

Low oxygen tension enhances the stimulation and proliferation of human T lymphocytes in the presence of IL-2

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Background

Optimization of the culture environment for the *ex vivo* expansion of T cells is crucial for obtaining the large doses of cells needed for cellular immunotherapy. O_2 tension is a key parameter that impacts the proliferation and quality of the expanded T cells.

Methods

Peripheral blood mononuclear cells were stimulated with either PHA or an anti-CD3 monoclonal antibody under 5% (low) or 20% (high) O_2 atmospheres. After stimulation, cells were cultured in the presence of IL-2 under either low or high O_2 conditions.

Results

T cells stimulated and grown under 5% O_2 exhibited higher proliferation rates and a mean ($n = 11$) of 5.8-fold greater total expansion over T cells grown under 20% O_2 . Stimulation under 5% O_2 produced a lasting proliferative effect even after a switch to 20% O_2 . Examination of apoptosis by the flow cytometry-based TUNEL assay showed a mean ($n = 9$) of 2.9-fold greater percentage of apoptotic

cells under 20% O_2 . Flow-cytometric analysis of the IL-2 receptor (CD25) showed that the normal downregulation kinetics – following stimulation-induced CD25 upregulation – were slowed under 5% O_2 such that the 5% O_2 cultures had a greater number of CD25⁺ cells, and those CD25⁺ cells expressed an average ($n = 6$) of 41% higher levels of CD25 receptor per cell. No significant O_2 tension effects were observed on other surface antigens (CD3, CD28, and CD62L) examined. The key metabolic parameters, specific glucose uptake rate, q_{glc} and specific lactate production rate, q_{lac} were both increased by a mean ($n = 5$) of 47% under 5% O_2 .

Discussion

Beyond the physiological significance, improved T-cell proliferation under 5% O_2 would allow for decreased culture times in expanding T cells for cellular immunotherapies. Evidence of increased IL-2R expression and reduced apoptosis levels under 5% O_2 may help explain this phenomenon.

Keywords

Oxygen, T lymphocytes, Interleukin-2 receptor, Apoptosis, Metabolism

Introduction

Cellular immunotherapy, in which a patient is treated with large doses of autologous *ex vivo*-expanded immune cells in order to eradicate aberrant—such as malignant or virally infected—cells, offers an alternative treatment for such diseases. The effectiveness of these therapies is considered to be dose dependent, as patient survival increases proportionately with larger doses of effector cells [1]. As a result, treatment regimens require large numbers of cells, typically 10^9 - 10^{11} cells per patient, making expansion of the cells via *ex vivo* cell culture necessary. In expanding T cells for immunotherapy, it is important not only to generate large numbers of cells, but also to ensure that the cell product is of high quality such that it will be biologically

active upon transfusion back to the patient. Thus, optimization of the cell culture parameters used for expansion of these cells is a crucial issue for the success of cellular immunotherapy. Currently, the majority of cellular immunotherapy protocols make use of static flasks or gas-permeable tissue culture bags housed in standard incubators with a gas atmosphere of 5% CO_2 and air (ca. 20% O_2 or a tension of 140 mmHg). Such static culture methods result in spatial and temporal variations in many parameters which may produce suboptimal conditions leading to decreased cell-expansion potential, and thus requiring a longer time for culture completion. Hematopoietic and immune cells experience a large spectrum of O_2 tension environments due to the physiology and vasculature of

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the bone marrow and the lymphoid organs [2, 3]. The mean O_2 tension in the hematopoietic and lymphoid-organ tissues is closer to 40 mmHg (or 5% O_2 in the gas atmosphere), while the anatomical architecture of these organs suggests substantial intraorgan gradients that might expose cells to even lower O_2 tensions. In recognition of this physiological O_2 tension milieu, several studies have established the profound effect of lower O_2 tensions on hematopoietic cells. O_2 effects on immune cells have been examined but with variable results.

Studies of myeloid hematopoietic cells have shown that greater cell proliferation is observed in a 5% O_2 environment [4]. More recent work [5] suggests that expansion and differentiation of different myeloid cell lineages are optimized under different O_2 conditions. Megakaryocytopoiesis and erythropoiesis are enhanced under high O_2 tensions (20%), while granulopoiesis is enhanced by low O_2 tensions (5%). In contrast, it has been reported that lower O_2 tensions may not have beneficial effects on lymphocyte cultures. Loeffler *et al.* [6] reported a significant reduction of lymphocyte proliferation under 1% O_2 when compared to 20% O_2 . Naldini *et al.* [7] reported that PBMCs are less susceptible to PHA activation under 2% O_2 . Andersen *et al.* [8] reported maximal thymidine incorporation by PHA-stimulated lymphocytes in a 20% O_2 environment, over a range of 0 to 70% O_2 concentrations. Krieger *et al.* [9] reported greater proliferation of PBMCs under 5% O_2 conditions than 20% O_2 when stimulated with Con A or PWM, but no differential O_2 effects when stimulated with PHA or staphylococcal enterotoxin B (SEB). It is worth noting that in all of these experiments no exogenous IL-2 was added to the cultures. It is also important to note that the optimal O_2 concentration for proliferation may not necessarily be the same as for other cell functions. For the case of PBMCs stimulated only with high levels of IL-2 in order to generate LAK cells, Ishizaka *et al.* [10] reported no difference in proliferation of the cells, but a reduction in the killing activity of the cells under 2% O_2 when compared to 20% O_2 .

We conducted experiments to test the hypothesis that a lower O_2 concentration – closer to that experienced by T cells *in vivo* (5% in the gas phase) – would provide a better proliferative environment than the 20% O_2 gas phase which is the standard culture condition used today. It is important to note that an O_2 tension of 5% as studied in these experiments is not considered to be

hypoxic. We also examined if the O_2 tension might affect T-cell apoptosis, the expression levels of key surface receptors and the metabolism of the T cells in culture.

Materials and Methods

Cells and culture medium

Cells for the study were obtained from two different populations. Samples of leukapheresis (a continuous-flow process for blood cell separation) products from non-hematological cancer patients who had undergone stem cell mobilization procedures consisting of treatment with G-CSF, with or without chemotherapy, (Response Oncology, Memphis, TN, USA) were used. While their potential exposure to chemotherapeutic agents could alter lymphocyte proliferation and susceptibility to oxidative damage, this nonetheless represents a population of significant interest as potential recipients of adoptive immunotherapy. Samples were also obtained from otherwise healthy donors undergoing therapeutic phlebotomies for treatment of hemochromatosis (Evanston Hospital, Evanston, IL, USA). Most hemochromatosis donors were in the 'maintenance' phase of treatment, and therefore did not have highly elevated serum iron levels. All cells were obtained after informed consent, under protocols approved by the respective institutional review boards. The PBMCs from the hemochromatosis patient samples were collected using a Histopaque density gradient, and were washed in culture medium prior to stimulation, which removes most of the potentially iron-enriched serum and erythrocytes. Cells were seeded at 1×10^6 cells/mL and cultured for 5-10 days in T-flasks using RPMI with 100 U/mL IL-2 (Chiron: Emeryville, CA, USA), 10% FBS (Hyclone: Logan, UT, USA), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 25 mM HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin and either 5 μ g/mL (PHA) or 20 ng/mL of soluble anti-human CD3 monoclonal antibody (PharMingen: San Diego, CA, USA) for activation. Following this cell activation period, the cells were observed to be >90% CD3⁺ by flow cytometry. Unless otherwise noted, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Culture protocols and O_2 evaluation

Following the initial activation in T flasks, cells were expanded in 100-mL spinner flasks (model 1965, Bellco Glass, Vineland, NJ, USA) in order to ensure a homo-

geneous culture environment. The spinner flasks were agitated at 60 rpm on stir plates in incubators that allowed for control of gas phase O_2 as well as CO_2 concentrations (Forma Scientific: Marietta, OH, USA). Low O_2 concentrations (5% in the gas phase) were obtained using a nitrogen purge and CO_2 was maintained at 5%. Using a dissolved O_2 probe (Ingold, Columbus, OH, USA) inserted into a spinner flask, we determined that following sampling, where a culture is briefly exposed to 20% O_2 , it takes ca. 4-5 hours for the culture to completely re-equilibrate to the low O_2 level. Therefore, sampling of the cultures was performed only once a day to minimize exposure of the low O_2 cultures to elevated O_2 concentrations. Cell concentrations were maintained between approximately 2×10^5 and 2×10^6 cells/mL by diluting the cultures using media which had been pre-equilibrated to the desired O_2 concentration. Counting of nucleated cells was carried out using a Coulter Multisizer (Coulter Electronics, Hialeah, FL, USA) after treatment with cetrimide (Sigma) to lyse the cells and release the nuclei. The impact of oxygen tension on T-cell proliferation was assessed based on differences in proliferation rate and total fold expansion resulting from the initial stimulation with PHA or anti-CD3 MAb. These differences can be readily observed on plots of T-cell expansion versus time. Cells were stimulated only once, and experiments concluded when cell proliferation stopped (usually 2 to 3 weeks). Total expansion (fold) was calculated based on percentage of $CD3^+$ cells in the initial blood sample.

Glucose and lactate metabolic analysis

Glucose and lactate concentrations were determined using a YSI model 2700 Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH, USA). Specific metabolic uptake and production rates (q_{glu} and q_{lac} in units of mmol (glucose or lactate)/cell/h) were determined by plotting the glucose or lactate concentration versus the integral under the cell growth curve (cumulative cell-hours) [11]. This method was used over relatively small 2-4 day segments of the experiments where periods of constant specific metabolic rate yield a straight line with a slope equal to that rate. For more detailed kinetic analysis of q_{glu} and q_{lac} changes over the course of a culture the second-order central slope method was used as previously described [12]. The yield coefficient describing the ratio of lactate production to glucose consumption, $Y_{lac/gluc}$, was calculated as q_{lac}/q_{gluc} .

The yield coefficients $Y_{cells/mmol\ glu}$ and $Y_{mmol\ lac/cell}$ were calculated as the respective ratios of changes in cell, glucose, or lactate concentrations over specific time intervals.

Apoptosis Assays

TUNEL assay

Flow cytometric determination of the apoptotic fraction of cells was performed using the terminal deoxy transferase mediated dUTP nick end labeling (TUNEL) assay [13] (In Situ Cell Death Detection Kit, Fluorescein, Boehringer Mannheim, Indianapolis, IN, USA). The basis of this assay is enzymatic incorporation of fluorescein-conjugated nucleotides into the DNA strand breaks characteristic of apoptotic cells. Briefly, cells were washed in PBS containing 1% BSA, fixed in 1% methanol-free formaldehyde, washed in PBS, and then resuspended in 70% ethanol and stored at $-20^\circ C$ until further processing. After all of the samples had been collected, cells were rewashed in PBS with 1% BSA, then resuspended in the label and enzyme solutions from the kit, and incubated at $37^\circ C$ for 1 h. Cells were then washed and resuspended in PBS with 1% BSA for analysis by flow cytometry. As a positive control, cells were incubated in DNase (1mg/mL) following the ethanol permeabilization to induce strand breaks.

Acridine orange/ethidium bromide assay

Some samples were also analyzed for apoptosis using the method described by Mercille and Massie [14]. Cells were labeled with a dye solution containing acridine orange (AO, 100 $\mu g/mL$) (Molecular Probes, Eugene, OR, USA) and ethidium bromide (EtBr, 100 $\mu g/mL$) (Molecular Probes, Eugene, OR, USA), which stain the DNA and allow for discrimination between apoptotic and non-apoptotic cells, based on breakdown of the nuclear morphology, as well as the viability of those populations. A volume of 8 μL of the dye solution was mixed with 60 μL of cell suspension, and viewed using a hemacytometer and fluorescence microscope. At least 200 cells were counted per sample at each timepoint.

Surface Antigen Staining and Flow Cytometric Analyses

Triplicate samples of approximately 5×10^5 cells were washed twice with cold PBA (phosphate buffered saline containing 1% BSA and 0.1% sodium azide). The pellets were resuspended in 100 μL of PBA containing an

amount of monoclonal antibody (Becton Dickinson: San Jose, CA, USA) that had been previously titrated to be saturating (CD3 – 25 μ L, CD25 – 15 μ L, CD28 – 20 μ L, CD62L – 20 μ L). Samples were incubated at 4°C for 30 min, washed twice more with cold PBA, and then resuspended in 500 μ L PBA for immediate analysis. Flow cytometric measurements were performed using a Becton Dickinson FACScan cytometer equipped with a 15 mW, 488 nm air cooled argon-ion laser. Approximately 7500 cells were analyzed per sample. Data acquisition was performed using the FACScan Research software and then analyzed using CellQuest version 1.2 (Becton Dickinson). The QuantiBRITE (Becton Dickinson) fluorescence quantitation beads were used at each acquisition session in order to provide a consistent calibration measure to relate fluorescence intensity to the number of PE molecules conjugated to the cell via the antibody staining process. While this does not necessarily give the exact number of receptors per cell (as the ratio of antibody binding to receptor is not known), the number of PE molecules per cell is proportional to the number of receptors per cell. This allowed for direct quantitative comparison of values of fluorescence intensity from samples stained and analyzed on different days in order to obtain kinetic information about surface receptor expression levels. Propidium iodide (PI, 2 μ g/mL) was added to one unstained sample on each day in order to exclude dead cells from the analyses. A gate on a plot of side scatter versus forward scatter (SSC vs FSC), which corresponded to the PI-negative population, was established from this sample and applied to all of the other samples on that day. This means of excluding dead cells (as opposed to adding PI to every sample) allowed for better comparison of fluorescence intensity with the QuantiBRITE calibration beads, as no PI was added to the bead samples.

Results

Reduced O₂ tension enhances T-cell activation and expansion

One of the culture protocols used to investigate the effects of oxygen tension during stimulation and culture of T cells was the oxygen 'crossover' culture experiment. PBMCs were activated with either PHA or anti-CD3 MAb and then cultured under 5% or 20% O₂ gas-phase environments. After approximately a week, half of the cells were maintained in the original culture environment

and the other half was switched to the alternate O₂ condition. Figures 1(a) & 1(b) are representative examples of these 'crossover' cultures which show the increased rate and greater extent of proliferation achieved by activating and culturing T cells in the 5% O₂ environment for both

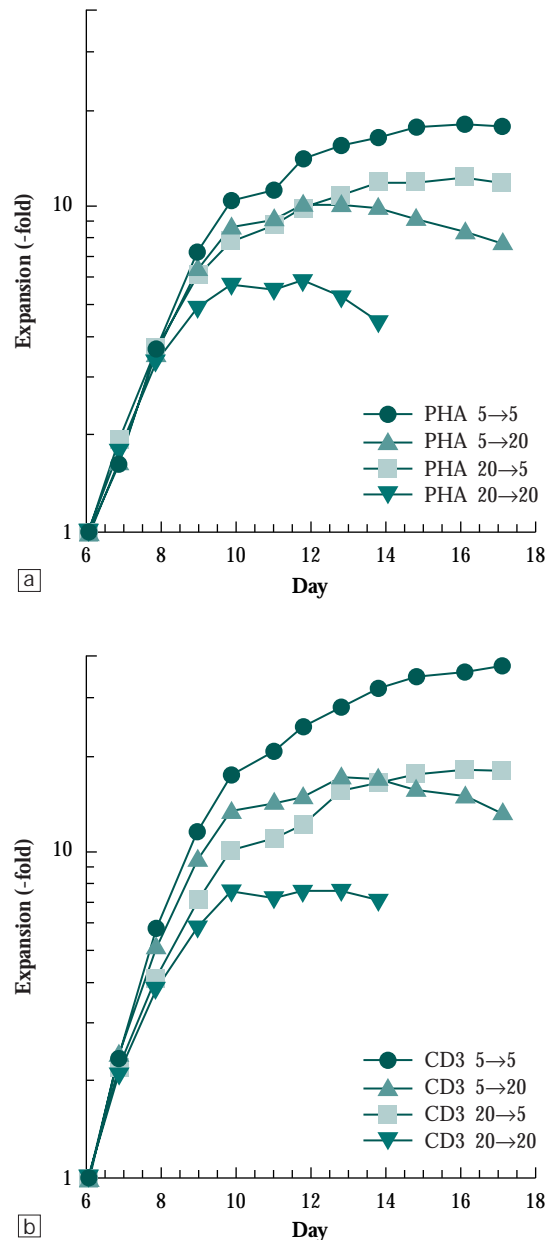


Figure 1. Growth curves of T cells from a representative 'O₂ crossover' experiment (sample D as identified in Tables 1–3). Cells were stimulated at day 0 with either (a) PHA or (b) anti-CD3 MAb, under either 5% or 20% O₂. At day 6, the activated T cells were split in half and either maintained in their original O₂ condition or switched to the alternate condition. O₂ tensions during 'stimulation→culture' are indicated in the symbol keys.

PHA and anti-CD3 MAb activated cells. Additionally, it appears that activation in the 5% O₂ environment provides a lasting protective effect, such that cells activated under the 5% O₂ and switched to 20% O₂ proliferate better than the cells that were both stimulated and maintained under 20% O₂. Similarly, cells stimulated under 20% O₂ and then switched to 5% O₂ failed to proliferate as well as cells that were both activated and cultured under 5% O₂. We conclude that the low O₂ environment improves the activation of the T cells. The greater proliferation seen in the anti-CD3 MAb-stimulated culture versus the PHA-stimulated is not a consistent effect and seems to vary between patient samples. Table 1 summarizes the differences in total fold expansion achieved for cultures of eleven experiments (9 blood samples) where cells were activated and cultured under 5% O₂ versus those activated and cultured under 20% O₂. Activation and culture under the low O₂ environment allows for a mean \pm SEM of 5.8 ± 0.95 -fold greater total expansion over that achieved by activation and culture under the high O₂ condition. The extent of variability in fold expansion between different donor samples, while large, is not unlike the variability shown in a number of other works [13-15]. There are no statistically significant

differences in total fold expansion (based on Student's *t*-tests) between the two sources of blood samples (non-hematological cancer or hemochromatosis patients) under 5% ($p = 0.44$) or 20% ($p = 0.70$) O₂ or in the ratio of proliferation between the two O₂ conditions ($p = 0.64$).

Experiments were also conducted to determine if a further decrease in O₂ concentration would provide an additional benefit in cell proliferation. Cells were activated with PHA and cultured for a week in T flasks in the 20% O₂ environment. The cells were then transferred into spinner flasks in incubators with 2.5%, 5%, and 20% O₂. We observed similarly increased proliferation in the 2.5% and 5% O₂ cultures, with both being superior to the 20% O₂ culture ($n = 2$; data not shown). Therefore no additional benefit was seen from culturing the cells under a 2.5% O₂ atmosphere.

Reduced O₂ tension reduces T-cell apoptosis

In an effort to understand the mechanism by which the cultures maintained under 5% O₂ were outperforming the cultures maintained under 20% O₂, we examined the kinetics of apoptosis over the course of cultures under the two conditions. The fractions of apoptotic cells were identified by flow cytometry using the TUNEL assay and

Table 1. Summary of effect of O₂ tension on T-cell expansion

Sample	Stimulation method	Expansion (-fold) in		Expansion ratio 5%:20% O ₂
		5% O ₂	20% O ₂	
A*	PHA	3175	582	5.5
B*	PHA	600	45	13.3
C*	PHA	640	235	2.7
C*	CD3	360	94	3.8
D*	PHA	106	35	3.0
D*	CD3	714	183	3.9
E [§]	PHA	2250	239	9.4
F [§]	CD3	180	30	6.0
G [§]	PHA	3505	730	4.8
H [§]	PHA	1647	232	7.1
I [§]	PHA	175	40	4.4
Mean \pm SEM		1214 \pm 374	222 \pm 70	5.8 \pm 0.95

Total fold expansion of T cells from nine blood samples stimulated with either PHA or anti-CD3 MAb, and maintained under either 5% or 20% oxygen. Expansion was based on the fraction of CD3⁺ cells in the initial samples.

* Sample from non-hematological cancer patient mobilized peripheral blood apheresis product.

[§] Sample from hemochromatosis patient therapeutic phlebotomy product.

also by fluorescence microscopy, using a dye solution of AO/EtBr to stain the DNA/. Figure 2 shows a representative example of the kinetics of the apoptotic populations, as detected by the two assays, for T cells which were stimulated and cultured under 5% or 20% O_2 . While the AO/EtBr assay almost always identifies a greater fraction of cells as being apoptotic, both assays show that T cells cultured under 20% O_2 have a greater fraction of apoptotic cells than those cultured under 5% O_2 . Table 2 summarizes the apoptosis data from nine cul-

ture experiments (8 blood samples). The values for each condition represent the averages of multiple timepoints measured using the TUNEL and AO/EtBr assays during the cultures. Apoptosis timepoints measured prior to 4 days following stimulation were excluded due to the large amount of cell death from non-T-cell populations. Averaged results show an increase in apoptosis levels in the 20% O_2 cultures of 2.9-fold based on the TUNEL assay, and 1.4- fold based on the AO/EtBr assay. The extent of the increase in apoptosis levels in the 20% O_2

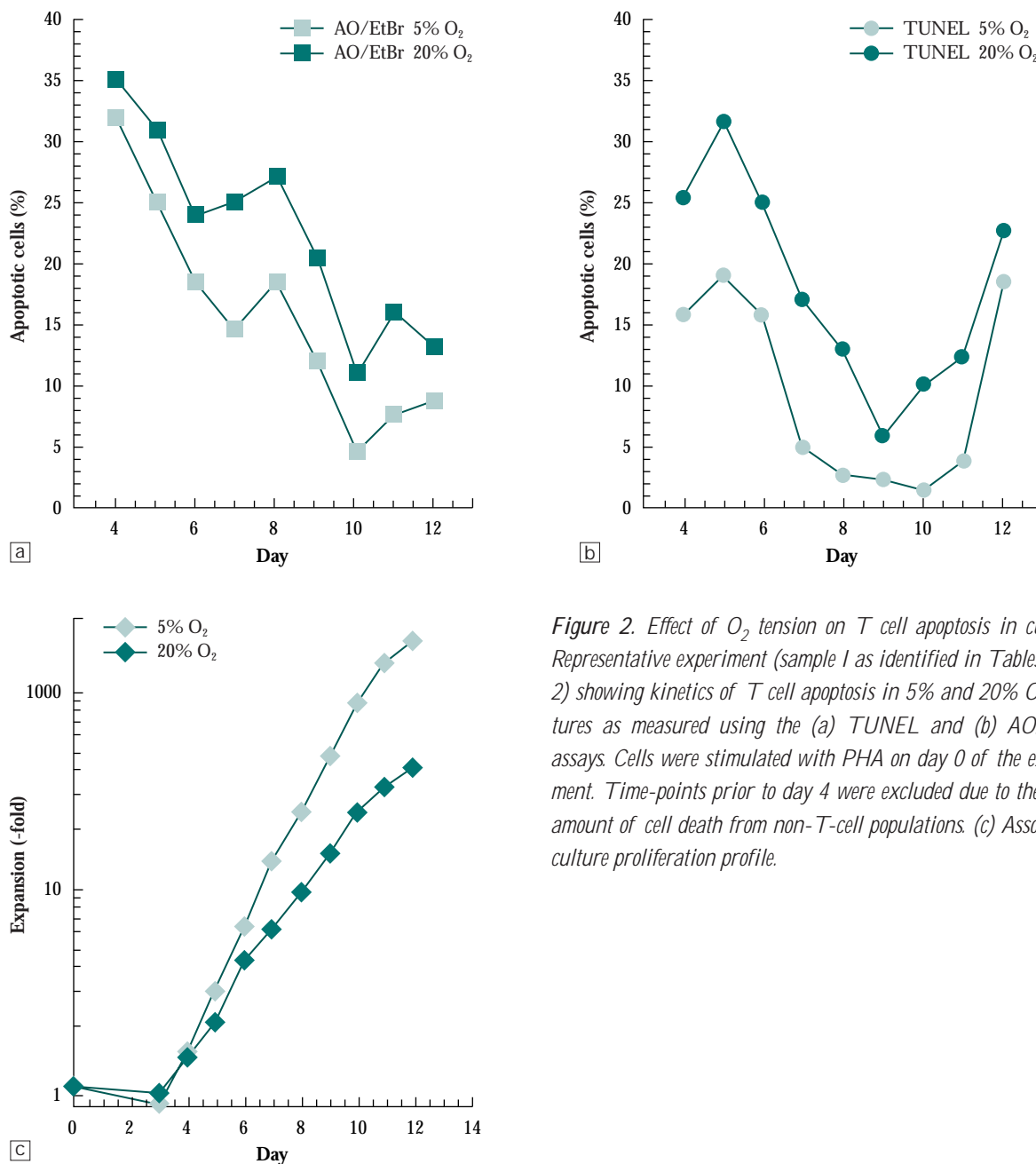


Figure 2. Effect of O_2 tension on T cell apoptosis in culture. Representative experiment (sample 1 as identified in Tables 1 & 2) showing kinetics of T cell apoptosis in 5% and 20% O_2 cultures as measured using the (a) TUNEL and (b) AO/EtBr assays. Cells were stimulated with PHA on day 0 of the experiment. Time-points prior to day 4 were excluded due to the large amount of cell death from non-T-cell populations. (c) Associated culture proliferation profile.

Table 2. Summary of the effect of O₂ tension on T-cell apoptosis in culture

Sample	Stimulation method	Apoptotic cells (%)				Ratio	
		5% O ₂		20% O ₂		20%:5% O ₂	
		TUNEL (AO/EtBr)	TUNEL (AO/EtBr)	TUNEL (AO/EtBr)	TUNEL (AO/EtBr)		
A*	PHA	1.7		3.7		2.2	
B*	PHA	3.4		15.8		4.6	
D*	PHA	1.6		10.3		6.4	
D*	CD3	1.6		6.7		4.2	
E [§]	PHA	2.6		5.2		2.0	
F [§]	CD3	2.7		5.0		1.9	
G [§]	PHA	3.0		4.6		1.5	
H [§]	PHA	5.5	(11.6)	9.7	(15.9)	1.7	(1.4)
I [§]	PHA	9.3	(15.8)	18.1	(22.5)	1.9	(1.4)
Mean ± SEM		3.5 ± 0.8	(13.7)	8.8 ± 1.7	(19.2)	2.9 ± 1.5	(1.4)

For each culture, the reported value is the percentage of apoptotic cells as detected by either the flow cytometry-based TUNEL assay or the fluorescence microscopy-based (AO/EtBr) assay. The values are the means of all measurements during that culture. No measurements were made during the first 4 days of culture due to the extensive apoptotic cell death of non-T cell populations.

* Sample from non-hematological cancer patient mobilized peripheral blood apheresis product.

§ Sample from hemochromatosis patient therapeutic phlebotomy product.

cultures (as measured by the TUNEL assay) does differ significantly ($p = 0.012$, based on Student's t -test) between the sources of blood samples (non-hematological cancer or hemochromatosis patients). The mean for the non-hematological cancer samples is a 4.4-fold increase, while the mean for the hemochromatosis patient samples is 1.8. It is not clear at this point why this difference was observed.

O₂ tension alters the levels of expression of the IL-2 receptor (CD25)

We also examined if the expression levels of several surface receptors known to be important for *in vivo* T-cell function were affected by O₂ concentration. Kinetic analysis of CD25, CD3, CD28, and CD62L expression was carried out using flow cytometry over the course of the cell expansion.

CD25

CD25 is the IL-2 receptor alpha chain (IL-2R- α). Stimulation of T cells upregulates the expression of CD25, with peak levels occurring between days 2 and 8 depending upon the stimulation method [16-18]. CD25 expression levels then decrease back to amounts equiva-

lent to background (isotype control) staining over the next several weeks of culture. For a representative experiment, Figure 3(a) shows that as CD25 is downregulated over the course of the culture, T cells growing under 5% O₂ had a greater fraction of cells which were positive for CD25 (defined as staining brighter than an isotype control) than the 20% O₂ cultures. Figure 3(b), from the same experiment, shows that the CD25 receptor content (per cell designated as CD25⁺) was higher on the 5% O₂ cultured cells than the 20% O₂ cultured cells during the course of the experiment. Table 3 summarizes the increased expression levels of CD25 on T cells in the 5% O₂ cultures for six culture experiments (4 blood samples), two of which were stimulated separately with both PHA and anti-CD3 MAb. Over the time period during culture in which CD25 was being downregulated, the samples cultured under low O₂ had an average of 56% greater fraction of cells which were positive for CD25. This difference was statistically significant, with $p < 10^{-6}$ as determined by the Student's paired t -test. Additionally, the average level of CD25 receptor content per cell during this time was also found to be 41% greater in the samples cultured under low O₂. This difference was also statistically significant (Student's paired t -test) with $p = 0.004$.

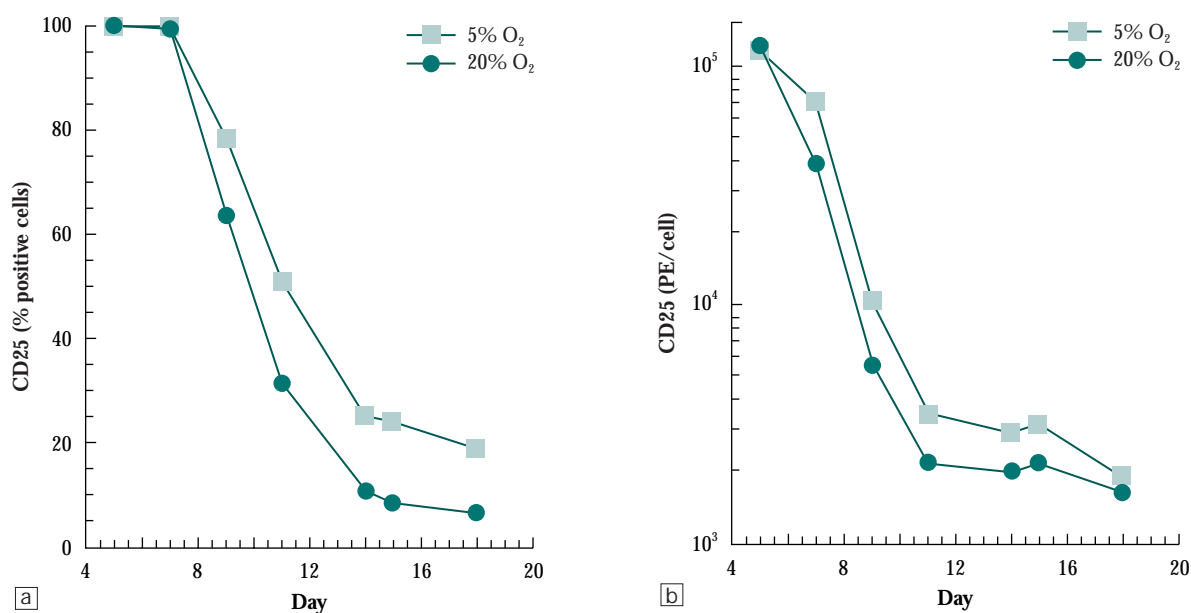


Figure 3. Effect of O₂ tension on expression kinetics of CD25 (IL-2 receptor). Cells (sample C as identified in Tables 1 & 3) were stimulated with PHA on day 0 of the experiment and maintained in either 5% or 20% oxygen environments. (a) Percentage of cells brighter than the isotype control; (b) amount of receptor per cell on those cells designated as CD25⁺ in (a) (quantified as PE molecules bound via antibody staining, using flow cytometry and the QuantiBRITE calibration beads as described in the Materials and Methods).

CD3, CD28, CD62L

The effect of O₂ tension on CD3 expression was examined because of its key role as a signal transduction molecule leading to T-cell activation. Analysis of CD3 expression levels on T cells cultured under 5% and 20% O₂ showed no consistent variation with O₂. However, while the expression levels of CD3 declined slightly over the course of the culture period, there were consistent increases in CD3 expression levels after each feeding with fresh medium (data not shown). This suggests that accumulation or depletion of some factor in the culture medium is causing downregulation of CD3 expression.

The effect of O₂ tension on the expression of CD28 and CD62L was also examined. CD28 is an important costimulatory molecule expressed on the majority of T cells and its expression is enhanced by activation [19, 20]. CD62L, also known as L-selectin, is a key protein involved in regulation of trafficking of T cells to the lymph nodes. Comparison of expression levels between T cells grown under 5% and 20% O₂ revealed no consistent trends for either CD28 or CD62L (data not shown).

O₂ tension affects the metabolism of T cells

Basic knowledge regarding the metabolism of T cells in culture is important for further optimization of culture

media and feeding strategies for T cell *ex vivo* expansion, therefore we also examined the effect of O₂ tension on the metabolic characteristics of T cells in culture. Important metabolic parameters including the specific rates of glucose uptake (q_{glu}) and lactate production (q_{lac}), as well as several key yield coefficients ($Y_{\text{lac}/\text{glu}}$, $Y_{\text{cells}/\text{mmol glu}}$, $Y_{\text{mmol lac}/\text{cell}}$) are vital for understanding the basic metabolism and the impact of O₂ tension [21]. These parameters were examined from cultures of T cells stimulated and grown under either 5% or 20% O₂. Over the duration of a culture, as the proliferation of the cells slowed, q_{glu} and q_{lac} decreased by as much as 10-fold. Both parameters were consistently higher in the culture stimulated and cultured under 5% O₂ tension. Examples of the variation in q_{glu} and q_{lac} for a representative experiment are shown in Figures 4(a) & 4(b) respectively. Both the q_{glu} and the q_{lac} were significantly higher under 5% O₂ culture conditions ($p < 10^{-6}$, as determined by the Student's paired *t*-test) for five patient samples which included both PHA and anti-CD3 MAb stimulated cultures (Figure 4(c)). The mean \pm SEM q_{glu} values were $(0.38 \pm 0.07) \times 10^{-10}$ mmol/cell/h for the 20% O₂ cultures and $(0.56 \pm 0.65) \times 10^{-10}$ mmol/cell/h for the 5% O₂ cultures, which is a 47% increase. The mean \pm SEM q_{lac} values were $(0.73 \pm 0.10) \times 10^{-10}$ mmol/cell/h

Table 3. Summary of effect of O₂ tension on CD25 (IL-2R) expression levels

Sample	Stimulation method	Ratio of CD25 expression	
		(% positive) ¹ 5%:20% O ₂ cultures	(PE/cell) ² 5%:20% O ₂ cultures
C*	PHA	1.95 ± 0.64	1.55 ± 0.26
C*	CD3	1.39 ± 0.17	1.62 ± 0.20
D*	PHA	1.79 ± 1.05	1.27 ± 0.10
D*	CD3	1.32 ± 0.22	1.27 ± 0.20
E [§]	PHA	1.77 ± 0.64	1.42 ± 0.14
F [§]	CD3	1.15 ± 0.16	1.31 ± 0.12
Mean ± SEM		1.56 ± 0.13	1.41 ± 0.06

For each culture the given ratio is the mean ratio of CD25 expression for 5% O₂:20% O₂ averaged over all of the time-points taken during a culture ± standard deviation. The overall means are the averages of the means from culture ± SEM. ¹Percent positive values indicate the fraction of cells staining brighter than the isotype control. ²Values are quantified as PE molecules bound via antibody staining using flow cytometry and the QuantiBRITE calibration beads as described in the Materials and Methods.

* Sample from non-hematological cancer patient mobilized peripheral blood apheresis product.

§ Sample from hemochromatosis patient therapeutic phlebotomy product.

for the 20% O₂ cultures and $(1.07 \pm 0.13) \times 10^{-10}$ mmol/cell/h for the 5% O₂ cultures, which is also a 47% increase. The mean values depicted in Figure 4(c) represent averages over the entire duration of the cultures and over five blood samples.

The apparent yield of lactate from glucose, $Y_{\text{lac}/\text{glu}}$, is indicative of the extent to which glucose is being metabolized anaerobically. The theoretical maximum yield is 2.0, as only two molecules of lactate can be obtained from a single molecule of glucose; however, production of lactate from other substrates such as glutamine can result in values larger than 2.0 [21]. We found no statistically significant difference (Student's paired *t*-test) in $Y_{\text{lac}/\text{glu}}$ under the two O₂ concentrations (mean ± SEM of 1.9 ± 0.05 for 5% O₂ cultures and mean ± SEM of 2.07 ± 0.11 for 20% O₂ cultures). The $Y_{\text{cells}/\text{mmol glu}}$ and $Y_{\text{mmol lac}/\text{cell}}$ were also calculated over the six experiments. No significant differences (Student's paired *t*-test) in these parameters were observed between cultures under 5% versus 20% O₂. For $Y_{\text{cells}/\text{mmol glu}}$ the mean ± SEM was $(3.0 \pm 0.15) \times 10^8$ for 5% O₂ cultures, and $(3.2 \pm 0.27) \times 10^8$ for 20% O₂ cultures. For $Y_{\text{mmol lac}/\text{cell}}$ the mean ± SEM was $(6.7 \pm 0.34) \times 10^{-9}$ for 5% O₂ cultures, and $(1.9 \pm 0.76) \times 10^{-8}$ for 20% O₂ cultures.

One of the important technical issues in performing these experiments was to limit sampling of the cultures (and hence exposure to elevated O₂ levels) to once per

day. In our initial efforts, more frequent sampling was used in order to better capture the growth kinetics; however after measuring the time needed to re-equilibrate to 5% O₂ following sampling (approx. 4-5 hrs), it became clear that this did not allow sufficient time for exposure to the low O₂ environment. Early metabolic data from these experiments (Carswell and Papoutsakis, 1998 – presentation at *Engineering Foundation: Cell Culture Engineering Conference*) showed no differences in q_{glu} and q_{lac} between cultures at 5% and 20% O₂, and exhibited an increased $Y_{\text{cells}/\text{mmol glucose}}$ for the 5% O₂ cultures. These results, which differ from our current results with the improved sampling technique, appear to be artifactual, and demonstrate the importance of the experimental techniques in examining the effects of O₂ tension on cell physiology.

Discussion

Our data demonstrate the profound positive effect of low O₂ tension (which is more representative of the *in vivo* tissue environment) on the *ex vivo* expansion of T cells. In addition to the physiological significance of this finding, the ability to significantly increase the rate and extent of proliferation by culturing under 5% O₂ conditions is an important improvement over the current methods of culturing T cells in a 20% O₂ atmosphere. The beneficial effects of low O₂ culture have been shown here to exist

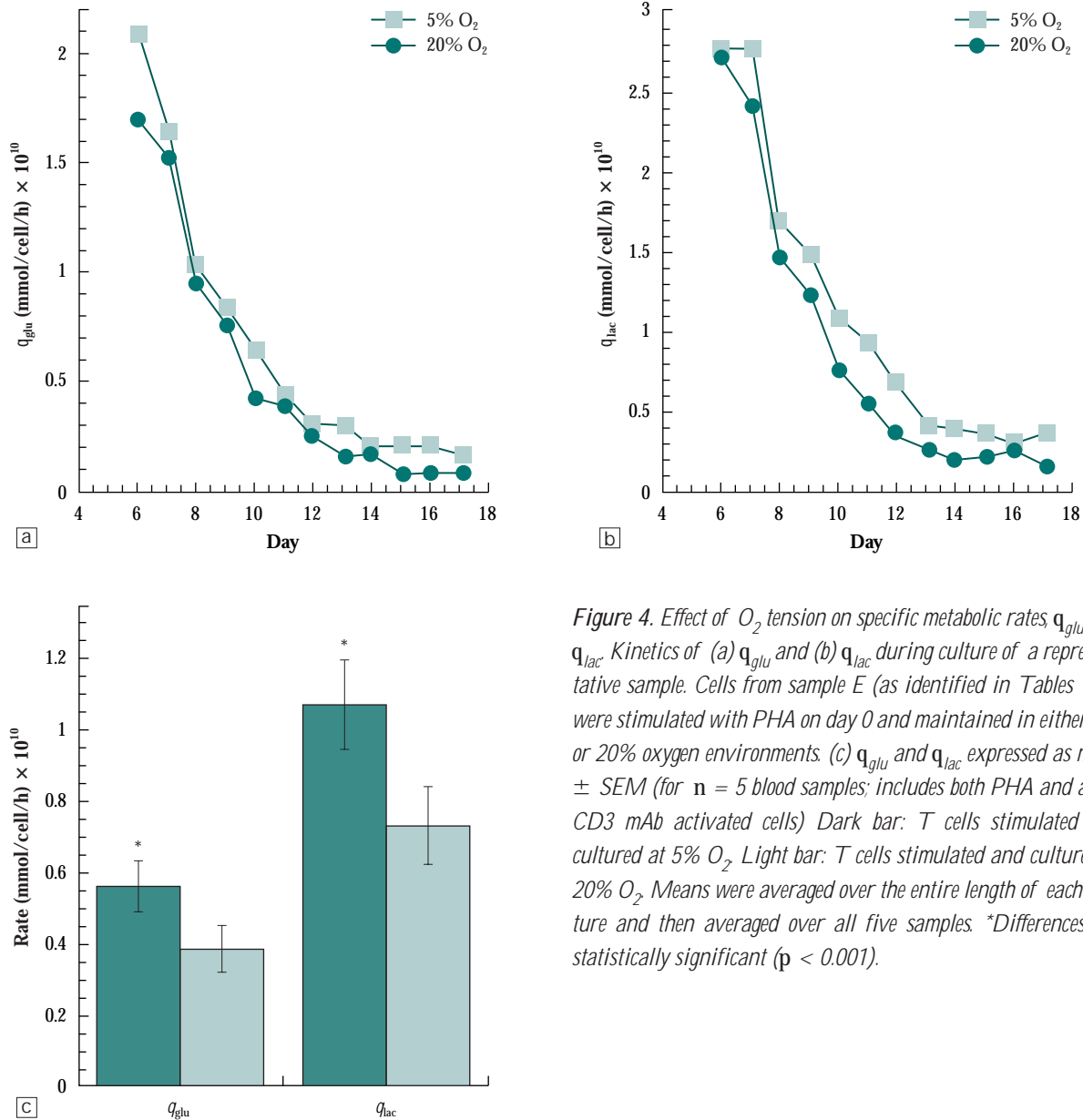


Figure 4. Effect of O_2 tension on specific metabolic rates q_{glu} and q_{lac} . Kinetics of (a) q_{glu} and (b) q_{lac} during culture of a representative sample. Cells from sample E (as identified in Tables 1–3) were stimulated with PHA on day 0 and maintained in either 5% or 20% oxygen environments. (c) q_{glu} and q_{lac} expressed as mean \pm SEM (for $n = 5$ blood samples; includes both PHA and anti-CD3 mAb activated cells) Dark bar: T cells stimulated and cultured at 5% O_2 . Light bar: T cells stimulated and cultured at 20% O_2 . Means were averaged over the entire length of each culture and then averaged over all five samples. *Differences are statistically significant ($p < 0.001$).

for T cells stimulated either with PHA or with an anti-CD3 MAb.

The O_2 crossover experiments, shown in Figures 1(a) & 1(b), were initially designed to investigate potential adaptation to damaging reactive oxygen species (ROS) by the T cells in the 20% O_2 environment during the activation period. The initial expectation was that one might observe an increased difference in proliferation between the 5% and 20% O_2 cultures if the cells were not exposed to the 20% O_2 tension during activation. Thus, cells stimulated in the 5% O_2 environment would have been more susceptible to the damaging effects of the 20% O_2 when they were switched into that condition. What was

observed however was the opposite. Cells stimulated in the 5% O_2 environment grew better after being switched into the 20% O_2 condition than the cells that were stimulated in the 20% O_2 environment and maintained there. This lasting protective effect seems to imply that the reason for the observed differences in proliferation rates is not simply the increased oxidative damage that the cells may suffer in the 20% O_2 environment. This is consistent with observations made by Zuckerberg *et al.* [22] who found that even brief hypoxic exposure had lasting effects on T-cell function and IL-2 mRNA levels.

Several studies have linked apoptosis to increased

levels of ROS. These studies include a proposed mechanism relating expression of p53 to increases in ROS which lead to cell death [23], and a proposed mechanism of action for Bcl-2 via an antioxidant pathway [24, 25]. Our results show consistently higher levels of apoptosis in the 20% O₂ cultures. While the increase in percentage of apoptotic cells is consistent, it is not clear if this small difference in apoptotic death is enough to account for the large differences observed in proliferation rates between the 5% and 20% O₂ cultures.

It is known that dissolved O₂ concentration can have dramatic regulatory effects on the expression of a number of different proteins expressed by lymphocytes and other cells including IL-2 [22], erythropoietin [26], IL-1[27], tumor necrosis factor [27], CD11b [28], CD18 [28], CD44 [29] and neural cell adhesion molecule [29]. Because the interactions of T cells via their surface receptors with target cells, cytokines, and localization target molecules are so crucial for their *in vivo* function, we examined whether culture under different O₂ tensions would affect the expression levels of key surface receptors. CD3, CD28, and CD62L exhibited no differences in expression due to O₂ concentration. This can be viewed as a positive result, as the increased proliferation under 5% O₂ is not offset by reduced expression of these key receptors. For CD25, the slowed downregulation kinetics observed during culture under 5% O₂ may help explain the increased growth rates and extent of proliferation.

A simple set of mathematical analyses was carried out in order to examine the possible impact on proliferation of the increased levels of apoptosis and faster downregulation kinetics of CD25 under the 20% O₂ atmosphere. The impact of the greater apoptotic fraction observed under 20% O₂ was assessed by making projections of the expected cell numbers at successive generations using the assumption that the apoptotic fraction would not divide. For these calculations we used the averages of apoptotic cell fractions from both the TUNEL and AO/EtBr assays over the whole culture duration: 9% apoptotic cells for the 5% O₂ cultures and 14% apoptotic cells for the 20% O₂ cultures. After eight generations the cells cultured under 5% O₂ would be predicted to have a 1.6-fold greater expansion over those cultures under 20% O₂. A similar analysis was performed based on the assumption that cells identified as CD25 negative (less bright than the isotype control) would not divide. Because CD25 expression was measured once a

day, it was assumed that one doubling occurred per day such that the CD25 positive fraction measured on a particular day was the fraction that was assumed to divide. Using this method and averaging the results from five sets of experimental data, after eight generations the cells under 5% O₂ would be predicted to have a 1.9 ± 0.14 (mean \pm SD) fold greater expansion over those cultures under 20% O₂. Coupled together, these two effects predict a 3.0 ± 0.2 fold greater number of cells in the 5% O₂ cultures after 8 doublings. Obviously these effects may not be truly independent, as removal of growth factors can induce apoptosis. However, the predicted effects are in the same general range as the observed effects. Additionally, it has been indicated that the rate of T cell proliferation may also be dependent on the expressed surface concentration of IL-2 receptor, with those expressing higher surface concentrations entering the cell cycle more rapidly [30, 31]. Therefore, T cells cultured under 5% O₂, which possess a higher CD25 surface receptor content per cell, may be proliferating faster and also adding to this effect.

The reported effects of O₂ concentration on the glucose metabolism of mammalian cells are somewhat variable. Miller *et al.* [34] reported decreasing specific glucose uptake rates with decreasing pO₂ over a range of 0.1–20% O₂ for a murine hybridoma cell line grown in continuous culture. Jan *et al.* [35] also reported decreasing specific glucose uptake and lactate production rates with decreasing pO₂ over a range of 2–25% O₂ with a murine hybridoma cell line grown in continuous culture. Lin *et al.* [36] reported increasing specific glucose uptake and lactate production rates with decreasing pO₂ for a recombinant CHO cell line grown in continuous culture. Our data also show an increased specific glucose uptake and lactate production rates in cultures grown at low (5%) O₂ tension as compared to the cultures grown at high (20%) O₂ tension. The variation in the rates of metabolism of T lymphocytes over the course of expansion is quite dramatic and it is important to understand the kinetics of this process as one attempts to develop optimal feeding protocols. The observation that proliferating lymphocytes have increased metabolic activity is well known [35–37] and it is believed that these increases are necessary to meet the energy demands of rapid cell division such as formation of new biomass. No differences in the examined metabolic yields were observed, although it might be expected that changes in the avail-

ability of O₂ to the cells would affect $Y_{lac/glu}$. However, activated lymphocytes are known to use glycolysis extensively as their source of energy [35].

Ex vivo stimulation and expansion of T cells in a 5% O₂ tension environment offers clear advantages over culturing under the standard 20% O₂ environment. Cells cultured under the 5% O₂ tension have reduced levels of apoptosis and have increased expression of IL-2R. The increased proliferative capacity under the lower O₂ tension is an important process improvement toward producing the large numbers of cells needed for cellular immunotherapy applications.

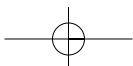
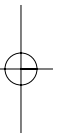
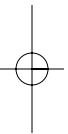
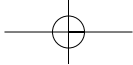
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