

# Effect of Oxygen Levels on the Physiology of Dendritic Cells: Implications for Adoptive Cell Therapy

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Dendritic cell (DC)-based adoptive tumor immunotherapy approaches have shown promising results, but the incidence of tumor regression is low and there is an evident call for identifying culture conditions that produce DCs with a more potent Th1 potential. Routinely, DCs are differentiated in CO<sub>2</sub> incubators under atmospheric oxygen conditions (21% O<sub>2</sub>), which differ from physiological oxygen levels of only 3–5% in tissue, where most DCs reside. We investigated whether differentiation and maturation of DCs under physiological oxygen levels could produce more potent T-cell stimulatory DCs for use in adoptive immunotherapy. We found that immature DCs differentiated under physiological oxygen levels showed a small but significant reduction in their endocytic capacity. The different oxygen levels did not influence their stimuli-induced upregulation of cluster of differentiation 54 (CD54), CD40, CD83, CD86, C-C chemokine receptor type 7 (CCR7), C-X-C chemokine receptor type 4 (CXCR4) and human leukocyte antigen (HLA)-DR or the secretion of interleukin (IL)-6, tumor necrosis factor (TNF)- $\alpha$  and IL-10 in response to lipopolysaccharide (LPS) or a cytokine cocktail. However, DCs differentiated under physiological oxygen level secreted higher levels of IL-12(p70) after exposure to LPS or CD40 ligand. Immature DCs differentiated at physiological oxygen levels caused increased T-cell proliferation, but no differences were observed for mature DCs with regard to T-cell activation. In conclusion, we show that although DCs generated under atmospheric or physiological oxygen conditions are mostly similar in function and phenotype, DCs differentiated under physiological oxygen secrete larger amounts of IL-12(p70). This result could have implications for the use of *ex vivo*-generated DCs for clinical studies, since DCs differentiated at physiological oxygen could induce increased Th1 responses *in vivo*.

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## INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (1,2) and are critical for the induction of immune responses to pathogens and cancer (1,3). Because of these properties, DCs are being widely used for vaccines and immunotherapeutic strategies (3–10). The most common approaches for tumor immunotherapy involve the use of DCs generated from the progenitors CD34<sup>+</sup> (11–13) or CD14<sup>+</sup> in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 *ex vivo* (14). Tumor antigens are delivered to DCs using many differ-

ent systems including whole tumor cells or lysates (15–17), RNA (18–21), peptides or viral vectors (22). Exposure to antigen is followed by the addition of a maturation stimulus *in vitro*, since mature DCs induce more potent immune responses than immature DCs, which can induce T-cell tolerance (23,24). One of the originally used maturation stimuli was monocyte-conditioned media (14), which was subsequently refined to a cocktail containing four components including prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-6 (8,25–27).

Although immune responses are generated to DC-based vaccine approaches

in patients, only in a few instances has tumor regression been observed in phase I clinical trials (28,29). Clearly, this result could have many explanations including the DC subtypes used, the maturation stimuli, the suppressive tumor microenvironment, treatment starting too late in the disease, and other explanations. An alternative possibility is that the *ex vivo*-generated DCs are not optimal for *in vivo* function because of the applied culture conditions. Optimization of the *in vitro* culture conditions should allow for the generation of DCs that will give the desired and maximal Th1-type immune response *in vivo*. In studies using *ex vivo* differentiated DCs, the DCs are generally differentiated in incubators that maintain atmospheric oxygen levels (21% O<sub>2</sub> and 5% CO<sub>2</sub>). However, *in vivo*, most cells including DCs do not encounter such high oxygen levels. DCs exist both in blood and tissue, and most of them reside in

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the tissue where the oxygen levels are 3–5% (30–32). The effect of physiological and atmospheric oxygen levels has recently been compared with regard to its effect on T cells (32–34). At physiological oxygen levels, primary T cells proliferated less in response to CD3/CD28 stimulation, which correlated with higher intracellular nitric oxide levels (33). Furthermore, cytotoxic T cells developed under 2.5% O<sub>2</sub> were more lytic but secreted lower amounts of IL-2 and interferon (IFN)- $\gamma$  (32). Although the effect of hypoxia on DCs has been investigated (35,36), since this is of interest to understand the effect of hypoxoxygenation on, for example, DCs in tumor tissue, no data exist on the effect of physiological oxygen levels on the differentiation of human DCs from progenitors and their maturation. Thus, we compared functionally and phenotypically monocyte-derived DCs that have been differentiated under physiological oxygen with those differentiated under atmospheric oxygen conditions with a goal to evaluate if we can generate more potent DCs for tumor immunotherapy.

## MATERIALS AND METHODS

### Cells

HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa cells that express human CD154 (HeLa-CD154) were generated by electroporation of an expression vector containing the full-length human CD154 into HeLa cells, followed by selection and cloning as described (37).

### Generation of Human Monocyte-Derived DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of normal volunteers (San Diego Blood Bank) over a Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient. To generate DCs, PBMCs were allowed to adhere to culture plates for 1 h. The nonadherent cells were washed off, and the adherent cells were cultured in RPMI 1640 medium supplemented

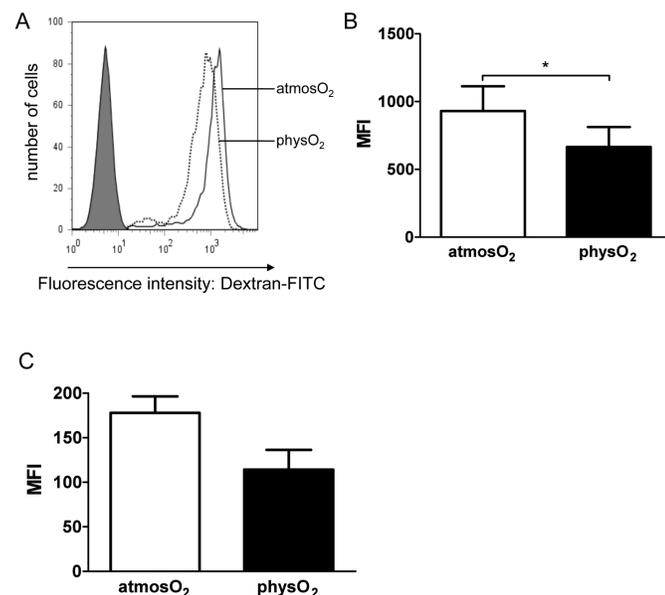
**Table 1.** The number of DCs recovered per well at atmospheric and physiological oxygen levels.

	atmosO <sub>2</sub>	physO <sub>2</sub>
Media	$3.3 \times 10^5 \pm 2.2 \times 10^5$	$4.1 \times 10^5 \pm 2.7 \times 10^5$
LPS	$5.5 \times 10^5 \pm 3.6 \times 10^5$	$4.5 \times 10^5 \pm 1.9 \times 10^5$
CyC	$4.4 \times 10^5 \pm 2.4 \times 10^5$	$5.4 \times 10^5 \pm 2.7 \times 10^5$

Data are the mean  $\pm$  SD from five separate donors.

with 2 mmol/L L-glutamine (GIBCO-BRL Life Technologies, Grand Island, NY, USA), 50  $\mu$ mol/L 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 10 mmol/L HEPES (GIBCO-BRL), penicillin (100 U/mL)-streptomycin (100  $\mu$ g/mL) (GIBCO-BRL) and 5% human serum (HS; Human AB serum, Gemini Bio Products, West Sacramento, CA, USA), supplemented with 1,000 units GM-CSF/mL (Bayer HealthCare Pharmaceuticals, Wayne, NJ, USA) and 200 units IL-4/mL

(R&D Systems, Minneapolis, MN, USA) at days 0, 2 and 4. Immature DCs were harvested on days 5–7. If not otherwise, the DCs were differentiated starting from day 0 under two different oxygen levels. Physiological oxygen tensions, or 5% O<sub>2</sub>, were generated in a Sanyo MCO-18M O<sub>2</sub>/CO<sub>2</sub> incubator (Sanyo Scientific, Bensenville, IL, USA). Gas phase O<sub>2</sub> levels were controlled by continuous injection of medical grade N<sub>2</sub> to reach the target oxygen level. DCs cultured in



**Figure 1.** Endocytic capacity of immature DCs generated at atmosO<sub>2</sub> and physO<sub>2</sub> conditions. Immature DCs differentiated at atmospheric or physiological oxygen levels were harvested and incubated with 1 mg/mL Dextran-FITC for 30 min at 37°C at atmospheric oxygen levels (A, B) or at physiological oxygen levels (C). Cells were subsequently washed, fixed and analyzed by flow cytometry. (A) Immature DCs exposed to media are shown in the shaded histogram as the control. PhysO<sub>2</sub> immature DCs (*dotted line*) and atmosO<sub>2</sub> immature DCs (*solid line*) were exposed to Dextran-FITC at atmosO<sub>2</sub>. One representative experiment out of two is shown. (B) The mean fluorescence intensities (MFI)  $\pm$  SD of four experiments, where DCs were exposed to Dextran-FITC at atmosO<sub>2</sub> using DCs from different donors. \*Statistically significant difference,  $P < 0.05$ , paired Student  $t$  test. (C) MFI  $\pm$  SD of four experiments conducted where DCs were exposed to Dextran-FITC at physO<sub>2</sub> levels using DCs from different donors. No significance was observed.

atmospheric oxygen levels (20% O<sub>2</sub>) were incubated in a standard incubator without the addition of N<sub>2</sub>. All cells were exposed to 5% CO<sub>2</sub>.

**Endocytosis Assay**

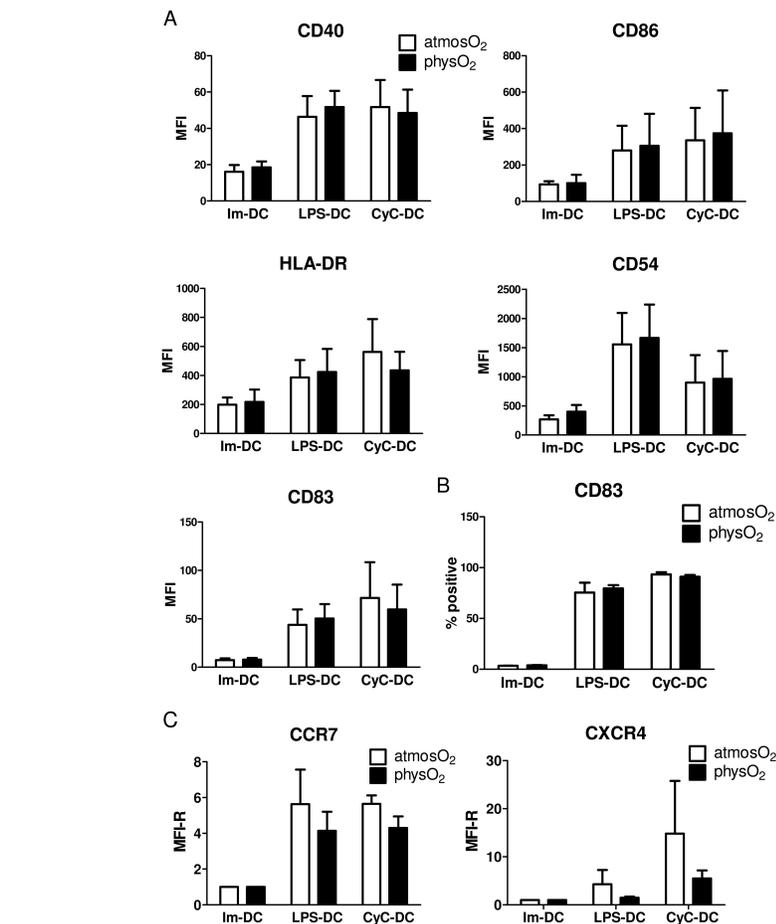
Immature DCs were collected on day 7 and incubated with 1 mg/mL Dextran-FITC (Molecular Probes, Eugene, OR, USA) for 30 min at 4°C or 37°C. The caps of the tubes were left opened to ensure that the cells were exposed to the respective oxygen levels. DCs were washed twice with 5% HS/RPMI, fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) (pH 7.2–7.4) and analyzed by flow cytometry using a FACSCalibur (Beckon Dickinson, San Jose, CA, USA). Data were analyzed using the FlowJo 7.2.2 software (Tree Star, Ashland, OR, USA).

**Stimulation of DCs**

At day 5 of culture, immature DCs were either left untreated (immature [IM]), stimulated with indicated doses of lipopolysaccharide (LPS) (*E. coli* serotype 026:B6; Sigma) or a cocktail of cytokines (CyC) consisting of TNF-α at 10 ng/mL, IL-1β at 10 ng/mL (all from R&D Systems) and PGE<sub>2</sub> at 1 μg/mL (Sigma). At 48 h after stimulation, cell-free culture supernatants were collected, and cytokines were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA).

**Analysis of DC Phenotype**

The 1 × 10<sup>4</sup> DCs were incubated for at least 20 min at 4°C in 100 μL PBS/5% fetal calf serum/0.1% sodium azide (staining buffer) with phycoerythrin (PE)-conjugated IgG specific for cluster of differentiation 54 (CD54), human leukocyte antigen (HLA)-DR (all from Becton Dickinson Immunostaining Systems, San Jose, CA, USA), CD83 (Immunotech-Beckman-Coulter, Marseille, France) and CD184 (C-X-C chemokine receptor type 4 [CXCR4]) (BD Biosciences, Franklin Lakes, NJ, USA) or fluorescein isothiocyanate (FITC)-conjugated IgG monoclonal antibody (mAb) specific for CD40



**Figure 2.** DCs differentiated and activated under atmosO<sub>2</sub> and physO<sub>2</sub> conditions show similar levels of surface molecule expression. Immature DCs were differentiated at atmosO<sub>2</sub> and physO<sub>2</sub> for 5 d. Subsequently, DCs were exposed to 10 ng/mL LPS (LPS-DC), a cytokine cocktail (CyC) containing IL-1β/TNF-α/PGE<sub>2</sub> (CyC-DC) or media only (Im-DCs) for 48 h. DCs were collected, stained and analyzed by flow cytometry for the expression of the depicted surface molecules (A). Data shown are the MFI ± SD from three independent experiments using DCs from different donors. (B) The percentage of CD83-positive DCs. Data shown are mean ± SD from three independent experiments using DCs from different donors. (C) Data shown are the MFI ± SD from five independent experiments using DCs from different donors.

and CD58 (all from Becton Dickinson Immunostaining Systems). Cells were washed 4x with staining buffer, fixed in 3.7% formaldehyde in PBS and examined by flow cytometry using a FACScan(Calibur) (BD Biosciences). In all experiments, isotype controls were included using PE- or FITC-conjugated irrelevant mAb of the same Ig class.

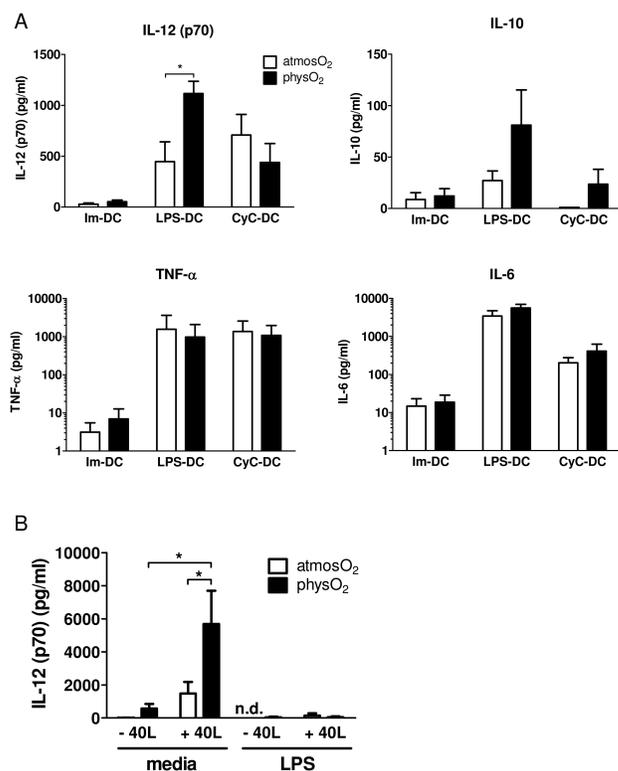
**T-Cell Isolation**

T cells were isolated by negative selection using the RosetteSep antibody

cocktail from StemCell Technologies (Vancouver, CA, USA) according to the manufacturer’s instructions. The purity of the isolated T cells was routinely ~99%.

**Mixed Leukocyte Reaction**

To assess levels of cellular activation and proliferation, cells were plated at 2 × 10<sup>5</sup> cells per well in a flat-bottomed 96-well tray at DC:T-cell ratios of 1:10 for 5 d in medium described above. T-cell proliferation was measured using the



**Figure 3.** Effect of oxygen levels on the cytokine profile of immature and mature DCs. (A) DCs were differentiated and activated as above with LPS or CyC. Cell culture supernatants were collected at 48 h, and the presence of the indicated cytokines and chemokines was measured by ELISA. Results shown are mean  $\pm$  SD of six independent experiments using DCs from different donors. \*Statistically significant difference;  $P < 0.05$ , paired Student  $t$  test. (B) Immature DCs differentiated at physO<sub>2</sub> showed higher secretion of IL-12 when stimulated with CD40L. DCs were differentiated and LPS matured at both oxygen levels and then were cocultured with HeLa (data not shown) or HeLa CD154 cells for 12 h. Supers were collected and IL-12p(70) was measured by ELISA. Results are of six independent experiments using DCs generated from different donors. n.d., IL-12 was not detected in those samples.

CellTrace CFSE Cell Proliferation Kit from Invitrogen (Eugene, Oregon, USA). Cells were stained in 0.1% bovine serum albumin/PBS for at least 10 min in a 37°C water bath, washed 3 $\times$  with culture media and then plated. Cells were harvested on days 2 and 5, fixed in 10% formaldehyde in PBS and analyzed by flow cytometry.

#### Activation of DCs Using CD154-Expressing HeLa Cells

Immature DCs were exposed to media or LPS at day 5 for 24 h. On day 6, immature and LPS matured DCs were collected and cocultured with HeLa or HeLa-CD154 cells. HeLa cells were

plated in a flat-bottomed 96-well plate at  $1.5 \times 10^4$  cells per well 24 h before addition of  $2.5 \times 10^5$  DCs. After 12 h, cell culture supernatants were collected and IL-12(p70) was measured by ELISA. HeLa-only controls did not contain measurable levels of IL-12.

#### ELISPOT

Ninety-six-well polyvinylidene fluoride (PVDF) plates (Millipore, Billerica, MA, USA) were coated overnight at 4°C with 5  $\mu$ g/mL of the primary anti-human IFN- $\gamma$  mAb (Mabtech, Cincinnati, OH, USA). The antibody-coated plates were washed 5 $\times$  with PBS and blocked with RPMI 1640 containing

5% human serum for 1 h at 37°C. Immature and mature DCs were pulsed with 100 ng/mL MART-1 peptide (ELAGIGILTV) for 1 h at 37°C. Unpulsed and peptide-pulsed DCs were cocultured with MART-1 specific T cells (generated in our laboratory from normal donor PBMCs) at a ratio of  $10^3$  DCs: $10^5$  T cells and incubated overnight (approximately 16–18 h) at 37°C. Enzyme-linked immunosorbent spot (ELISPOT) plates were washed 5 $\times$  with PBS containing 0.05% Tween-20 followed by a 2-h incubation at room temperature with 1  $\mu$ g/mL biotinylated anti-IFN- $\gamma$  mAb (Mabtech). Plates were washed 5 $\times$  in PBS with 0.1% Tween-20. Streptavidin-horseradish peroxidase (1:500) was added to wells and incubated for 1 h at room temperature. The plates were washed 5 $\times$  in PBS with 0.1% Tween-20 and then 2 $\times$  in PBS only, followed by a 2- to 3-min incubation in tetramethylbenzidine (TMB [Mabtech]) to develop the reaction. Plates were washed with tap water to stop the reaction. IFN- $\gamma$ -secreting T cells were counted using an automated image analysis ELISpot reader (ImmunoSpot Series 1 Analyzer; Cellular Technology, Cleveland, OH, USA).

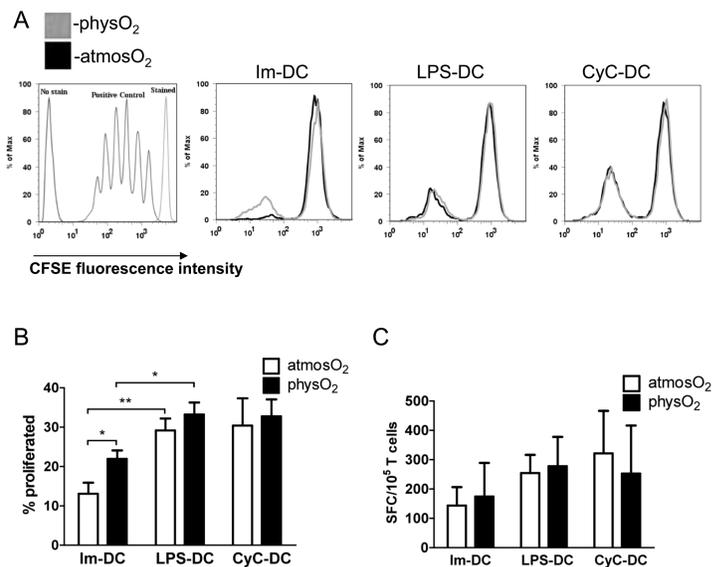
#### Statistical Analysis

Data are represented as mean  $\pm$  SD. Data were analyzed for statistical significance using a Student  $t$  test.  $P$  values  $< 0.05$  were considered statistically significant.

## RESULTS

### Immature DCs Differentiated Under Physiological Oxygen Levels Show Decreased Endocytic Activity

Because cell death during the culture period can negatively influence the outcome of an immunization, we first evaluated the yield/recovery of viable DCs after differentiation from blood monocyte precursors at physiological oxygen (physO<sub>2</sub>) levels or atmospheric oxygen (atmosO<sub>2</sub>) levels. The yield of viable immature and mature DCs generated under



**Figure 4.** DCs differentiated at atmosO<sub>2</sub> and physO<sub>2</sub> induced similar levels of T-cell activation. Immature and mature atmosO<sub>2</sub> DCs and physO<sub>2</sub> DCs were analyzed for the ability to stimulate allogeneic T-cell proliferation in a mixed lymphocyte reaction (MLR). Allogeneic T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and cocultured with IM-DCs, LPS-DCs or CyC-DCs for 5 d at atmosO<sub>2</sub>. Cells were collected and T-cell proliferation was analyzed by flow cytometry gating on lymphocytes. (A) A representative result of three is shown. (B) The mean ± SD percentage of proliferating allogeneic T cells from three independent experiments using DCs and T cells from different donors. \*Statistically significant difference, *P* < 0.05, Student *t* test. (C) Immature and mature DCs were generated and activated as above from HLA-A\*0201-positive donors, pulsed with 100 ng/mL HLA-A\*0201-specific MART1 peptide and cocultured with a MART1-specific T-cell clone at a DC:T-cell ratio of 1:100 for 18 h at atmosO<sub>2</sub>. Controls included non-peptide-pulsed DCs. The number of spot-forming cells (SFCs) for IFN-γ secretion was assessed by ELISPOT. Data are shown as mean ± SD of the number of SFCs per 10<sup>5</sup> T cells from three independent experiments.

physO<sub>2</sub> or atmosO<sub>2</sub> levels were comparable (Table 1).

Next, the endocytic capacity of immature DCs differentiated at physO<sub>2</sub> or atmosO<sub>2</sub> was compared by measuring the uptake of Dextran-FITC at both oxygen levels. Although 100% of the immature DCs differentiated under the two oxygen conditions took up Dextran-FITC (Figure 1A), DCs differentiated under physO<sub>2</sub> conditions endocytosed less Dextran-FITC per DC than atmosO<sub>2</sub> DCs when endocytosis took place at atmospheric oxygen levels (Figure 1B, *P* < 0.05). However, when the uptake of Dextran-FITC was evaluated in physO<sub>2</sub> levels, no significant difference was noted between DCs differentiated at physO<sub>2</sub> and at atmosO<sub>2</sub> (Figure 1C).

#### DCs Differentiated and Matured Under Atmospheric and Physiological Oxygen Levels Show Similar Expression of Maturation Markers

To examine whether oxygen levels influence DC maturation, the expression of cell surface molecules characteristic of DC maturation and functionality was analyzed in DCs that were differentiated under and subsequently matured by exposure to LPS or a cocktail of inflammatory cytokines (CyC: containing PGE<sub>2</sub>, TNF-α and IL-1β) under physO<sub>2</sub> or atmosO<sub>2</sub> conditions. As expected, increased expression of CD54, CD40, CD83, CD86 and major histocompatibility complex class II was observed in response to both stimuli (Figure 2). However, no difference in the expression of

these molecules was observed between immature or mature physO<sub>2</sub> DCs or atmosO<sub>2</sub> DCs with regard to mean fluorescence intensity (see Figure 2A) or percentage of cells positive for the respective molecules (see Figure 2B). One of the noted deficiencies in DC-based therapies is the inability of the majority of injected DCs to migrate to the draining lymphoid tissue. Expression of C-C chemokine receptor type 7 (CCR7), a receptor needed for lymph node homing of DCs, was upregulated by LPS and CyC exposure as expected. However, no difference in expression was noted between physO<sub>2</sub> DCs or atmosO<sub>2</sub> DCs (see Figure 2C). In addition, similar expression of the receptor CXCR4 was seen in immature DCs differentiated at both oxygen levels. Although mature atmosO<sub>2</sub> DCs showed higher expression of CXCR4, the difference was not significant (see Figure 2C).

#### Mature DCs Differentiated Under Physiological Oxygen Secrete Larger Amounts of IL-12

In addition to expression changes in cell surface molecules, mature DCs are also characterized by the secretion of inflammatory cytokines promoting the immune response. DCs differentiated under physO<sub>2</sub> or atmosO<sub>2</sub> when exposed to either LPS or CyC showed increased production of IL-6, IL-12(p70) and TNF-α under both conditions (Figure 3). No significant difference was observed in IL-6, TNF-α and IL-10 secretion between immature or mature DCs differentiated at the different oxygen levels. However, physO<sub>2</sub> DCs showed a significant increase in IL-12(p70) expression after exposure to LPS but not CyC (see Figure 3A). Although DCs differentiated at physO<sub>2</sub> or atmosO<sub>2</sub> conditions show similar expression of the costimulatory molecule CD40, we wanted to determine the influence of oxygen levels on CD40 stimulation with CD40L, which is mimicking the receptor and ligand interaction occurring between DCs and T cells. DCs were differentiated and LPS matured at the different oxygen levels and was sub-

sequently cocultured with HeLa or HeLaCD154 cells. Significantly higher levels of IL-12(p70) secretion were observed in physO<sub>2</sub> DCs after stimulation with CD40L (see Figure 3B). DCs that were exposed prior to LPS for 24 h, washed and subsequently stimulated with CD40L showed reduced secretion of IL-12(p70) compared with those only exposed to CD40L (see Figure 3B). This result is consistent with findings by Sinistro *et al.*, showing that LPS desensitizes macrophages to CD40L stimulation (38). PhysO<sub>2</sub> DCs stimulated with LPS for 24 h, washed and cultured for an additional 12 h after the wash in new media showed low levels of IL-12(p70) (42.9 pg/mL) (see Figure 3B, sixth bar from left) in contrast to DCs stimulated in the presence of LPS for 48 h (see Figure 3A). Because IL-12(p70) production peaks around 12–18 h, the reason is most likely that after the removal of all accumulated IL-12 24 h after the first stimulation, no significant amount of additional IL-12 was released in the following 12 h of culture, which is 36 h after the initial stimulation.

#### DCs Differentiated Under Physiological or Atmospheric Oxygen Levels Induce Similar Levels of T-Cell Activation

Mature cytokine-producing DCs elicit T-cell activation and proliferation, leading to the development of adaptive immunity (38,39). DCs differentiated and matured under physO<sub>2</sub> or atmosO<sub>2</sub> conditions were cocultured with allogeneic T cells for 3 d (data not shown) and 5 d (Figures 4A, B). As expected, increased T-cell proliferation was observed with mature DCs compared with immature DCs at day 5 of the coculture at both oxygen levels. Interestingly, immature physO<sub>2</sub> DCs induced significantly higher proliferation in allogeneic T cells than immature atmosO<sub>2</sub> DCs when the proliferation was performed at atmospheric oxygen levels. Immature physO<sub>2</sub> DCs induced proliferation in 22 ± 4% of T cells and immature atmosO<sub>2</sub> DCs in 13 ± 5% of T cells (see Figures 4A, B). However, no difference was noted in mature DCs.

To examine whether antigen-specific CD8<sup>+</sup> T-cell activation was affected by DCs differentiated under different oxygen conditions, we used HLA-A\*0201-positive donors. Immature DCs, LPS-DCs and CyC-DCs generated at the different oxygen conditions were pulsed with MART-1 peptide and cocultured with a MART-1-specific CD8<sup>+</sup> T-cell clone for 18 h, and secretion of IFN-γ was measured by ELISPOT (see Figure 4C). The result shows that oxygen levels had no influence on the assay. No difference was observed between immature physO<sub>2</sub> or atmosO<sub>2</sub> DCs or mature physO<sub>2</sub> or atmosO<sub>2</sub> DCs with regard to their ability to induce MART1-specific T-cell responses.

#### DISCUSSION

Because DCs are the most potent initiators of antigen-specific T-cell responses, they have been applied toward immunotherapy of cancer and chronic infectious diseases. For DC-based adoptive immunotherapy, DCs are differentiated *ex vivo* from CD14<sup>+</sup> or CD34<sup>+</sup> progenitors, pulsed with tumor antigen, exposed to a maturation stimulus and reinjected back into patients (7,40). For these studies, DCs were differentiated in CO<sub>2</sub> incubators containing atmospheric oxygen levels of 20–21%. However, these levels are two to four times higher than physiological oxygen levels, which are approximately 12% in arterial blood and 3–5% in tissue (30–32), where most DCs reside.

In light of a recent finding showing altered T-cell responses at atmosO<sub>2</sub> compared with physO<sub>2</sub> (33), we sought to determine if differentiation of DCs from CD14<sup>+</sup> progenitors under physiological oxygen levels would alter their physiology and antigen-presenting capacity, with the hope to identify improved conditions for DC-based studies. Surprisingly, no difference in expression of the surface molecules CD54, CD40, CD83, CD86, HLA-DR, CXCR4 and CCR7 was observed in immature DCs or mature DCs. In addition, the secretion of TNF-α, IL-6 and IL-10 from either immature or mature DCs did not differ between the different oxygen culture conditions.

We found that LPS-matured physO<sub>2</sub> DCs secreted higher levels of IL-12(p70) than LPS-matured atmosO<sub>2</sub> DCs. IL-12 is an important cytokine mediating Th1 polarization of CD4<sup>+</sup> T cells, which provides help for the activation of cytotoxic CD8<sup>+</sup> T cells. Although physO<sub>2</sub> DCs did not elicit increased CD8<sup>+</sup> T-cell responses *in vitro*, as measured by IFN-γ secretion, this could be due to the use of a CD8<sup>+</sup> T-cell clone, which is easier to activate than, for instance, naive T cells. Another possibility is that the levels of IL-12 secreted in the two different oxygen conditions were sufficiently high to support T-cell activation; thus, no difference could be observed. The increased capacity of immature physO<sub>2</sub> DCs to activate allogeneic T cells could be due to their increased secretion of IL-12 after ligation with CD40L on the T cells, as seen when DCs were stimulated by CD40L.

We found that the cytokine cocktail-matured DCs, when generated under physiological or atmospheric oxygen conditions, did not differ with regard to their ability to induce proliferation of allogeneic T cells or activation of antigen-specific CD8<sup>+</sup> T cells. It was recently shown that cytokine cocktail-matured DCs can also induce immunosuppressive regulatory T cells (Tregs) (41), and it remains to be tested what effect oxygen levels will have on the induction of Tregs by DCs.

Overall, we show that DCs differentiated and stimulated with LPS or CD40L under physO<sub>2</sub> conditions secreted higher amounts of IL-12(p70), suggesting that it may be more beneficial to culture and activate DCs at physO<sub>2</sub> levels because higher IL-12 secretion may cause a more pronounced T-cell response *in vivo*. Overall, only a few differences were observed owing to oxygen levels and other parameters, such as the DC subtype, the maturation stimulus, or the presence of the immunosuppressive microenvironment of the tumor are more likely influencing the poor clinical outcome of the currently used adoptive DC immunotherapy protocols.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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