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Research paper

Oxygen tension regulates the in vitro maturation of GM-CSF expanded murine bone marrow dendritic cells by modulating class II MHC expression

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Abstract

Conventional culture conditions for GM-CSF expanded murine bone marrow derived dendritic cells (BMDCs) uses ambient (hyperoxic) oxygen pressure (20% v/v, 152 Torr) and medium supplemented with the thiol 2-mercaptoethanol (2-Me). Given the redox activities of O_2 and 2-Me, the effects of 2%, 5%, 10%, and 20% v/v O_2 atmospheres and omitting 2-Me from the medium were tested upon the generation of GM-CSF expanded BMDCs. DC yield, phenotype and function were compared to BMDCs grown using conventional conditions. All cultures yielded DC subsets with $CD11c^+$ MHC II^{NEG}, $CD11c^+$ MHC II^{INT}, $CD11c^+$ MHC II^{HI} expression phenotypes, classed as precursor, immature, and mature DCs (IDC, MDC). Low O_2 tensions generated significantly fewer precursor DCs, and more IDCs and MDCs. Cytometer sorted precursor DCs expressed surface class II MHC after transfer to low, but not high O_2 atmospheres. Expression of myeloid markers was similar between BMDC cultures generated in 5% O_2 or conventional conditions, and MDCs from low O_2 cultures had the morphology typical of mature myeloid DCs. IDCs and MDCs from low O_2 and conventional culture conditions were similarly potent allostimulatory APCs. The O_2 tension (but not 2-Me addition) in vitro significantly influences overall DC subset frequencies and yield, and governs DC maturation by regulating the surface class II MHC expression of GM-CSF expanded BMDC cultures.

Keywords: Dendritic cell; Bone marrow; Myeloid; Oxygen tension; Class II MHC; 2-mercaptoethanol

Abbreviations: BMDCs, bone marrow derived dendritic cells; DCs, dendritic cells; rmGM-CSF, recombinant murine granulocyte-macrophage colony stimulating factor; MHC II, class II MHC; IDC, immature DC; MDC, mature DC; 2-Me, 2-mercaptoethanol; APC, antigen presenting cell; Ag, antigen; IFN-γ, interferon gamma; IL-12, interleukin-12; FcRI, Fc receptor epsilon I; R10, RPMI 1640 medium supplemented with 10% serum; HBSS, Hank's balanced salt solution; PBS, phosphate buffered saline; FBS, fetal bovine serum; FITC, fluorescein isothiocyante; PE, phycoerythrin; APC, allophycoerythrin; PI, propidium iodide; ANOVA, analysis of variance; FSC, forward scatter; SSC, side scatter; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha.

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1. Introduction

Dendritic cells (DCs) acquire antigens from self or nonself sources and efficiently present them to naïve and resting T cells. Antigen (Ag) loaded DCs respond to Ag-specific T cells by increasing their intracellular oxidation state (Matsue et al., 2003) and by generating a reducing microenvironment (Angelini et al., 2002). The antioxidant Ebselen inhibits T cell proliferation and cytokine production induced by DCs as well as DC cytokine production induced by T cells (Matsue et al., 2003). Cellular redox state therefore appears to be an important mediator of Ag-specific, bidirectional DC-T cell communication. Tissue normoxia ranges from 2.5% to 9% v/v, but most, if not all, in vitro studies of DCs use conventional culture conditions employing ambient (hyperoxic) oxygen pressure (O_2=20.9% v/v, 152 Torr) and medium supplemented with 2-mercaptoethanol (2-Me; 50 μM). Ambient O_2 tension or thiol concentration in the culture medium significantly influences the secretion of IFN-γ and IL-12 (Murata et al., 2002) and enhances expression of FcR1 (Novak et al., 2002) by DCs. Given the role of ambient O_2 in mediating redox reactions and the thiol activity of 2-Me, we adapted the protocol by Lutz et al. (1999) by generating murine bone marrow-derived DCs (BMDCs) using a range of O_2 tensions and omitting 2-Me from the culture medium. We examined the precursor, immature and mature (IDC, MDC) DC subsets that arose and compared them to DCs grown using conventional conditions. Under normoxic and 2-Me free culture conditions, GM-CSF expanded murine BMDCs are readily grown with yields of class II MHC^+ myeloid DCs that are 2 to 3 fold greater than cultures grown using conventional conditions. DCs cultured by this method are virtually identical in surface phenotype and allostimulatory capacity compared to cells grown using conventional conditions. In addition, we find the expression of surface class II MHC, a marker of DC maturation status, is regulated on $CD11c^+$ BMDCs by the O_2 tension in vitro.

2. Materials and methods

2.1. Chemicals and antibodies

[^{3}H]thymidine (activity 20 Ci/mmole) was purchased from Perkin Elmer Life Sciences (Boston, MA). Recombinant murine granulocyte-macrophage stimulating factor (rmGM-CSF) was purchased from Sigma (St. Louis, MO) or R&D Systems (Minneapolis, MN). Antibodies (all purchased from BD-Pharmingen, San Diego, CA) to the following murine marker epitopes are as follows with the clone name in parenthesis: class II MHC (2G9 or M5/114.15.2), CD11c-APC and -PE (HL3), CD86-PE (GL1), biotinylated anti-CD8α (53-6.7), CD45R/B220-PE (RA3-6B2), CD11b-FITC (M1/70), and unconjugated anti-invariant chain (In-1) and anti- CD16/32 (2.4G2). Fluorophore conjugated isotype control rat IgG2a (R35–95), IgG2b (A95-1), monoclonal antibodies (mAb) and hamster IgG (G235–2356) were purchased from Pharmingen, isotype control mouse IgG2a (5–205) mAb was purchased from Accurate Chemical (Westbury, NY). Unconjugated hamster Ig, streptavidin-FITC and -PE were also purchased from Pharmingen. Prolong antifade reagent was purchased from Molecular Probes, Inc. (Eugene, OR). Propidium iodide was purchased from Sigma.

2.2. Animals and cell culture

Female C57BL/6 and BALB/c mice aged 6 to 8 weeks were obtained from JAX West, Inc. (Davis, CA) and were euthanized in accordance with a protocol approved by the UC Davis Animal Resources Service. Extracted bone marrow was depleted of red blood cells by ammonium chloride lysis. R10 medium used to culture bone marrow and sorted DCs was RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 2 mM sodium pyruvate, and 1000 IU penicillin and 1000 μg streptomycin (Invitrogen) per 500 ml of medium. 2-Mercaptoethanol (2-Me, Sigma; 50 μM final) was added to R10 for cells cultured at 20% O_2 and 37 °C (standard culture conditions). HBSS and PBS (without calcium or magnesium) were purchased from Invitrogen.

Generation of bone marrow derived DCs was then carried out according to the protocol published by Lutz (Lutz et al., 1999) using either 10 or 15 cm diameter uncoated sterile plastic petri dishes for culture. For every 78 cm^2 of culture area, 2×10^6 red cell depleted marrow cells were plated in 10 ml of R10 medium supplemented with 20 ng/ml rmGM-CSF. After 3 days an equal volume fresh R10 with 20 ng/ml rmGM-CSF was added per plate. Every two days, one half of the culture supernatant was removed, the cells spun down, resuspended in R10 with 20 ng/ml rmGM-CSF and returned. Nonadherent cells were harvested and prepared for flow cytometry between days 6 and 8. Cells were maintained at 37 °C in a humidified Thermo Forma model 3130 tissue culture incubator (Thermo Forma, Marietta, OH) equipped with built-in

CO_2 and O_2 monitors and attached nitrogen and carbon dioxide gas supplies. Carbon dioxide was set to 5% v/v, and oxygen to 2%, 5%, 10% v/v or ambient (20.9%). The oxygen and carbon dioxide contents of the incubator atmosphere were periodically verified using Fyrite gas analyzers (Bacharach Inc, New Kensington, PA).

2.3. Flow cytometric cell sorting and analysis

For flow sorting, bulk or gradient fractionated (see below) nonadherent cells were preblocked with 2 μl anti CD16/32 and 0.5 μl hamster IgG per 1×10^6 for 10 min on ice. Then saturating anti class II MHC (2G9) and anti CD11c mAb were added and allowed to bind for 15 min. Cells were washed once with PBS supplemented with 2% FBS and resuspended in PBS:FBS for every 1×10^6 cells for 15 min. The stained cells were washed, passed through a sterile 35 μm nylon mesh, and propidium iodide added to a final concentration of 0.5 μg/ml immediately before aseptic sorting on a MoFlo cytometer (Cytometric, Fort Collins, CO). Single stained and unstained controls were used to define sorting gates and to adjust fluorescence compensation. CD11c positive cells were considered DCs, which were then further graded as precursor DCs, IDCs or MDC depending on their staining intensity (negative, intermediate, or high) for class II MHC.

Nonadherent cells were either directly labeled with antibodies as described above or enriched for low density class II MHC^+ DCs before labeling and cell sorting/analysis by passage over a discontinuous iodixanol (Optiprep, Accurate Chemical) gradient. Bulk nonadherent cells ($1–2 \times 10^8$) were resuspended in 4 ml of 3/1 v/v HBSS:Optiprep (final density = 1.085 g/ml) and transferred to a polypropylene centrifuge tube. This was overlaid with 5 ml of a mixture of 3.2 volumes diluent (0.88% (w/v) NaCl, 1 mM EDTA, 0.5% (w/v) bovine serum albumin, 10 mM Hepes–NaOH, pH 7.4) and one volume Optiprep (final density = 1.065 g/ml). This layer was overlaid with 3 ml HBSS. Cells were centrifuged for 20 min at 20 °C with no braking. Cells (usually 50×10^6 from bone marrow from 2 mice) at the HBSS and the diluent:Optiprep interface were collected for antibody staining. Low density cell populations showed increased cell granularity (SSC, associated with mature DCs), 2-fold more $CD11c^+$ DCs, and reduced sorting time needed to obtain $CD11c^+$ class II MHC^+ compared to non-fractionated populations (not shown).

For flow cytometric analysis bulk or gradient fractionated nonadherent cells were transferred to polystyrene tubes, cooled on ice, centrifuged 5 min at 400 $\times g$ in a chilled rotor, aspirated, washed with ice cold 2% FCS:PBS, centrifuged again, and blocked with anti CD16/32 mAb and hamster IgG for 10 min on ice. Directly conjugated mAbs were used to label at a concentration of 0.3 $\mu g/10^6$ cells, unlabeled and biotinylated primary mAbs at 0.1 $\mu g/10^6$. Specie and Ig isotype matched antibodies and FITC- and PE-conjugated streptavidin were used at the same concentrations for labeling control cells. Propidium iodide was added to a final concentration of 0.5 μg/ml before analysis on a FACScan® flow cytometric analyzer (Becton Dickinson, Palo Alto, CA). Phenotyping of the bulk nonadherent cells from the 2%, 5%, 10% and 20% O_2 cultures for simultaneous CD11c and class II MHC expression in Fig. 1 was performed using three independent cultures. Cell surface phenotyping of the PI-negative, FSC/SSC gated low density cells in Fig. 4 (cultured in 5% O_2 or conventional culture conditions of 20% O_2 + 2-Me) was performed with two independent cultures with similar results.

2.4. Fluorescence micrography

Sorted MDC were spun onto glass slides using a Cytospin apparatus (Statspin Inc., Norwood, MA) fixed for 10 min with ice cold 4% paraformaldehyde: PBS, washed 3 times with PBS, mounted with Prolong antifade, coverslipped, dried, and sealed with nail polish. Light from a mercury arc lamp was filtered through a 510 nm bandpass filter (± 20 nm) to provide fluorophore excitation. Fluorescence images using a 580 nm long pass filter were taken using a digital camera (Kodak, Rochester, NY) mounted on a Nikon Diaphot (Melville, NY) inverted microscope. Images were processed using Adobe Photoshop software (San Jose, CA). For confocal imaging of invariant chain, cytospun cells were fixed with 4% paraformaldehyde: PBS for 20 min at 4°C, then were permeabilized with three exchanges of 0.2% Tween-20 in PBS (TPBS) for 10 min each. Nonspecific binding was blocked using goat IgG (50 μg/mL) in TPBS for 1 h. Cells were washed and incubated with In-1 antibody in TPBS for 1 h. Cells were washed three times with TPBS for 10 min each. Goat anti-rat secondary antibody conjugated to FITC was diluted 1/1000 in TPBS and allowed to bind for 1 h. Cells were washed three times with TPBS, then twice with PBS. After mounting with Prolong antifade supplemented with 5 μg/mL propidium iodide, images were acquired using a Bio-Rad MRC 600 laser scanning confocal microscope (Richmond, CA). Fluorescence PMT thresholds and gains were set using cells

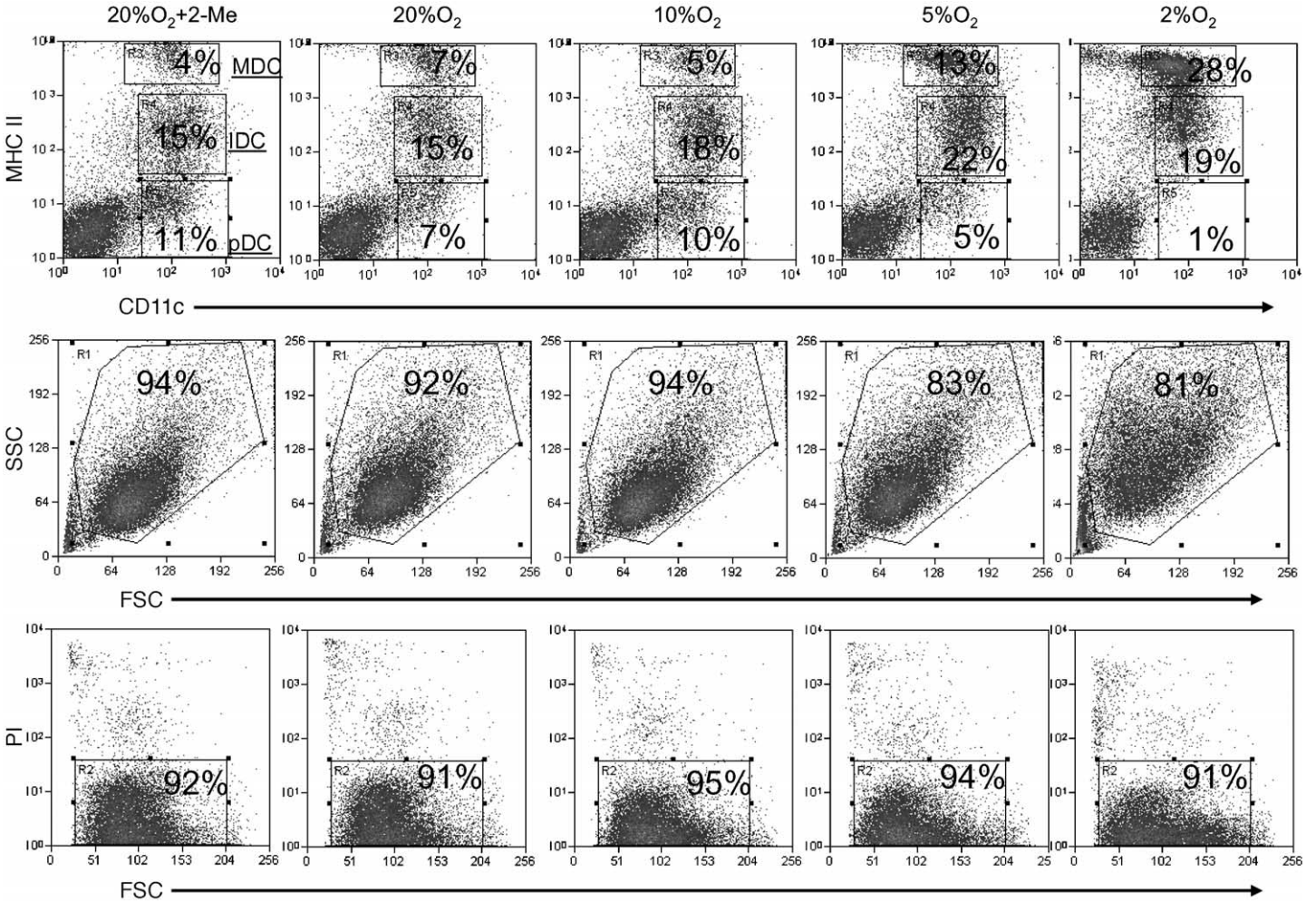

Fig. 1. Flow cytometric analysis dot plots of nonadherent cells from GM-CSF expanded bone marrow cultures. Columns from left to right: cells cultured in conventional conditions (20% O_2 and 2-Me), 20%, 10%, 5%, or 2% O_2. Top row: co-expression of CD11c and class II MHC on PI-negative cells. Regions encompassing $CD11c^+$ DCs with high, intermediate, and no class II MHC expression are denoted MDC, IDC, and precursor DC (pDC) in the first top left hand panel. The percentage of each DC subset is given within each region. Note the inverse relationship between class II MHC expression and increasing O_2 tension. Middle row: cell size (FSC) versus granularity (SSC). Note the increased granularity in the 2% O_2 population, due to an increase in MDC (data not shown). Bottom row: viability (propidium iodide (PI) uptake) versus cell size (FSC). 30,000 events are shown per plot and are representative of 3 independent culture sets.

stained with fluorescent secondary antibody alone. Confocal immunophenotyping analyses were performed on cells obtained from at least two separate cultures.

2.5. DC maturation assay

Sorted DC subsets were cultured onto perfluorocarbon containers to avoid adhesion-induced signaling events that could potentially confound readouts of cell status and function (Lu et al., 1994). DCs were resuspended in R10 supplemented with 5 ng/ml GM-CSF to 1×10^6 cells/ml, and aliquoted into perfluorocarbon tissue culture vials or 96 well format plates (Savillex, Minnetonka, MN), placed in a 37 °C incubator and harvested by gentle pipetting. DCs were harvested 48 h after plating, antibody stained and analyzed for CD11c and class II MHC expression (using M5/114.15.2 mAb) by flow cytometry.

2.6. Allogeneic T cell proliferation assay

Splenic $CD4^+$ T cells were obtained from 6 to 8 week old female BALB/c mice after ammonium chloride lysis and antibody mediated negative depletion of other leukocytes using a kit purchased from R&D Systems. Sorted IDCs and MDCs subsets were treated for 30 min with mitomycin C (50 μg/ml) washed 4 times with PBS, resuspended in medium, counted, and plated in 96 well round bottom plates (Nalge Nunc International, Rochester, NY), with 10^5 purified $CD4^+$ T cells added per well in a final volume of 0.2 ml. DC and T cell only wells were plated to provide background counts. DCs originally grown in 2-Me-free or -supplemented medium were replated in the same for these cocultures; i.e., cocultures with 5% O_2 DCs used medium without 2-Me supplementation. 54 h after coculture initiation, 1 μCi [^{3}H]thymidine was added per well. 18 h later cells were harvested onto glass fiber filters using a cell harvester (Brandel, Gaithersburg, MD). Filter associated radioactivity was determined in a scintillation counter, and net T cell thymidine incorporation recorded after subtraction of combined DC and T cell only counts (<3000 dpm). Stimulation assays were performed twice independently with similar results.

2.7. Data analysis

One-way ANOVA was performed using Origin 6.0 (Northampton, MA) software to test for statistical significance.

3. Results

3.1. Oxygen tension regulates the proliferation of $CD11c^+$ DCs from GM-CSF expanded murine bone marrow cultures

Pooled bone marrow cells from C57BL/6 female mice were plated in GM-CSF containing medium as described in Materials and methods and individual cultures placed in separate incubators where the atmospheric oxygen tensions were adjusted to 2%, 5%, 10%, and 20% by nitrogen purging. 2-Me was added to the culture medium to 50 μM where indicated. 6 days later, nonadherent cells were harvested, counted, antibody stained and analyzed by flow cytometry (Fig. 1) where cell size, granularity, viability, and class II MHC and CD11c expression levels for the different culture conditions are shown as dot plots. The top row of panels shows that all culture conditions generated four distinct cell subpopulations based on antibody epitope expression: double negative cells, $CD11c^+$ class II MHC^{NEG}, $CD11c^+$ class II MHC^{INT}, and $CD11c^+$ class II MHC^{HI}. DC populations were identified by CD11c expression and DC maturation status by their class II MHC expression (Lutz et al., 1999; Jackson et al., 2002; Diao et al., 2004). Thus $CD11c^+$ class II MHC^{NEG}, $CD11c^+$ class II MHC^{INT}, and $CD11c^+$ class II MHC^{HI} were classed as precursor DCs, immature, and mature DCs (IDCs, MDCs). As the O_2 tension was increased, the percent class II MHC positive DCs (IDCs and MDCs) decreased, and class II MHC negative DC (precursor) increased within the culture (Fig. 1, top row of panels). Cell granularity (SSC) (Fig. 1, middle row) was consistently higher for cells from the 2% O_2 cultures, and this was due to the increased proportion of MDCs in these cultures (data not shown). Cell size and granularity did not differ between the other cultures. All cultures had high viability, with over 90% of cells excluding PI (Fig. 1, bottom row).

The overall yield of nonadherent cells and $CD11c^+$ cells varied according to O_2 tension and whether 2-Me was in the culture medium (Table 1). Cultures maintained at 5% and 10% O_2 (no 2-Me) or conventional conditions generated the most, and the 2% and 20% O_2 conditions the fewest nonadherent cells. $CD11c^+$ cell yields significantly varied with the O_2 tension only, and 2-Me had no effect. This was somewhat unexpected given that cultures grown at 20% O_2 without 2-Me generated significantly fewer nonadherent cells (39% less) than those grown with 2-Me supplemented media (Table 1). DC subset yields also varied significantly

Table 1
Yields of nonadherent cells and dendritic cells from murine bone marrow cultured in varying oxygen concentrations

O_2 (%)	Nonadherent	precursor DC	IDC	MDC	IDC+MDC
2	11.03 ± 2.28 (.013)	0.25 ± 0.16 (.007)(0.010)[a]	2.74 ± 0.42 (.040)	1.99 ± .63 (.682)(.028)[a]	4.73 ± .76 (.019)
5	21.22 ± 3.47	1.41 ± 0.37	5.44 ± 1.50	2.35 ± .44	7.79 ± 1.19
10	22.80 ± 1.44 (.504)	2.64 ± 0.68 (.051)	4.51 ± 0.77 (.396)	1.17 ± .34 (.021)(.097)[a]	5.69 ± .83 (.066)
20	12.93 ± 1.48 (.019)	1.50 ± 0.57 (.82)	2.11 ± 0.58 (.023)	0.69 ± .16 (.003)	2.81 ± .50 (.003)
20+2−Me	21.28 ± 4.85 (.986)(.046)[b]	3.00 ± 1.03 (.006)(.091)[b]	3.10 ± 0.92 (.083)(.193)[b]	0.74 ± .06 (.003)(.629)[b]	3.84 ± .86 (.010)(.146)[b]

The mean in millions of nonadherent cells or DC per 50 ml of bone marrow cell culture ± SD after 6 days of culture. Data were obtained from 3 independent cultures. IDC=immature DC, MDC=mature DC. 2−Me, 2-mercaptoethanol (50 μM) supplemented medium. First p value in parentheses derived from one-way ANOVA in comparison to 5% O_2.

[a] ANOVA p value compared to 20% O_2+2−ME.

[b] ANOVA p value compared to 20% O_2.

with the O_2 tension but again not with 2-Me supplementation. Precursor DC counts ranged the most, with a 12 fold difference between the 2% O_2 and conventional culture condition values (Table 1). IDC yields were less variable, with a 2.6 fold maximal difference between 5% and 20% O_2 cultures (Table 1). MDC counts were slightly more variable, with a 3.4 fold difference between the 5% and 20% O_2 cultures (Table 1). 5% O_2 generated more $CD11c^+$ cells than conventional conditions, but significant differences were achieved for the MDC subset only (Table 1).

3.2. Oxygen tension regulates the expression of surface class II MHC on precursor DCs

As noted above, a trend emerged where the frequency and number of precursor DCs increased, and that of IDCs and MDCs decreased, as the O_2 culture tension rose (Fig. 1 and Table 1). We explored a possible link between the O_2 tension and precursor DC development by culturing sorted precursor DC in their original O_2 tensions and measuring the expression of surface class II MHC^+, with the expectation that as O_2 tension increased, relative class II MHC expression should decrease.

Sorted precursor DC subsets from all cultures were recultured under their original conditions and examined for CD11c and class II MHC expression 2 days later. Surface class II MHC expression occurred only on precursor DCs originating from the 2% and 5% O_2 cultures, precursor DCs re-cultured at 10% and 20% O_2 showed little or no class II MHC expression (Fig. 2, $n=2$). We wondered then if the acquisition of surface class II MHC expression was a property unique to precursor DC generated under low (2% or 5%) oxygen, or if precursor DC from the higher O_2 tension cultures could also be induced to express surface class II MHC by transfer to a lower O_2 environment. To test this hypothesis, sorted precursor DC from 10%, 20%, and 20% O_2+2-Me cultures were returned to a low (2%) or high (20%) O_2 atmosphere, and 2 days later stained and analyzed for CD11c and class II MHC expression. Precursor DC originating from higher O_2 cultures can be induced to express surface class II MHC only after transfer to a low, but not high O_2 environment (Fig. 3). 2-Me supplementation slightly decreased the mean fluorescence intensity and the proportion of precursor DC induced to express class II MHC ($n=2$), and precursor DC re-cultured in 2% O_2 expressed 2-fold higher CD11c than cells re-cultured in 20% O_2.

3.3. Physiologic O_2 tension (5% v/v) generates BMDCs with normal phenotype and function

We undertook further phenotypic and functional studies upon DCs generated from 5% O_2 (and 2-Me free) cultures, since those conditions consistently had the greatest positive effect upon $CD11c^+$ cell yield. After 6–8 days culture, non-adherent bone marrow derived cells were harvested and prepared for flow cytometric cell analysis by density gradient floatation (to enrich for DCs, see Materials and methods) and antibody staining. The low density portion of gradient fractionated cells from the BMDC cultures grown under conventional conditions or 5% O_2 generated cells ($n=2$ independent cultures) did not differ significantly in size (forward scatter (FSC), granularity (side scatter, SSC), or viability (PI exclusion, data not shown). We compared the co-expression of cell surface markers associated with $CD11c^+$ DCs between the low density cell fractions from the two culture conditions with a panel of antibodies specific for CD11b, Ly-$6G^+$/Gr-1, CD54/ICAM-1, CD80, CD86, CD40, CD45R/B220 and CD8α.

In the PI-negative, FSC/SSC gated populations, CD11c was expressed more intensely and by more cells (64% vs. 52%) from the 5% O_2 cultures, which was noted earlier in Table 1. The increased CD11c-positive cells in the 5% O_2 cultures did not always

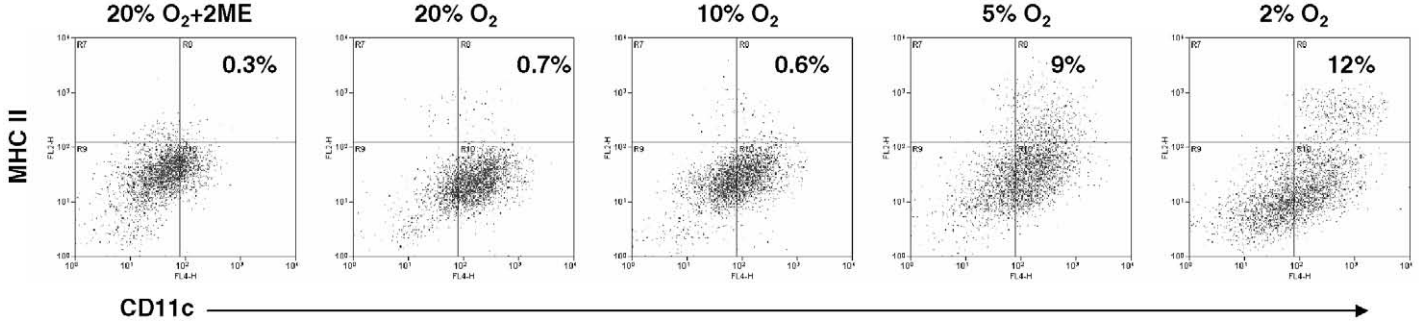

Fig. 2. Flow cytometric analysis dot plots of CD11c and class II MHC coexpression on precursor DCs ($CD11c^{+}$, class II MHC^{NEG}) sorted from conventional conditions (20% O_2+2-Me, first panel on the left), 20% O_2 (second panel), 10% O_2 (third panel), 5% O_2 (fourth panel), 2% O_2 (fifth panel) and recultured for 48 h in their original O_2 concentrations. Cells were then harvested and stained with direct fluorophore conjugated mAbs HL3-APC (α-CD11c) and M5/115.14.2-PE (α-class II MHC). Precursor DCs that acquire class II MHC expression are in the upper right dot plot quadrant together with the percentage. Quadrant gates were set using unstained and single stained cells. 3000 PI negative events are shown per dot plot.

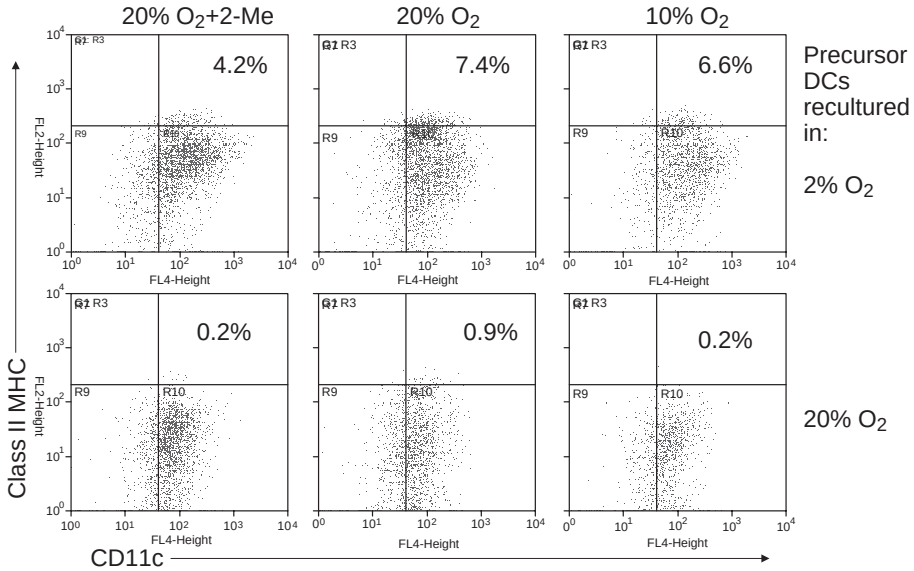

Fig. 3. Flow cytometric analysis dot plots of CD11c and class II MHC coexpression on precursor DCs ($CD11c^+$, class II MHC^{NEG}) sorted from conventional conditions (20% O_2+2-Me, left column), 20% O_2 (middle column) and 10% O_2 (right column) recultured for 48 h in 2% O_2 (top row of panels) or 20% O_2 (bottom row). Precursor DCs that acquire class II MHC expression are in the upper right dot plot quadrant together with the percentage. Cells were harvested, stained and analyzed as in Fig. 2. An equal number (2000 to 3500) of PI negative events are shown in each matched pair of dot plots.

correlate with an increased expression of other DC associated surface markers. Compared to those grown under conventional conditions, the percent of $CD11c^+$ cells from the 5% O_2 cultures co-expressing CD54 or CD11b increased, those co-expressing CD86 decreased, and those co-expressing Ly6G or CD80 were the same.

In both cultures, the cells with the most intense CD80 or CD86 expression (Fig. 4, upper right hand quadrants) tended to be large and granular, indicative of a more mature DC phenotype. Conversely, $CD11c^+$ cells with more moderate CD80 or CD86 expression tended to be smaller and less granular, indicative of a less mature DC phenotype (data not shown). 5% O_2 cultures generated nearly 30% more $CD11b^+$ cells that also expressed it more intensely, as well as 30% more $CD54^+$ cells than conventional culture conditions.

The myeloid lineage specific marker CD11b is expressed by $CD11c^+$ cells in both populations in a bimodal manner (upper right quadrants, Fig. 4) with the 5% O_2 cultures yielding 30% more positive cells. The ratio of $CD11c^+/CD11b^{INT}$ to $CD11c^+/CD11b^{HI}$ expressors in the 5% O_2 cultures was 1:5.8 and was 1:2.3 in the conventionally cultured cells. Backgating these two populations from the 5% O_2 and the conventional O_2 culture reveals that the $CD11b^{HI}/CD11c^+$ cells are more granular, while the $CD11b^{INT}/CD11c$ cells are less granular (data not shown). Interestingly, reanalysis of IDC+MDC and precursor DC in Fig. 1 from both cultures also showed the same trend of decreasing granularity from the IDC+MDC to the precursor DC subsets.

CD40 staining was absent, indicating a lack of activated mature myeloid DCs, which can be induced in MDCs with LPS or TNFα (Lutz et al., 1999). The lack of staining for CD45R indicates an absence of B cells. We tested if O_2 tension (and omitting 50 μM 2-Me) influenced the development of $CD8\alpha^+$ $CD11c^+$ DCs in our cultures as Brasel et al. (2000) identified $CD8\alpha^+$ $CD11c^+$ DCs in Flt3L cytokine-expanded murine bone cultures. We found no CD8α expressing cells under any of the culture conditions we tested.

Low density gradient fractionated BMDCs were used to expedite flow cytometric sorting of class II MHC^+ DCs for further morphological and functional assays. The yield of sorted low density IDCs and MDCs from cultures generated at 5% O_2 compared to conventional conditions were 1.5 fold greater due to more MDCs ($n=3$, data not shown). This is nearly the same difference found in the unfractionated nonadherent cells (2 fold difference, Table 1) therefore the low density cell fraction accurately represents the DCs found in the unfractionated cultures. In Fig. 5, fluorescence micrographs of sorted MDCs generated in 5% O_2 stained with anti-class II MHC and -CD11c mAbs (panels A and B) or with anti-invariant chain mAb

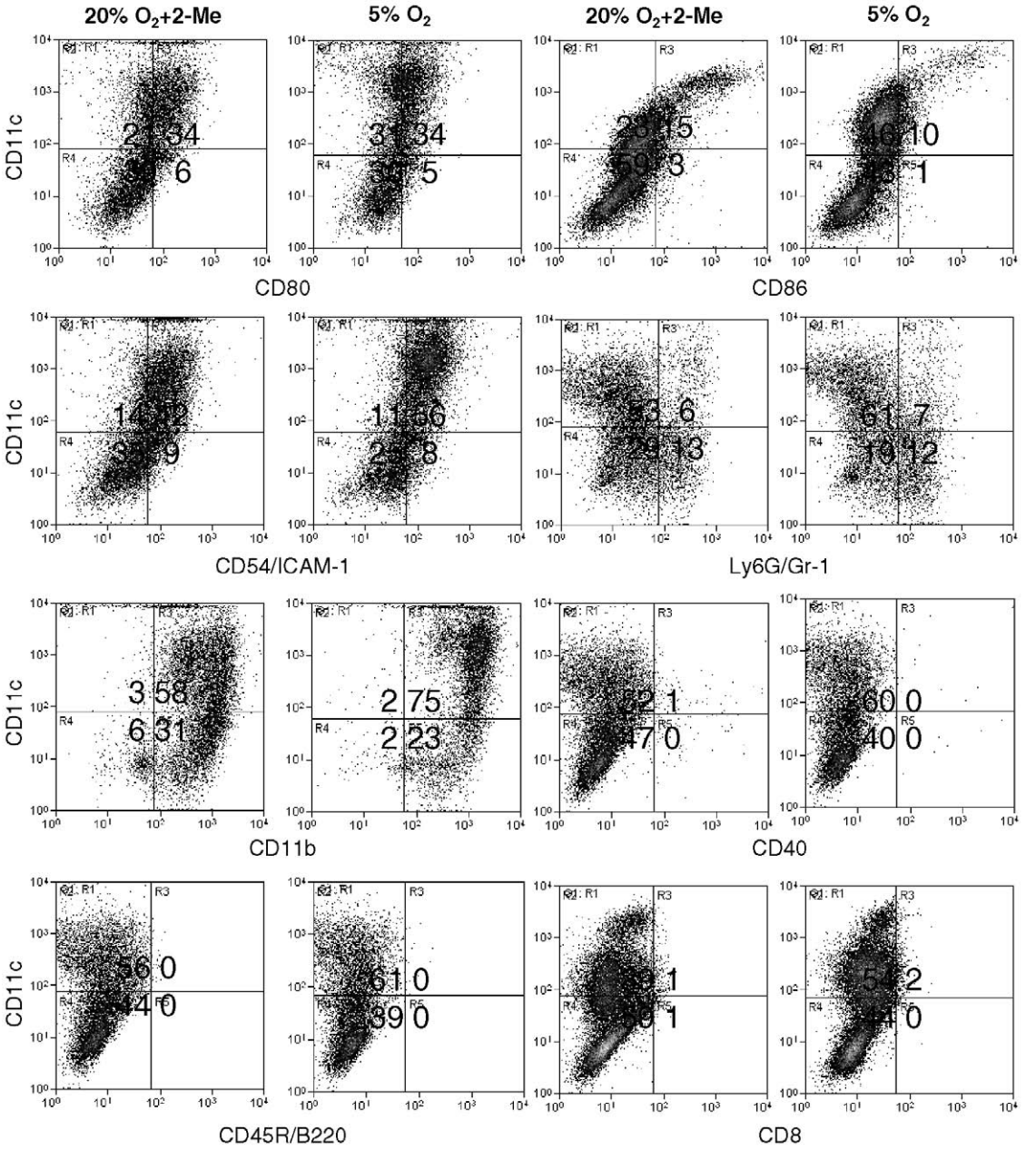

Fig. 4. Flow cytometric analysis dot plots of PI-negative, FSC/SSC gated low-density nonadherent GM-CSF derived bone marrow cells cultured in conventional conditions with 20% oxygen + 2-Me (first and third columns from the left) or 5% O_2 (second and fourth columns). 20,000 propidium iodide negative events shown were analyzed for coexpression of CD11c (y-axis) and myeloid-granulocyte lineage cell surface markers CD11b, Ly-6G/Gr-1, cell adhesion marker CD54/ICAM-1, costimulatory markers CD80, CD86 and CD40, B cell marker CD45R, and the DC subset marker CD8α (x-axes). Numbers within each quadrant indicate the percent positive cells. Cells stained with isotype matched control antibodies using hamster IgG, rat IgG2a, rat IgG2b, and mouse IgG2a were used to set quadrant boundaries.

and PI (panel C) display the typical myeloid DC cellular characteristics of stellate morphology, numerous cell processes, and lobulated, excentric nuclei. These results show the majority of the low-density cells grown in 5% O_2 or under conventional conditions are myeloid DCs (>50% $CD11c^+$) with minor qualitative or quantitative differences in the expression of key myeloid markers ($n = 2$ experiments).

Low density DCs generated in 5% O_2 or from conventional conditions were sorted and then compared for antigen presentation function in an allogeneic T cell stimulation assay. BALB/c $CD4^+$ T cells were added to

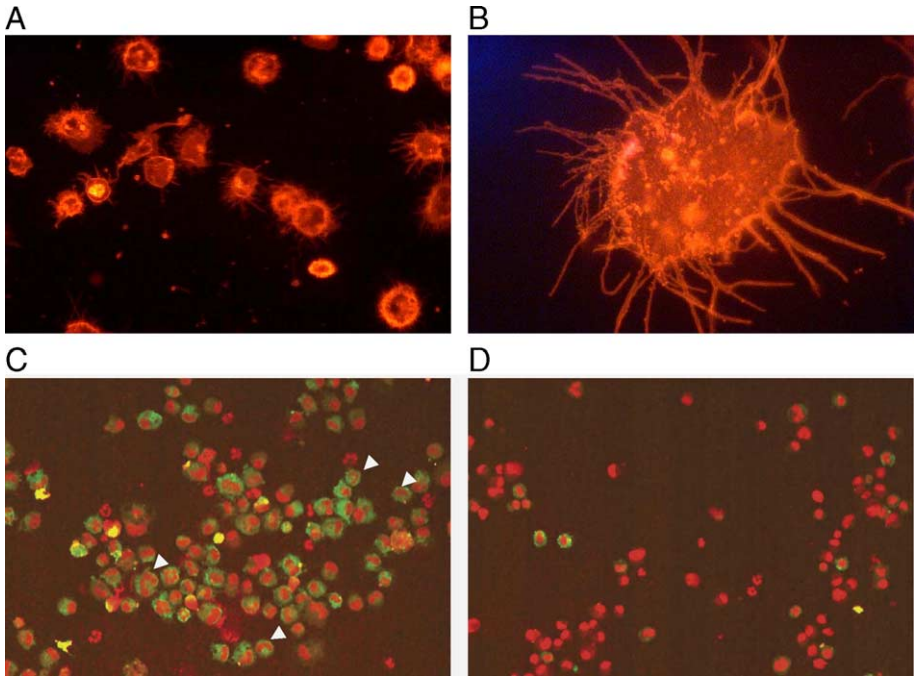

Fig. 5. Fluorescence micrographs of flow sorted, CD11c$^+$ and class II MHC$^+$ stained (panels A and B) mature dendritic cells from 5% O_2 cultures displaying morphologic characteristics typical of dendritic cells. Light from a mercury arc lamp was filtered through a 510 nm bandpass filter (±20 nm) to provide fluorophore excitation. Images shown in panels A and B were obtained with a 550 nm long pass filter and represent UV-excited fluoresence from both the anti-class II MHC-PE and anti-CD11c-APC mAb conjugates. Panel C is a confocal image of MDC visualized for invariant chain (cytoplasmic) expression and propidium iodide (nuclear) fluorescence. Panel D shows control MDC stained with FITC conjugated second step Ab and propidium iodide. Arrow heads in C indicate nuclei, note lobulation and excentric localization. (Panels A, C, D) 40× original magnification, (Panel B) 100× original magnification.

graded numbers of C57BL/6 IDCs or MDCs and the cultures maintained at 5% or under conventional conditions. DCs originally grown in 2-Me-free or -supplemented medium were replated in the same for these cocultures; i.e., cocultures with 5% O_2 DCs used medium without 2-Me supplementation.T cell stimulation did not differ significantly with IDCs generated in either O_2 tension, nor if the co-cultures were kept in a 5% or 20% O_2 atmosphere (Fig. 6A and C). MDCs generated from conventional cultures were significantly more stimulatory regardless of the O_2 tension in which the cocultures were maintained (Fig. 6B and D). Why these MDCs have a greater allostimulatory capacity may reflect the presence of 2-Me in the coculture medium and/or the greater number of CD86$^+$ cells in these cultures (Fig. 4). T cells require cysteine to proliferate, but are unable to import its precursor cystine. 2-Me allows T cells to import cystine as a cystine and 2-Me mixed disulfide, and intracellular cleavage of the disulfide releases cystine (Ishii et al., 1981). Nevertheless, all the DCs used in these studies were potent APCs, inducing T cell proliferation at the highest T:DC ratio tested (243:1). We conclude murine bone marrow cells cultured with GM-CSF at a physiologic oxygen tension (5% v/v) and without 2-Me supplementation generate myeloid DCs that have the same leukocyte surface marker expression and nearly equal allostimulatory capacity as DCs produced using ambient oxygen and 50 μM 2-Me supplemented medium.

4. Discussion

We adapted a widely cited protocol describing an improved method of culturing DCs from GM-CSF expanded murine bone marrow (Lutz et al., 1999) by using a range of O_2 tensions (2%, 5%, 10%, 20% v/v) and omitting 2-mercaptoethanol (2-Me) from the culture medium. BMDC cultures grown with 5% or 10% O_2 (no 2-Me) generated significantly more IDCs and MDCs than conventional conditions using ambient O_2 and 50 μM 2-Me (2 fold increase, Table 1). BMDC cultures grown in a 5% O_2 atmosphere (tissue normoxia range is 2.5–9%, ambient O_2=20.9% v/v) and in the absence of 2-Me possess the morphology and surface antigen markers consistent with myeloid DC. Importantly DC prepared in 5% O_2 maintained significant efficacy in activating allogeneic CD4$^+$ T cells isolated from BALB/c mice (243:1 T:DC ratio, Fig. 6). Col-

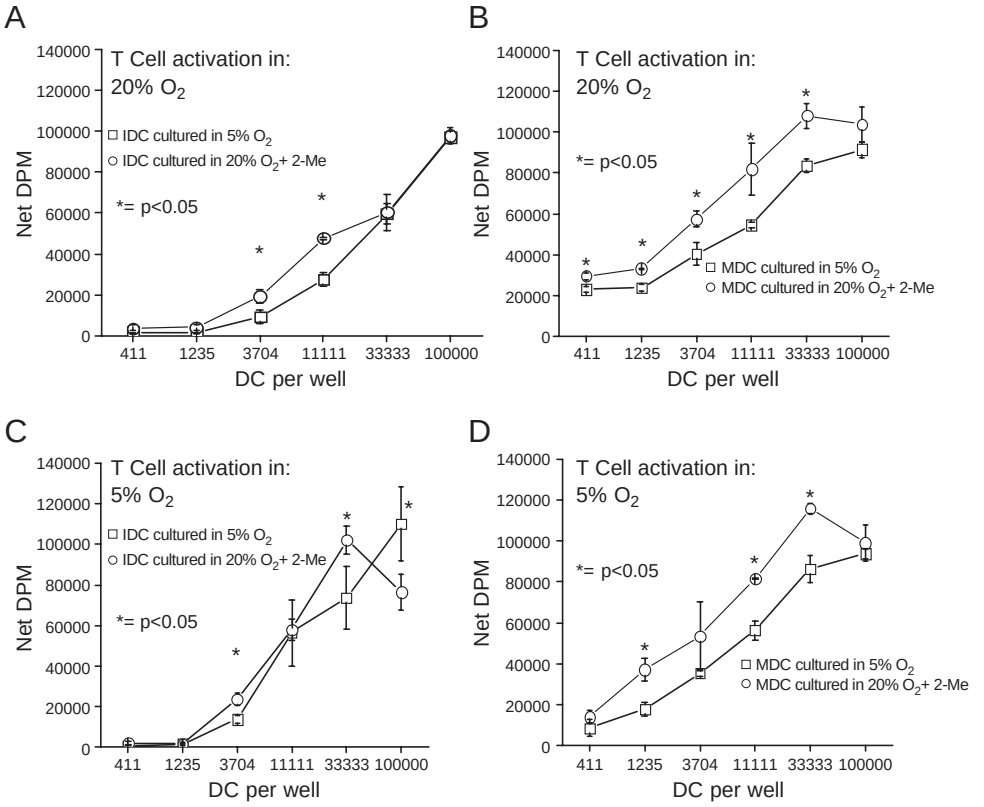

Fig. 6. Three day allogeneic presentation assay to $CD4^+$ T cells by BMDCs generated in 5% O_2 or conventional conditions (20%O_2+50 μM 2-Me). DCs originally grown in 2-Me-free or -supplemented medium were replated in the same for these cocultures; i.e., cocultures with 5% O_2 DCs used medium without 2-Me supplementation. T cell activation was subsequently performed in either a 20% (A and B) or 5% (C and D) O_2 atmosphere. (Panels A and C) Sorted immature B6 DCs APCs cocultured with 10^5 CD4+BALB/c splenocytes. (Panels B and D) Sorted mature B6 DCs cultured with 10^5 CD4+BALB/c splenocytes. The mean values of triplicate 96 wells plus one standard deviation from one of two similar experiments are shown.

lectively our data indicate that phenotypically and functionally, DCs grown under the more physiologic conditions of normoxia and without 2-Me supplementation are equivalent to DCs generated using conventional conditions (20% O_2 and 2-Me supplemented media), with the additional benefit of increased yields of class II MHC^+ DCs.

Interestingly, precursor DCs ($CD11c^+$, class II MHC^{NEG}) tended to accumulate in the BMDC cultures grown at higher (10% and 20%) O_2 tensions, implicating O_2 or a metabolite in the regulation of precursor DC class II MHC expression. We tested this possibility by culturing sorted precursor DCs under different O_2 tensions. Regardless of their original culture conditions, all precursor DCs were capable of generating $CD11c^+$ class II MHC^+ DCs, but would do so only after transfer to low (2% and 5%) but not high (10% and 20%) O_2 (Figs. 2 and 3), thus linking the regulation of precursor DC surface class II MHC expression to the environmental O_2 tension. The very low numbers of class II MHC^+ DCs that arose under conditions of high O_2 concentrations may reflect a dependence of these precursor DCs upon adherent cells for maturation induction, since intact cultures generate class II MHC^+ DCs. The increased numbers of class II MHC^+ in the low O_2 bone marrow cultures we observed were probably due to accelerated DC maturation that accompanied the enhanced surface class II MHC expression. A longer lifespan of the IDCs and MDCs from the lower O_2 environments versus their counterparts generated in higher O_2 may also play a role in this increase, but we did not test that possibility.

Compared to conventional conditions, BMDC grown in 5% O_2 yielded more $CD11c^+$ cells that expressed the marker more intensely (Table 1 and Fig. 4). We compared $CD11c^+$ cells grown in 5% O_2 to those grown under conventional conditions for co-expression of other myeloid DC associated markers. 5% O_2 cultures generated 30% more $CD11b^+$ cells that in addition expressed the marker more intensely,

as well as 30% more $CD54^+$ cells. Conversely, 50% fewer $CD86^+$ DC were found in the 5% O_2 cultures (Fig. 4). Staining for CD80 and Ly6G/Gr-1 revealed no differences in marker co-expression either in intensity or percent positive cells (Fig. 4). It would be interesting to vary the O_2 concentration and test whether this regulates CD11b, CD80, and CD86 expression in bone marrow derived DCs as we have shown it does for surface class II MHC.

CD11b was expressed by $CD11c^+$ cells from the 5% O_2 and conventional conditions in a bimodal manner (Fig. 4, 2nd row from bottom, upper right hand quadrants). Reanalysis of the $CD11b^{HI}/CD11c^+$ cells from both cultures revealed they were more granular (higher SSC), and the $CD11b^{INT}/CD11c^+$ cells were less granular (data not shown). As stated earlier on page 12 in Results, IDC+MDC and precursor DC in Fig. 1 from both cultures showed the same trend of decreasing granularity from the IDC+MDC to the precursor DC subsets, suggesting the possibility that the granular, $CD11b^{HI}/CD11c^+$ cells represent IDC and MDC, and the less granular, $CD11b^{INT}/CD11c^+$ cells represent precursor DC. In support of this hypothesis, the ratios of precursor DC to IDC+MDC in conventionally cultured BMDCs and those grown in 5% O_2 (from Fig. 1) are 1:1.7 and 1:7. The ratios of the $CD11b^{INT}/CD11c^+$ (proposed precursor DC) to $CD11b^{HI}/CD11c^+$ (proposed IDC+MDC) in conventionally cultured BMDCs and those grown in 5% O_2 are 1:2.3 and 1:5.8, respectively, a fairly close fit. Although we did not perform an analysis of the capacity of the $CD11b^{INT}/CD11c^+$ cells to generate IDCs or MDCs, our hypothesis that they contain precursor DCs could be readily tested by the method employed in this manuscript.

It is noteworthy that 2-Me supplementation did not improve DC yields despite significantly increasing (39% more, 20% O_2 vs. conventional conditions, Table 1) the nonadherent cell yield in the conventional conditions. The BMDC cultures grown in 20% O_2 without 2-Me had a higher percentage of class II MHC^+ cells, which offset the conventional cultures' increased cell count. 2-Me was also not essential for T cell proliferation in an allogeneic activation assay, although cocultures using 2-Me supplemented medium showed greater T cell activation (Fig. 6A–D). Activated T cells require cysteine to proliferate, but are unable to transport its precursor cystine which is abundant in serum. Human myeloid DCs secrete low levels of cysteine that is dramatically upregulated upon coculture with allogeneic T cells (Angelini et al., 2002). It is likely our murine myeloid DCs grown at 5% O_2 and without 2-Me supplemented medium also secrete cysteine as they supported robust T cell proliferation (Fig. 6A–D), though we did not test their (2-Me free) media for thiols. The greater T cell proliferation we observed in the cocultures containing 2-Me are likely due to two causes: one, the presence of a higher percentage of $CD86^+$ DC, and two, 2-Me forming a mixed disulfide with cystine which T cells transport, which is then followed by cysteine release after intracellular cleavage of the disulfide bond (Ishii et al. 1981). 2-Me supplementation slightly decreased the mean fluorescence intensity and the proportion of precursor DC induced to express class II MHC, and precursor DC recultured in 2% O_2 expressed 2-fold higher CD11c than cells recultured in 20% O_2.

DC activation and associated immune functions are known to be subject to regulation by their redox environment (Angelini et al., 2002; Matsue et al., 2003). For these reasons, DCs grown under tightly regulated O_2 in the absence of exogenous reducing agent are likely to provide a physiologically relevant baseline from which to study the role of the local redox environment in regulating DC development in general, and class II MHC expression in particular.

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