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## Differential Effects of Physiologically Relevant Hypoxic Conditions on T Lymphocyte Development and Effector Functions

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# Differential Effects of Physiologically Relevant Hypoxic Conditions on T Lymphocyte Development and Effector Functions

Charles C. Caldwell, Hidefumi Kojima, Dmitriy Lukashev, John Armstrong, Mark Farber, Sergey G. Apasov, and Michail V. Sitkovsky<sup>1</sup>

Direct measurements revealed low oxygen tensions (0.5–4.5% oxygen) in murine lymphoid organs *in vivo*. To test whether adaptation to changes in oxygen tension may have an effect on lymphocyte functions, T cell differentiation and functions at varying oxygen tensions were studied. These studies show: 1) differentiated CTL deliver Fas ligand- and perforin-dependent lethal hit equally well at all redox conditions; 2) CTL development is delayed at 2.5% oxygen as compared with 20% oxygen. Remarkably, development of CTL at 2.5% oxygen is more sustained and the CTL much more lytic; and 3) hypoxic exposure and TCR-mediated activation are additive in enhancing levels of hypoxia response element-containing gene products in lymphocyte supernatants. In contrast, hypoxia inhibited the accumulation of nonhypoxia response element-containing gene products (e.g., IL-2 and IFN- $\gamma$ ) in the same cultures. This suggests that T cell activation in hypoxic conditions *in vivo* may lead to different patterns of lymphokine secretion and accumulation of cytokines (e.g., vascular endothelial growth factor) affecting endothelial cells and vascular permeabilization. Thus, although higher numbers of cells survive and are activated during 20% oxygen incubation *in vitro*, the CTL which develop at 2.5% oxygen are more lytic with higher levels of activation markers. It is concluded that the ambient 20% oxygen tension (plus 2-ME) is remarkably well suited for immunologic specificity and cytotoxicity studies, but oxygen dependence should be taken into account during the design and interpretation of results of *in vitro* T cell development assays and gene expression studies *in vivo*. *The Journal of Immunology*, 2001, 167: 6140–6149.

Immune cell development, activation, and effector functions take place in primary, secondary, and tertiary lymphoid organs (1) with different infrastructure, vasculature, and oxygen supply. Immune cells and their progenitors could be exposed to both relatively high (normoxia) and low (hypoxia) oxygen tensions as they develop and migrate in massive numbers between blood and various tissues (1, 2). Hypoxic extracellular environments were demonstrated in some normal tissues (3, 4) and during chronic inflammatory and malignant diseases (5–10). Under such conditions mechanisms of lymphocyte adaptation to hypoxia are likely to exist. Lymphocytes in general, and cytotoxic cells in particular, have been shown to adapt to hypoxia, as reflected in observations of lymphocytic infiltration and cytotoxicity under hypoxic conditions in solid tumors (3). The cell adaptation to hypoxia is believed to be accomplished by stabilization of levels and increase in transcriptional activity of hypoxia inducible factor (HIF)<sup>3</sup>-1 $\alpha$  and HIF-2 $\alpha$ , which are required for activation of genes encoding glucose transporters and glycolytic enzymes, as well as for synthesis of such biologically active molecules as vascular endothelial growth factor (VEGF) and inducible NO synthase (11, 12).

In addition to changes in the patterns of gene expression and cell metabolism, low oxygen tension may affect the balance of SH and S-S groups implicated in maintenance of the properly folded state of proteins in the cytosol (13). Hypoxia may also cause changes in regulation of cellular processes due to the decrease in reactive oxygen species formation and action in many types of cells including T cells (14, 15).

Although secondary lymphoid organs are of utmost importance in immune cell development and functions, it was not known whether lymphocytes are exposed to hypoxic or normoxic environment in different compartments of thymus, spleen, and lymph nodes, and whether hypoxic exposure may affect immune response.

In this study, we have evaluated the effects of hypoxia on lymphocyte differentiation and effector functions and addressed the following questions. 1) What is the oxygen tension in different compartments of murine lymphoid organs? 2) What are the effects of physiologically relevant hypoxia on T cell differentiation vs effector functions of differentiated T cells? 3) What is the effect of hypoxia on hypoxia response element (HRE)-containing gene products (e.g., VEGF) vs non-HRE-containing gene products (e.g., IL-2 and IFN- $\gamma$ ) in TCR-activated lymphocytes?

The observations suggest that hypoxic exposure of T cells *in vivo* may significantly alter TCR-triggered lymphokine secretion patterns and CTL development without an appreciable effect on the lethal hit delivery by CTL.

## Materials and Methods

### Animals

C57BL/6 and DBA/2 mice were maintained in pathogen-free National Institutes of Health animal facilities. Mice were 6–10 wk old, and four to eight animals were used in each experiment.

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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<sup>1</sup> Address correspondence and reprint requests to Dr. Michail V. Sitkovsky, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 10/11N311, 10 Center Drive, Bethesda, MD 20892-1892. E-mail address: mvsitkovsky@helix.nih.gov

<sup>2</sup> Abbreviations used in this paper: HIF, hypoxia inducible factor; HRE, hypoxia response element; KLH, keyhole limpet hemocyanin; VEGF, vascular endothelial growth factor.

### Oxygen measurements

Oxygen tension measurements in organs of DBA/2 mice were conducted as previously described (16) using a recessed-tip oxygen microelectrode with a tip diameter of 9–12  $\mu\text{m}$  (17) and a micromanipulator (model M01202E; Narishige, Tokyo, Japan). Electrode current was measured with a microsensor (Chemical Microsensor model 201; Diamond General, Ann Arbor, MI) and recorded on a computer using CODAS software (AT-codas; Dataq Instruments, Akron, OH). Measurements were conducted in a Faraday cage which minimized extraneous electronic noise. The electrode was calibrated both before and after the measurements with four certified gas mixtures in saturated water. After each animal was sacrificed, the electrode remained in the organ to verify the null point of zero oxygen tension in the organ. At least three mice were used for each organ measured, while two to four tracks were taken sequentially of each organ.

### Cells and medium

Splenocytes were isolated from adult spleens *ex vivo* and incubated in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 5% dialyzed FCS (heat inactivated) and 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 1 mM sodium pyruvate, 1 mM HEPES, and nonessential amino acids (RP5). CTL clone BK1 (an anti-keyhole limpet hemocyanin (KLH) peptide), CD4<sup>+</sup> CTL clone, and anti-H2d CTL clone GD1 (FasL deficient, derived from *gld* mice) were maintained according to published procedures (18, 19).

Unless otherwise indicated, medium for cultures conducted at 20% oxygen contained 50  $\mu\text{M}$  2-ME, while medium for cultures conducted at 1 or 2.5% oxygen concentrations did not. Culture vessels for MLCs were six-well plates filled with 9 ml of RP5 medium and a cell density of 2.5 million cells/ml medium (2.0 million effector and 0.5 million stimulators). Cells to be cultured at 1 or 2.5% oxygen were centrifuged and resuspended in culture medium saturated with a certified gas mixture containing 1 or 2.5% oxygen, 5.0% CO<sub>2</sub>, and 94 or 92.5% N<sub>2</sub> (Robert's Oxygen Company, Rockville, MD). Cells were incubated at various oxygen tensions in a NAPCO 7000 (NAPCO, Winchester, VA) three-gas (CO<sub>2</sub>, N<sub>2</sub>, and O<sub>2</sub>) incubator.

### Cell culture and CTL assay

T cell development was studied during incubation of splenocytes isolated from C57BL/6 mice (responders) with irradiated (3,000 rads) splenocytes from DBA/2 mice stimulators at various oxygen tensions.

CTL cytotoxicity was measured in a routine 4-hr, 37°C, <sup>51</sup>Cr-release assay and calculated as described earlier (20). The proportions of CD4<sup>+</sup> and CD8<sup>+</sup> cells in cultures were evaluated in parallel by flow cytometry.

### Monoclonal Abs

FITC- or allophycocyanin-conjugated rat anti-mouse CD4 (clone RM-4-5), R-PE -conjugated rat anti-mouse CD25 (clone PC61), FITC- or allophycocyanin-conjugated rat anti-mouse CD8a (clone 53-67), PE-conjugated armenian hamster anti-mouse TCR  $\beta$ -chain (clone H57-597), PE-conjugated rat anti-mouse LFA-1 (CD11a, clone 2D7), PE- or allophycocyanin-conjugated rat anti-mouse IL-2 (clone JES6-1A12), PE- or allophycocyanin-conjugated rat anti-mouse IFN- $\gamma$  (clone XMG1.2), and anti-mouse CD3 mAb (clone 145-2C11) were purchased from BD PharMingen (San Diego, CA).

### Cytokine analysis

Intracellular staining of TCR-activated cells to evaluate cytokine production *in situ* was done in permeabilized cells using anti-IFN- $\gamma$  and IL-2 mAb as described recently (21) using 2  $\mu\text{M}$  (final concentration) monensin (CalBiochem, La Jolla, CA) and saponin buffer (PBS containing 0.1% (w/v) saponin, 0.1% BSA, 0.01 M HEPES, and 0.1% sodium azide) after 4 h of 2C11 anti-CD3 mAb restimulation. After staining with indicated anti-cytokine mAb, cells were analyzed by flow cytometry.

Concentrations of IL-2 and IFN- $\gamma$  in cell-free supernatants from MLC were determined using ELISA kits (Amersham Pharmacia Biotech, Piscataway, NJ). VEGF production was assayed using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

### Flow cytometry

Analysis of cell surface Ag expression and of cytokine expression *in situ* was performed as described earlier (21, 22). Flow cytometry data acquisition and analysis were done on a FACSCalibur using FACS research software and CellQuest programs (BD Biosciences, Mountain View, CA).

### Statistics

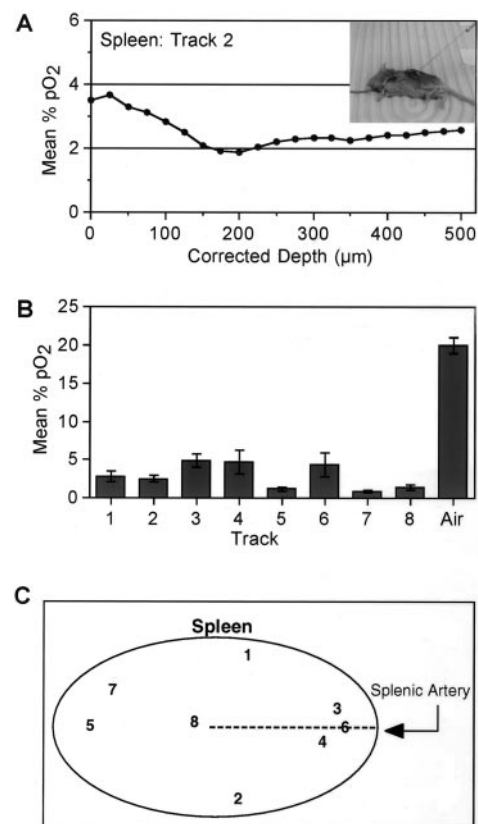
Standard errors were determined using the Microsoft Excel spreadsheet program (Microsoft, Redmond, WA).

## Results

### Direct measurements of oxygen tension in lymphoid organs *in vivo*

Standard *in vitro* culture systems for CTL generation have a microenvironment with a pH of 7.3, a CO<sub>2</sub> concentration of 5% (v/v), and an oxygen tension of ~20%, with 50  $\mu\text{M}$  2-ME added to the culture. Measurements of pH in spleen tissue yielded values of 7.20 and 7.21 in rat and cat, respectively (23, 24), and by extrapolating data on measurements of CO<sub>2</sub> in blood, it is accepted that the tissue CO<sub>2</sub> tension is ~5% (25, 26). It was important to evaluate the distribution of oxygen tension in different compartments of lymphoid organs. Therefore, we proceeded to directly measure oxygen tension in several lymphoid tissues, including the spleen.

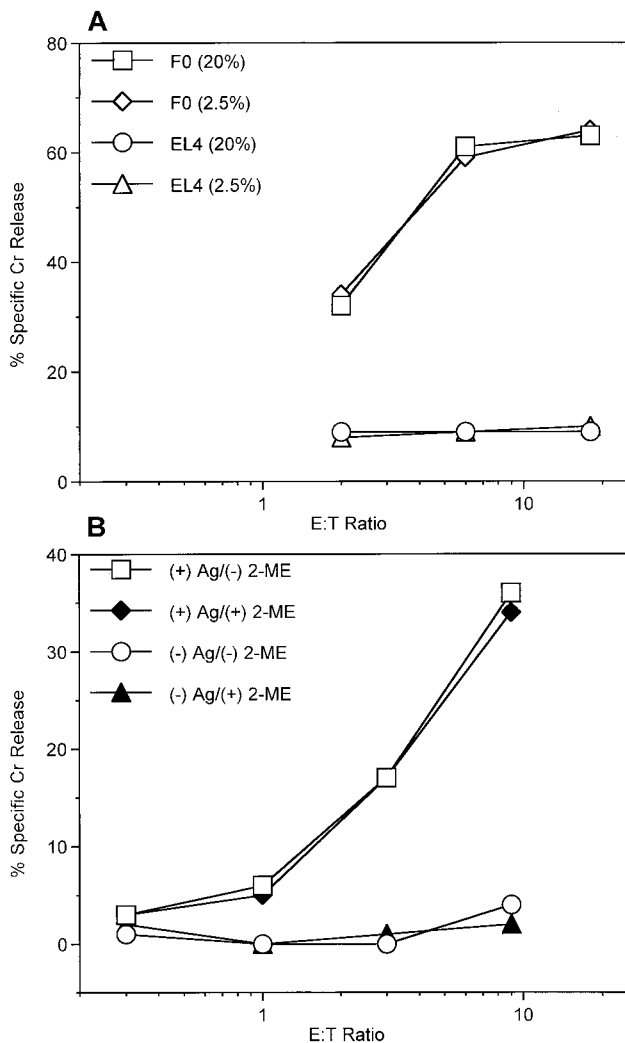
We observed low (between 4 and 34 torr, or 0.5–4.5%) oxygen tensions in lymphoid organs (Fig. 1) as compared with 150 torr or



**FIGURE 1.** Direct oxygen tension measurements in lymphoid organs *in vivo*. *A*, Representative track of measurements at increasing distances from the original electrode point insertion into the spleen. The corrected depth was calculated by taking into account the penetration angle coupled with 50- $\mu\text{m}$  insertion increments. An individual track is defined as a series of step-wise-increasing measurements. Each point represents the average oxygen tension over a 10–30 s period. *A, inset*, Use of the Clark microelectrode for oxygen measurements in spleen of anesthetized mice. *B*, Demonstration of low levels of oxygen tension in spleen *in vivo*. Eight independent tracks are presented to illustrate the range of measurements of mean oxygen tension in spleen. It is indicated for comparison purposes that ambient atmospheric oxygen tension is ~20%. *C*, Schematic presentation of electrode locations during oxygen tension measurements within the spleen. The square represents the s.c. incision, while the irregular oval represents the spleen. Each number is the point of insertion for the indicated tracks. The splenic artery is represented by the dashed line.

~20% oxygen concentration in the atmosphere (27). Fig. 1A shows oxygen level measurements as the electrode is incrementally (50  $\mu\text{m}$  per step) inserted into the spleen. The oxygen tension is relatively higher near the splenic artery (Fig. 1, B and C, tracks 3, 4, and 6) compared with the more distal locations in the spleen (Fig. 1, B and C, tracks 5 and 7). Similarly low oxygen tensions were detected in other lymphoid organs (data not shown).

The demonstration of predominantly hypoxic conditions in lymphoid organs led us to reexamine the effect of changes in the bioreductive potential on T cell effector functions and on T cell development in vitro either by changing the oxygen tensions (Figs. 2–5) or by incubating cells at the same oxygen tension (20%) but in the presence or absence of reducing agent 2-ME (data not shown).



**FIGURE 2.** Effect of changes of redox potential on the lethal hit delivery by differentiated CTL. *A*, Perforin-mediated lethal hit delivery by CTL is not redox potential dependent and is not affected by low oxygen tension. FasL-deficient GD1 clone (19) CTL (derived from *gld* mice) were incubated in parallel with  $^{51}\text{Cr}$ -labeled non-Ag-bearing EL4 cells (control) or with Fas-negative A20.FO cells in a 4-hr  $^{51}\text{Cr}$  release assay at 2.5 vs 20% oxygen, as described in *Materials and Methods*. *B*, FasL/Fas-mediated lethal hit delivery by CTL is not redox potential dependent. BK1, an anti-KLH peptide CD4-CTL clone with only the FasL mechanism of lethal hit delivery, was incubated with  $^{51}\text{Cr}$ -labeled A20.HL cells (pre-pulsed with TNP KLH, 0.01  $\mu\text{g}/\text{ml}$  to allow for the expression of an Ag) or in the absence of added Ag, at 20% oxygen tension with or without reducing agent (50  $\mu\text{M}$  2-ME). Lysis of target cells was determined as described in *Materials and Methods*. Data are representative of two independent experiments.

It was found that while routinely used 20% oxygen plus 2-ME incubations were best in supporting the cell growth and differentiation in vitro, the culture of cells at 1% oxygen plus 2-ME or 2.5% oxygen plus 2-ME resulted in dramatic decrease in the number of cells and differentiation of T cells (data not shown). The 2.5% oxygen tension represents a good approximation of physiological levels of oxygen in secondary lymphoid tissues. Therefore, in subsequent studies we compared lymphocyte development and functions at 1 or 2.5% oxygen in the absence of reducing agent with routinely used cultures at 20% with 50  $\mu\text{M}$  2-ME.

#### *Fas ligand- and perforin-mediated lethal hit delivery by differentiated CTL are not affected by an oxygen tension*

Experiments taking advantage of genetic tools to discriminate between possible differences in effects of changes in redox environment on FasL/Fas- vs perforin-mediated cytotoxicity are described in Fig. 2. Incubation of Fas-expressing cells at hypoxic conditions with anti-Fas mAb established that the Fas-triggered cell death pathway is not dependent on redox potential (data not shown); however, because FasL-mediated cytotoxicity by CTL requires the TCR-triggered up-regulation of Fas ligand (28), we directly tested whether CTL FasL-mediated cytotoxicity is dependent on redox potential (Fig. 2B). In these experiments, the redox dependence of FasL-mediated cytotoxicity by CTL, which requires TCR-triggered up-regulation of Fas ligand (28), was tested using a BK1 anti-KLH peptide, CD4<sup>+</sup> CTL clone that operates using only the FasL-mediated mechanism of lethal hit delivery (Fig. 2B). A similar result was observed in studies of the anti-Fas mAb/Fas-triggered cell death and in experiments using FasL-deficient CTL clone GD1, which uses only perforin-mediated cytotoxicity and kills equally well at 2.5 and 20% oxygen (Fig. 2A and data not shown). These experiments show that FasL-mediated cell death is not affected by redox changes in the environment.

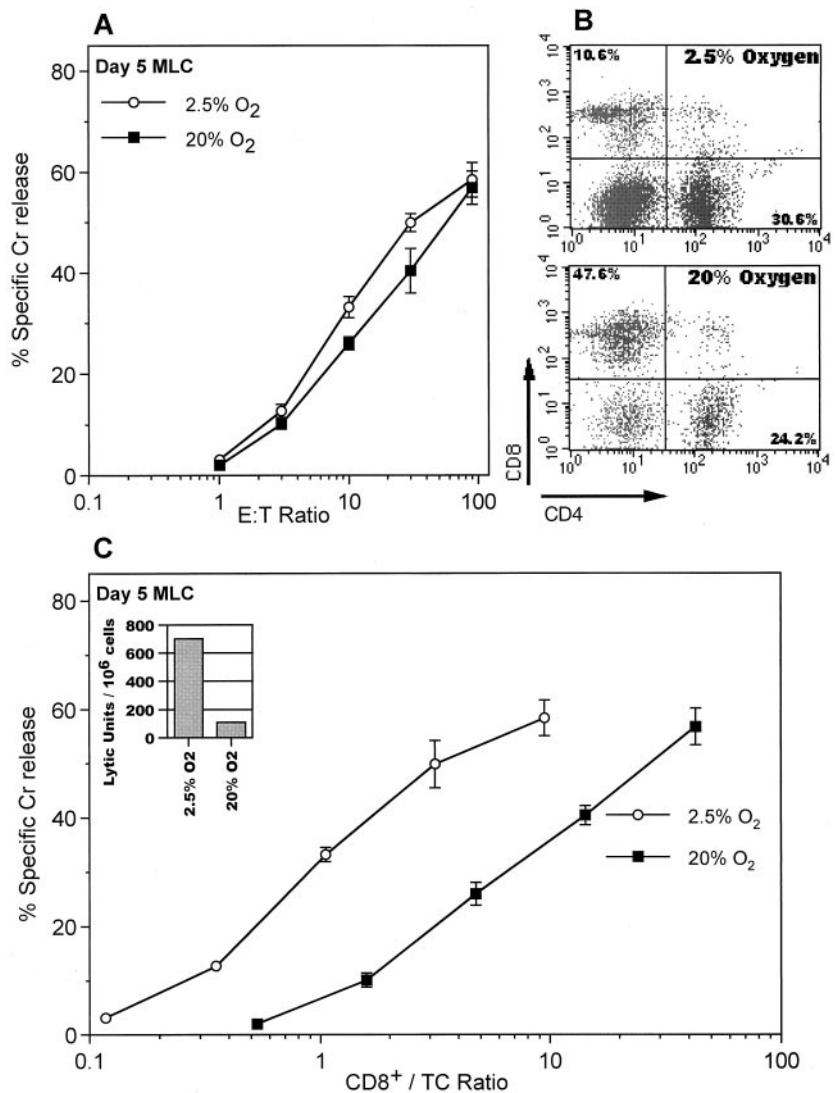
Such insensitivity of both FasL- and perforin-mediated lethal hit delivery pathways to changes in redox potential is in stark contrast to the oxygen tension dependence of processes of CTL development.

#### *Physiologically low oxygen tensions favor more lytic CD8<sup>+</sup> CTL development*

Routinely used assays of CTL development are performed at ambient oxygen tension (20% plus 2-ME), and this allows for accumulation of CTL after 5 or 6 days of culture in CO<sub>2</sub> incubators. We confirmed that the addition of 2-ME (29, 30) was necessary for CTL development at ambient (20%) oxygen tension (data not shown), while the presence of  $5 \times 10^{-5}$  M 2-ME at lower oxygen tensions was not conducive for CTL development (1 or 3% oxygen, data not shown). It was important to compare the outcome of routinely used in vitro 20% oxygen with 50  $\mu\text{M}$  2-ME vs more physiologically relevant conditions of incubation of lymphocytes at 2.5% oxygen without 2-ME.

It is shown in Fig. 3A that at day 5 there is no difference between cytolytic activity of CTL developed at 2.5 vs 20% oxygen if calculations of numbers of effector cells in E:T ratios were performed per total viable cells. However, it was noticed that the CD4:CD8 ratio in these cultures was different between hypoxic and normoxic conditions (Fig. 3B). Although there were more CD8 than CD4 at 20% oxygen tensions (CD4:CD8 ~ 1:2), the opposite was observed at hypoxic conditions as the CD4/CD8 ratio was much higher (~3:1) among MLC cells. This, taken together with equal lytic activity of both total cell cultures (Fig. 3A), suggested that while there were fewer CD8<sup>+</sup> T cells developed at hypoxic conditions, their lytic activity could be much higher per CD8<sup>+</sup> CTL basis. This prompted an investigation where we tested both cytotoxicity and CD4 vs CD8 numbers of cells by flow cytometry to

**FIGURE 3.** Physiologically low oxygen tensions favor delayed but more lytic CD8<sup>+</sup> CTL development. **A**, Comparison of results of <sup>51</sup>Cr cytotoxicity assay of CTL developed at 2.5 vs 20% oxygen, with E:T ratios calculated as total viable cells:TC after 5 days of MLC. Spleen cells were incubated for 5 days at 2.5 vs 20% oxygen tension and then assayed for cytotoxic potential by incubation with <sup>51</sup>Cr-labeled P815 H-2<sup>d</sup> cells for 4 h at 20% oxygen. Nonspecific lysis was determined not to exceed 5% <sup>51</sup>Cr release using <sup>51</sup>Cr-labeled EL4 cells (H-2<sup>b</sup>). **B**, Ratio of CD4<sup>+</sup> cells to CD8<sup>+</sup> cells is markedly different under the varying oxygen incubations. CD4<sup>+</sup> and CD8<sup>+</sup> cells were incubated in the above conditions with percentages determined by flow cytometry. CD8<sup>+</sup> CTL developed at 2.5% oxygen tension have higher lytic activity than do CD8<sup>+</sup> CTL at 20% oxygen. Results of <sup>51</sup>Cr cytotoxicity assay of CTL developed at 2.5 vs 20% oxygen were compared with E:T ratios calculated as CD8<sup>+</sup> CTL:TC ratio. The proportion and number of CD8<sup>+</sup> CTL in cytotoxicity assay was determined in parallel flow cytometry measurements of cell samples. **C**, *inset*, Comparison of CD8<sup>+</sup> CTL lytic units per million cells after 5-day MLC development at 2.5 vs 20% oxygen. The ratios of CD8<sup>+</sup> CTL to targets that were required to accomplish the 30% <sup>51</sup>Cr release from Ag-specific P815 cells were calculated from the CD8<sup>+</sup>:TC ratio in **B**, and the number of units per million cells developed at these oxygen tensions was calculated. Results are representative of three independent experiments.



enable the recalculation of cytotoxicity per CD8<sup>+</sup> CTL basis. The lytic potential of CTL in these experiments was estimated by recalculating the results of the <sup>51</sup>Cr release assay as the CD8<sup>+</sup> CTL:target cell ratio in addition to routinely used numbers of total viable effector cells.

With these calculations, it was found that hypoxic conditions favor the development of much more lytic CD8<sup>+</sup> CTL (see Fig. 3C). Indeed, it is shown that CD8<sup>+</sup> CTL cells developed under hypoxic conditions possess a 7-fold higher killing potential as measured by lytic units per million cells (Fig. 3C, *inset*). Thus, the much larger number of less lytic CD8<sup>+</sup> cells that accumulate at 20% oxygen may misleadingly mask differences between the development of lytic CTL under these two conditions at day 5 of MLC (Fig. 3A) if results are not compared as CD8<sup>+</sup> CTL:target ratio.

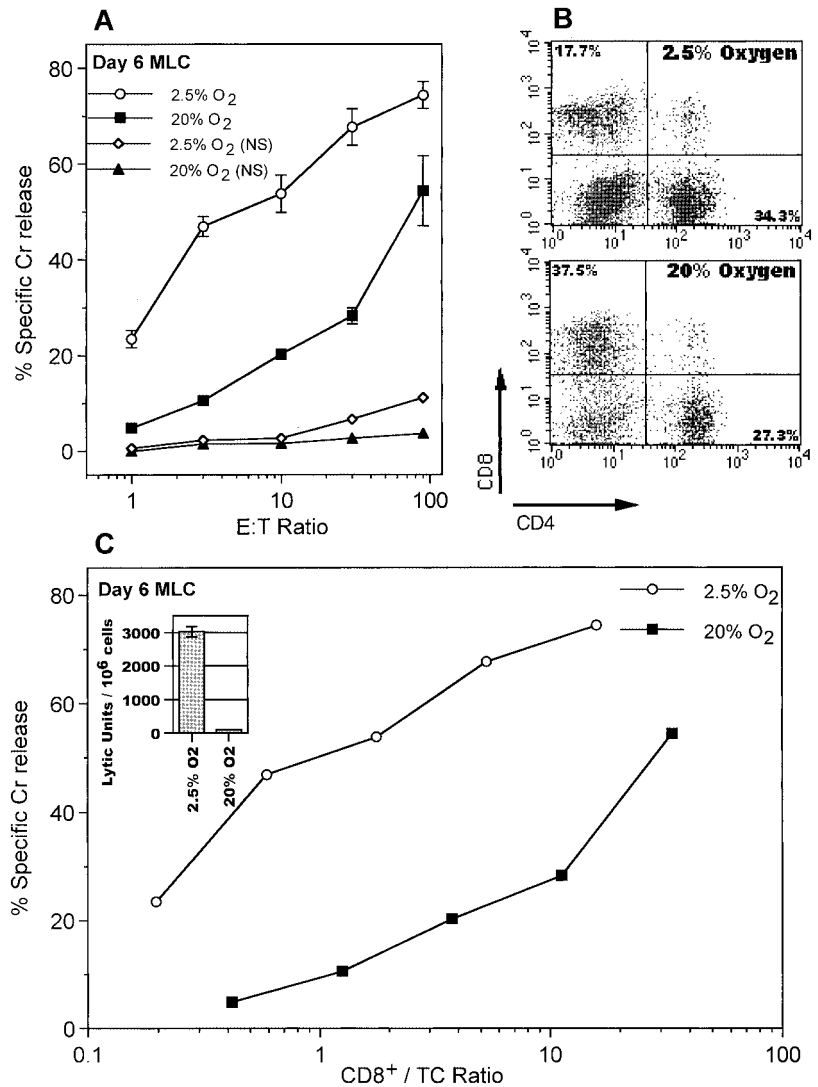
To differentiate between Ag-driven expansion of specific TCR-expressing T cells and the possible expansion of bystander CD8<sup>+</sup> T cells, we used the OVA-specific TCR-transgenic mice. Using this system we found, in agreement with our MLC data in Figs. 3 and 4, that OT-1 CTLs expanded more quickly under 20% oxygen than under 2.5% oxygen tension when stimulated with the OT-1 specific peptide, SIINFEKL (Table I).

Further time-dependent studies of CTL development showed that between days 5 and 6 of incubation there was a significant acceleration in development of CD8<sup>+</sup> CTL with high lytic activity

at hypoxic 2.5% oxygen. This is reflected in both CD4/CD8 ratios and in cytotoxic activities on a CD8<sup>+</sup> cell basis (Fig. 4, A and B). Furthermore, the numbers and proportions of CD8<sup>+</sup> T cells continued to increase from day 5 to day 6 (Fig. 4B and data not shown) at hypoxic 2.5%. In contrast, it appears that the numbers and proportions of CD8 at day 6 at 20% are decreasing due to increased cell death (data not shown). Even more striking differences were observed at day 6 of MLC in regard to higher CD8<sup>+</sup> CTL activity (Fig. 4C). When recalculated on per CD8<sup>+</sup> CTL basis a dramatic increase in lytic units from 700 to 3000 was observed with CD8<sup>+</sup> CTL at hypoxic conditions, while at 20% CD8<sup>+</sup> CTL remain the same (Fig. 4C, *inset*).

The data above lead us to investigate both numbers and the activation state of the CD8<sup>+</sup> CTL cells. Table II shows that at least three times more CD8<sup>+</sup> cells are developed in MLC at 20% than at 2.5% oxygen. Not only are there more CD8<sup>+</sup> cells, but a 5-fold larger number of CD8<sup>+</sup> cells also express the IL-2R (CD25) at 20% oxygen. However, flow cytometry analysis indicates that even though there are fewer CD25<sup>+</sup>CD8<sup>+</sup> T cells at 2.5%, those CD8<sup>+</sup> T cells that are activated at low oxygen tension have a higher surface density of CD25<sup>+</sup> (mean fluorescence intensity = 807 at day 5 at 2.5% vs 146 at 20% oxygen) (see Table II). Thus, although more cells survive and are activated at 20% oxygen, cells

**FIGURE 4.** Low oxygen tension favors a delayed but more potent CTL response during MLC. **A**, Comparison of results of  $^{51}\text{Cr}$  cytotoxicity assay of CTL developed at 2.5 vs 20% oxygen, with E:T ratios calculated as total viable cells:TC after 6 days of MLC. Spleen cells were incubated for 6 days at 2.5 vs 20% oxygen tension and then assayed for cytotoxic potential by incubation with  $^{51}\text{Cr}$ -labeled P815 H-2<sup>d</sup> cells for 4 h at 20% oxygen. **B**, Differences in proportion of CD8<sup>+</sup> and CD4<sup>+</sup> cells from days 5–6. Changes in CD4<sup>+</sup> and CD8<sup>+</sup> proportions as determined in parallel flow cytometry measurements of cell sample. **C**, Dramatic increase in lytic units of CD8<sup>+</sup> CTL developed at 2.5% oxygen tension as compared with CD8<sup>+</sup> CTL at 20% oxygen on day 6 of the MLC. Results of  $^{51}\text{Cr}$  cytotoxicity assay of CTL developed at 2.5 vs 20% oxygen were compared with E:T ratios calculated as CD8<sup>+</sup> CTL:TC after staining with anti-CD8 mAb. **D**, inset, Comparison of CD8<sup>+</sup> CTL lytic units per million cells after 6-day MLC development at 2.5 vs 20% oxygen. The ratios of CD8<sup>+</sup> CTL to targets that were required to accomplish the 30%  $^{51}\text{Cr}$  release from Ag-specific P815 cells were calculated from the CD8<sup>+</sup>:TC ratio in **C**, and the number of lytic units per million cells developed at these oxygen tensions was calculated. E:T ratios were calculated as CD8<sup>+</sup> CTL:TC. Results are representative of three independent experiments.



at 2.5% oxygen develop more slowly, have a higher level of activation, and are much more lytic.

#### Differential effects of hypoxia on production of hypoxia-dependent HRE-containing and hypoxia-independent gene products during TCR-driven differentiation of CTL

To evaluate the effect of oxygen tension changes on cytokine secretion and consumption, the levels of VEGF, IFN- $\gamma$ , and IL-2 were determined in MLC culture supernatants by ELISA (Fig. 5, A and B) and on the single-cell level in situ by flow cytometry (Fig. 6).

Table 1. Flow cytometric estimation of numbers of OT-1 CD8<sup>+</sup> at days 2 and 3 of MLC<sup>a</sup>

Day	Number of OT-1 CD8 <sup>+</sup> Cells	
	2.5% O <sub>2</sub>	20% O <sub>2</sub>
2	0.02	0.12
3	0.03	1.42

<sup>a</sup> The percentage of viable CD8<sup>+</sup> cells was determined by flow cytometry as described in *Materials and Methods*. The number of cells (in millions) with the indicated surface marker was calculated from direct cell counts and viable cell counts (propidium iodide exclusion test, flow cytometry), and by estimation of the percentage of CD8<sup>+</sup> cells. These data are representative of two experiments. The MLC was started (day 0) by adding 20 million effector splenocytes which included 1.5 million CD8<sup>+</sup> cells.

Differentiation of T cells in vivo and in vitro is both TCR-driven and cytokine-dependent. It is based upon the complex and yet to be fully understood interplay of different cytokines and cytokine receptor-expressing immune cells at different stages of differentiation (31). Therefore, it was important to determine whether cytokine secretion by T cells is oxygen tension dependent.

It was shown with different types of cells, although not with T lymphocytes, that the adaptation to low oxygen tension proceeds through activities of HIF-1 and HIF-2, which trigger transcription of HRE-containing genes (11). One of these genes is VEGF (11), and we compared the effect of hypoxia on the accumulation of VEGF and the non-HRE-containing IL-2, IFN- $\gamma$  genes (Fig. 5A) in activated lymphocytes.

The hypoxia-induced enhancement of cytokine accumulation was observed in studies of VEGF during MLC at 2.5 vs 20% oxygen (Fig. 5B). No VEGF accumulation was found in supernatants of T cells until the day 4 of incubation at 20% oxygen (Fig. 5B). In contrast, VEGF was detected in supernatants as soon as the day 2 at hypoxic, 2.5% oxygen cultures. The normoxic, 20% oxygen incubation did eventually result in accumulation of VEGF, but the total amount of VEGF at hypoxic 2.5% was higher, suggesting that there is a combined, additive effect of HRE-dependent and TCR-activation-dependent VEGF production by T cells. The accumulation of IL-2 in the 20% oxygen MLC supernatant at its

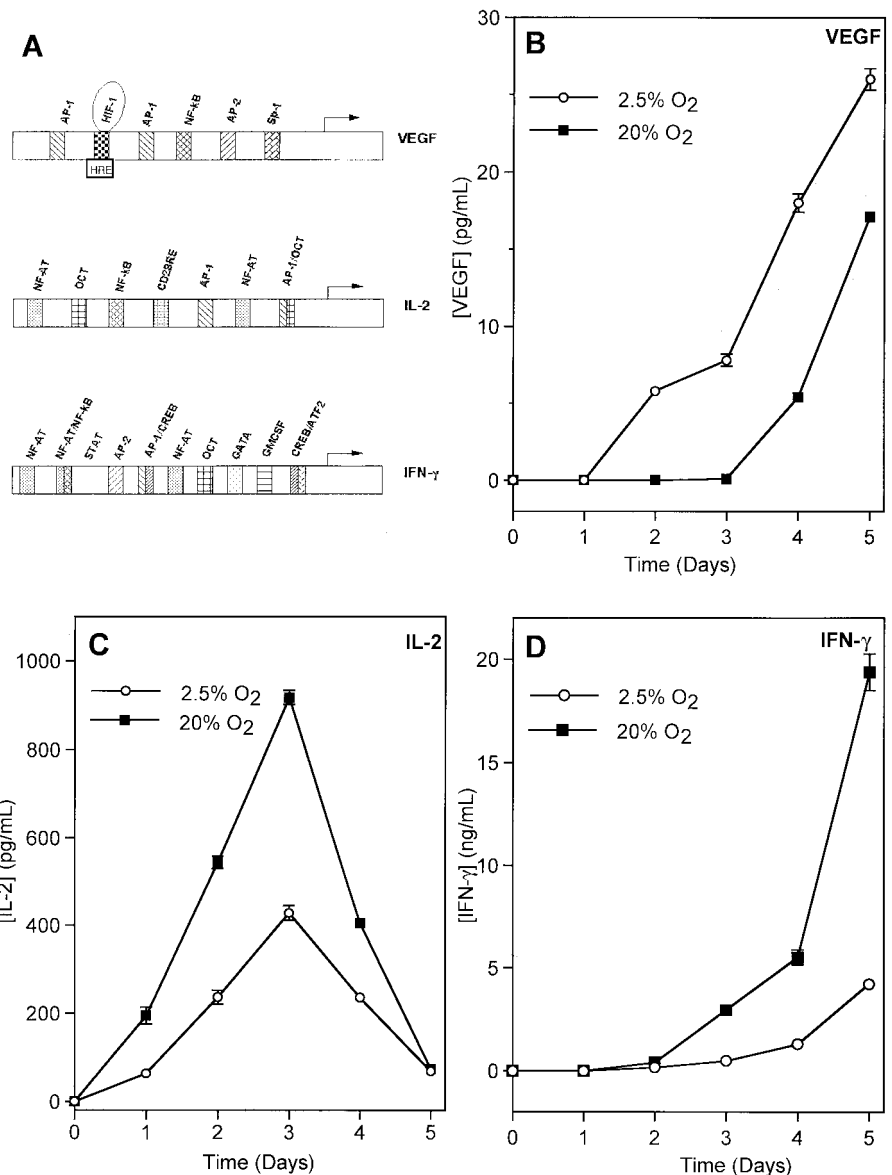
Table II. Flow cytometric estimation of proportion of CD8<sup>+</sup> and activated CD8<sup>+</sup> CD25<sup>+</sup> cells at days 4, 5, and 6 of MLC<sup>a</sup>

Day	Number of CD8 <sup>+</sup> Cells		Number of CD8 <sup>+</sup> /CD25 <sup>+</sup> Cells		CD25 MFI on CD8 <sup>+</sup> Cells	
	2.5% O <sub>2</sub>	20% O <sub>2</sub>	2.5% O <sub>2</sub>	20% O <sub>2</sub>	2.5% O <sub>2</sub>	20% O <sub>2</sub>
4	0.27	1.03	0.02	0.45	588	211
5	0.23	1.18	0.07	0.66	807	146
6	0.21	0.78	0.04	0.22	192	80.1

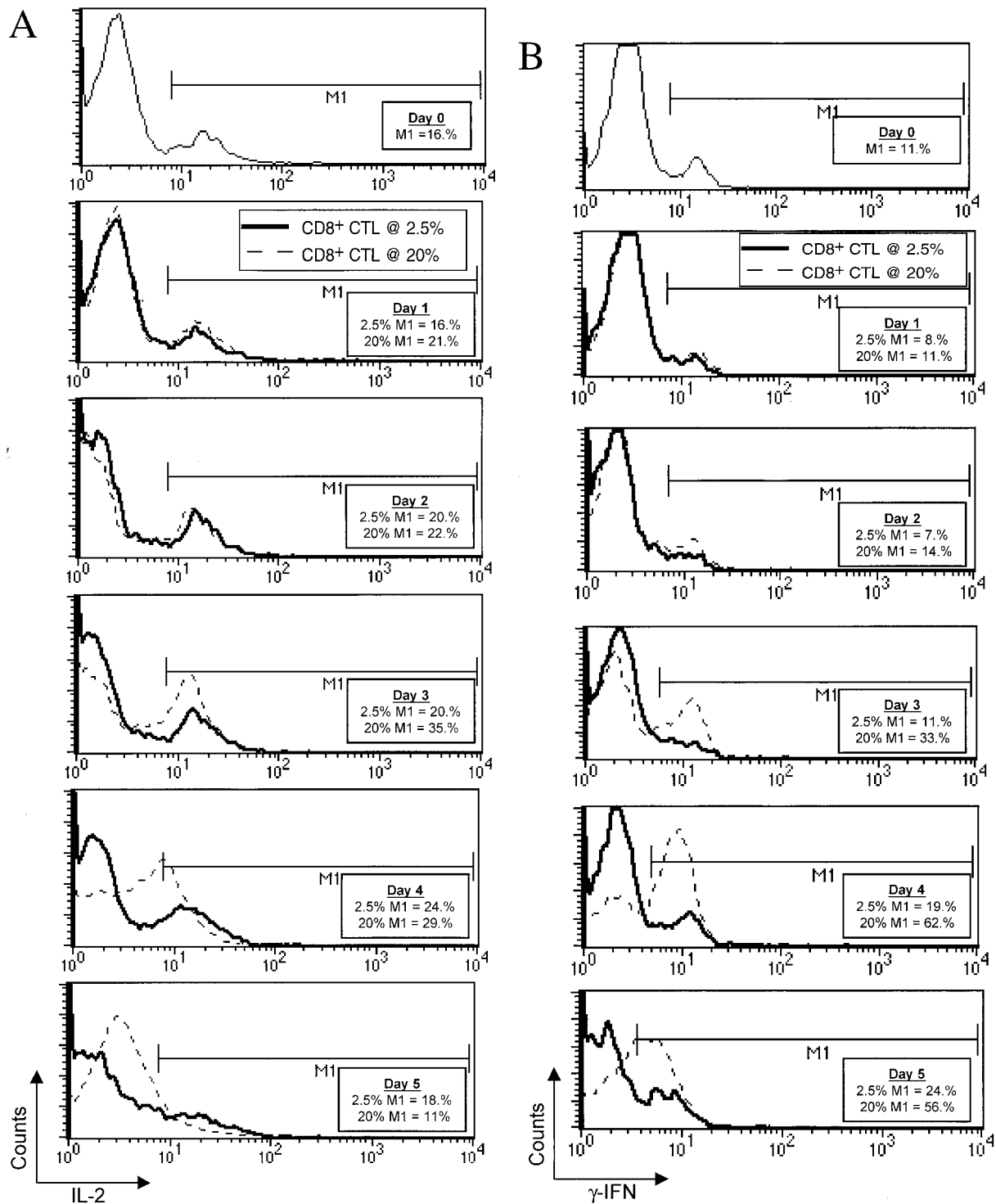
<sup>a</sup> The percentage of viable, CD8<sup>+</sup> and CD25<sup>+</sup> cells was determined by flow cytometry as described in *Materials and Methods*. The number of cells (in millions) with the indicated surface marker was calculated from direct cell counts and viable cell counts (PI exclusion test, flow cytometry), and by estimation of the percentage of CD8<sup>+</sup> or CD25<sup>+</sup> cells. These data are representative of six experiments. The MLC was started (day 0) by adding 20 million effector splenocytes which included 1.5 million CD8<sup>+</sup> cells with 20,000 of activated CD8<sup>+</sup>CD25<sup>+</sup> cells with CD25 mean fluorescence intensity (MFI) = 170.

peak was about 2-fold higher than that found in the 2.5% MLC supernatant (Fig. 5C). Furthermore, the increase in the accumulation and the decrease in IL-2 was also much greater in the 20% oxygen tension MLC supernatant. Similarly, the rate of increase and the total amount of IFN-γ in the 20% oxygen supernatant was much higher compared with the 2.5% culture (Fig. 5D).

The experiment in Fig. 6 was designed to compare the production of IL-2 and IFN-γ at different oxygen tensions on a per cell basis. It is shown that the rate of production, as measured by the intensity of cytokine labeling by mAb, is about equal on all days for both cytokines in both culture conditions. Furthermore, it appears that the percentage of IL-2 secreting T cells is about the same



**FIGURE 5.** Differential effects of low oxygen tension on production of HRE-containing (VEGF) vs non-HRE-containing (IL-2, IFN-γ) cytokines. Cell-free supernatants were collected at the indicated times from cell cultures incubated at 2.5 vs 20% oxygen and analyzed for the presence of cytokines by ELISA as described in *Materials and Methods*. *A*, Schematic promoter region and nuclear transcription factor binding sites of the cytokines tested. *B*, Measurements of VEGF (picograms per milliliter). *C*, Measurements of IL-2 (picograms per milliliter). *D*, Measurements of IFN-γ (nanograms per milliliter).



**FIGURE 6.** In situ measurements of IL-2 and IFN- $\gamma$  in CD8<sup>+</sup> T cells during time course of MLC. Cell samples were taken from both 2.5 and 20% culture conditions at the indicated time points. The cells were restimulated with anti-TCR/CD3 Ab for 4 h in the presence of 2  $\mu$ M monensin. The cells were stained with the indicated anti-cytokine Ab as described in *Materials and Methods*. Viable CD8<sup>+</sup> T cells were gated and analyzed for IL-2 and IFN- $\gamma$  expression. *A*, Time-dependent increases in IL-2-producing cells. IL-2 expression studies as described in *Materials and Methods*. *B*, Time-dependent increases in IFN- $\gamma$ -producing cells. IFN- $\gamma$  expression studies were performed as described in *Materials and Methods*.

on all days. However, because the absolute number of TCR-activated, IL-2-producing cells is much higher at 20% oxygen (as shown in Table II), the ELISA measurements of IL-2 in supernatants yielded a higher concentration of IL-2 at 20% than at 2.5% oxygen. In contrast, the proportion of IFN- $\gamma$ -producing cells is much higher at 20% than at 2.5% oxygen. It is shown in Fig. 6 that

the proportion of IFN- $\gamma$ -producing CTLs is at least 3-fold higher during days 3 and 4 of incubation.

Altogether, these experiments demonstrate that less VEGF, but much more IFN- $\gamma$  and IL-2 as well as much more IFN- $\gamma$ -producing CD8<sup>+</sup> and CD4<sup>+</sup> T cells were accumulated under normoxic conditions than at hypoxic conditions (Figs. 5 and 6). This may



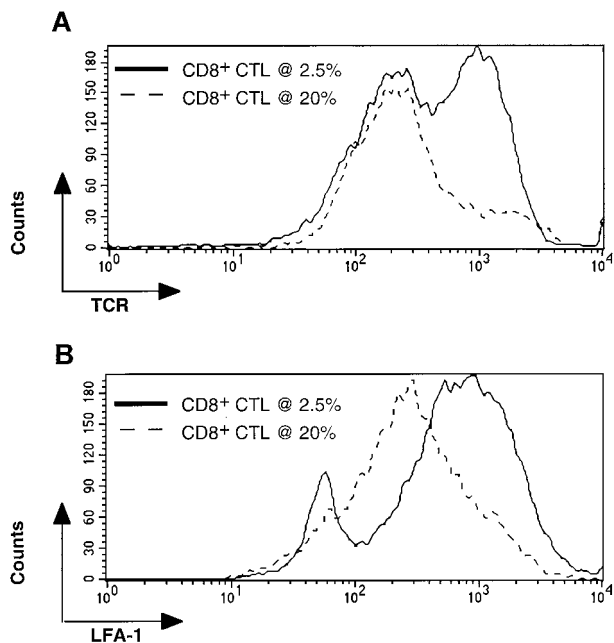
partially explain differences in cell numbers (Table II) as due to the IL-2-dependent growth and the proliferation advantage of T cells at 20% oxygen.

#### High cell surface density of TCR/CD3 complex and cell adhesion LFA-1 molecules on CD8<sup>+</sup> T cells at hypoxic conditions of MLC

Attempts to explain the increase in lytic activity of CTL from day 5 to day 6 at 2.5 vs 20% oxygen incubations (Figs. 3 and 4) included evaluations of expression of recognition and cell adhesion molecules (Fig. 7), of lytic molecules perforin and FasL, as well as comparison of levels of CTLA-4 (data not shown). No differences between hypoxic and normoxic conditions of incubations were found in levels of expression of perforin, FasL, and regulatory CTLA-4 molecules as determined by flow cytometry (data not shown). In contrast, levels of expression of TCR and LFA-1 were higher on CD8<sup>+</sup> cells at 2.5% oxygen tension, and much higher proportions of CD8<sup>+</sup> T cells could be classified as TCR<sup>high</sup>LFA-1<sup>high</sup> at 2.5% oxygen than at 20% oxygen (Fig. 7). Importantly, the greatest increase in proportion of TCR<sup>high</sup>LFA-1<sup>high</sup> CD8<sup>+</sup> T cells was observed at the time (days 5–6) when the most lytic CD8<sup>+</sup> CTL were detected at 2.5 vs 20% oxygen (see Figs. 3 and 4).

## Discussion

The main observations of our studies measuring oxygen tensions in lymphoid tissues and attempting to mimic these conditions in vitro are: 1) the demonstration of predominantly hypoxic conditions in different compartments of lymphoid tissues in vivo; 2) differential effect of hypoxia on CTL lethal hit delivery vs CTL differentiation in vitro; and 3) differential effects of hypoxia on transcription of HRE-containing (e.g., VEGF) vs non-HRE-containing cytokines genes (e.g., IFN- $\gamma$  and IL-2) in lymphocytes.



**FIGURE 7.** Hypoxic (2.5% oxygen tension) conditions of cell culture favor development of CD8<sup>+</sup> T cells with higher surface density of TCR and LFA-1 molecules. Cell samples were taken from both 2.5 and 20% culture conditions at different time points and analyzed by flow cytometry (data on day 6 are presented). Viable CD8<sup>+</sup> T cells were gated and analyzed for TCR and LFA-1 expression. *A*, TCR expression studies using anti-TCR-CD3 complex mAb were performed as described in *Materials and Methods*. *B*, LFA-1 expression studies were performed using anti-LFA-1 mAb as described in *Materials and Methods*.

Indeed, the lethal hit delivery by CTL is not affected by hypoxia (Fig. 2). The observations of similar lethal hit delivery by CTL at 1, 2.5, and 20% oxygen (Fig. 2 and data not shown) are conceptually appealing and may reflect the fidelity of functions of this effector arm of immune system in all tissue compartments. Otherwise, hypoxic areas, such as areas of inflammation, would be providing “safe havens” for infected cells. These data may also settle the longstanding controversy regarding studies of the effect of oxygen tension on CTL lethal hit delivery and processes of cell death (3, 32–34). It appears that processes of cell death do require very low levels of oxygen, but there are no differences in CTL-induced target cell death when oxygen tension is varied between 1 and 20%.

In contrast to effector lytic functions of differentiated CTL, the development of CTL and patterns of lymphokine accumulation were very different at 2.5 vs 20% oxygen (Figs. 3–5). An unexpected observation was that low oxygen tension conditions result in development of CD8<sup>+</sup> CTL (Figs. 3 and 4), which were more lytic. Studies of lymphokine accumulation in supernatants, in parallel with estimations of IFN- $\gamma$  and IL-2 on a per cell basis (Figs. 5 and 6), are consistent with the model in which greater numbers of less activated and less lytic CD8<sup>+</sup> T cells are developed at normoxic conditions, while hypoxia favors sustained expansion of more lytic CD8<sup>+</sup> CTL. Subsequent experiments (Fig. 6) suggested that the higher IL-2 concentration found in cell supernatants at 20% oxygen is not because there is more IL-2 production per cell at 20%, but because there is a higher number of IL-2-producing cells at 20% than at 2.5% oxygen. However, the reasons for higher IFN- $\gamma$  accumulation are most likely due to both higher number of T cells and a higher proportion of IFN- $\gamma$ -producing T cells at 20% oxygen than at 2.5% oxygen.

Demonstration of much higher proportions of TCR<sup>high</sup>LFA<sup>high</sup> cells among CD8<sup>+</sup> 2.5 vs 20% oxygen (Fig. 7) provides a possible explanation as to why these CTL are more lytic. The higher surface density of TCR and LFA-1 recognition and cell adhesion molecules at 2.5% oxygen may result in more lytic CTL, because these surface molecules are important in lethal hit delivery by CTL due to their role in CTL-target cell conjugate formation and lethal hit delivery (35). It is much less clear why there are more TCR<sup>high</sup>LFA<sup>high</sup> CD8<sup>+</sup> T cells at 2.5% than at 20% oxygen. One possibility is that a higher proportion of TCR<sup>high</sup>LFA<sup>high</sup> reflects their better (preferential) expansion or survival at 2.5% oxygen than at 20% oxygen. This, in turn, could reflect differences in cytokine repertoire and concentrations in MLC conducted at different oxygen tensions. For example, observations of increased VEGF accumulation at 2.5% oxygen (Fig. 5) suggest that the hypoxia-enhanced accumulation of VEGF and/or of other yet to be determined gene products may create more favorable conditions for the accumulation of TCR<sup>high</sup>LFA<sup>high</sup> CTL. Indeed, it was reported that VEGF is able to induce increased levels of LFA-1 molecules (36), which are important for CTL lytic hit delivery. Interestingly, “hypoxic” CD8<sup>+</sup> T cells could not be enriched for studies using negative selection and the AutoMACS separator by removing CD4 and B cells most likely due to their more adhesive properties (data not shown). Future studies may address in detail whether culturing cells under hypoxic conditions change the ability of cells to participate in cell-cell interactions and adhesion. In support of such an interpretation are the observations of increased expressions of cell surface adhesion molecules LFA-1 on the CD8 from 2.5% oxygen (Fig. 7).

Thus, effects of hypoxia on T cells could be inhibiting (e.g., decrease in IFN- $\gamma$ , IL-2 secretion; see Fig. 5, *C* and *D*) or enhancing (e.g., improvement of CD8<sup>+</sup> CTL development at 2.5 vs 20% oxygen, as shown in Figs. 3 and 4; and of VEGF secretion, as shown in Fig. 5*B*). Other functions, such as lethal hit delivery by

CTL, are oxygen independent (Figs. 2 and 3). The explanation of these opposite and cell differentiation-dependent effects of oxygen tension could involve effects of hypoxia-associated accumulation of extracellular adenosine (21, 22, 37–40) and activities of HIF-1 and HIF-2 (11). Indeed, differences between 1, 2.5, and 20% oxygen tensions described here could be at least partially explained by the patterns of HIF-1 expression (41–44) and by HIF-1-mediated changes in cellular response. It was shown that both HIF-1 and HIF-2 activate transcription of hypoxia-inducible genes. The common feature of these genes is the presence of HRE, and among HRE-containing genes are VEGF, erythropoietin, and enzymes of glycolytic pathway (11), which enable the switch of ATP generation from oxidative phosphorylation in resting lymphocytes to glycolysis in activated lymphocytes (45). It remains to be determined in future studies whether HIF-1 or HIF-2, or both, contribute to transcription of hypoxia-dependent genes in immune cells.

These observations point to the need for better understanding of the mechanisms of lymphocyte adaptation to hypoxia and of the role of HIF-1 and HIF-2 in these processes. Indeed, observations of the augmenting effects of hypoxic exposure and of TCR triggering (VEGF vs IL-2 and IFN- $\gamma$ ; see Fig. 5) do support the view that the overall effect of hypoxia on T cell functions is to alter the proportion of HRE- and non-HRE-containing cytokines. In contrast, genes that do not have an HRE are expressed later and at levels which are higher in normoxic conditions (20%) than at hypoxic conditions (2.5%). The accelerated accumulation of VEGF in hypoxic conditions (Fig. 5) is best explained by our recent findings that HIF-1 is an immediate-early response gene (D. Lukashev, C. C. Caldwell, and M. V. Sitkovsky, unpublished observations). Thus, the early presence of HIF-1 $\alpha$  gene product in activated T cells ensures earlier commencement of transcription of hypoxia-dependent genes.

The observations of increased densities of TCR and LFA-1 on CD8<sup>+</sup> CTL at hypoxic conditions (Fig. 7) point to the need in further studies of molecular mechanisms of this phenomenon. Especially interesting are questions about the possible role of VEGF in CD8<sup>+</sup> CTL development. Even though VEGF has been demonstrated to be a cause of increase of LFA-1 on NK cells (36), it is not clear whether it was the direct effect of VEGF or of other VEGF-induced cytokines on CD8<sup>+</sup> T cells in the experiment described in Fig. 5. Indeed, it is well established that VEGF receptors are expressed mostly on endothelial cells, and future detailed studies of VEGF receptor expression among different immune cells may address the question as to what is the mechanism of increase in the TCR and LFA-1 levels of expression on CD8<sup>+</sup> T cells in hypoxic conditions of MLC. Thus, it is possible, although it remains to be further investigated, that levels of TCR and LFA-1 in CTL are at least partially controlled by VEGF levels and that the better lytic ability of CTL developed at hypoxic conditions may be due to the functioning of hypoxia-dependent, VEGF-mediated T cell recognition and cell adhesion molecules.

The data described demonstrate that the established routine conditions of lymphocyte culture (20% oxygen in the presence of reducing agent) are remarkably well suited to studies of immunologic specificities. Indeed, the importance of mimicking the in vivo redox potential in in vitro assays was well recognized in the original studies of lymphocytes in vitro. The use of a 7% oxygen mixture (46) as well as the addition of reducing agent 2-ME (29, 47) to incubations at 20% oxygen resulted in dramatic success in studies of Ag specificity in cellular immunologic assays in vitro. The observations of differences in lymphokine production between low oxygen and normoxic conditions could be helpful in studies of lymphokine-driven differentiation of Th1 vs Th2 and of Tc1 vs Tc2 cells (31), as well as in studies of T cell activation-dependent gene expression using DNA chip technology (48). The strong im-

pact of in vitro oxygen tension on the outcome of T cell development adds yet another facet in considering the dramatically better than expected expansion of Ag-specific CTL in vivo using MHC class I/peptide tetramer detection assays (49, 50).

The question about the oxygen tension dependence of IFN- $\gamma$  secretion by human monocytes has also been addressed by Krieger et al. (51), and together with our observations the data support the model where immune cell development and functions proceed differently at low hypoxia vs normoxia. Use of 1 and 2.5% oxygen tensions, which were shown to be promoting the expression and functions of HIF-1 (42), may represent a better approximation of the hypoxic conditions in vivo. Furthermore, the observations reported here suggest the use of hypoxic oxygen tensions in some in vitro assays to better approximate in vivo conditions in studies of T cell functions. The pH at  $\sim$ 7.2 appears to be the most optimal and physiological way, because direct measurements of pH in spleen of cats (23) and rats (24) show the pH values of 7.20 and 7.21 respectively. The direct measurements of CO<sub>2</sub> in tissues were performed in studies of brain. It was shown that brain pCO<sub>2</sub> is normally  $\sim$ 5%, yet even when the brain becomes hypoxic, the pCO<sub>2</sub> remains unchanged (25, 26). These data suggest, in the absence of direct measurements in spleen, that pCO<sub>2</sub> is maintained at relatively constant levels in tissues in vivo.

Our data also point to the need in further evaluation of cell culture of hematopoietic stem cells and committed progenitors (52). Indeed, the demonstration of differential effects of hypoxia on  $\gamma\delta$  vs  $\alpha\beta$  T cells (data not shown) suggests that similar lineage-specific effects could be taking place during the expansion of marrow-repopulating progenitors in bone marrow.

Taken together, these observations may help in designing experiments to test the model of in vivo T cell expansion where more CD8<sup>+</sup> CTL are formed in vivo in more vascularized tissue compartments with a sufficient network of capillaries and oxygen supply, while fewer CD8<sup>+</sup> CTL but more potent CD8<sup>+</sup> killers would accumulate in hypoxic areas.

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