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*J Immunol* 2015; 194:5282-5293; Prepublished online 27 April 2015; doi: 10.4049/jimmunol.1402827

http://www.jimmunol.org/content/194/11/5282

Supplementary Material

http://www.jimmunol.org/content/suppl/2015/04/25/jimmunol.1402827.DCSupplemental

References

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Allogeneic Mature Human Dendritic Cells Generate Superior Alloreactive Regulatory T Cells in the Presence of IL-15

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Expansion of Ag-specific naturally occurring regulatory T cells (nTregs) is required to obtain sufficient numbers of cells for cellular immunotherapy. In this study, different allogeneic stimuli were studied for their capacity to generate functional alloantigen-specific nTregs. A highly enriched nTreg fraction (CD4+CD25+CD127low) T cells) was alloantigen-specific expanded using HLA-mismatched immature, mature monocyte-derived dendritic cells (moDCs), or PBMCs. The allogeneic mature moDC-expanded nTregs were fully characterized by analysis of the demethylation status within the Treg-specific demethylation region of the FOXP3 gene and the expression of both protein and mRNA of FOXP3, HELIOS, CTLA4, and cytokines. In addition, the Ag-specific suppressive capacity of these expanded nTregs was tested. Allogeneic mature moDCs and skin-derived DCs were superior in inducing nTreg expansion compared with immature moDCs or PBMCs in an HLA-DR– and CD80/CD86-dependent way. Remarkably, the presence of exogenous IL-15 without IL-2 could facilitate optimal mature moDC-induced nTreg expansion. Allogeneic mature moDC-expanded nTregs were at low ratios (<1:320), potent suppressors of alloantigen-induced proliferation without significant suppression of completely HLA-mismatched, Ag-induced proliferation. Mature moDC-expanded nTregs were highly demethylated at the Treg-specific demethylation region within the FOXP3 gene and highly expressed of FOXP3, HELIOS, and CTLA4. A minority of the expanded nTregs produced IL-10, IL-2, IFN-γ, and TNF-α, but few IL-17–producing nTregs were found. Next-generation sequencing of mRNA of moDC-expanded nTregs revealed a strong induction of Treg-associated mRNAs. Human allogeneic mature moDCs are highly efficient stimulator cells, in the presence of exogenous IL-15, for expansion of stable alloantigen-specific nTregs with superior suppressive function. The Journal of Immunology, 2015, 194: 5282–5293.

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D4+ regulatory T cells (Tregs) are important in controlling autoimmune diseases and are key players in the countermechanism for immune responses by exerting suppressive function either by direct cell–cell contact or soluble factors. Tregs can either originate directly from the thymus, that is, thymic-derived, naturally occurring Tregs (nTregs), or develop in the periphery, termed induced Tregs (1). Stable expression of FOXP3 is key to the suppressive function of Tregs (2). This is achieved by constitutive demethylation of the Treg-specific demethylation region (TSDR) in the promoter of the FOXP3 gene and this is characteristic for nTregs. For this reason, nTregs have a more stable suppressor function than induced Tregs and constitute a suitable candidate cell population for immunotherapy.

Recent studies have shown the therapeutic potential of Tregs given as cellular immunotherapy to control unwanted immune responses such as autoimmune diabetes mellitus (3–5) or graft-versus-host disease (6–8). Data from transplantation models using animals have also shown the efficacy of Tregs in controlling the alloreactive immune response, thereby preventing or delaying allograft rejection. Moreover, in a humanized mouse skin transplant model, alloantigen-specific Tregs were superior in controlling skin rejection compared with polyclonal-expanded Tregs (9, 10).

Thymic-derived (n)Tregs can be efficiently isolated from PBMCs via FACS using their cell-surface characteristics, that is, CD3+, CD4+, CD25bright, and CD127low/− (11). These Tregs reveal a highly demethylated TSDR within the FOXP3 gene and show a high expression of FOXP3 (11). However, nTregs represent only a minor (5–10%) population of peripheral CD4+ T cells, and large numbers of nTregs are needed for clinical applications. The Tregs proposed for therapeutic trials rely on anti-CD25 microbead-isolated Tregs, which may contain substantial numbers of effector T cells (12, 13). Efficient in vitro expansion protocols generating large numbers of polyclonal Tregs have been developed using anti-CD3– and anti-CD28–coated beads and a high dose of IL-2 (14, 15). Addition of rapamycin to this cell culture is used to diminish outgrowth of the contaminating effector T cells (16, 17). Alloantigen-specific Tregs with a much greater specificity can be generated by expansion in the presence of allogeneic PBMCs (18, 19) or B cells (20, 21). The potential...
benefit of these alloantigen-specific Tregs is targeted suppression rather than general immunosuppression and increased suppressive potency (9, 21–26). This may translate in fewer Tregs needed to have a therapeutic effect in the patient.

Mature dendritic cells (DCs) are considered to be the most potent stimulator cells for allogeneic effector T cells, but relatively little is known about their capacity to support proliferation of stable alloantigen-specific nTregs in comparison with standard PBMCs. Results from animal studies indicated that DCs can be used to expand alloreactive Tregs (27–29). Human immature DCs can stimulate the expansion of Tregs (19), but some degree of maturation leading to semimature DCs (30) yielded better results. In all protocols, there is a need for exogenous IL-2 to obtain optimal Treg expansion, but the requirements for exogenous cytokines may be different when mature monocye-derived DCs (moDCs) are used as stimulator cells (31). In this study, we show that human allogeneic mature moDCs are superior stimulator cells to generate very potent alloantigen-specific nTregs in the presence of exogenous IL-15 only.

Materials and Methods

Blood samples

PBMCs were isolated from healthy donor-derived buffy coats obtained from the local blood bank (Sanquin Blood Bank, Rotterdam, The Netherlands), as described previously in detail (32). This study did not require approval from any ethical committee according to the Dutch Medical Research Involving Human Subjects Act (WMO). It was conducted according to the principles of the Declaration of Helsinki and in compliance with Internationa Conference on Harmonization/Good Clinical Practice regulations.

Cell sorting experiments

PBMCs were enriched for T lymphocytes using the PanT cell isolation kit (Miltenyi, Bergisch Gladbach, Germany). Enriched T lymphocytes were prepared for sorting experiments using the following mAbs: Anti-CD14-labeled CD3 (BD, Erembodegem, Belgium), Pacific blue-labeled CD4 (BD Pharmingen, Erembodegem, Belgium), PeCy7-labeled CD25 (epitope A; BD), PE-labeled CD127 (BD Pharmingen), and inclusion of a live-dead marker ViaProbe (7- amino-actinomycin D; BD Pharmingen). After staining, the cells were washed and resuspended at 20–25 × 10^6/ml and sorted (BD FACSAria II SORP; BD) into Tregs (CD3^-CD4^-CD25^+CD127^+ and T effector cells depleted for Tregs (CD3^+Teff consisting of both CD4^- T cells depleted for Tregs and CD8^- T cells) (11). The isolated fractions were tested for purity and were used immediately, after an overnight rest at 37˚C in standard culture medium containing 20% of heat-inactivated pooled human serum, or frozen until further use in standard medium containing a final concentration of 10% of DMSO. The purities amounted to 60 ± 0.4% and 98.5 ± 0.7% for Tregs (4.3 ± 0.1% of CD4^- T cells in the presort sample) and T cells depleted of Tregs, respectively. The enriched Treg fraction was >90% FOXP3^+ with a >95% demethylated TSDR in the FOXP3^+ gene for male donors (11) and >45% for female donors because of the position of the FOXP3 gene on the X chromosome. Typically, on average, 1 × 10^6 nTregs could be sorted from 10 ml freshly drawn peripheral blood of a healthy donor, representing >75% recovery of the total nTreg population within the blood sample.

Preparation of allogeneic stimulators

Allogeneic PBMCs were either used immediately as stimulus to expand nTregs as described later or further enriched for monocytes using magnetic cell sorting and culture of moDCs. In brief, monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. FACs analysis revealed a purity of >95% of the isolated fraction (data not shown). MoDC differentiation was performed as described in detail previously (33). In brief, monocytes were cultured for 6 d in the presence of IL-4 (40 ng/ml; Peprotech, London, U.K.) and GM-CSF (50 ng/ml; Peprotech) at 37˚C in a humidified atmosphere containing 5% CO2. After 6 d of culture, immature moDCs were either used immediately to expand Tregs or maturation was induced in 48 h, by adding a mixture of the following cytokines: TNF-a (20 ng/ml; Tebu-bio), IL-1β (10 ng/ml; Tebu-bio), IL-6 (10 ng/ml; Tebu-bio), and PGE2 (1 μg/ml Sigma–Aldrich; Biolegend Europe BV, Uithoorn, The Netherlands). The differentiation of monocytes to immature moDCs as well as maturation of moDCs was evaluated by staining the immature and mature moDCs using mAbs directed against CD14 (BD Pharmingen), HLA-DR (BD), CD40 (BD Pharmingen), CD80 (BD Pharmingen), CD86 (BD Pharmingen), and CD83 (Serotec; Kidlington, Oxford, U.K.). Samples were measured on the FACSCanto II (BD) and analyzed using FACSdiva software version 6.1.2. (BD), and mature moDCs typically consisted of a (uniform) population of CD14^+, HLA-DR^+, CD40^+, CD80^+, and CD83^+ cells as described previously (34).

Alloregeneic skin DC isolation

Human skin was obtained from patients undergoing corrective breast or abdominal plastic surgery at the Erasmus Medical Center (Rotterdam, The Netherlands). Skin-derived DCs were allowed to migrate out of skin biopsies during 48 h and subsequently harvested as previously described by de Grajil et al. (35). Numbers of skin-derived DCs varied from 0.5 to 1.5 × 10^6/explant (data from three to five independent experiments). These skin-derived DCs were immunophenotyped using mAbs directed against CD1a (eBioscience, Vienna, Austria), HLA-DR (BD), and CD83 (Serotec). Skin-derived DCs, consisting of CD1a^-, HLA-DR^-, and CD83-expressing cells, were compared with monocytes with respect to cytokine production and alloreactive nTregs as described later. For this purpose, the harvested skin-derived cells, among which are also non-DCs, were irradiated (30 Gy) to prevent outgrowth of these non-DCs (i.e., lymphocytes).

Expansion of sorted fractions

Tregs were expanded either in an allogenic (2.5 × 10^6/well) or polyclonal manner (1 × 10^6/well) for 11 d. In brief, optimal conditions for allogeneic nTreg expansion were determined by performing kinetics, varying ratios of APCs, that is, 4:1 (only for allogeneic PBMC), 1:1, 1:1:10, and 1:100, and investigating cytokine requirements (none added, IL-2, IL-15, or IL-2/IL-15). Alloreactive Nreg expansion induced by alloregeneic immature or mature moDCs was compared with that induced by irradiated (30 Gy) allogeneic PBMCs. To determine the fraction of APCs within PBMCs, we used staining using mAbs directed to CD14 and CD19 (BD). Polyclonal expansion was performed using anti–CD3/anti–CD28 T cell expander Dynabeads (Invitrogen Dynal, Oslo, Norway) at a 1:1 ratio. Expansion was performed in standard culture medium alone or supplemented with different concentrations of recombinant human IL-2 (Alloga BV) alone or in combination with IL-15 (10 ng/ml; Peprotech, Rocky Hill, NJ) (11, 18) to investigate the possibility of synergistic effects on nTreg expansion. Every 2–3 d, fresh culture medium supplemented with cytokines was added to the wells, and if necessary the wells were split. At various time points, the numbers of Treg/well were counted under the microscope.

Additional experiments were performed to study in more detail the cytokine requirements for alloreactive Treg expansion using alloregeneic mature moDCs. For this purpose, Tregs were expanded at a 1:1 ratio with alloregeneic mature moDCs in the presence of IL-15 alone or IL-15 in combination with 20 μg/ml anti–IL-2R α Ab (basiliximab; Novartis Pharma BV, The Netherlands), known to fully block signaling of IL-2 via CD25 (36–38), or 20 μg/ml IgG1 (isotype control; R&D Systems Europe Ltd, Abingdon, U.K.). Kinetics and Treg numbers were compared for the different culture conditions.

To gain more insight into the mechanism of expansion of nTregs by alloregeneic mature moDCs, we preincubated alloregeneic mature moDCs for 1 h at 37˚C with blocking Abs directed to HLA class II (clone Ti29; BD Biosciences Pharmingen), CD80/CD86 (clone anti-CD80.1; Sigma–Aldrich), HLA-DR (clone DR10; Sigma–Aldrich), CD40 (clone 5D12; gift from Dr. J. Kwekkeboom), or a combinations of these. As a control, preincubation with total human IgG (Sigma–Aldrich) was included. Optimal concentrations of all blocking Abs were used to ensure complete blockade of the signal (39–42). Subsequently, these alloregeneic mature moDCs were added at a ratio of 1:10 (DC/Tregs) to nTregs in the presence of exogenous IL-15 (10 ng/ml).

Phenotype of expanded Treg fractions

The phenotype of the different Treg fractions expanded in an allogenic (Ag-specific) or polyclonal manner was studied in more detail using Abs directed toward CD3, CD4, CD45RO, and CCR7 at the cell surface and intracellularly using FITC-labeled HELIOS (Biologend Europe BV, Uithoorn, The Netherlands), PE-labeled FOXP3 (eBioscience), and allopolyacycin-labeled CTLA4 (BD) according to the manufacturer’s instruction, and expression was compared with that of the nTregs at day 0 (not-expanded Tregs). Samples were measured on the FACSCanto II (BD) and analyzed using FACSdiva software version 6.1.2. (BD).

Methylation analysis TSDR FOXP3

Cell pellets of FACS-sorted not-expanded and the different expanded nTregs were digested with proteinase K and treated with bisulphite using the EZ DNA Methylation-Direct Kit (Zymo Research from Base Clear Lab}
products) according to the manufacturer’s instructions. During bisulphite treatment, unmethylated cytosines were converted into uracil, whereas methylated cytosines remained unchanged. The TSDR of FOXP3 was amplified by quantitative PCR using the StepOnePlus Real-Time PCR system and the TaqMan Genotyping Master Mix (both from Applied Biosystems BV Europe) with methylation- and demethylation-specific primers and probes as previously published (43). The percentage of TSDR demethylation was calculated as the ratio of demethylated DNA copy numbers and the sum of methylated and demethylated DNA copy numbers. All Treg donors examined in this study were female, and no correction was done to compensate for the X-linked nature of FOXP3.

RNA sequencing

Next-generation sequencing was performed on not-expanded (n = 4), anti-CD3/anti-CD28–coated bead-expanded (n = 3) and allogeneic mature moDC-expanded Tregs (n = 4) at the Erasmus Center for Biomics. For this purpose, expansion of nTregs was performed in the presence of only exogenous IL-15 for 11 d, and allogeneic mature moDCs or anti-CD3/anti-CD28–coated beads were used at a ratio of 1:1 (stimulus/nTreg). Subsequently, RNA was isolated from these samples (containing 1–2 million cells/sample) using the Qiagen RNeasy kit (Qiagen, Valencia, CA). Samples with a RNA-integrity >8 were used. Samples were prepared with TruSeq RNA, sequenced SR43 bp on HiSeq2500 rapid mode, demultiplexed with CASAVA 1.8.4, aligned with TopHat 1.3.1 (http://tophat.cbcb.umd.edu; with Bowie 0.12.7) against the human genome hg19 with RefSeq gene annotation (Illumina iGenomes), per sample expression level calling with Cuffdiff 1.0.3. A differential expression analysis was performed using edgeR (Bioconductor package; http://bioconductor.org) and Cuffdiff (Tophat/Cufflinks/Cuffdiff). The samples were generated in four experiments making use of one Treg donor. A batch-effect term has been included in the EdgeR design matrix, to separate sample generation batch-effect differences from differences among the three groups compared.

Cytokine-producing capacity of expanded Treg fractions

The potential of the different Treg fractions, not-expanded and expanded in an allogeneic (Ag-specific) or polyclonal manner, to produce cytokines was studied by stimulating the cells at a 1:1 ratio with anti-CD3/anti-CD28–coated beads or leaving them unstimulated. After a 12 h-stimulation in the presence of the cytokine secretion inhibitors GolgiPlug (brefeldin A; BD) and Golgi-Stop (monensin; BD), the cell surface was stained using Abs directed to CD3 and CD4. After fixation and permeabilization, cells were divided into three parts and subsequently stained intracellularly using Abs directed to IL-2, IFN-γ, and either TNF-α, IL-10, or IL-17 (all from BD). Samples were measured on the FACS Canto II (BD) and analyzed using FACS Diva software version 6.1.2. (BD). Frequencies of cytokine-producing T cells, corrected for background cytokine-producing T cells, were compared with those observed in nTregs at day 0 (not-expanded Tregs), and as a control for cytokine-producing cells, non-effector T cells were also included in this part and treated in a similar manner. The RNA sequencing data may be accessed using accession number GSE66385 (http://www.ncbi.nlm.gov/geo/).
Suppression assay
Suppressive capacity was tested using the different Treg fractions. In brief, Tregs were transferred in triplicate to the wells and used at different ratios, that is, 1:5 to 1:320. Subsequently, 5 × 10^5 CD3^+ Teffs (responder) were added in triplicate to the wells. Irradiated autologous, allogeneic, or third-party (2-2-2 MM to both responder and allogeneic stimulus) PBMCs were used as stimulators at a concentration of 5 × 10^4/well. At day 5, [3H] thymidine was added, and after 16–18 h of incorporation, the plates were harvested and radioactivity was counted using a liquid scintillation counter. Proliferation in the presence of nTregs is depicted as cpm, corrected for the autologous-induced proliferation, or transferred to a percentage of inhibition using the following formula: (cpm in absence of Tregs − cpm in presence of Tregs)/cpm in absence of Tregs × 100.

Statistical analyses
For comparisons between groups, the t test, Mann–Whitney U test, one-way ANOVA, or Kruskal–Wallis test were used, as appropriate. Post hoc analysis was performed using Bonferroni test for multiple comparisons or Mann–Whitney U test. The p values at α < 0.05 for two sides were considered statistically significant.

Results
Allogeneic mature moDCs are superior in expanding nTregs
Initial experiments, using the standard conditions of irradiated allogeneic PBMC-supported nTreg expansion (18), showed the potency of allogeneic stimuli to expand nTregs in a dose-dependent manner because a significant positive association was observed between the ratio of allogeneic stimulus to nTregs used.

FIGURE 2. Exogenous rIL-15, but not rIL-2, is needed for optimal immature moDC-induced nTreg expansion. Mature moDCs of allogeneic origin were used to expand FACS-sorted nTregs at a ratio of 1 (mature moDC):10 (nTregs) in the presence of no additional cytokine, increasing concentrations of recombinant human IL-2 (25, 300, 600, or 2000 IU/ml) recombinant human IL-15 (10 ng/ml), or the combination of both rIL-2 and rIL-15 (A). Asterisks indicate significant differences in fold expansion when compared with no exogenous cytokine added (*p < 0.05, **p < 0.01). In a next set of experiments we determined whether IL-2, either produced by the mature moDCs or nTregs, could enhance nTreg expansion in the presence of IL-15. For this purpose, an IL-2Rα–blocking Ab (basiliximab, 20 μg/ml) was added to the coculture of nTregs and allogeneic immature moDCs (at a 1:1 ratio) and exogenous IL-15 (10 ng/ml). As a control, we added a similar concentration of the isotype (IgG1) to the coculture and compared this with the situation with only exogenous IL-15 added. The numbers of nTregs obtained after 11 d of expansion are given in (B) (black bars: no blocking Ab; white bars: isotype control; gray bars: IL-2Rα–blocking Ab). Data are presented as mean ± SEM of at least three independent experiments.

FIGURE 3. Stable high FOXP3-expression and TSDR-demethylation status within FOXP3 gene of expanded nTregs. Expanded nTregs were stained intracellularly for FOXP3, and expression was compared with that in the starting population (A and B). (A) A typical flow-cytometric example is given where the fluorescence minus one is represented by the gray solid histogram, the FOXP3 expression of the starting population by histogram with the gray line, that of the allogeneic mature moDC-expanded nTregs by histogram with the black solid line, and that of the anti-CD3/anti-CD28–coated bead expansion by histogram with the black hatched line. (B) Mean ± SEM FOXP3 expression (MFI of PE-labeled FOXP3) of three to five independent experiments is given on the left y-axis for the different nTreg fractions. In addition, on the right y-axis, the corresponding transcript counts for FOXP3 are given. In addition, the TSDR-demethylation status within the FOXP3 gene was analyzed for the different nTreg fractions obtained from three to five independent experiments and depicted as mean ± SEM in (C).
for expansion and number of nTregs obtained (Fig. 1A). Allogeneic immature moDCs showed the lowest potential to expand nTregs followed by irradiated allogeneic PBMCs, and the highest numbers were obtained using allogeneic mature moDCs ($p < 0.05$). At the highest ratio, the (mean ± SEM) fold increases amounted to 8 ± 3, 16 ± 2, and 62 ± 4 for immature moDCs, allogeneic PBMCs, and mature moDCs, respectively. Autologous mature moDCs did not expand nTregs (data not shown). Immature moDCs are less capable of stimulating/activating T cells when compared with mature moDCs. As expected (44), DC maturation leads to significantly increased median fluorescence intensity (MFI) levels of HLA class II and costimulatory molecules CD40, CD80, and CD86 by at least 1–2 log (data not shown). Blocking the interaction of HLA class II molecules with the TCR (Fig. 1B) and CD80/86 with CD28 (Fig. 1B) reduced the expansion capacity of nTregs by allogeneic mature moDCs by ~67% ($p < 0.05$) and 90% ($p < 0.01$), respectively. Interestingly, we did not observe an effect of blocking the interaction of CD40 with CD154 with respect to fold expansion rates, even in the presence of an excess of anti-CD40 Ab (40, 42). Combining the blockade of HLA class II and costimulatory pathways fully suppressed expansion of nTregs by allogeneic mature moDCs.

Next, we compared the kinetics of allogeneic-induced Treg expansion and polyclonal (anti-CD3/anti-CD28–coated bead)–induced Treg expansion. Peak numbers of nTregs after alloantigen-specific expansion were observed at a later time point compared with a polyclonal stimulus (Fig. 1C). In conclusion, allogeneic mature moDCs were superior in inducing nTreg expansion when compared with immature moDCs or PBMCs as source of alloantigen-specific stimulus and are therefore used throughout the rest of this article as the source for inducing alloantigen-specific nTregs.

**Allogeneic mature moDC-induced expansion of nTregs is facilitated in the presence of exogenous IL-15 without the need for IL-2**

Adding both IL-2 and IL-15 to the cell culture yields optimal numbers of expanded Tregs (18). In particular, exogenous IL-2 is an essential part of all current Treg-expansion protocols (14, 45). Bead-induced expansion of Tregs in the presence of exogenous IL-15 alone resulted in a 33% reduction of fold expansion ($p < 0.05$; i.e., fold expansion in the presence of IL-2/IL-15 and IL-15 amounted to 9.7 ± 2.5 and 6.5 ± 2.7, respectively). Without adding IL-2 and/or IL-15 to the cell culture, allogeneic mature moDCs were unable to induce significant Treg expansion. Surprisingly, exogenous recombinant human IL-15 without the presence of optimal concentrations of exogenous IL-2 was sufficient to induce optimal mature moDC-stimulated Treg expansion.
Because mature moDCs can produce IL-2 (46), this may obviate the need for exogenous IL-2 during allogeneic mature moDC-induced Treg expansion. However, frequencies of IL-2-producing allogeneic mature moDCs within the Treg culture were always <1% (data not shown). Moreover, addition of an IL-2Rα blocker at a high dose (20 μg/ml) that totally prevents IL-2 signaling (36–38) did not significantly affect Treg expansion (Fig. 2B). In conclusion, allogeneic mature moDCs are superior cells to induce nTreg expansion, and the presence of IL-15 obviates the need for exogenous IL-2.

Mature moDC-expanded nTregs express high levels of FOXP3 and maintain the TSNDR-demethylation status within the FOXP3 gene

Long-term cultures of nTregs may result in loss of the nTreg-phenotype identified by diminished FOXP3 expression, which is associated with loss of suppressive capacity (47–49). In accordance with a previous publication (11), >90% of the freshly isolate nTregs expressed FOXP3, in contrast with a very low expression in T cells depleted for nTregs (effector T cells) (11). Upon expansion of nTregs, expression of FOXP3 increased (Fig. 3A, 3B). The MFI of PE-labeled FOXP3 (mean ± SEM) increased from 1770 ± 357 to 3464 ± 795 and 3743 ± 673 for not-expanded, bead-expanded, and allogeneic mature moDC-expanded nTregs, respectively. These MFI values for FOXP3 corresponded to the data obtained for FOXP3 mRNA using next-generation sequencing (Fig. 3B). In addition, the high demethylation status of the TSNDR within the FOXP3 gene of nTregs (all female donors) did not decrease upon expansion (Fig. 3C). Lyonization of one of the X chromosomes (50) in these females might not be complete, explaining the somewhat higher percentages of demethylated TSNDR within FOXP3 upon expansion (Fig. 3C). These results did not change after prolonging our expansion period by another round of stimulation for 6–10 d by applying either an allogeneic or polyclonal expansion protocol (data not shown). In conclusion, allogeneic mature moDC-expanded nTregs maintained their nTreg-phenotype after prolonged culture and expansion. In agreement with the lack of FOXP3+ effector T cells, the percentage of demethylated TSNDR within the FOXP3 gene of effector T cells was very low (0.1 ± 0.07%).

Allogeneic mature moDC-expanded nTregs have a memory phenotype and express HELIOS and CTLA4 at high levels

Naive nTregs were present in the starting population of nTregs, but upon expansion of these cells, especially in an Ag-specific manner, the naive T cell fraction diminished significantly (p < 0.05; Fig. 4A). However, no significant changes within the memory T cell subsets were noticed (Fig. 4A). All nTregs before expansion consisted of ~30% of HELIOS+ T cells (51). Upon expansion this frequency did not change significantly. Interestingly, within the HELIOS-expressing nTregs, a shift was noticed toward more FOXP3highHELIOS+ cells if allogeneic mature moDCs were used to expand nTregs (p < 0.001). This shift was more pronounced within the allogeneic mature moDC-expanded nTregs when compared with the bead-expanded ones (Fig. 4B). CTLA4, one of the important negative regulators used by nTregs to scale down
immune responses both in vitro (52) and in vivo in the transplant setting (53), is highest expressed on allogeneic mature moDC-expanded nTregs (Fig. 4C, right histogram and graph) when compared with both not-expanded and bead-expanded nTregs ($p < 0.001$). In conjunction with the result obtained with the analysis of FOXP3 expression, nTreg expansion by allogeneic mature moDCs resulted in the greatest enhancement of typical nTregs markers.

Allogeneic mature moDC-expanded nTregs are superior in suppressing proliferation

Allogeneic mature moDC-expanded nTregs exhibited superior suppression of the alloantigen-induced T cell proliferation compared with that induced by 3rdP (Fig. 5A, 5C; $p = 0.02$ when comparing the slopes of the lines describing percentages of inhibition). This difference in suppressive capacity was most evident at a low ratio of nTregs to Teff, and significant suppression was still observed at ratios <1:320. The suppressive capacity of nTregs expanded in a polyclonal manner (Fig. 5B, 5D) was not different when comparing the lines describing the inhibition of alloantigen-induced proliferation with that of 3rdP-induced proliferation (Fig. 5B).

Cytokine profile of allogeneic mature moDC-expanded nTregs

Few nTregs produced IL-17 immediately upon isolation, and this was not affected upon long-term expansion (Fig. 6A, 6B). In addition, the low frequencies of IL-2 and IL-10 producing nTregs at day 0 were also maintained upon expansion for 11–12 d (Fig. 6A, 6B). Interestingly, nTregs at day 0 were able to produce TNF-$\alpha$ and small amounts of IFN-$\gamma$, albeit at lower frequencies than those observed by CD4$^+$Teffs (Fig. 6A, 6B). Again, the measured frequencies of these proinflammatory cytokines did not significantly change upon expansion of these nTregs. Taken together, expanding nTregs in an Ag-specific manner with allogeneic mature moDCs did not significantly affect the cytokine-producing potential, and no indication for transition from Treg to effector T cell was found.

In vivo skin-derived DCs have a similar functional capacity as mature moDCs to support expansion of alloantigen-specific nTregs

Skin-derived DCs are the in vivo equivalent of moDCs and are essential in presenting captured Ag to effector T cells after migration to T cell areas in secondary lymph nodes (34). Therefore, it is of interest to test whether these DCs also have the potency to expand nTregs in an alloantigen-specific manner. Cells allowed to migrate out of skin in 48 h consisted of both lymphocytes (non-DC) and DCs (Fig. 7A, 7B). Upon migration of DCs from the skin (Fig. 7A), these cells acquired a mature phenotype, that is, $\sim$80% expressed CD1a, almost all coexpressed HLA-DR, and $\sim$60% coexpressed CD83 (Fig. 7C) and had a superior capacity to support the expansion of alloantigen-specific nTregs. The numbers of alloantigen-specific nTregs after expansion with skin-derived DCs, at a 1:1 ratio, were similar to the results obtained with in vitro-generated allogeneic mature moDCs used at a 1:10 ratio, that is, fold expansion amounted to 25 ± 6 versus 24 ± 3, respectively. The kinetics of skin-derived, DC-induced nTreg expansion (Fig. 7D) is similar to other allogeneic stimuli used for Treg ex-

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**FIGURE 6.** Cytokine-producing capacity allogeneic mature moDC-expanded nTregs similar to not-expanded nTregs. FACS-sorted nTregs were left unexpanded or expanded using mature moDCs (ratio 1:10) or polyclonal-expanded (using anti-CD3/anti-CD28–coated beads, at a ratio of 1:1 bead/nTreg) and subsequently tested for their cytokine-producing capacity upon stimulation with anti-CD3/anti-CD28–coated beads. (A) A typical flow-cytometric example is shown for analysis of IL-2, IFN-$\gamma$, TNF-$\alpha$, IL-10, and IL-17–producing cells, and CD4$^+$Teffs (last column) were included as a positive control. (B) The different frequencies of total cytokine-producing cells corrected for background (unstimulated) are shown in a graph for the different expansion protocols, and frequencies of cytokine-producing effector CD4$^+$ T cells were also included.
pansion and not different for the two conditions tested, that is, in the presence of both exogenous IL-2 and IL-15 or only IL-15. In addition, skin-derived DCs, similar to mature moDCs, drove nTreg expansion in an IL-2–independent manner as exogenous IL-15 alone resulted in similar numbers of alloantigen-specific nTregs (Fig. 7E). These data indicate that in vivo mature DCs may act as potent stimulators of nTreg proliferation.

Next-generation RNA sequencing

In addition to the earlier described findings, we performed gene-expression profiling on RNA samples obtained from not-expanded nTregs (= A), allogeneic mature moDC-expanded (= B), and polyclonal (anti-CD3/anti-CD28–coated bead)-expanded (= C) nTregs using next-generation sequencing. More than 85% of the reads could be aligned to the reference (human genome) as listed in the Supplemental Table I. A multidimensional scaling plot for all samples is shown in Fig. 8A. All samples for group B cluster nicely together, whereas for groups A and C we see a distinction between sample generation batches. This suggests that the batch effect term is important. Cuffdiff does not incorporate this effect; we report only the EdgeR results in this article. Using a false discovery rate (FDR) value <0.05 as cutoff and no restriction with respect to the fold change (FC) revealed the largest number of genes differentially expressed to occur when comparing not-expanded nTregs with those that were expanded using anti-CD3/anti-CD28–coated beads, that is, 3062 genes as illustrated in

**FIGURE 7.** Skin-derived, DC-induced nTreg expansion. (A) A typical example of the microscopic view of skin-derived DCs is given (original magnification ×400; arrows indicate a typical skin-derived DC as well as a lymphocyte). The cells were harvested and immunophenotyped (typical flow-cytometric example is given in B and C). Based on forward and sideward characteristics, DC-like cells are identified (B) and subsequently these are further characterized using Abs directed against CD1a, HLA-DR, as well as CD83 (C, black lines), and expression is compared with the unstained sample (C, gray lines). Next these cells were irradiated (30 Gy) and used at a ratio of 1:1 to expand nTregs in presence of both IL-2 and IL-15 (●) or IL-15 alone (○) for a period of 11 d. Kinetics of nTreg expansion induced by skin-derived DCs is given in (D). Fold expansion of three independent experiments is depicted in (E), with fold expansion on the y-axis, whereas on the x-axis the different expansion conditions are depicted.
The lowest number of genes differentially expressed were observed when comparing the two expansion protocols, that is, 1184 (Fig. 8B, right panel). We zoomed in on differentially expressed genes in allogeneic mature moDC-expanded nTregs versus not-expanded nTregs and polyclonal (bead)-expanded nTregs, and focused on expression of genes typically upregulated or downregulated in nTregs either in a resting state or upon activation/expansion based on the literature (54–56) and grouped them into five categories (Fig. 8C, Table I).

Both CD25 and CD127, markers used to identify Tregs, are differently expressed when comparing allogeneic mature moDC-expanded nTregs versus not-expanded nTregs and polyclonal (bead)-expanded nTregs, and focused on expression of genes typically upregulated or downregulated in nTregs either in a resting state or upon activation/expansion based on the literature (54–56) and grouped them into five categories (Fig. 8C, Table I).

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Discussion

The results of this study show that allogeneic mature human DCs, both monocyte or skin derived, are able to support the generation of alloantigen-specific nTregs by exogenous IL-15 alone without

Fig. 8B (middle panel). The lowest number of genes differentially expressed were observed when comparing the two expansion protocols, that is, 1184 (Fig. 8B, right panel). We zoomed in on differential expressed genes in allogeneic mature moDC-expanded nTregs versus not-expanded nTregs and polyclonal (bead)-expanded nTregs, and focused on expression of genes typically upregulated or downregulated in nTregs either in a resting state or upon activation/ expansion based on the literature (54–56) and grouped them into five categories (Fig. 8C, Table I).

Both CD25 and CD127, markers used to identify Tregs, are differently expressed when comparing allogeneic mature moDC-expanded nTregs with not-expanded nTregs. Expansion increased mRNA expression of CD25 by >25-fold, whereas that of CD127 was almost 5-fold lower when comparing the average counts per million values of allogeneic mature moDC-expanded nTregs with those of not expanded (Fig. 8C, Table I). mRNA expression of homing receptors was not different for the Tregs expanded in an Ag-specific manner and those not expanded, except for CCR4, CD62L, and CD103, whose mRNA expression was increased >6-fold and almost 2-fold for CCR4 and CD103, respectively, and decreased almost 2-fold for CD62L. Molecules associated with nTreg function are expressed at higher levels upon expansion, and expression is higher for Ag-specific expanded versus polyclonal expanded nTregs. Expression of molecules associated with activation/cell death, for example, GARP and LAG3, was upon expansion and the former is higher expressed if an Ag-specific expansion protocol was used when compared with polyclonal expansion (Fig. 8C, Table I). Important transcription factors for nTregs, like FOXP3 and HELIOS, higher expressed upon expansion, but the highest levels were observed for nTregs expanded in an Ag-specific manner (Fig. 8C, Table I).
compromising the typical nTreg characteristics. Using mature moDCs as stimulator cells, it is possible to generate large numbers of allogeneic-specific nTregs necessary for cell therapy from an acceptable volume of peripheral blood.

Compared with previously published protocols, the proportion of effector T cells in the enriched Treg population was reduced to a minimum (<2%) by using multiparameter flow-cytometric cell sorting. Percentages of contaminating effector T cells in the Treg fraction amounted to 20–50% (12, 13, 18) using the anti-CD25 microbead isolation method. The expansion of these bead-isolated Tregs was mostly performed in the presence of rapamycin to prevent outgrowth of these effector T cells (17), but such a relative large percentage of effector T cells may very well confound findings with respect to phenotype and function of Tregs after expansion. In addition, phenotypic and cytokine profiles of expanded nTregs did not indicate any outgrowth of effector T cells or expansion. In contrast, phenotypic and cytokine profiles of expanded nTregs showed the typical nTreg characteristics, like FOXP3, HELIOS, and CTLA4 expression, were upregulated, whereas the expanded nTregs showed a stable profile of low frequencies of IFN-γ, TNF-α, IL-2, IL-10, and IL-17–producing cells when compared with the nonexpanded ones.

The stability of the nTreg population after a prolonged period of expansion may be compromised and plasticity of Tregs has been observed due to factors present in the environment (57). One of the most important factors for an nTreg to exert immunosuppressive activity is stable and high expression of FOXP3. This is ensured by a demethylated TSDR within the promotor of the FOXP3 gene. Upon repetitive stimulations of Tregs, the expression of FOXP3 may decrease accompanied by a loss of demethylated TSDR within the FOXP3 gene (47). In this study, this feature of nTregs was not compromised upon one expansion round of 11–12 d, implying the safe use of allogeneic mature moDC-expanded nTregs.

In addition, a unique feature of Treg expansion supported by allogeneic mature moDCs, but not that supported by anti-CD3/anti-CD28–coated beads, was the complete independence of IL-2, either exogenously added or potentially produced during cell culture. Allogeneic skin-derived DCs were also able to induce Treg expansion without exogenous IL-2 added to the culture, indicating that this phenomenon was not limited to the artificially created moDCs in vitro. The role of cytokines in development, homeostasis, and function of Tregs remains to be elucidated. IL-2 is known to be essential for Treg homeostasis and activation (58–60), and most Treg-expansion protocols make use of high concentrations (>200 U/ml) of IL-2. However, IL-2–deficient mice had normal numbers of functional Tregs (61, 62), but mice lacking both IL-2 and IL-15 showed a decrease in functional Tregs (63). We observed that exogenous IL-15 is sufficient to promote allogeneic mature (mo)DC-induced nTreg expansion and survival without the need for IL-2. Production of small amounts of IL-2 by mature moDCs upon microbial stimulation (46, 64) and/or T cell–mediated stimuli (65) was not responsible for Treg expansion as we have shown that blocking of the IL-2Rα-chain in the presence of IL-15 did not significantly affect Treg numbers. However, in accordance with the results obtained with all other nTreg expansion protocols, even such highly efficient APCs like mature DCs still need exogenous IL-2 or IL-15 to support nTreg proliferation. The result of blocking experiments indicated that, in particular, the increased expression of HLA-DR, CD80, and CD86 on mature DCs is essential for the expansion of nTregs.

### Table I. Differentially expressed genes specific for nTregs

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<tr>
<th>Treg Markers</th>
<th>Gene ID</th>
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Italic values represent a FDR < 0.05.

A, not-expanded nTregs; B, allogeneic mature moDC-expanded nTregs; C, polyclonal (anti-CD3/anti-CD28–coated bead)–expanded nTregs.
DENDRITIC CELL–INDUCED EXPANSION OF ALLOREACTIVE Tregs


