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IL-10–Modulated Human Dendritic Cells for Clinical Use: Identification of a Stable and Migratory Subset with Improved Tolerogenic Activity

Fanny Kryczanowsky,[1] Verena Raker,[1] Edith Graulich, Matthias P. Domogalla, and Kerstin Steinbrink

Dendritic cells (DCs) are key regulators of protective immune responses and tolerance to (self-)Ags. Therefore, the scientific rationale for the use of tolerogenic DC therapy in the fields of allergies, autoimmunity, and transplantation medicine is strong. In this study, we analyzed the tolerogenic capacity of IL-10–modulated DC (IL-10DC) subpopulations to identify a DC subset that combines potent immunosuppressive activities with valuable immune properties for clinical implementation. IL-10DCs consist of two phenotypically distinct subpopulations: CD83^{high}CCR7^{+} IL-10DCs and CD83^{low}CCR7^{-} IL-10DCs. Suppressor assays with activated effector T cells revealed that CD4^{+} regulatory T cells generated by CD83^{high} IL-10DCs (iTreg^{+}) exhibited a significantly higher suppressive capacity compared with CD4^{+} regulatory T cells generated by CD83^{low} IL-10DCs (iTreg^{-}). In this context, iTreg^{+} displayed a more activated phenotype (proliferation, cytokine production) compared with iTreg^{-}. In contrast to CD83^{low} IL-10DCs, CD83^{high} IL-10DCs exerted a strong migratory capacity toward the secondary lymphoid organ chemokine CCL21 and retained a functionally stable phenotype under inflammatory conditions. In addition, CD83^{high} IL-10DCs expressed significantly higher levels of surface and soluble CD25. Functional analysis demonstrated that IL-10DC–related soluble CD25 efficiently inhibited the proliferation of activated T cells and that blockade of CD25 function abolished the induction of regulatory T cells by IL-10DCs, indicating a critical role for IL-10DC–related CD25 in shifting the immune response toward an iTreg^{-} controlled tolerance reaction. In conclusion, the selective use of the CD83^{high} IL-10DC subset may result in a higher efficacy of tolerance induction in vivo and may support the development of novel DC vaccination strategies for transplantations, as well as for allergic and autoimmune diseases. ***The Journal of Immunology*****, 2016, 197: 3607–3617.**

Dendritic cells (DCs) are sentinels of the immune system and are essentially involved in the induction of protective immunity and the development and maintenance of tolerance. The function of DCs, immunogenic versus tolerogenic, is defined by distinct maturation processes and is influenced by soluble factors and interaction with immune cells during maturation (1–3).

Because of their physiological properties and the availability of clinical-grade reagents, ex vivo–generated DCs have been safely and successfully used in clinical trials aimed at stimulating an efficient immune response against tumors (4, 5). Moreover, it was demonstrated that Ag-specific tolerance in humans can be induced in vivo via vaccination with Ag-pulsed ex vivo–generated tolerogenic DCs (6, 7). The majority of clinical studies were carried out with ex vivo–generated monocyte-derived DCs, taking advantage of their plasticity. Several protocols to generate tolerogenic DCs were described using different agents. In addition to immune-modulatory drugs, including dexamethasone (8, 9), rapamycin (9–11), TGF-β (9, 11, 12), and vitamin D3 (9, 11, 13), several studies showed that human IL-10–modulated DCs (IL-10DCs) are potent inducers of induced regulatory T cells (iTregs) (9, 11, 14–16). Intriguingly, a comprehensive study revealed that IL-10DCs are superior to other in vitro–generated human tolerogenic DCs in terms of their tolerogenic properties and induction of potent iTreg-mediated immune regulation, identifying them as a promising therapeutic tool (11).

The important role of IL-10 in tolerance induction in vivo also was documented. Gregori et al. (17) identified IL-10–modulated tolerogenic DCs in peripheral blood of humans that induce type I Tregs. In addition, tumor-derived IL-10 converts immunogenic DCs into tolerogenic DCs in tumor tissues, facilitating immune escape (18). Furthermore, DCs obtained from patients with hyper-IgE syndrome caused by STAT3 mutations showed defective responses to IL-10 and, thus, displayed an impaired ability to induce iTregs, confirming the crucial role of IL-10DCs for control of immune responses in vivo (19). Thus, ex vivo–generated tolerogenic IL-10DCs might be an important therapeutic strategy to promote or restore tolerance in T cell–mediated diseases.

Recently, we identified two subpopulations of human IL-10DCs: CD83^{high}CCR7^{+} IL-10DCs and CD83^{low}CCR7^{-} IL-10DCs. In this study, we investigated the tolerogenic properties of these IL-10DC subpopulations to identify a clinical-grade IL-10DC subset

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Abbreviations used in this article: DC, dendritic cell; [^{3}H]TdR, [^{3}H]thymidine deoxyribose; iDC, immature DC; IL-10DC, IL-10–modulated DC; iTreg, induced regulatory T cell; iTreg^{+}, Treg generated by CD83^{high} IL-10DC; iTreg^{-}, Treg generated by CD83^{low} IL-10DC; mDC, mature DC; sCD25, soluble CD25; Teff, effector T cell; Treg, regulatory T cell.

that is best suited for tolerance-vaccination therapies in humans. Notably, both iTreg subpopulations were able to suppress activated effector T cells (Teffs), but CD83^{high} IL-10DCs generated significantly stronger iTreg-mediated immune suppression. Importantly, CD83^{high} IL-10DCs exhibited a stable tolerogenic and pronounced migratory phenotype under inflammatory conditions, including high CCR7 expression enabling migration to lymphatic tissues in vivo. Therefore, in contrast to CD83^{low} IL-10DCs, CD83^{high} IL-10DCs are of great interest for inducing or re-establishing immunological tolerance in different clinical settings, including allogeneic transplantation, allergies, and autoimmune diseases.

Materials and Methods

Cell isolation

Leukapheresis products and buffy coats were obtained from healthy adult volunteers with the approval of the local ethics committee of Rhineland-Palatinate.

Flow cytometry analysis

DC populations were stained with the following anti-human Abs: CD83-PE (clone HB15e; eBioscience), CD14-PE (clone M5E2; BD Biosciences), CD80-PE (clone MAB104; Beckman Coulter), PD-L1–PE (clone MIH18; eBioscience), ILT3-PE (clone ZM4.1; eBioscience), ILT4-PE (clone 42D1; eBioscience), CD25-PE (clone 4E3; BD Biosciences), anti-human HLA-DR–FITC (clone L243; BD Biosciences), and anti-human CCR7-allophycocyanin (clone 150503; R&D Systems).

Flow cytometry analysis of primed T cell populations was performed on day 5 of primary culture and 48 h after polyclonal restimulation. T cells were stained with anti-human CD4-allophycocyanin (clone RPA-T4; BD Biosciences) in combination with anti-human CD25-PE (clone 4E3; Miltenyi Biotec), CD45RO-PE (clone UCHL1; BD Biosciences), CD45RA-FITC (clone HI100; BD Biosciences), CD28-FITC (clone KOLT-2; ImmunoTools), Foxp3-PE (clone PCH101; eBioscience), ICOS-PE (clone ISA3; eBioscience), PD-1–PE (clone cBIOJ105; eBioscience), or CTLA-4-PE (clone BNI3; BD Biosciences).

IL-2–producing T cells were identified by intracellular staining with a BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). Therefore, cells were first stained with Fixable Viability Dye eFluor 780 (eBioscience), CD4-allophycocyanin (clone RPA-T4; BD Biosciences), and CD25–PE–Cy5 (clone M-A251; BD Biosciences). Subsequently, fixed cells were stained with anti-human IL-2–PE (clone N7-48A; BD Biosciences).

Generation and stability of monocyte-derived DCs

For the generation of human DCs, PBMCs were isolated using Ficoll density gradient centrifugation and incubated in 3 ml of RPMI 1640 supplemented with 1% autologous plasma for 40 min in six-well plates (Costar) at a density of 5×10^6/ml. After removal of nonadhering cells, plastic-adherent cells were cultured with IMDM (Lonza) supplemented with 400 U/ml GM-CSF (LEUKINE; Berlex Laboratories), 150 IU/ml IL-4 (ImmunoTools), and 5% autologous plasma. In some experiments, purified CD14$^+$ precursor cells were used in place of adherent cells for IL-10 DC generation. Cells were isolated using 5 μl of CD14 beads/1×10^7 PBMCs (Miltenyi Biotec) (purity > 95%) and were further cultured in a similar manner as plastic-adherent cells. At day 5, cells were collected, and 1×10^6 cells were resuspended in 3 ml of IMDM supplemented with 5% autologous plasma, 400 U/ml GM-CSF, and 150 IU/ml IL-4 for the generation of immature DCs (iDCs) or were additionally stimulated with a maturation mixture containing 5 ng/ml IL-1β, 5 ng/ml TNF-α (both from Miltenyi Biotec), 50 IU/ml IL-6 (Strathmann Biotec), and 1 μg/ml PGE_2 (Cayman Chemical) to generate mature DCs (mDCs) in six-well plates. The simultaneous (in the presence of the maturation mixture) addition of 20 ng/ml recombinant human IL-10 (CellGenix) generated tolerogenic DCs (IL-10DCs). On day 7, CD83^{high}CCR7$^+$HLA-DR^{high} and CD83^{low} CCR7$^-$HLA-DR^{low} IL-10DC subsets were separated by FACS, labeling IL-10DCs with allophycocyanin-conjugated anti-human-CCR7 mAb (R&D Systems). For migration experiments, tolerogenic DC subsets were sorted using PE-conjugated anti-human CD83 mAb (eBioscience) on IL-10DCs to avoid modulatory effects on the migratory capacity of IL-10DCs by addressing CCR7 directly. We obtained sort-purified CD83^{high}CCR7$^+$ and CD83^{low}CCR7$^-$ IL-10DC subpopulations at high purity (≥97%) and in sufficient numbers in both settings (Supplemental Fig. 1).

The stability of the phenotype was tested by stimulating 1×10^6 DCs with 5 ng/ml IL-1β, 5 ng/ml TNF-α (both from Miltenyi Biotec), or 50 IU/ml IL-6 (Strathmann Biotec) in 3 ml of DC culture medium in six-well plates. After 24 h, cells were stained for the expression of CD14, CD83, CCR7, and HLA-DR. Purity, maturation, and stability of marker expression of mDCs and IL-10DCs, as well as the purity of separated IL-10DC subsets (≥97%), were analyzed by flow cytometry.

For IL-10 titration experiments, 0.2, 2, 20, or 200 ng/ml IL-10 was added at day 5 in the presence of 400 U/ml GM-CSF, 150 IU/ml IL-4, and the maturation mixture containing 5 ng/ml IL-1β, 5 ng/ml TNF-α (both from Miltenyi Biotec), 50 IU/ml IL-6 (Strathmann Biotec), and 1 μg/ml PGE_2 (Cayman Chemical).

Migration assays

In vitro Transwell (5 μm pore) migration analysis was performed using Transwell Permeable Supports (Corning). Inserts were equilibrated, prior to use, with culture medium (Iscove's medium + 5% autologous DC serum) for ≥1 h at 37°C. The lower wells contained rCCL21 (100 ng/ml; PeproTech). A total of 2×10^5 IL-10DCs, CD83^{high} IL-10DCs, or CD83^{low} IL-10DCs was added to the upper well for 3 h (5% CO_2, 37°C). Migrated cells were transferred to flow cytometry tubes and resuspended in 150 μl of PBS/3 mM EDTA/0.5% human serum albumin. Subsequently, migrated cells in the bottom wells were counted for 60 s with high flow rate (60 μl/min) on a BD LSR II cytometer and analyzed with DIVA software. For assessment of cell viability, cells were stained with Fixable Viability Dye eFluor 780 (eBioscience) for 30 min at 4°C. The number of dead cells in the samples was determined using an LSR II cytometer and DIVA software.

Assessment of soluble CD25 in DC cultures

On day 7 of culture, DC populations were harvested and cultured for 24 h in 3 ml of IMDM supplemented with 5% autologous plasma in the absence of stimuli in six-well plates. Soluble CD25 (sCD25) secretion in supernatants was quantified by ELISA (BD Biosciences).

Isolation of resting CD4$^+$ T cells

PBMCs were isolated using Ficoll density gradient centrifugation. Thereafter, CD4$^+$CD45RA$^+$ T cells were obtained from PBMCs by depletion of CD14$^+$ CD45RO$^+$CD25^{high} cells and following enrichment of CD4$^+$ T cells via magnetic beads (MACS; Miltenyi Biotec). Purity of isolated naive T cells (≥95%) was examined by flow cytometric analysis and staining of T cells with anti-human mAbs against CD8 (Beckman Coulter), CD4/CD25 (BD Biosciences, Miltenyi Biotec), CD4/CD45RO/CD45RA (all from BD Biosciences), CD4/CD45RA/CCR7 (BD Biosciences/BD Biosciences/R&D Systems), and CD4/CD45RA/CD62L (BD Biosciences/BD Biosciences/ImmunoTools).

Primary stimulation

Naive T cells (3×10^6) were cocultured with mDCs as control (for induction of Teffs) and with each subpopulation of IL-10DCs (for induction of Tregs generated by CD83^{high} IL-10DCs [iTreg$^+$] and Tregs generated by CD83^{low} IL-10DCs [iTreg$^-$]) in 3 ml of X-VIVO 20 (Lonza) supplemented with 1% autologous plasma and 2 U/ml IL-2 (Chiron) in six-well plates. DC/T cell ratios (1:10 or 1:20) were used. In some experiments, the anti-human CD25 mAb basiliximab (20 μg/ml; Novartis) or large amounts of IL-2 (200 ng) were added during the coculture of IL-10DCs and T cells. In some experiments, preincubation of IL-10DCs with anti-human CD25 Ab (20 μg/ml) was performed for 1 h. Subsequently, DCs were washed and used for T cell coculture experiments, as described. After 5 d of coculture, T cells were rested for 3–4 d in 3 ml of medium (at a density of 1×10^6/ml). Annexin V/7- aminoactinomycin D staining was performed to assess apoptosis. A total of 1×10^5 cells was stained with Annexin V–PE and 7-aminoactinomycin D in 1× binding buffer (Apoptosis Kit; BD Biosciences) for 15 min at room temperature. Cells were washed with binding buffer and analyzed on a BD LSR II cytometer within 1 h.

MLR

Isolated naive T cells (2.5×10^4) were stimulated with mDCs and each subset of IL-10DCs at ratios of 1:10, 1:20, 1:40, and 1:80 in 200 μl X-VIVO 20 supplemented with 1% autologous plasma and 2 U/ml IL-2 (Chiron) in 96-well plates (Costar). On day 3 of coculture, primed T cells were pulsed with [^{3}H]thymidine deoxyribose ([^{3}H]TdR) for 18 h for assessment of T cell proliferation by [^{3}H]thymidine incorporation.

Restimulation assay

A total of 0.5×10^5 cells of each T cell population was restimulated for 72 h with 1 μg/ml anti-CD3 mAb + 2 μg/ml anti-CD28 mAb (BD Biosciences) in 200 μl of X-VIVO 20 (Lonza) in 96-well plates. Addition of [^{3}H]TdR for 18 h enabled the assessment of T cell proliferation by [^{3}H] thymidine incorporation.

CTLL-2 assay

On day 7 of culture, DC populations were harvested and cultured in IMDM supplemented with 5% autologous plasma in the absence of stimuli. DC supernatants containing sCD25 were collected 24 h later and incubated or not for 1 h with IL-2 (100 U/ml; Chiron). A total of 1×10^4 CTLL-2 cells was cultured with DC supernatants in a 1:1 volume ratio with CTLL-2 culture medium (RPMI

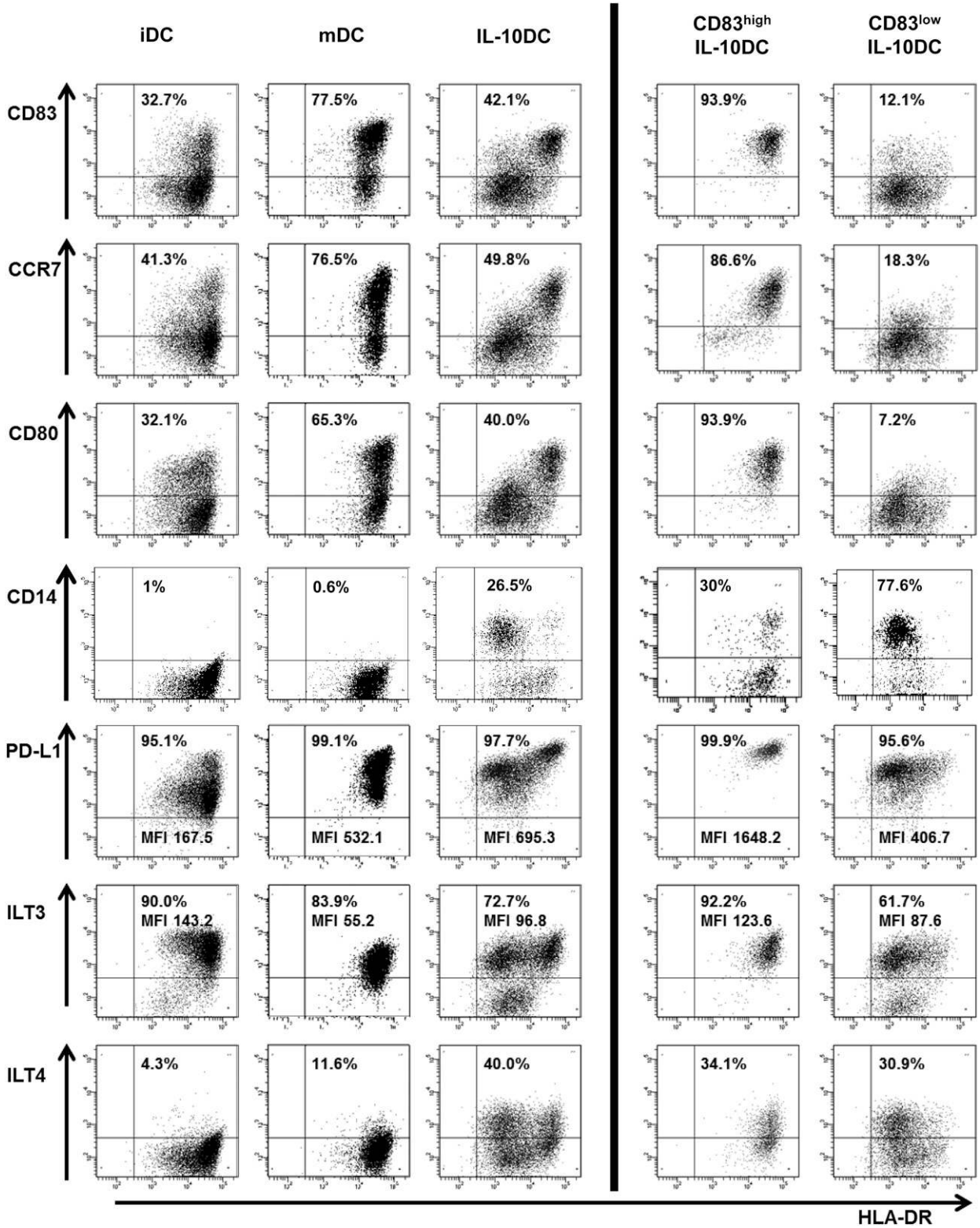

FIGURE 1. Phenotype of IL-10DC subpopulations. DC populations (iDCs, mDCs, IL-10DCs, and sort-purified CD83^{high} and CD83^{low} IL-10DCs) were harvested on day 7 of culture, and expression of surface molecules was determined by flow cytometry. One representative experiment of 5–10 independent experiments is shown.

1640 + 10% FCS + 2 mM L-glutamine) in 96-well plates in a total volume of 200 μl. Treatment of CTLL-2 cells with IMDM + 5% autologous DC plasma was used as control. After 48 h of coculture, [^{3}H]TdR was added for 18 h, and proliferation of CTLL-2 cells was assessed by [^{3}H]thymidine incorporation.

Cytokine production of primed T cell populations

Measurement of cytokine production was performed by ELISA. IFN-γ and IL-13 (both from ImmunoTools) were quantified in supernatants of primary culture on day 5, as well as in supernatants after 24 h of polyclonal restimulation.

Real-time PCR analysis

After 5 d of coculture and a resting period of 3 d, 1.5×10^6 cells of each T cell population were restimulated with 1 μg/ml anti-CD3 mAb + 2 μg/ml anti-CD28 mAb (BD Biosciences) in 1.5 ml in six-well plates, and mRNA was obtained at the indicated time points. After polyclonal restimulation, the

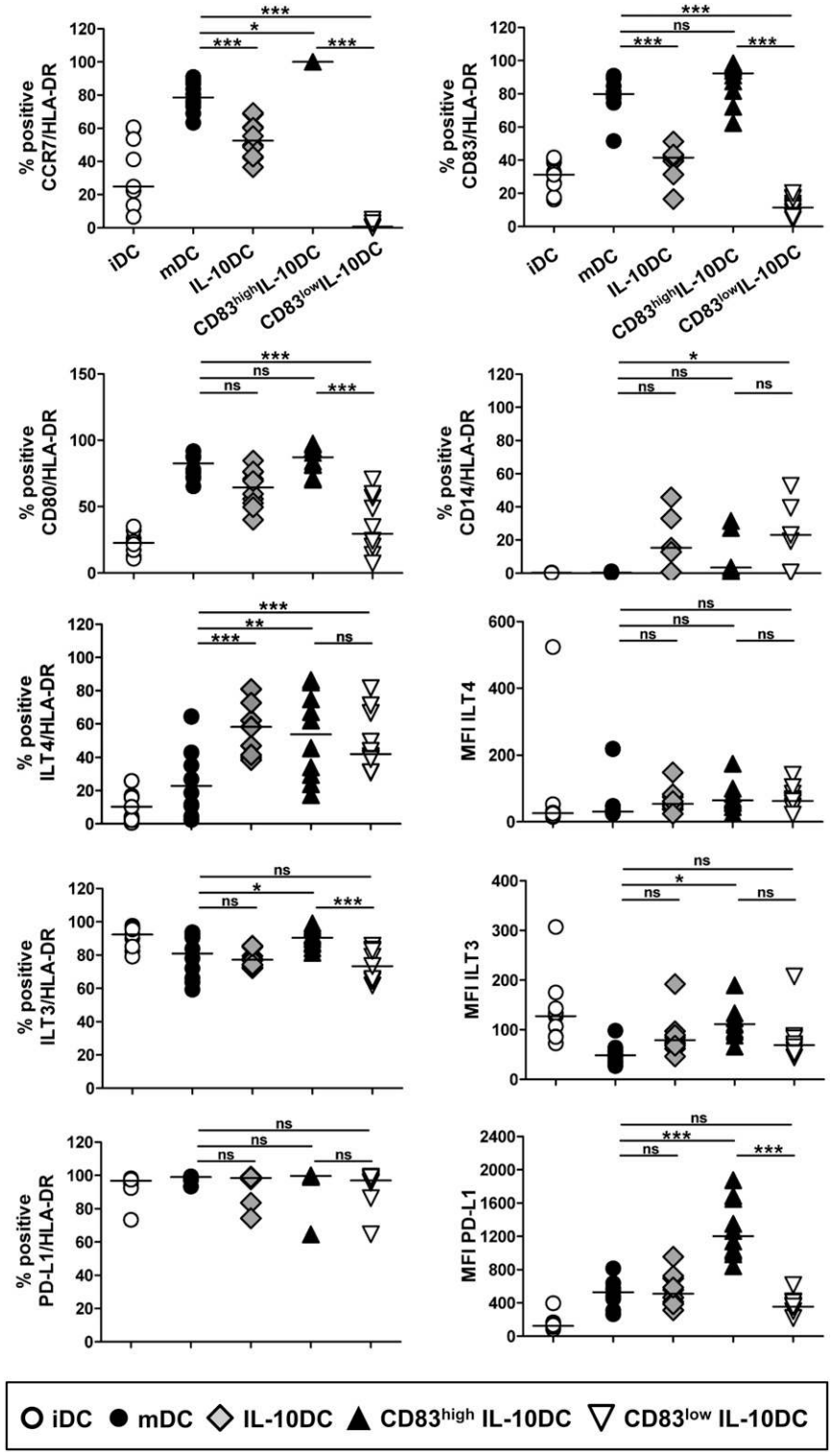

FIGURE 2. Expression of surface molecules of IL-10DC subpopulations (immature DCs [iDCs], mature DCs [mDCs], IL-10-modulated DCs [IL-10DCs], and sort-purified CD83^{high} and CD83^{low} IL-10DCs) was determined by flow cytometry on day 7 of DC culture. Pooled data of 5–10 independent experiments are presented as median of HLA-DR double positive cells and MFI (ILT3, ILT4, and PD-L1). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant.

relative expression of *IL-2* (t = 0, 5, and 24 h) and the T cell–differentiation transcription factors *T-bet*, *GATA-3*, and *Foxp3* (t = 0 and 5 h) of DC-primed T cell subpopulations was analyzed by real-time PCR (Qiagen). Data are normalized to values obtained from mDC-stimulated T cells (t = 0 h).

Suppressor assays

T cell populations were harvested on day 3 of the resting phase, washed, and stained with proliferation dyes (1 μM Cell Proliferation Dye eFluor 670 [eBioscience] for responder T cells; 0.5 μM CellTrace Violet [Life Technologies] for iTreg subsets) for 15 min (1×10^7/ml) or 20 min (1×10^6/ml), respectively, at 37°C. A total of 1×10^5 responder T cells was cocultured with 1×10^5 of each induced Treg subset (1:1) stimulated with mDCs (syngeneic to primary stimulation of IL-10DCs, DC/T cell ratio of 1:50), irradiated PBMCs (T cells 3:1 ratio) + 1 μg/ml anti-CD3 mAb, or 0.5 μg/ml anti-CD3 mAb + 0.25 μg/ml anti-CD28 mAb (BD Biosciences) in a total volume of 200 μl in 96-well plates. In some experiments, Treg/responder T cell ratios of 3:1 and 2:1 were used. For identification and gating of APCs (PBMCs, mDCs), cells were stained with 1 μM CFSE (Invitrogen) for 20 min at 37°C at a density of 1×10^7/ml. On day 5 of coculture, T cells were harvested, and proliferation of responder T cells, as well of each iTreg subset, was measured by flow cytometry and analyzed with BD DIVA software.

Statistical analysis

Statistical significance was analyzed with the paired Student *t* test or the Wilcoxon matched-pairs test (cytokine production and real-time PCR analysis of primed T cells) using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA). In cases in which multiple groups were analyzed, one-way ANOVA followed by a Tukey post hoc test for multiple comparisons, was used. The *p* values < 0.05 were considered statistically significant.

Results

Tolerogenic IL-10DCs consist of two phenotypically distinct subpopulations

Tolerogenic human IL-10DCs exhibit a strong ability to induce iTregs (11, 12, 14–17, 20). Like inflammatory mDCs that were applied successfully in vaccination studies in cancer patients, tolerogenic DCs may be used in therapeutic strategies to induce or restore immune tolerance in autoimmune and allergic diseases. Therefore, the goal of this study was the identification of the most suitable subpopulation of IL-10DCs for in vivo applications.

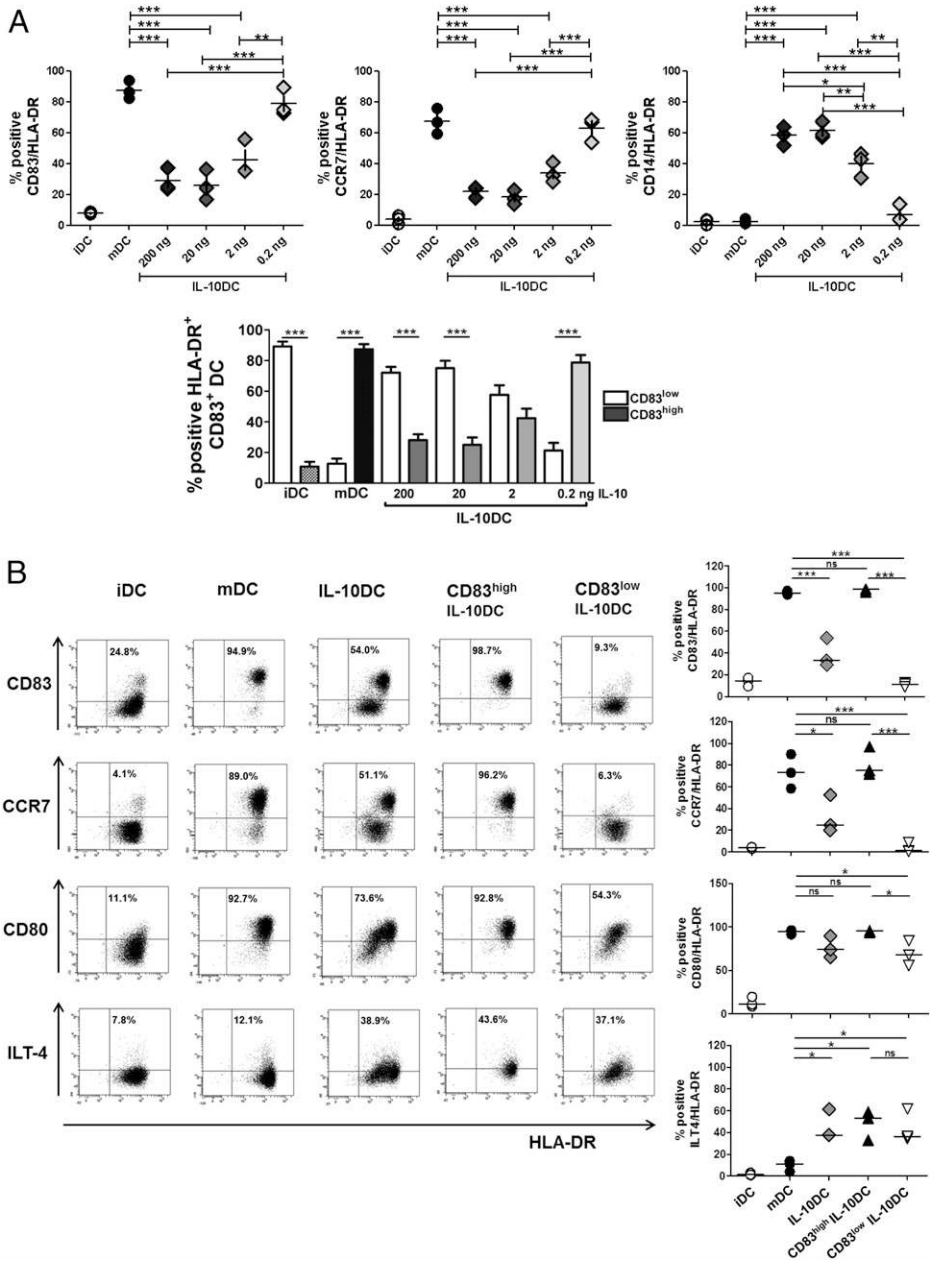

FIGURE 3. (**A** and **B**). Alteration of IL-10DC culture conditions revealed optimal IL-10 levels and identified $CD14^+$ myeloid immune cells as precursor cells. iDCs, mDCs, and IL-10DCs (matured in the presence of the indicated concentrations of IL-10) were harvested at day 7 of culture and analyzed for surface marker expression by flow cytometry. (A) Data represent the mean of three independent experiments. Percentages of HLA-DR double-positive cells (upper panels). Ratio of $CD83^{low}$ and $CD83^{high}$ DC subsets within $CD83^+HLA\text{-}DR^+$ DCs was determined (lower panel). (B) CD14 immunomagnetically enriched immune cells were isolated from PBMCs and cultured as described previously for IL-10DCs generation. At day 7 of culture, flow cytometric analysis of DC surface marker expression was performed. Results of one representative experiment (left panel) and pooled data from three independent experiments are shown as mean ± SD of HLA-DR double-positive cells (right panels). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

In contrast to inflammatory mDCs, which are characterized by increased expression of CD80, CD83, and MHC class II molecules, IL-10 induced a significant reduction in these maturation-related surface molecules and an increase in the monocyte/macrophage-specific marker CD14 in IL-10DCs (Figs. 1, 2). However, a more detailed analysis revealed the existence of two subpopulations of IL-10DCs: $CD83^{high}CCR7^{+}HLA\text{-}DR^{high}$ IL-10DCs (referred as to $CD83^{high}$ IL-10DCs) and $CD83^{low}CCR7^{-}HLA\text{-}DR^{low}$ IL-10DCs (referred as to $CD83^{low}$ IL-10DCs) (Figs. 1, 2).

To determine which IL-10DC subpopulation exhibits the more feasible properties for tolerance induction, we purified both subsets by Ab-mediated flow cytometry–based cell sorting. We took advantage of the high correlation of expression of the molecule CD83 and the chemokine receptor CCR7 on IL-10DCs, which defined the two distinct IL-10DC populations (Fig. 1, Supplemental Fig. 1). Because signaling via CD83 was shown to contribute to DC function, whereas DC-expressed CCR7 is not involved in the T cell–stimulatory function of DCs (21–23), the surface molecule CCR7 was used as target for the Ab-mediated cell sorting experiments (Fig. 1, Supplemental Fig. 1). We obtained sort-purified $CD83^{high}CCR7^{+}$ and $CD83^{low}CCR7^{-}$ IL-10DC subpopulations with high purity (≥97%) and sufficient number (Supplemental Fig. 1).

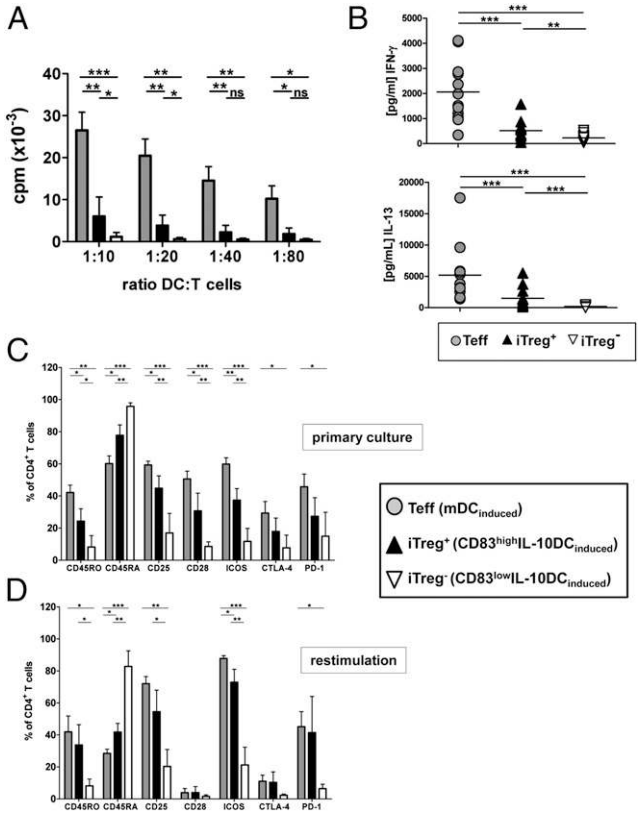

FIGURE 4. (**A–D**) Reduced T cell stimulatory capacity of IL-10DC subpopulations and phenotype of iTreg populations. (A) After 3 d of coculture, T cell populations primed by mDCs, $CD83^{high}$ IL-10DCs, or $CD83^{low}$ IL-10DCs were pulsed with [^{3}H]TdR for 18 h to assess T cell proliferation. Pooled data are shown as mean ± SEM of five independent experiments. (B) Secretion of IFN-γ and IL-13 in supernatants of cocultures was measured after 5 d by ELISA (n = 12). The indicated panel of T cell activation markers was analyzed on day 5 of primary culture (C) and at 48 h after polyclonal restimulation (D). Pooled data (mean ± SD) are from four independent experiments (C) or from three independent experiments (D). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns. not significant.

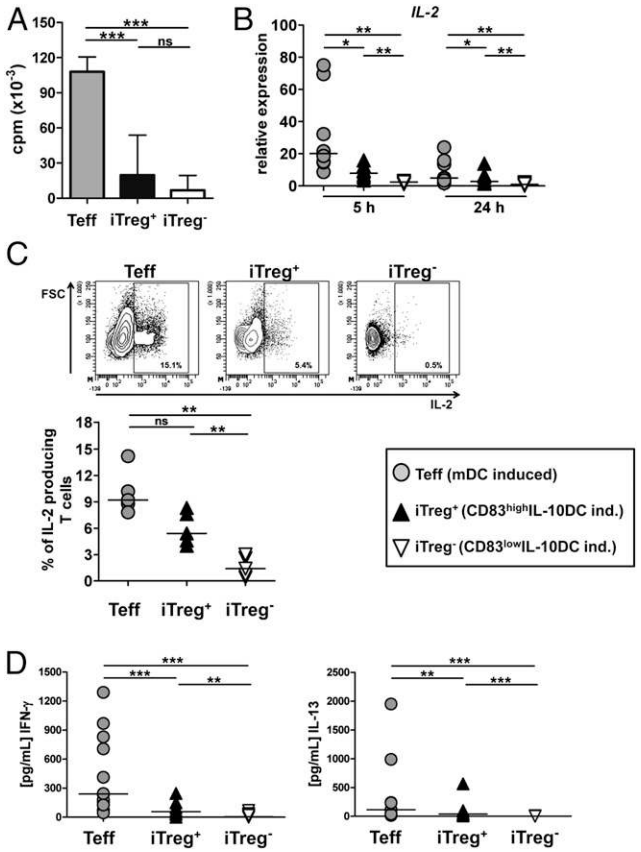

FIGURE 5. (**A–D**) Impaired activation of iTreg subsets after restimulation. After 5 d of coculture with mDCs, $CD83^{high}$ IL-10DCs, or $CD83^{low}$ IL-10DCs and a resting phase of 3 d, T cell populations were restimulated with anti-CD3/anti-CD28-mAb, as described in *Materials and Methods*. (A) T cell proliferation was measured by [^{3}H]TdR incorporation. Pooled data (mean ± SEM) from 17 independent experiments. (B) Relative expression of *IL-2* was analyzed by real-time PCR after 5 and 24 h of restimulation. Data are normalized to *IL-2* expression of mDC-stimulated Teffs (t: 0 h = 1) and are shown as median values from eight independent experiments. (C) The frequency of IL-2–producing T cells 24 h after restimulation was measured by flow cytometry. One representative experiment (upper panel) and pooled data (mean ± SD) from four independent experiments (lower panel) are shown. (D) Assessment of IFN-γ and IL-13 production in supernatants of T cell cultures (24 h) after polyclonal restimulation was performed by ELISA. Pooled data from 12 independent experiments are shown as median values. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

Analysis of the phenotype illustrated that the $CD83^{high}$ IL-10DC subset expressed maturation molecules (CD80, CD83, HLA-DR) to a similar degree as the inflammatory mDCs (Figs. 1, 2). In contrast, $CD83^{low}$ IL-10DCs exhibited a greater reduction in the expression of CD80, CD83, and HLA-DR, as observed for iDCs (Figs. 1, 2). Compared with inflammatory mDCs and iDCs, we found increased expression of the coinhibitory molecules ILT4, ILT3, PD-L1, and CD14 on both IL-10DC subsets. Notably, >90% of $CD83^{high}$ IL-10DCs expressed the chemokine receptor CCR7, which is required for DC homing to lymphoid tissues and, thereby, displayed an important feature for efficient induction of T cell tolerance by in vivo applications of tolerogenic DCs (Figs. 1, 2; Supplemental Fig. 1) (24, 25).

Titration experiments for IL-10 revealed that 20 ng (as shown in previous studies) and higher concentrations of IL-10 (200 ng) induced the typical phenotype of tolerogenic IL-10DCs with reduced CD83/CCR7 expression and increased CD14 expression

compared with mDCs (Fig. 3A, upper panels) (14–16). In contrast, generation of DCs in the presence of lower amounts of IL-10 (0.2, 2 ng) resulted in the development of mature CD83^{high}CCR7^{high} CD14^{low} DCs rather than tolerogenic IL-10DCs (Fig. 3A, upper panel). These data were reflected by similar ratios of CD83^{high} versus CD83^{low} IL-10DCs after incubation with 20 or 200 ng IL-10 (Fig. 3A, lower panel).

CD14$^+$ cells are the precursor cells of CD83^{high} and CD83^{low} IL-10DC subsets

Next, we wanted to identify the type of precursor cells of the CD83^{high} and CD83^{low} IL-10DC subsets. For this purpose, purified CD14$^+$ immune cells were used for IL-10DC generation instead of the adherent cell fraction of human PBMCs. In these experiments, we did not observe any differences in the phenotype of CD83^{high} and CD83^{low} IL-10DC subpopulations (Fig. 3B) compared with IL-10DCs generated from adherent myeloid cells (Figs. 1, 2). In addition, IL-10DCs generated from CD14$^+$ precursor cells induced the development of low-proliferating, anergic iTregs in a similar manner as shown for IL-10DCs generated from adherent cells (data not shown) (14–16). Thus, these experiments indicate that CD83^{high} and CD83^{low} IL-10DCs differentiate from CD14$^+$ precursor cells.

iTreg$^+$ display a more activated phenotype

To analyze the T cell response induced by both IL-10DC subsets, DCs were cocultured with allogeneic CD4$^+$CD45RA$^+$CD25^{low} T cells. In contrast to inflammatory mDCs as inducers of Teffs, we observed significantly diminished T cell proliferation and IFN-γ and IL-13 production by iTreg$^+$ and iTreg$^-$, reflecting a reduced efficacy in T cell activation by both IL-10DC subsets (Fig. 4A, 4B).

The expression of T cell activation/effector markers CD45RO, CD25, CD28, and ICOS, as well as of inhibitory molecules PD-1 and CTLA-4, was reduced on both iTreg subsets compared with Teffs, but significant differences were noted between the two iTreg populations after induction (Fig. 4C, Supplemental Fig. 2) and restimulation (Fig. 4D). The restimulation experiments were performed to investigate the stability of the T cell populations induced by both IL-10DC subsets (Fig. 5). Compared with Teffs, both iTreg subpopulations displayed significantly inhibited proliferation (Fig. 5A) and reduced production of IL-2 (Fig. 5B, 5C). In line with these results, impaired Th1 and Th2 responses of the iTreg subsets were reflected by significantly inhibited secretion of IFN-γ and IL-13, respectively, after restimulation (Fig. 5D). The viability of both iTreg subpopulations did not differ from Teffs, excluding an enhanced death rate of iTregs (data not shown).

In conclusion, the vast majority of parameters (proliferation, cytokine production, surface parameters) revealed significant differences between the two iTreg subsets, indicating an activated phenotype of iTreg$^+$ and a stronger T cell stimulatory capacity of CD83^{high} IL-10DCs compared with CD83^{low} IL-10DCs.

iTreg$^+$ exhibit a significantly higher suppressive activity

To analyze the suppressive activity of both iTreg subpopulations, we used a flow cytometry–based analysis combined with two fluorescent dyes for assessment of responder T cell proliferation. The experiments clearly revealed that iTreg$^+$ and iTreg$^-$ exhibited a strong capacity to inhibit the proliferation of responder T cells (Fig. 6A, 6B), indicating a potent suppressive activity (Fig. 6C). This regulatory function was shown for different stimuli (syngeneic mDCs, PBMCs + anti-CD3 mAb, anti-CD3 mAb + anti-CD28 mAb) (Fig. 6A–C). Notably, iTreg$^+$ exhibited significantly enhanced suppressive activity compared with iTreg$^-$, as shown for a Treg/Teff ratio of 1:1 (Fig. 6A–C), as well as for increasing ratios (2:1 and 3:1) (Supplemental Fig. 3). These data identify CD83^{high} IL-10DCs as promising candidates for in vivo vaccinations studies.

To investigate the induction of Foxp3$^+$ Tregs by IL-10DCs, Foxp3 expression of iTregs was analyzed. We found an impaired (iTreg$^-$) or similar (iTreg$^+$) percentage of Foxp3$^+$ T cells compared with Teffs (Supplemental Fig. 4). Foxp3 expression is also induced during activation of human Teffs, but to a lesser extent, as seen in Foxp3$^+$ Tregs (26). Therefore, these data identified Foxp3 expression as a T cell–activation marker rather than a Treg indicator, which excluded the generation of Foxp3$^+$ Tregs by IL-10DCs.

Stability of the tolerogenic phenotype of CD83^{high} IL-10DCs under inflammatory conditions

Tolerogenic DC used for vaccination studies in humans should exhibit a stable phenotype because, upon challenge with pathogens or proinflammatory factors, they might further differentiate into immunogenic DCs, thereby augmenting, rather than preventing, immune responses. We tested the effect of an inflammatory

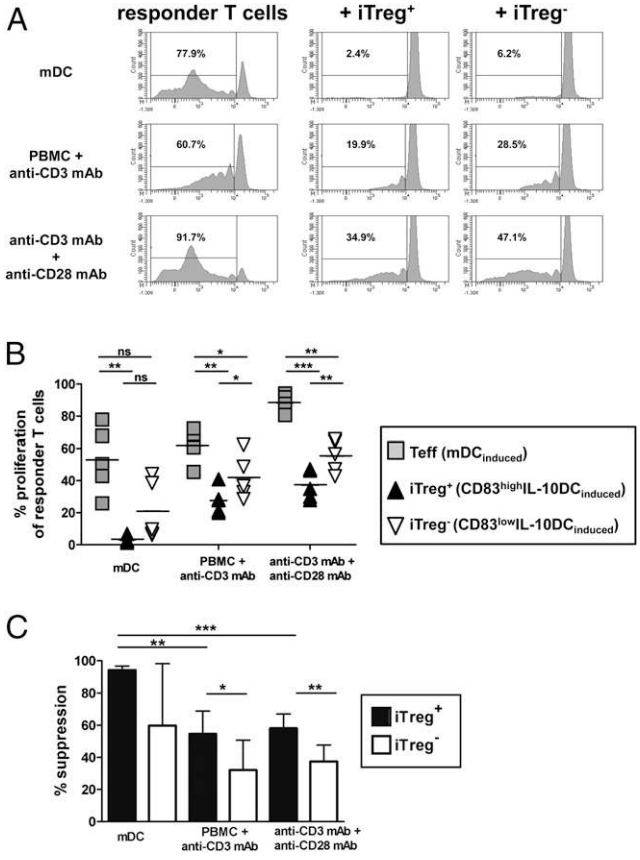

FIGURE 6. (**A**–**C**) Stronger suppressive capacity of iTregs induced by CD83^{high} IL-10DCs. iTreg subpopulations were cocultured with CD4$^+$ CD25$^-$ responder T cells after staining with proliferation dyes (iTregs were stained with CellTrace Violet, responder T cells were stained with Cell Proliferation Dye eFluor 670). mDCs (syngeneic to primary stimulation), PBMCs + 1 μg/ml anti-CD3 mAb, or 0.5 μg/ml anti-CD3 mAb + 0.25 μg/ml anti-CD28 mAb were used for restimulation. On day 5, proliferation of responder T cells was measured by flow cytometry. (**A**) Proliferation of responder T cells (percentage). One representative of five independent experiments is shown. (**B**) Pooled data (mean ± SD) from five independent experiments. (**C**) Suppressive capacity of both iTreg subpopulations (mean ± SD) from five independent experiments. Percentage of suppression was normalized to proliferation of responder T cells in the absence of iTreg (0% suppression). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significant.

environment by use of the proinflammatory cytokines IL-1β, IL-6, and TNF after generation and sorting of both IL-10DC subsets. Flow cytometric analysis revealed that CD83^{high} IL-10DCs exhibited a stable phenotype, as demonstrated by unaltered CD83/HLA-DR, CCR7/HLA-DR, and CD14/HLA-DR expression (Fig. 7A–C). In contrast, the percentage of CD83/HLA-DR double-positive cells was enhanced in unseparated IL-10DCs, CD83^{low} IL-10DCs, and iDCs (control) (Fig. 7A), revealing a maturation within the inflammatory environment induced by the cytokine mixture.

High migratory capacity of CD83^{high} IL-10DCs

Induction of tolerance processes, including modulation of the T cell response and induction of Tregs, is an important feature of therapeutic vaccination studies with tolerogenic DCs in vivo. Therefore, homing of tolerogenic IL-10DC subsets to secondary lymphoid tissues is required after, for example, i.v. or s.c. applications. Secretion of chemokines CCL19/21 by lymph node cells is an important mechanism to induce directed migration of immune cells, such as DCs, to secondary lymphoid organs (25, 27). Therefore, we tested the migration of nonseparated IL-10DCs, CD83^{high} IL-10DCs, and CD83^{low} IL-10DCs toward CCL21 (Fig. 7D). These experiments revealed that CD83^{high} IL-10DCs exhibited a significantly increased directed migration compared with IL-10DCs and, in particular, with CD83^{low} IL-10DCs lacking a relevant migratory capacity, identifying CD83^{high} IL-10DCs as good candidates for in vivo applications (Fig. 7D). Analysis excluded a decreased viability of the CD83^{low} IL-10DC subset (data not shown).

Novel mechanism for tolerogenic functions: detection of CD25 and sCD25 in tolerogenic DC cultures

Despite differences in maturation status, both IL-10DC subpopulations were potent inducers of iTregs with regulatory properties. However, iTreg^{+} displayed a more potent suppressive activity compared with iTreg^{-}, as described above. As demonstrated previously, inflammatory mDCs expressed the α-chain of IL-2R (CD25) (Fig. 8A, 8B) (28–30). Notably, compared with inflammatory mDCs, we found a significantly higher expression of CD25 on nonseparated IL-10DCs, as well as on CD83^{high} IL-10DCs, but not on CD83^{low} IL-10DCs, and significant differences between CD83^{high} and CD83^{low} IL-10DCs (Fig. 8A, 8B). Furthermore, we assessed the secretion of sCD25 in the supernatants of DC populations (Fig. 8C). These experiments revealed a 2-fold greater secretion by IL-10DCs and, a 12-fold greater secretion by the CD83^{high} IL-10DC subset compared with mDCs and CD83^{low} IL-10DCs.

Functional analysis using the IL-2–dependent CTLL-2 cell line revealed that sCD25 in the supernatants of IL-10DCs significantly inhibited IL-2–mediated T cell proliferation more strongly than did the supernatants from inflammatory mDCs. Thus, these results suggest that neutralization of IL-2 is a potential mechanism through which tolerogenic IL-10DCs block activation of Teffs (Fig. 8D). Furthermore, we tested the function of IL-10DC–related CD25 on iTreg induction by use of an anti-human CD25 mAb (31, 32). For this purpose, we incubated IL-10DCs with the anti-CD25 mAb or performed priming experiments on IL-10DCs with naive T cells in the presence of the anti-CD25 mAb (Fig. 8E). In addition, we

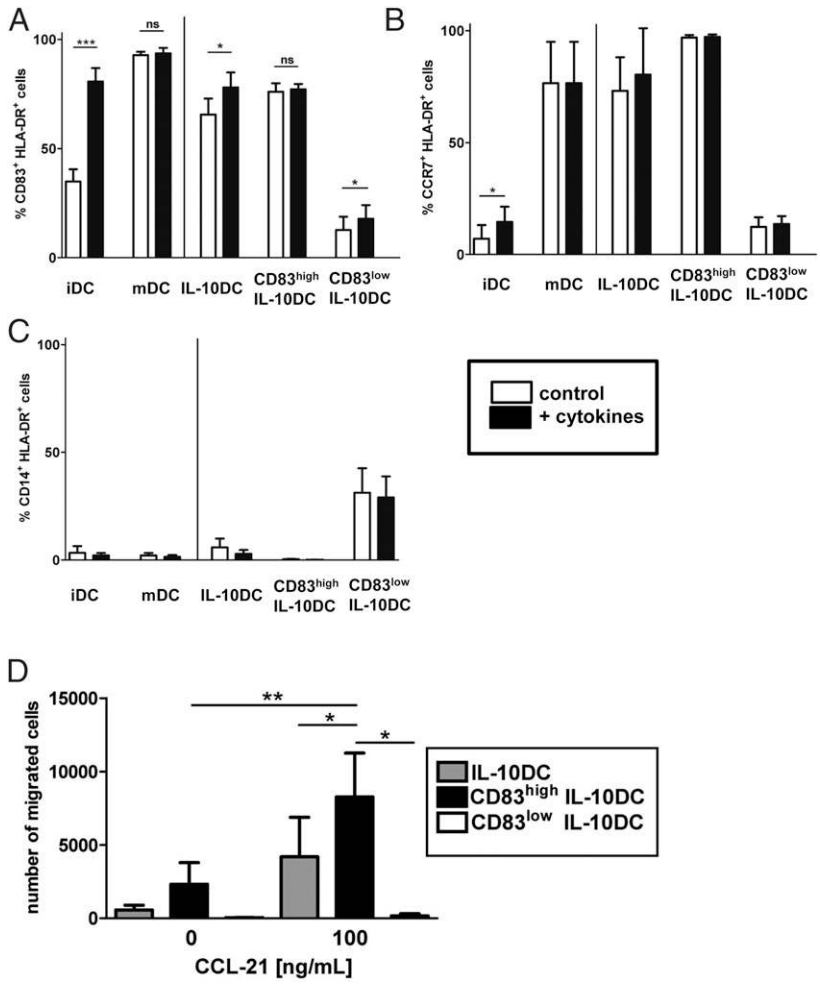

FIGURE 7. (**A**–**D**) CD83^{high} IL-10DCs exhibited a stable phenotype under inflammatory conditions, as well as a significantly greater migratory capacity toward CCL21 compared with IL-10DCs and CD83^{low} IL-10DCs. IL-10DCs were harvested on day 7 of culture and purified by flow cytometry–based sorting into CD83^{high} and CD83^{low} IL-10DCs. iDCs and mDCs served as controls. (A–C) After an additional incubation with TNF, IL-1β, and IL-6 for 24 h, expression of surface molecules was determined by flow cytometry. Percentages of HLA-DR double-positive cells are shown. Pooled data (mean ± SD) from four independent experiments. (D) The migratory capacity of IL-10DCs and sort-purified CD83^{high} and CD83^{low} IL-10DCs was analyzed in CCL21-directed migration assays, as described in *Materials and Methods*. Pooled data (mean ± SD) from four independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

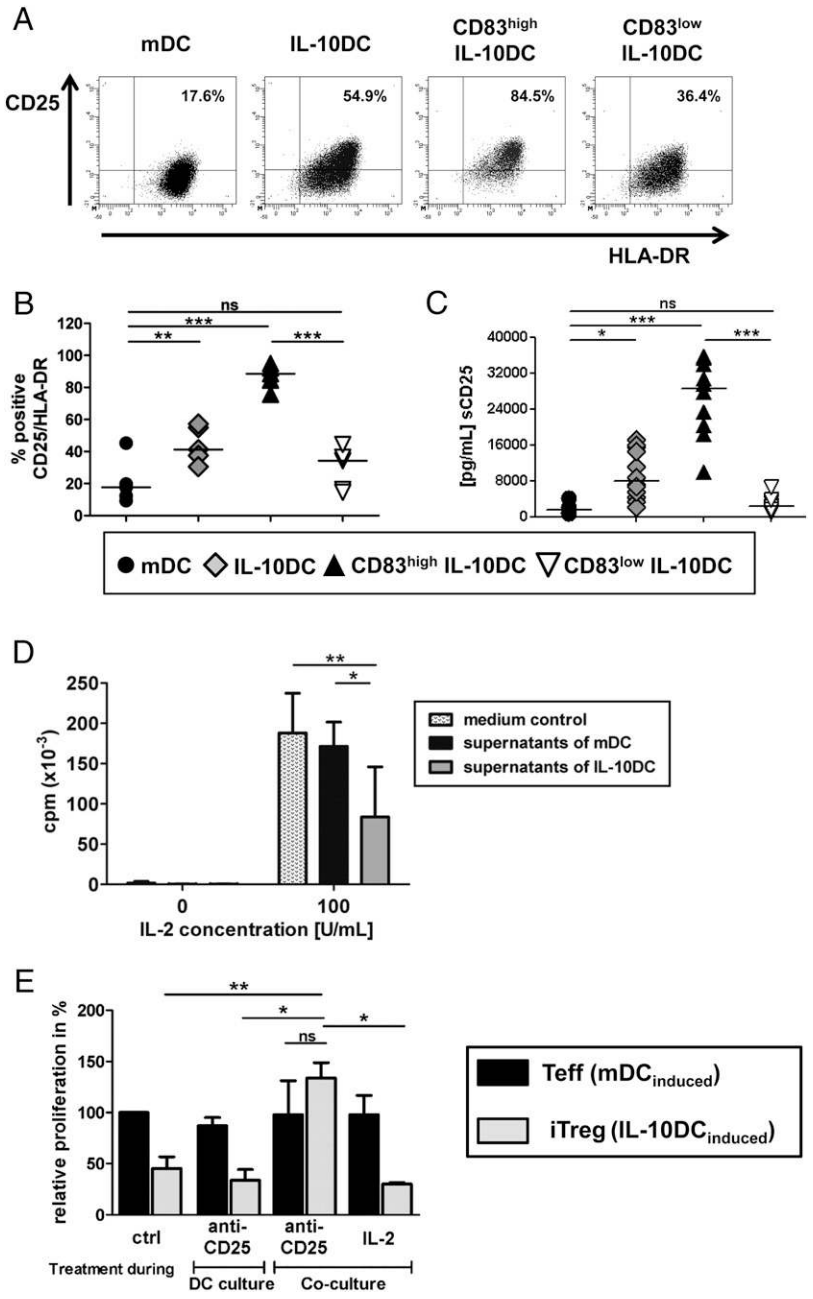

FIGURE 8. (**A**–**D**) Enhanced expression of surface CD25 and sCD25 by CD83^{high} IL-10DCs. On day 7 of DC culture, expression of the α-chain of IL-2R (CD25) on mDCs, IL-10DCs, and CD83^{high} and CD83^{low} IL-10DC subsets was analyzed by flow cytometry. (A) One representative of five independent experiments is shown. Percentages of CD25/HLA-DR double-positive cells are demonstrated. (B) Pooled data (median ± SD) from five independent experiments. (C) On day 7 of culture, DC populations were harvested and cultured for an additional 24 h. Detection of sCD25 in supernatants was performed by ELISA. Pooled data as median ± SD from four to nine independent experiments. (D) DC supernatants were incubated without or with IL-2 (100 U/ml) for 1 h. The IL-2–dependent CTLL-2 cell line was cultured or not with DC supernatants for 48 h in the presence of IL-2, as indicated, and pulsed with [^{3}H]TdR for 18 h. CTLL-2 proliferation was assessed by [^{3}H]TdR incorporation. Pooled data (mean ± SEM) from three independent experiments. (**E**) IL-10DCs were incubated with the anti-human CD25 mAb basiliximab and subsequently cocultured with naive T cells for iTreg induction. In addition, coculture experiments were performed in the presence of the anti-CD25 mAb or high amounts of IL-2 (200 U/ml). mDCs served as controls for Teff stimulation. After restimulation, T cell proliferation of Teffs (induced by mDC) and iTregs (induced by IL-10DCs) was assessed by [^{3}H]TdR incorporation. Pooled data (mean ± SD, normalized to Teff proliferation = 100%) from three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

analyzed the impact of IL-2 on the induction of iTregs by IL-10DCs by adding high doses of IL-2 (Fig. 8E). Incubation of IL-10DCs with the anti-CD25 mAb or addition of IL-2 did not affect the induction of anergic iTregs by IL-10DCs, as demonstrated after restimulation compared with mDC-generated Teffs (Fig. 8E). However, functional blocking of CD25 during coculture experiments significantly abrogated the induction of anergic iTregs by IL-10DCs compared with effector T cells and with iTregs (iTregs generated in the absence of anti-CD25 mAb, by anti-CD25 mAb–preincubated DCs or in the presence of IL-2) (Fig. 8E). In conclusion, these results indicate that IL-10–related sCD25 can block the activation of Teffs by IL-2 neutralization and that functional blockade of CD25 results in inhibition of iTreg induction by IL-10DCs, thereby shifting the T cell ratio toward iTreg-mediated immunosuppression.

Discussion

In this study, the tolerogenic properties of CD83^{high} IL-10DC and CD83^{low} IL-10DC subsets were investigated to identify the IL-10DC subpopulation that is better suited for tolerance-vaccination therapies in vivo. CD83^{high} IL-10DCs exhibited a stable phenotype in the presence of additional proinflammatory stimuli, including high CCR7 expression, and displayed a significantly higher migratory capacity, enabling migration to secondary lymphoid organs. In addition, functional experiments revealed that CD83^{high} IL-10DC–induced iTregs possessed a significantly stronger suppressive capacity. Therefore, CD83^{high} IL-10DCs may be excellent candidates for tolerance-inducing vaccination studies in vivo.

Most of our knowledge about using tolerogenic DCs, including IL-10DCs, for the treatment of autoimmune and allergic diseases or transplant rejections is derived from extensive work in animal models or in vitro studies (33); however, translation into humans is conceptually and technically possible. Application of inflammatory mDCs in clinical trials in cancer patients demonstrated that DC vaccination in humans is generally well tolerated and has minimal side effects (4). Moreover, vaccination studies with

monocyte-derived iDCs revealed the induction of Ag-specific tolerance by generation of $CD8^+$ Tregs and inhibition of Teff responses in vivo (6, 7). Transfer of immature tolerogenic DCs into healthy volunteers, genetically engineered tolerogenic DCs into diabetic patients, or peptide-loaded DCs into patients with rheumatic arthritis in phase I studies was well tolerated (6, 7, 34, 35).

Common features of most tolerogenic DCs are an immature phenotype with low expression of costimulatory molecules and chemokine receptors and reduced secretion of proinflammatory cytokines, including IL-12 and IL-23, as inductors of Th1/Tc1/Th17 immune responses (33). In our study, the $CD83^{low}$ IL-10DC subset exhibited these features of tolerogenic DCs, whereas the $CD83^{high}$ IL-10DC subpopulation exhibited high levels of CD80, CD86, and HLA-DR, comparable to inflammatory mDCs. However, $CD83^{high}$ IL-10DCs did not produce functionally relevant IL-12/IL-23 (data not shown); they expressed coinhibitory molecules, such as ILT3/4, which were reported to contribute to IL-10DC–mediated tolerance (17).

Gregori et al. (17) demonstrated the existence of IL-10DCs in vitro that induce type I Tregs as a result of high IL-10 secretion and IL-10–dependent ILT4/HLA-G signaling of IL-10DCs. In contrast, we did not detect any HLA-G expression on IL-10DCs and did not observe functionally relevant IL-10 secretion by IL-10DCs (data not shown). These differences may result from differences in the DC culture protocols. In this study, we added IL-10 only for the last 2 d of the maturation step, whereas other groups described generation of DCs in the presence of IL-10 during the entire culture period (17, 36, 37).

A relevant criterion for the identification of tolerogenic DCs for vaccination studies is their resistance to inflammatory stimuli in vivo, which can occur in the setting of autoimmunity, allergy, or transplant rejection. Therefore, we established a protocol for IL-10DC generation by incubation of iDCs with IL-10 in the presence of a maturation mixture to generate a stable phenotype of tolerogenic IL-10DCs (14). Moreover, we found that $CD83^{high}$ IL-10DCs were highly resistant to additional proinflammatory stimuli after removal of IL-10, reflecting the state after application of tolerogenic DCs in vivo.

Notably, the study by Boks et al. (11) clearly demonstrated that IL-10DCs exerted optimal properties for tolerance induction compared with all other tested in vitro–generated human tolerogenic DC populations. Particularly, they found that IL-10DC–induced iTregs exhibited the most efficient capacity to inhibit Teff responses. Intriguingly, in this study we significantly improved the tolerogenic properties of IL-10DCs by sort purifying $CD83^{high}$ IL-10DCs as generators of iTregs with significantly stronger regulatory functions compared with $CD83^{low}$ IL-10DCs. In addition, $CD83^{high}$ IL-10DCs exhibited highly efficient migration toward the lymphoid organ chemokine CCL21, which is absolutely required for induction of efficient T cell tolerance in vivo. Expression of CCR7 varied between the different tolerogenic DC populations (dexamethasone, vitamin D3: ~10%, TGF-β: ~15%, rapamycin: ~50%), but it never reached high expression on $CD83^{high}CCR7^+$ IL-10DCs (85–95%), resulting in an optimal migratory capacity of $CD83^{high}$ IL-10DCs.

Because of their different phenotypes, both IL-10DC subsets seem to use several mechanisms to exert their tolerogenic functions. Notably, the $CD83^{high}$ IL-10DC subset exhibited up to a 4-fold increased expression of CD25 and a 12-fold increased secretion of sCD25. The function of CD25 with regard to tolerogenic IL-10DCs is not completely understood. On mDCs, the expression of membrane-bound CD25 is reported to be involved in IL-2 *trans*-presentation to stimulate $CD25^-$ (naive) T cells (29). In contrast, it was reported that supernatants of DCs containing increased levels of sCD25 induced a diminished T cell response by blockade of IL-2 (38). In agreement with these data, we found that IL-10DC–related sCD25 abrogated the IL-2–dependent proliferation and activation of Teffs. Intriguingly, functional blocking of CD25 by use of an anti-CD25 Ab revealed an abrogation of iTreg induction by IL-10DCs (31, 32) These data suggest that IL-10DC–related CD25 critically contributes to tolerance induction by skewing the balance between activated Teffs/Tregs toward iTreg-controlled immunosuppression.

This study revealed that $CD83^{high}$ IL-10DCs may be promising candidates for induction or restoration of tolerance in vivo because of their stable phenotype under inflammatory conditions, their pronounced migration to secondary lymphatic organs, and their ability to induce highly potent iTregs. The scientific rationale for implementation of tolerogenic DC therapy to induce and promote tolerance in humans is strong. Evaluation of technical and therapeutic logistical issues is an important next step before tolerogenic DCs can be used in clinical studies. In this context, further evaluations to optimize vaccinations strategies with tolerogenic IL-10DCs may lead to improved tolerance-promoting regimens in the context of allergy, autoimmune disease, and transplant rejection.

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Disclosures

The authors have no financial conflicts of interest.

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