded using Fixable Live/Dead Yellow Stain (Invitrogen), according to the manufacturer’s specifications. All Abs were obtained from eBioscience (San Diego, CA). Data acquisition was performed on a Gallios flow cytometer, and Kaluza software (both from Beckman Coulter, Analis, Suarèlc, Belgium) was used for data analysis.

Determination of cytokine/chemokine secretion

The supernatant of iDC, Ctrl-mDC, or 1,25D3-mDCs cultures was analyzed for the levels of cytokines/chemokines using the FlowCytomix Mouse Chemokine 6-Plex Kit (eBioscience), in combination with additional FlowCytomix Simplex Kits for IL-10, IL-6, TNF-α, and IP-10. Samples were analyzed on a Gallios flow cytometer, and FlowCytomix Software (eBioscience) was used for the analysis. Supernatants from the DC:T cell cocultures were analyzed for the levels of secreted IL-2 using the FlowCytomix Simplex Kit for IL-2, as described above.

Isolation of T cells from TCR-Tg mice

Purified T cells were prepared from homogenized splenocytes and lymph nodes from Tg mice using magnetic separation. Briefly, purified total CD45^+ cells (from OT-II or BDC2.5 mice) were prepared by negative selection using an Ab mixture to CD16/CD32 to minimize nonspecific binding. Thereafter, 2.5 × 10^5 cells were labeled directly with the following conjugated Abs: CD11c, I-Ad (clone 39-10-8 for NOD), I-Ab (clone AF6-120.1 for C57BL/6), H-2kd (clone SF1-1.1 for NOD), H-2kb (clone AF6-88.5 for C57BL/6), CD80, CD86, PD-L1, and matching isotype controls. Signal height and widths were used to exclude doublets. Dead cells were excluded using Fixable Live/Dead Yellow Stain (Invitrogen), according to the manufacturer’s specifications. Total Abs were obtained from eBioscience (San Diego, CA). Data acquisition was performed on a Gallios flow cytometer, and Kaluza software (both from Beckman Coulter, Analis, Suarèlc, Belgium) was used for data analysis.

2.5(OH)2D3 PROMOTES MIGRATION-COMPETENT TOLEROGENIC DCs

In vitro lymphocyte-proliferation assays and FACS analysis

Negatively purified total CD45^+ or CD45^+CD25^+ lymphocytes from Tg mice were cocultured with Ctrl-mDCs or 1,25D3-mDCs primed with appropriate peptide (0.1–1 μg/ml) at a 1:10 DC:T cell ratio in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 1 mM glutamine, 1 mM Na-pyruvate, and 50 μM 2-ME. Cells were cultured at 37°C in 5% CO2 for 1–3 d before Ab staining and analysis by FACS. Cells were gated based on the forward and side scatter (lymphocyte gate) and signal height and
widths (doublet exclusion). Thereafter, T cell activation was assessed by simultaneous staining for CD4, CD25, CD44, and CD69 (all from ebioscience). Induction/expansion of Tregs was assessed after direct staining with Abs against CD4 and CD25 and intracellular staining against Foxp3 (all from ebioscience). For the detection of intracellular cytokine production, cells were incubated for 6 h with 25 ng/ml PMA and 0.5 μM ionomycin. GolgiStop was added during the last 4 h of incubation to block intracellular protein transport. Leukocytes, after staining for surface molecules, were treated with Cytofix/Cytoperm (BD Biosciences), and rat anti-mouse IL-10 mAb (BD Biosciences) was used for intracellular staining. Dead cells were excluded using Fixable Live/Dead Yellow Stain, according to the manufacturer’s specifications. Data acquisition was performed on a Gallios flow cytometer, and Kaluza software (Beckman Coulter) was used for data analysis.

Quantification of T cell apoptosis
T cell apoptosis was determined after staining with an annexin V (ANXAX5) probe (BD Biosciences) and propidium iodide (PI; eBioscience), according to the manufacturers’ specifications. Briefly, cocultured leukocytes were harvested after 24 h in culture, washed two times with PBS, and stained in 1× binding buffer with allophycocyanin-conjugated ANXV and PI for 15 min at room temperature. T cells were analyzed within 1 h on a Gallios flow cytometer, after exclusion of DCs based on forward and side scatter. Kaluza software was used for data analysis.

Histology and immunofluorescence
Insulins and insulin positivity were assessed by histological screening of pancreatic glands from 12–14-wk-old NOD mice. The organs were preserved by snap-freezing in 2-methyl-butane 99%-% (ACROS Organics, Geel, Belgium) and liquid nitrogen. Further processing and staining occurred according to standard histological techniques, as previously described (23).

In vivo migration assay
BDC2.5 mimotope-loaded (10 μg/ml) NOD-derived Ctr-mDCs or 1,25D3−mDCs were labeled with a PKH26 Red Fluorescent Cell Linker Kit for Gentec (Mitsubishi Chemical) Labeling solution (green), according to the manufacturer’s specifications. DCs were extensively washed in RPMI 1640 medium containing 10% FCS and resuspended in PBS. Thereafter, 1 × 106 cells were injected i.p. into recipient NOD-SCID mice. After 24–48 h, recipient mice were perfused with cold PBS, and RBCs were lysed after incubation with NH4Cl. The spleens (or pancreas) or enzymatically digested (pancreas and liver) into single-cell suspensions prior to staining and analysis by FACS. For the enzymatic digestions, pancreases were minced into small fragments and treated with 1 mg/ml collagenase NB8 (SERVA, Heidelberg, Germany) and 20 mg/ml DNase I for 30 min at room temperature. T cells were analyzed within 1 h on a Gallios flow cytometer, after exclusion of DCs based on forward and side scatter. Kaluza software was used for data analysis.

Statistical analysis
All comparisons were tested for statistical significance with the unpaired t test or one-way ANOVA, with subsequent Tukey posttest for multiple comparisons, using GraphPad Prism software (GraphPad, La Jolla, CA). For all tests, data were considered significantly different at p < 0.05.

Results
BM-derived NOD DCs exhibit phenotypical maturation abnormalities in vitro
Comparing BM-derived DCs from diabetes-prone NOD mice and diabetes-resistant C57BL/6 mice showed a significantly lower yield of viable iDCs from cultures generated from NOD mice (data not shown). Following maturation, a minor loss of CD11c+ cell recovery was observed in both mouse strains (data not shown).

With regard to the expression of molecules involved in Ag presentation, NOD-derived iDCs exhibited a hyperinflammatory phenotype, with elevated levels of CD80 and CD86 compared with control C57BL/6 iDCs (Fig. 1A; gating strategy shown in Supplemental Fig. 1). Interestingly, however, once exposed to a maturation stimulus, NOD mDCs failed to further upregulate both costimulatory molecules, in addition to the percentage of MHCII+ cells. Nevertheless, the basal levels of both CD80 and CD86 in NOD mDCs remained higher than the ones observed in C57BL/6 mDCs (Fig. 1A). Furthermore, the ability to produce proinflammatory mediators and cytokines is altered in NOD mDCs, with slightly higher mRNA levels of inos and lower protein secretion levels of both IL-6 and TNF-α (versus C57BL/6 mDCs) (Fig. 1B). More importantly, NOD iDCs secreted greater levels of different monocyte/macrophage and T cell–attractant chemokines, such as CCL3, CCL4, CCL5, and CXCL10, into the culture supernatants compared with C57BL/6 iDCs (Fig. 1C). Following maturation, NOD mDCs failed to upregulate CCL3 and CCL5, a phenomenon that was clearly observed in C57BL/6-derived mDCs. In contrast, the production of CXCL10 was more pronounced in NOD mDCs after maturation compared with C57BL/6 mDCs.

1,25(OH)2D3 restraints the inflammatory response of NOD and C57BL/6 BM-derived DCs
Given the intrinsic differences between NOD- and C57BL/6-derived DCs, we investigated whether 1,25(OH)2D3 treatment could similarly modulate the DC morphology and phenotype from both mouse strains. Ctr-mDCs generated from the BM of NOD and C57BL/6 mice characteristically developed as mostly non-adherent cells with numerous dendrites on their cell surface. Exposure of NOD-derived DC cultures to 1,25(OH)2D3 typically favored the appearance of spindle-shaped adherent cells, an attribute that was similarly promoted in DCs derived from the C57BL/6 strain (Supplemental Fig. 2A). Additionally, in DCs generated in the presence of 1,25(OH)2D3, upregulation of the VDR-specific target gene, cyp24a1, hydroxylase, was observed in a similar extent in NOD- and C57BL/6-derived 1,25D3−mDCs, confirming the full responsiveness of both cell types to the active compound (Supplemental Fig. 2B).

Exposure of either NOD- or C57BL/6-derived mDCs to 1,25(OH)2D3 caused the downregulation of CD80 and CD86, as well as the percentage of MHCII+ cells (Fig. 2A; gating strategy shown in Supplemental Fig. 3). Moreover, expression of inos was suppressed to a greater extent after 1,25(OH)2D3 treatment in...
FIGURE 1. NOD-derived DCs present maturation abnormalities in vitro. Control iDCs or mDCs were obtained in vitro from BM precursors derived from NOD or C57BL/6 mice, as described in Materials and Methods. NOD-iDCs (light gray bars or white triangles), NOD mDCs (white bars or white circles), C57BL/6-iDCs (dark gray bars or black triangles), and C57BL/6 mDCs (black bars or black circles) were analyzed for their percentage positivity or surface marker expression and different inflammatory parameters. (A) Percentage positivity and surface marker expression evaluation was performed by FACS analysis of different molecules (MHCII, CD80, CD86) after gating on viable and single CD11c+ cells. Values depicted in the bar graph represent the percentage of positive cells in the CD11c+ population (MHCII) and the levels of the mean fluorescence intensity (MFI) minus the MFI of the corresponding isotype controls ($\Delta$MFI) ± SEM from CD80 and CD86 in control iDCs or mDCs ($n = 4–7$). (B) Relative mRNA expression of inos was analyzed by quantitative real-time PCR. Cytokine production levels of IL-6 and TNF-α into the culture supernatants was analyzed by FACS ($n = 3$). (C) The levels of different chemokines was analyzed by FACS in the supernatant of the DC cultures ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. 

$1,25(OH)_2D_3$ PROMOTES MIGRATION-COMPETENT TOLERGENIC DCs
mDCs derived from NOD mice, in contrast to IL-6 and TNF-α, for which suppression of secretion was less pronounced in NOD-derived 1,25D$_3$-mDCs compared with C57BL/6-derived 1,25D$_3$-mDCs (Fig. 2B). Furthermore, we detected differential regulation of chemokine production by 1,25(OH)$_2$D$_3$ when comparing mDCs derived from the two mouse strains. Treatment with 1,25(OH)$_2$D$_3$ decreased the secretion levels of CCL3, CCL4, CCL5, and CXCL10 and increased the secretion levels of CCL2 and CCL7.

**FIGURE 2.** 1,25(OH)$_2$D$_3$ modulates the surface marker and cytokine/chemokine profile of NOD- and C57BL/6-derived mDCs. NOD Ctr-mDCs (white bars or white circles), NOD 1,25D$_3$-mDCs (diagonally striped bars or white squares), C57BL/6 Ctr-mDCs (black bars or black circles), or C57BL/6 1,25D$_3$-mDCs (horizontally striped bars or black squares) were obtained, as described in Materials and Methods, and analyzed for their surface marker expression or percentage positivity (A) and expression/production of proinflammatory mediators/cytokines (B) and chemokines (C) 24 h after exposure to the maturation stimulus. The vertical dashed line has been added to provide clear distinction between C57BL/6 on the right and NOD on the left. Data are mean ± SEM of at least three independent experiments. Measurements presented for Ctr-mDCs from both NOD and C57BL/6 mice are equal to the ones presented in Fig. 1. *p < 0.05, **p < 0.01, ***p < 0.001 versus Ctr-mDCs.
(Fig. 2C, Supplemental Fig. 2C) in C57BL/6-derived 1,25D₃-mDCs compared with Ctr-mDCs. However, in NOD-derived mDCs, 1,25(OH)₂D₃ failed to regulate the levels of CCL2, CCL4, and CCL7, even triggering a slight upregulation of CCL3 compared with Ctr-mDCs (Fig. 2C, Supplemental Fig. 2C). The secretion levels of CCL5 and CXCL10 by NOD-derived 1,25D₃-mDCs followed the same downward trend as in 1,25D₃-mDCs derived from C57BL/6 animals.

1,25(OH)₂D₃ reduces the T cell stimulatory capacity of C57BL/6 and NOD BM-derived mDCs

Because 1,25(OH)₂D₃ modulated the expression of molecules involved in T cell stimulation in mDCs, we next investigated the functional impact of these changes on T cell proliferation and activation. For this purpose, we cocultured Ag-loaded NOD- or C57BL/6-derived Ctr-mDCs and 1,25D₃-mDCs with CD4⁺CD25⁻ T cells originated from two Tg models: BDC2.5-Tg animals (expressing a Tg TCR recognizing I-A<sup>v</sup>-bound BDC2.5 mimotope in the NOD background) and OT-II-Tg mice (expressing a TCR recognizing I-A<sup>b</sup>-bound OVA323–339 peptide in the C57BL/6 background) for 72 h. As illustrated in Fig. 3A, 1,25D₃-mDCs clearly present a T cell hypostimulatory capacity compared with their control counterparts, both at 0.1 and 1 μg/ml of appropriate peptide (p < 0.0001, versus Ctr-mDCs). In addition to the lower proliferation rates of CD4⁺ T cells in the presence of 1,25D₃-mDCs, IL-2 secretion into the culture supernatants was similarly decreased by >50% (p < 0.01, versus Ctr-mDCs) (Fig. 3B). This was followed by a comparable decrease in the activated CD44⁺CD69⁺ double-positive T cells (p < 0.05, versus Ctr-mDCs) (Fig. 3C) at the end of the coculture period. Importantly, all of these inhibitory actions on T cell proliferation, IL-2 secretion, and activation were evident for both C57BL/6- and NOD-derived 1,25D₃-mDCs.

NOD-derived 1,25D₃-mDCs interfere with the T cell division cycle, exhibit Treg-promoting parameters, and foster the expansion of Tregs in vitro

We next sought to determine the mechanism by which 1,25D₃-mDCs restrain responder T cell proliferation specifically in NOD animals. To investigate whether increased T cell apoptosis and/or interference with cell cycle entry could account for the inhibition of T cell proliferation, we first measured the apoptotic marker ANXA5, as well as membrane permeability to PI, in CD4⁺ T cells from BDC2.5-Tg mice cultured with NOD-derived Ctr-mDCs or 1,25D₃-mDCs for 24 h. Clearly, no differences were observed in the frequency of early apoptotic (ANXA5⁺), late apoptotic (ANXA5⁺PI⁺), or necrotic (PI⁺) CD4⁺ T cells cultured in the presence of either type of DC (Fig. 4A). We then followed the rate of eFluor 670 proliferation dye dilution of labeled BDC2.5-Tg CD4⁺ T cells cultured with peptide-loaded Ctr-mDCs or 1,25D₃-mDCs for 72 h. An overall reduction in T cell proliferation from 79.0 ± 2.2% by Ctr-mDCs to 65.5 ± 4.1% by 1,25D₃-mDCs was observed (n = 4, p < 0.05) (Fig. 4B). Interestingly, T cells cultured with 1,25D₃-mDCs were characterized by slightly higher numbers of resting T cells at cycle 0 (11.8 ± 2.9% versus 9.7 ± 2.1% for Ctr-mDCs, not significant) and lower numbers of proliferating T cells reaching later division cycles (Fig. 4B).

Tolerogenic DCs can orchestrate T cell responses by modulating T cell proliferation, as well as by inducing the generation of Tregs. First, we evaluated different features of NOD-derived 1,25D₃-mDCs that would point to an increased Treg-promoting capacity, such as an increased surface expression of the PD-L1/CD80 ratio, an important parameter for the induction of Tregs by 1,25D₃-mDCs (12), and the production of immunoregulatory molecules, such as IL-10, TGF-β, and IDO. As depicted in Fig. 4C, treatment with 1,25(OH)₂D₃ significantly upregulated the surface expression of the PD-L1/CD80 and the secreted protein levels of IL-10 (p < 0.01 versus controls) and also increased the mRNA levels of tgfβ and ido.
FIGURE 4. Mechanisms of T cell suppression and Treg expansion by 1,25D$_3$-mDCs in the NOD mouse model. (A) Total CD4$^+$ T cell viability was measured after 24 h in coculture with Ctr-mDCs (white bars) or 1,25D$_3$-mDCs (striped bars). Staining for ANXA5 and/or PI was analyzed by FACS, after exclusion of DCs based on the forward and side scatter (lymphocyte gate). Data are mean ± SEM of positive T cells in three independent experiments. (B) T cell division cycle entry and progression were analyzed after 72 h in coculture with Ctr-mDCs or 1,25D$_3$-mDCs using eFluor 670 cell proliferation dye dilution on CD4$^+$ T cells. The graph represents one of three experiments performed. (C) Surface expression of PD-L1/CD86 ratio and secretion of IL-10 into the culture supernatants were assessed after 24 h of exposure of Ctr-mDCs (white bars and circles) or 1,25D$_3$-mDCs (striped bars and white squares) to the maturation stimulus. Data are mean ± SEM of at least three independent experiments. mRNA expression levels of tgf$eta$ and ido were analyzed by quantitative real-time PCR in Ctr-mDCs or 1,25D$_3$-mDCs (n = 3). (D and E) Treg generation/expansion is shown as the relative frequency of double-positive CD25$^+$Foxp3$^+$ T cells in the total CD4$^+$ population (n = 6). The stippled bar represents the frequency of Tregs in freshly (Figure legend continues)
Next, we investigated whether NOD-derived 1,25D$_3$-mDCs have the functional capacity to promote Tregs in vitro by analyzing the frequency of typical CD4$^+$CD25$^+$Foxp3$^+$ Tregs following coculture with peptide-loaded Ctr-mDCs or 1,25D$_3$-mDCs. Significantly increased numbers of Tregs were encountered when total CD4$^+$ T cells were cocultured in the presence of 1,25D$_3$-mDCs at a concentration of 1 µg/ml BDC2.5 mimotope (10.8 ± 1.3% versus 7.1 ± 0.96% for Ctr-mDCs, n = 6, p < 0.05), and was even more pronounced at a lower Ag concentration of 0.1 µg/ml (18.8 ± 0.4% [1,25D$_3$-mDCs] versus 10.4 ± 1.1% [Ctr-mDCs], n = 6, p < 0.0001) (Fig. 4D). To assess whether this phenomenon resulted from an expansion of pre-existing Tregs or instead, conversion from naive CD4$^+$ T cells, the starting total CD4$^+$ T cell population was depleted of Tregs prior to coculture with Ctr-mDCs or 1,25D$_3$-mDCs. 1,25D$_3$-mDCs failed to reproduce this increase in the frequency of the CD4$^+$CD25$^+$Foxp3$^+$ Treg population following a coculture period with CD4$^+$CD25$^+$ naive T cells (Fig. 4D). Of note, the 1,25D$_3$-mDC–expanded Treg population produced higher intracellular levels of the anti-inflammatory cytokine IL-10 (p < 0.05, versus total CD4$^+$ T cells cocultured with Ctr-mDCs) (Fig. 4E).

1,25(OH)$_2$D$_3$ promotes migration-competent tolerogenic DCs in the NOD mouse model

Given the tolerogenic potential that NOD-derived 1,25D$_3$-mDCs presented in vitro, we next analyzed whether these cells were able to migrate in vivo, in particular to the vicinity of the autoimmune inflammation. For this purpose, we first performed a kinetic study in which Ctr-mDCs or 1,25D$_3$-mDCs loaded with the disease-relevant Ag, BDC2.5 mimotope, were fluorescently labeled with PKH26 Red and administered i.p. in 12–14-wk-old NOD animals. Between 24 and 96 h after DC transfer, different organs (pancreas, pLN, spleen, and liver) were harvested and analyzed by FACS for the relative frequency of double-positive PKH26 Red and CD11c$^+$ cells within the CD11c$^+$ population. Of note, both Ctr-mDCs and 1,25D$_3$-mDCs were able to actively migrate in vivo toward all organs analyzed, with maximal retrieval after 72 h. Interestingly, 1,25D$_3$-mDCs migrated at a greater rate, with 2.40- and 3.99-fold higher numbers detected in the pancreas and liver, respectively, of NOD mice compared with Ctr-mDCs, 72 h after transfer (Fig. 5A).

To investigate whether the preferential migration pattern of 1,25D$_3$-mDCs toward the pancreas and liver of NOD mice was linked to the occurrence of inflammation in this area (Supplemental Fig. 4), we performed a similar in vivo migration experiment using immunoincompetent NOD.Scid mice, which lack T and B cells, as recipients. In this context, Ctr-mDCs and 1,25D$_3$-mDCs showed an altered migratory profile compared with control NOD mice, with higher numbers of either type of DC retrieved from the pancreas, but not from the liver, 72 h after transfer (Fig. 5B). Differences between the migratory profiles of Ctr-mDCs and 1,25D$_3$-mDCs toward the pancreas or liver of NOD.Scid mice were not observed.

1,25D$_3$-mDCs restrain autoreactive T cell reactivity in vivo

Given that 1,25(OH)$_2$D$_3$ generates mDCs that inhibit proinflammatory T cell responses in vitro, we speculated that these cells are suppressive upon transfer in vivo. To test this hypothesis, NOD.Scid animals were adaptively transferred with activated BDC2.5 CD4$^+$ T cells and either NOD-derived Ctr-mDCs or 1,25D$_3$-mDCs. After 72 h, dilution of eFlour 670 proliferation dye–labeled donor T cells was assessed in the pancreas, spleen, pLN, and iLN of recipient mice. Interestingly, 1,25D$_3$-mDCs significantly inhibited the proliferation of autoreactive CD4$^+$ T cells in the pLN but not in the nondraining iLN of NOD.Scid mice (Fig. 6). Low numbers of donor T cells were recovered from the pancreas and spleen of recipient mice, and values remained similar between the 1,25D$_3$-mDC and Ctr-mDC groups (data not shown).

Discussion

Therapeutic strategies to combat autoimmune diabetes ideally involve restoration of tolerance toward β-cell–derived Ags without compromising the ability of the immune system to efficiently respond to foreign threats. The capacity of DCs, especially iDCs, to dampen autoimmune reactivity in an Ag-specific manner highlights their potential as cell-based immunotherapies (24–32). However, clinical applications of DC-based therapies in autoimmune disease intervention are hampered by the concern that iDCs will develop into immunostimulatory cells upon encountering inflammatory stimuli in vivo. In this sense, we (9, 14, 18) and other investigators (6, 7, 12) showed that mDCs differentiated and matured in vitro in the presence of the biologically active form of vitamin D, 1,25(OH)$_2$D$_3$, produced a stable phenotype, typically characterized by low levels of Ag-presenting and costimulatory molecules (MHCI, CD80, CD86), as well as a high ratio of PD-L1/CD86 and anti-inflammatory cytokines (IL-10, TGF-β). In this study, we further demonstrated the capacity of 1,25(OH)$_2$D$_3$ to imprint a similar tolerogenic profile in mDCs derived from diabetes-prone mice and diabetes-resistant animals. This was evidenced by the ability of NOD-derived 1,25D$_3$-mDCs to hinder autoreactive T cell proliferation in vitro while expanding the Foxp3$^+$ Treg population from resting CD4$^+$ T cells in vitro, which was accompanied by increased intracellular production of IL-10. Furthermore, NOD-derived 1,25D$_3$-mDCs dampened the proliferation of activated CD4$^+$ T cells in vivo and showed an intact capacity to home to secondary draining lymphoid organs and pancreas as the site of inflammation.

However, exploring the capacity of 1,25(OH)$_2$D$_3$ to reproduce the characteristic tolerogenic DC phenotype in the context of a T1D setting is not a trivial matter. Several reports indicated disease-associated DC abnormalities that may compromise using the immunomodulatory action of 1,25(OH)$_2$D$_3$, on DC function for further development of autologous cell transfer therapies. For example, DCs isolated from the spleen and lymph nodes of NOD mice exhibit phenotypic and functional abnormalities (33, 34). In addition, BM-derived DCs arising from experimental T1D models exhibit a heightened proinflammatory profile, with increased levels of NF-κB, acid phosphatase, and IL-12 and low expression levels of IL-10 (35–40). These DCs are incapable of sufficiently sustaining the proliferation of Treg populations in animal models of T1D (41, 42). Furthermore, human T1D patients have fewer circulating DCs that exhibit minor dysregulated NF-κB activity, as well as phenotypic defects (43–46).

Despite such inherent disease-associated DC abnormalities that promote a hyperinflammatory DC profile, we showed that treatment with 1,25(OH)$_2$D$_3$ promoted fully functional tolerogenic
DCs from NOD animals. C57BL/6- or NOD-derived 1,25D3-mDCs featured a similar decrease in the expression of MHCII, CD80, or CD86. In addition, NOD-derived 1,25D3-mDCs were slightly less effective in preventing the maturation-induced production of proinflammatory cytokines and chemokines compared with C57BL/6-derived 1,25D3-mDCs. These discrepancies could influence the modulation of T cell responses, because the ability of DCs to activate T cells is tightly regulated (47, 48) by the engagement of the TCR by the Ag-bound MHC complex and the delivery of costimulation to T cells (49), as well as by the delivery of T cell–polarizing factors (i.e., cytokines) that determine the fate and balance among Th1, Th2, and Th17 cells and Tregs, representing a major determinant of the nature of subsequent cellular and humoral responses (50, 51). Despite these differences, NOD-derived 1,25D3-mDCs showed a potent capacity to interfere with the proliferation and activation of aggressive autoreactive BDC2.5

FIGURE 5. NOD-derived 1,25D3-mDCs exhibit an intact ability to actively migrate in vivo. (A) Ctr-mDCs (white circles) or 1,25D3-mDCs (white squares) were loaded with the BDC2.5 mimotope (10 μg/ml) for 2 h. Thereafter, DCs were fluorescently labeled with PKH26 and injected i.p. (1 x 10⁶ cells/mouse) in 12–14-wk-old adult NOD mice. At the time points indicated, the specified organs were extracted, mechanically or enzymatically processed, and analyzed by FACS for the presence of viable PKH26+CD11c+ DCs after exclusion of CD3+, CD19+, and Ly6G+ cells. Graphs show the relative frequency of PKH26+CD11c+ double-positive DCs in the total CD11c+ population. Symbols represent individual mice analyzed over at least four independent experiments. (B) Migration of Ctr-mDCs or 1,25D3-mDCs toward pancreas and liver was evaluated 72 h after DC transfer via i.p. injections into NOD or NOD.SCID mice. Data are the relative frequency of viable PKH+CD11c+ DCs in the total CD11c+ population. Symbols represent individual mice analyzed in at least three independent experiments. PKH26+CD11c+ frequencies shown for the NOD mice in (B) are the same as shown in (A). *p < 0.05, **p < 0.01.
1,25(OH)2D3 PROMOTES MIGRATION-COMPETENT TOLEROGENTIC DCs

CD4+ T cells in vitro. More importantly, these 1,25D3+ mDCs restraigned proliferation of activated CD4+ T cells in vivo in a T1D transfer model into NOD.SCID mice, which supports the notion that these 1,25D3+ mDCs are promising tools to arrest the ongoing autoimmune response in T1D.

Mechanisms of tolerance induction by DCs could include T cell silencing or deletion, as well as the induction/expansion of Tregs (52). Interestingly, decreased T cell proliferation rates in the presence of 1,25D3+ mDCs were not associated with increased levels of early and/or late apoptotic T cells in culture, as shown by van Halteren et al. (9); rather, they were associated with interference with the process of T cell division. A previous study (9) reported similar findings, supporting the notion that 1,25D3+ mDCs promote a state of T cell hyporesponsiveness in vitro. Additionally, we investigated whether NOD-derived 1,25D3+ mDCs have the ability to induce or expand Tregs, because the active compound triggered the production/expression of several Treg-promoting parameters in 1,25D3+ mDCs (e.g., high PD-L1/CD86 ratio, IL-10, TGFβ, and IDO). Our data point to the expansion of pre-existing Tregs, rather than the conversion from naive CD4+ T cells, because 1,25D3+ mDCs failed to reproduce the characteristic increase in Treg frequency in vitro following coculture with CD25-depleted CD4+ T cells. In contrast, different groups (11, 12, 53) reported that 1,25(OH)2D3-treated DCs favor the conversion of naïve CD4+ T cells to Foxp3+ Tregs or Tr1 cells. The use of different protocols involving multiple rounds of Ag stimulation and expansion may explain these discrepancies. Alternatively, other DC sources or subsets thereof may be differentially programmed by 1,25(OH)2D3 to support conversion/expansion of different Treg subsets (54).

In summary, the present results greatly expand our knowledge about the tolerogenic profile and T cell inhibitory capacity of 1,25D3+ mDCs derived from diabetes-prone NOD animals. Furthermore, we show that 1,25D3+ mDCs from NOD mice have an intact migratory capacity and successfully dampened proliferation of activated T cells in vivo. Although their features are very promising, many questions remain to be answered regarding the use of 1,25D3+ mDCs as vaccination therapy in T1D in terms of dose, frequency of administration, safety, and therapeutic efficacy. However, these questions can be definitively addressed only by assessing similar experimental information in DCs derived from T1D patients, with subsequent human clinical trials. Nevertheless, this study highlights the potential of 1,25D3+ mDCs to restore immune tolerance in an autoimmune disease setting, representing, to our knowledge, a first and essential step in the development of safe and efficient DC-based vaccination strategies in the prevention/intervention of T1D.

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Disclosures
The authors have no financial conflicts of interest.
Supplemental Figure 1. Gating strategy used and histograms for the analysis of MHCII-CD80-CD86 molecules and matching isotype controls in iDCs and Ctr-mDCs derived from C57BL/6 animals. Signal height and widths were used to exclude doublets. Dead cells were excluded by using the Fixable Live/Dead Yellow stain. Only living, single and CD11c⁺ DCs were included in the analysis.
Supplemental Figure 2. A) Morphological features of NOD- (left panels) and C57BL/6-derived (right panels) Ctr- (top) or 1,25D$_3$-mDCs (bottom) at the end of the differentiation and maturation period; B) Relative mRNA expression of cyp24a1 was analyzed by real-time quantitative PCR (RT-qPCR) in NOD or C57BL/6-derived Ctr-iDCs and Ctr- or 1,25D$_3$-mDCs. Values depicted in the bar graph represent the mean ± S.E.M of the relative mRNA levels of the cyp24a1 gene (n=3); C) The production levels of CCL2 and CCL7 were analyzed in the supernatants of NOD- or C57BL/6-derived Ctr- or 1,25D$_3$-mDC cultures. Values are represented as the mean ± S.E.M of 3 independent experiments (**p<0.01 vs. Ctr-mDCs).
Supplemental Figure 3. Gating strategy used and histograms for the analysis of MHCII-CD80-CD86 molecules and matching isotype controls in Ctr- and 1,25D3-mDCs derived from C57BL/6 animals. Signal height and widths were used to exclude doublets. Dead cells were excluded by using the Fixable Live/Dead Yellow stain. Only living, single and CD11c+ DCs were included in the analysis.
Supplemental Figure 4. H&E staining was performed on cryosections of pancreas of 12-14 week old NOD mice. Panels show the typical islet infiltrate and destruction of insulin-producing beta cells in pancreatic glands of NOD mice from our colony.