Immunology Letters 167 (2015) 72–86

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Effects of extracellular pH and hypoxia on the function and development of antigen-specific cytotoxic T lymphocytes

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ARTICLE INFO

Article history:
Received 21 January 2015
Received in revised form 29 June 2015
Accepted 10 July 2015
Available online 21 July 2015

Keywords:
Cytotoxic T lymphocytes
Hypoxia
Extracellular pH
Low pH

ABSTRACT

The major effector cells for cellular adaptive immunity are $CD8^+$ cytotoxic T lymphocytes (CTLs), which can recognize and kill virus-infected cells and tumor cells. Although CTLs exhibit strong cytolytic activity against target cells *in vitro*, a number of studies have demonstrated that their function is often impaired within tumors. Nevertheless, CTLs can regain their cytotoxic ability after escaping from the tumor environment, suggesting that the milieu created by tumors may affect the function of CTLs.

As for the tumor environment, the patho-physiological situation present *in vivo* has been shown to differ from *in vitro* experimental conditions. In particular, low pH and hypoxia are the most important microenvironmental factors within growing tumors. In the present study, to determine the effect of these factors on CTL function *in vivo*, we examined the cytolytic activity of CTLs against their targets using murine CTL lines and the induction of these cells from memory cells under low pH or hypoxic conditions using antigen-primed spleen cells. The results indicated that both cytotoxic activity and the induction of functional CTLs were markedly inhibited under low pH. In contrast, in hypoxic conditions, although cytotoxic activity was almost unchanged, the induction of CTLs *in vitro* showed a slight enhancement, which was completely abrogated in low pH conditions.

Therefore, antigen-specific CTL functions may be more vulnerable to low pH than to the oxygen concentration *in vivo*. The findings shown here provide new therapeutic approaches for controlling tumor growth by retaining CTL cytotoxicity through the maintenance of higher pH conditions.

1. Introduction

The major effector cells involved in cellular adaptive immunity are CD8-expressing cytotoxic T lymphocytes (CTLs), which can recognize and directly eliminate virus-infected cells and tumor cells. Therefore, these antigen-specific $CD8^+$ CTLs have been widely accepted to be a central player in preventing the spread of viruses and the progression of tumors. Although CTLs exhibit strong cytolytic activity against tumor cells *in vitro*, a number of studies have shown that the CTL activity within tumors is often impaired by various mechanisms. Nevertheless, CTLs can regain their cytotoxic activity after escaping from the tumor environment, suggesting that the milieu created by growing tumors may influence the cytotoxicity of $CD8^+$ CTLs.

The patho-physiological conditions in tumors *in vivo* have been shown to markedly differ from *in vitro* experimental conditions. For example, the physiological oxygen concentration in various tissues *in vivo* was found to be significantly lower than the oxygen concentration in the air because of insufficient oxygen supply, a state known as hypoxia [1]. The atmospheric pressure of oxygen (pO_2) is approximately 21.2 kPa (approximately 21% of total atmospheric pressure), whereas, pO_2 in the thymus and spleen are 0–2.3 kPa (0–2.3%) and 0.5–4.5 kPa (0.5–4.5%), respectively [1]. In the skin epidermis [2] and the hematopoietic stem cell niche [3,4], pO_2 is often less than 1 kPa (<1%). Furthermore, hypoxia has been identified as an important by-product of a wide range of pathological states such as solid tumors [5,6], colic lesions [7], wound healing [8], and ischemic injury [9].

Abbreviations: CFSE, 5-(and 6-)carboxyfluorescein diacetate succinimydylester; CTLs, cytotoxic T lymphocytes; CTM, complete T-cell medium; GrB, granzyme B; IFN-γ, interferon-γ; mAbs, monoclonal antibodies; pO_2, partial pressure of oxygen; PBS, phosphate-buffered saline; Tg, transgenic mice; vSC25, recombinant vaccinia virus expressing HIV-1 IIIB gp160 genes.

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http://dx.doi.org/10.1016/j.imlet.2015.07.003

In the early 1920s, Warburg demonstrated that tumor cells used glycolysis rather than oxidative phosphorylation to acquire energy, even in the presence of oxygen [10,11]. Hypoxia in the tumor environment has been shown to increase the expression of many genes, including those encoding glycolysis-associated proteins and enzymes. Accordingly, tumor cells produce and release large amounts of lactic acid, which creates an acidic microenvironment [12,13]. Therefore, the interstitial fluid of tumors and abscesses has a pH less than 6.0 that averages 0.2–0.6 units lower than the mean extracellular pH of normal tissues [14,15]. Therefore, hypoxia and acidic conditions are both important factors that create microenvironmental features, especially within tumors.

Several studies have demonstrated that the function of immune cells, including CTLs, depends on patho-physiological conditions. Redegeld et al. and Mendler et al. reported that the cytotoxic activity of CTLs against target cells was decreased in an acidic environment using murine and human CTL lines [16,17]. Fischer et al. also detected an increase in serum lactate levels in one-third of 140 cancer patients with high tumor burden [12]. These authors also showed that the proliferation of and cytokine production by CTLs stimulated with PMA/ionomycin were significantly impaired by the addition of 20 mM lactic acid to the culture medium [12]. In contrast, Caldwell et al. reported that although the differentiation of $CD8^+$ CTLs in 2.5% O_2 was slower than that in atmospheric O_2 concentration, cytolytic activity against target cells was stronger than in atmospheric O_2 concentration [18]. However, the effects of hypoxia or an acidic milieu on immune cell function have not yet been elucidated in detail.

In the present study, to analyze the effects of these environmental factors on CTL function, we examined the cytolytic activity of CTLs against target cells and the induction of CTLs from memory cells under low pH or hypoxic conditions using murine antigen-specific CTL lines and antigen-primed spleen cells, respectively. The results obtained here indicated that cytotoxic activity against target cells and the induction of functional CTLs *in vitro* were both inhibited in low pH medium. In contrast, in hypoxic conditions, cytolytic activity against target cells was not changed, and the induction of CTLs *in vitro* showed a slight enhancement. However, this enhancing effect on CTL induction by hypoxia was impaired under both low pH and low oxygen conditions.

Therefore, antigen-specific CTL functions may be more vulnerable to low pH than to oxygen concentration *in vivo*. The findings shown here provide new therapeutic approaches for controlling tumors by maintaining CTL activity as a result of retaining a physiological pH even under pathological conditions.

2. Materials and methods

2.1. Mice

Female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan Inc. (Tokyo, Japan). Transgenic mice (Tg-RT-1) expressing the TCR α and β chain (Vα 42H11 and Vβ8.1) genes of the murine CTL clone RT-1 [19] specific to P18IIIB (RIQRGPGRAFVTIGK) restricted by a class I MHC molecule [20,21] in the genetic background of BALB/c (H-$2D^d$) were established and bred in our colony [22,23]. The F1 mice of Tg-RT-1 and BALB/c mice were used in these experiments. All mice used in this study were maintained in a specific pathogen-free micro-isolator environment. All experiments were performed according to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Recombinant vaccinia virus

vSC25 (recombinant vaccinia virus expressing the HIV-1IIIB gp160 envelope glycoprotein), which has been previously described [20], was used to immunize mice to induce envelope-specific CTLs. Mice were intravenously (i.v.) inoculated with 1×10^7 PFU/mouse of vSC25 for infection experiments.

2.3. Synthetic peptides and reagents

The peptides used in this study were all purchased from TaKaRa Bio Co. (Tokyo, Japan). The peptide P18IIIB (aa: RIQRGPGRAFVTIGK) represents the immunodominant CTL epitope presented by the murine class I MHC molecule H-$2D^d$ in the HIV-1 IIIB gp160 envelope glycoprotein [20]. The peptide OVA-8 (aa: SIINFEKL) represents the immunodominant CTL epitope of ovalbumin.

Recombinant HIV-1 IIIB envelope glycoprotein gp120 (Baculovirus) was purchased from Immuno Diagnostics, Inc. (Woburn, MA, USA). The AbISCO adjuvant (AbISCO-100) was purchased from ISCONOVA AB (Uppsala, Sweden). L-(+)-Lactic acid solution (30% in H_2O) was purchased from Sigma–Aldrich (St. Louis, MO). A Cell Trace TM CFSE cell proliferation kit (fluorescein-based dye 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester; CFSE) was purchased from Life Technologies Corporation (Carlsbad, CA, USA) and dissolved in dimethylsulfoxide at a concentration of 5 mM according to the manufacturer's instructions.

2.4. Generation of milieu conditions

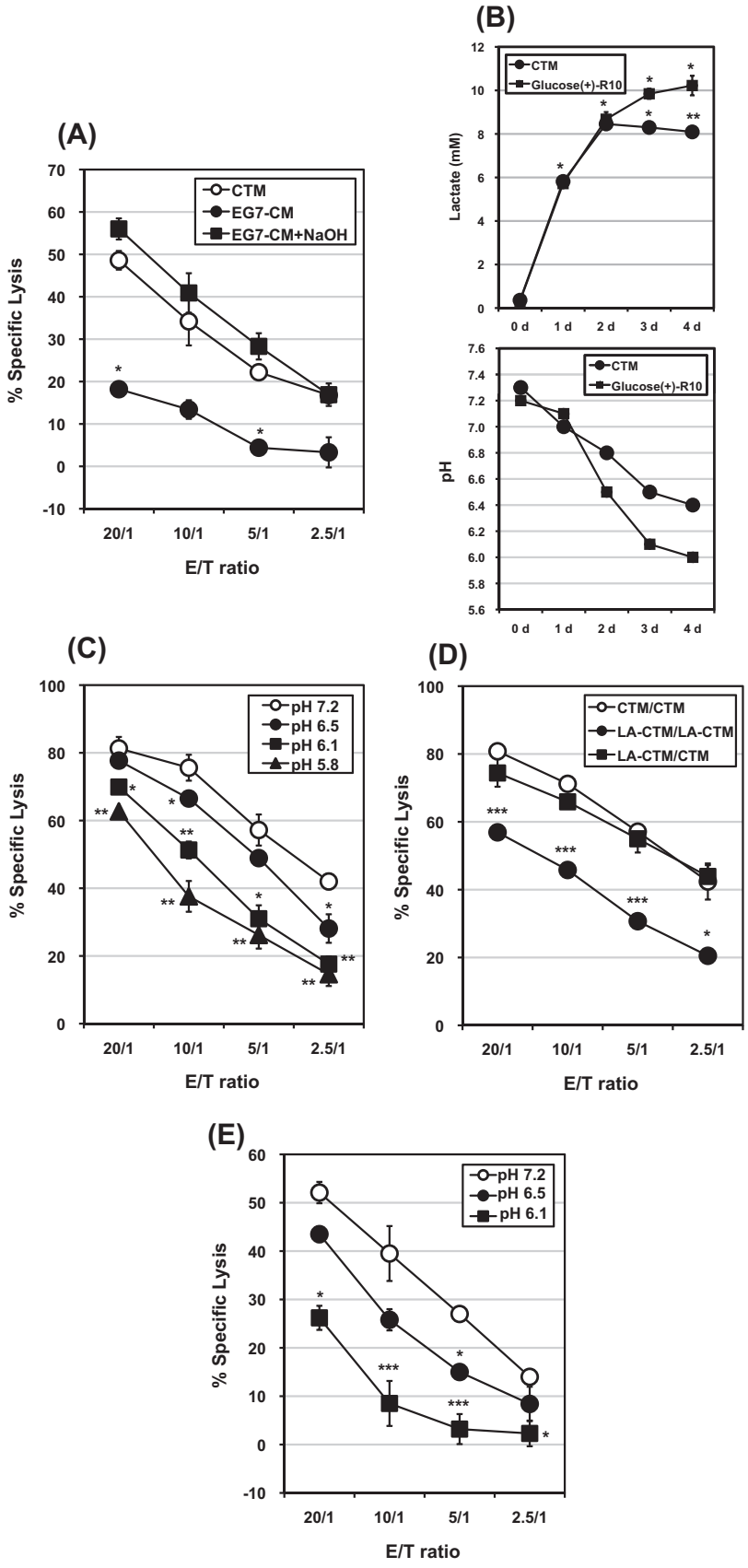
The normal CTL milieu used in this study was complete T-cell medium (CTM) (RPMI1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES, 100 μM non-essential amino acids, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol) [20]. The pH of this medium was generally 7.2 ± 0.1, and the medium was used until 2 days after preparation. The lactic acidosis milieu was created by directly adding lactic acid solution to CTM and was adjusted to an objective pH immediately before usage.

2.5. Generation of hypoxic conditions

The BIONIX-1 hypoxic cell culture kit (Sugiyamagen, Tokyo, Japan) was used to generate hypoxic conditions [24,25]. This system comprises an Anaero Pack-Anaero 5% (oxygen absorber; Mitsubishi Gas Chemical, Tokyo, Japan), OXY-M oxygen monitor (JIKCO, Tokyo, Japan), Anaero Pouch (Mitsubishi Gas Chemical), and plastic clips to seal the pouch. To reduce the pO_2 of the air, plastic plates containing the cells, oxygen absorber, and oxygen monitor were placed in the pouch, and the open side was sealed with a clip and left until the desired pO_2 in the pouch was reached. At the desired pO_2, the pouch was further sealed between the culture plates and the oxygen absorber using another clip to stop the absorption of oxygen. Immediately before separation by the clip, the air in the plates was replaced with the air in the pouch by opening the lid of the plates to accelerate equilibration. According to the manufacturer's instructions, this kit creates conditions of low oxygen concentration and approximately 5% CO_2 within 1 h.

2.6. Cell lines and in vitro CTL induction

EL4 cells (mouse thymoma cell line; H-2^b) and P815 cells (DBA/2-derived mastocytoma cell line; H-2^d) were maintained in CTM. The EG7-OVA transfectant [26] was maintained in CTM


(A)
% Specific Lysis
CTM
EG7-CM
EG7-CM+NaOH
E/T ratio
20/1
10/1
5/1
2.5/1
(B)
Lactate (mM)
CTM
Glucose(+)-R10
pH
0 d
1 d
2 d
3 d
4 d
(C)
pH 7.2
pH 6.5
pH 6.1
pH 5.8
(D)
CTM/CTM
LA-CTM/LA-CTM
LA-CTM/CTM
(E)
pH 7.2
pH 6.5
pH 6.1

with 400 μg/ml Geneticin (Sigma–Aldrich). The BALB/c.3T3 (H-2^d) fibroblast transfectant expressing HIV-1 gp160 of the IIIB isolate (f15-12) [20] and the control transfectant containing selectable marker genes (Neo) were maintained in CTM containing 150 μg/ml Geneticin.

To induce the ovalbumin (OVA)-specific CTL line (LINE-OVA), 5×10^6 spleen cells from C57BL/6 mice previously immunized with peptide OVA-p-pulsed bone marrow-derived dendritic cells were re-stimulated *in vitro* with mitomycin C (MMC)-treated EG7-OVA cells in 24-well culture plates containing 1.5 ml of CTM for 5 days at 37 °C. A long-term OVA-specific CTL line (LINE-OVA) was generated by bi-weekly stimulation with MMC-treated EG7-OVA cells and 10% Rat Con A supernatant-containing medium (Rat T-STIM; Collaborative Biomedical Products, Bedford, MA). This CTL line contained approximately 95% OVA-specific cells, as determined by H-2K^b/OVA-tetramer staining.

To induce HIV-IIIB P18-I10-specific CTLs, 5×10^6 spleen cells from mice previously immunized with 1×10^7 PFU/mouse of vSC25 or 5 μg of recombinant HIV-1 IIIB envelope glycoprotein gp120 mixed with the AbISCO adjuvant were re-stimulated *in vitro* with 1×10^5 MMC-treated f15-12 cells in 24-well culture plates for 5 days at 37 °C. The HIV-IIIB P18IIIB-specific CTL line LINE-IIIB was generated as described previously [20], and this line contained approximately 85% epitope P18IIIB-specific cells, as determined by H-2D^d/P18IIIB-tetramer staining.

2.7. Flow cytometric analysis

Flow cytometric analysis was performed to determine the surface molecule expression of cells using a FACS Canto II six-color cytometer (BD Bioscience) with FlowJo software (Tree Star, Inc., Ashland, OR, USA). Half a million cells were pelleted and resuspended in 100 μl PBS containing 1% FCS and 0.1% sodium azide. FITC- or PE-labeled monoclonal antibodies (mAbs) were added to the pellet and incubated for 30 min at 4 °C. Cells were then washed twice and resuspended in PBS. The antibodies FITC-labeled anti-mouse CD3ϵ (clone 145-2C11) (BioLegend, Inc., San Diego, CA), FITC-labeled anti-mouse CD8β (clone VTS156.7.7; BioLegend, Inc.), FITC-labeled anti-mouse CD25 (IL-2Rα chain p55, clone 7D4; BD Biosciences Pharmingen, San Jose, CA), FITC-labeled anti-mouse CD69 (clone H1.2F3; eBioscience Inc., San Diego, CA), FITC-labeled anti-mouse T cell-receptor β chain (clone H57-597; BioLegend, Inc.), FITC-labeled anti-mouse H-2K^b/H-2D^b (clone 28-8-6; BioLegend, Inc.), FITC-labeled anti-mouse H-2D^d (clone 34-2-12; BD Biosciences Pharmingen), PE-labeled anti-mouse Vβ8.1, 8.2 TCR (MR-5-2; BD Biosciences Pharmingen), PE-labeled anti-mouse CD274 (PD-L1) (clone 10F.9G2; BioLegend, Inc.), PE-labeled anti-mouse CD273 (PD-L2) (clone TY25; BioLegend, Inc.), PE-labeled anti-mouse CD279 (PD-1) (clone J43; BD Biosciences Pharmingen) were used. T-Select H-2K^{b}OVA Tetramer-SIINFEKL-PE or T-Select H-2D^d HIV Tetramer (Medical & Biological laboratories Co., Ltd. Nagoya, Japan) was used to stain antigen-specific CTLs.

2.8. Cytotoxicity assay

The cytolytic activity of CTLs was measured as described previously [20] using a standard 4 h ^{51}Cr-release assay with various ^{51}Cr-labeled targets as indicated in the figure legends.

2.9. Cell proliferation assay

Cell proliferation was measured as previously described [27] using CFSE. Splenic CD8$^+$ T cells were labeled with 2.5 μM CFSE and incubated with MMC-treated f15-12 cells in 200 μl of various culture media in a 96-well flat-bottom plate for 4–5 days. After incubation, re-stimulated cells were harvested, and the CFSE concentration of proliferating cells was analyzed by flow cytometry.

2.10. Cytokine detection

The amount of mouse IFN-γ in the culture supernatants was determined with an ELISA Kit (ELISA MAX^{TM} Standard Mouse IFN-γ; BioLegend, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

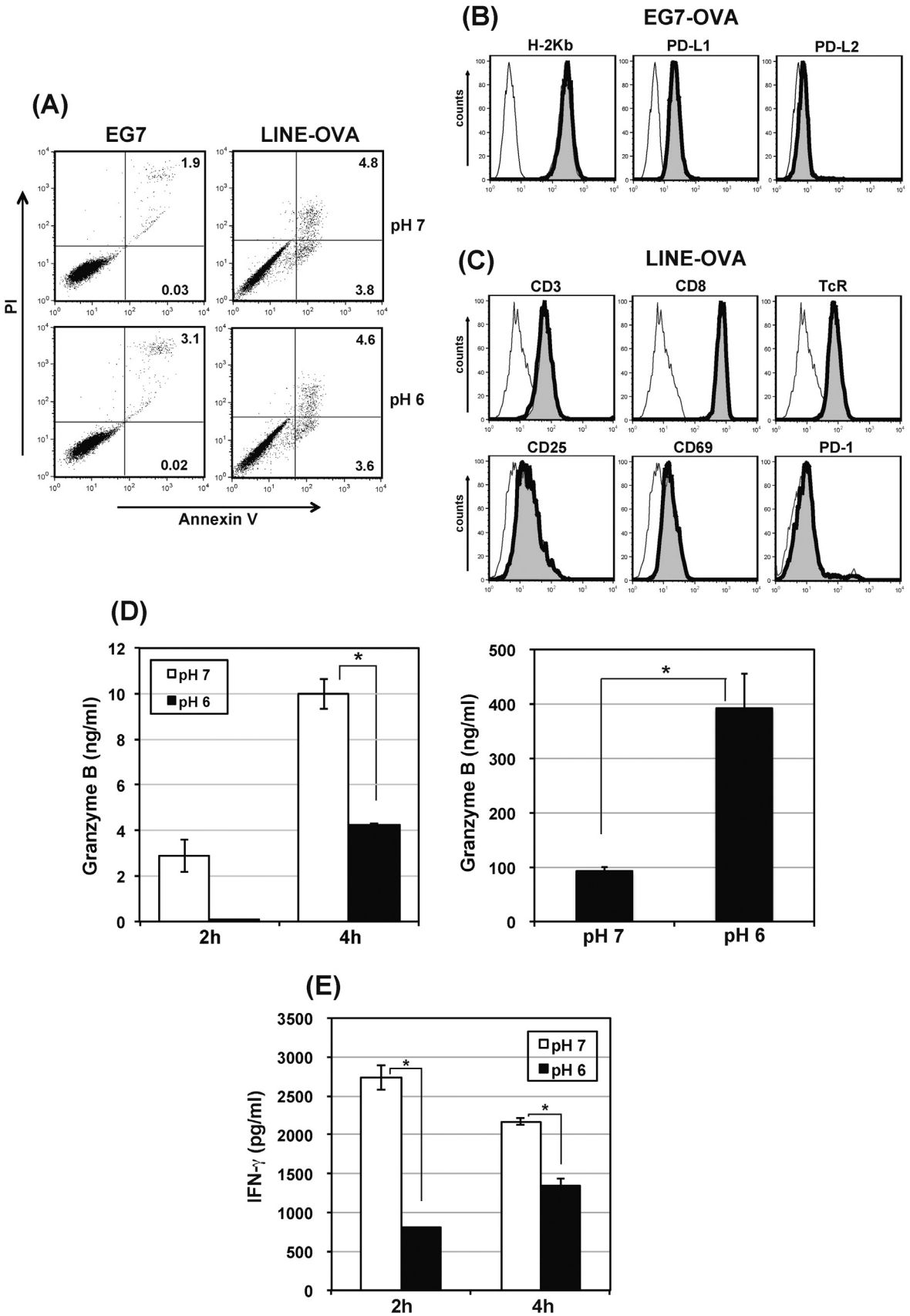
2.11. Colorimetric lactate assay

A total of 4×10^5 of EG7-OVA cells was incubated in 24-well culture plates in CTM or high-glucose R-10 medium (RPMI1640 medium containing 10% heat-inactivated FCS, 10 mM HEPES, 25 mM D-glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol) for 4 days at 37 °C. The culture supernatant was collected, and the lactate content was measured using a Lactate Colorimetric Assay Kit II (BioVision Research Products, Mountain View, CA) according to the manufacturer's instructions.

2.12. Colorimetric granzyme B assay

For measurements of granzyme B (GrB) release, 2×10^6 line OVA cells were added to 5×10^5 EG7-OVA in 200 μl CTM. The cells were incubated for 4 h at 37 °C. Thereafter, cells were centrifuged, and the supernatants were aspirated and stored at −20 °C. For measurements of intracellular GrB, 2×10^6 line OVA cells were incubated with 5×10^5 EG7-OVA for 4 h at 37 °C. Cells were then harvested and incubated with 0.5% NP-40 in PBS for 30 min at 4 °C to prepare cell lysates. GrB activity was measured by the GrB-specific hydrolysis of substrates. A total of 180 μl of the reaction mixture (200 μM Boc-Ala-Ala-Asp thiobenzyl ester (BLT; Calbiochem, San Diego, CA) in a reaction buffer containing 50 mM HEPES, pH 7.5; 10% sucrose; 0.05% 3-((3-cholamidopropyl) dimethylammonio) propanesulfonate (CHAPS); and 5 mM dithiothreitol (DTT)) was added to 20 μl of cell lysate. The absorbance at 405 nm was measured after incubation for 24 h at 37 °C. The concentrations of samples were determined using a standard curve with recombinant mouse GrB (Sigma–Aldrich, St. Louis, MO).

Fig. 1. Effect of medium pH on the cytotoxic activity of antigen-specific CTLs. (A) LINE-OVA cells were incubated with ^{51}Cr-labeled EG7-OVA target cells in normal CTM (pH 7.2, open circles), the 3-day-culture supernatant of EG7-OVA cells (pH 6.2, closed circles), or culture supernatant buffered with 1 N sodium hydroxide solution (pH 7.3, closed squares) for 4 h at the indicated effector-target ratios for a standard ^{51}Cr-release assay. *$p < 0.05$ compared with cultures in CTM (open circles). (B) EG7-OVA cells (4×10^5 cells/well) were incubated in CTM or high-glucose R-10 medium for 4 days at 37 °C. The pH value and lactate content of the culture supernatant were measured every day. *$p < 0.05$, **$p < 0.01$ compared with medium alone. (C) LINE-OVA cells were incubated with ^{51}Cr-labeled EG7-OVA target cells in CTM with various pH values (open circles, pH 7.2; closed circles, pH 6.5; closed squares, pH 6.1; closed triangles; pH 5.8) for 4 h at the indicated effector-target ratios. *$p < 0.05$, **$p < 0.01$ compared with cultures in CTM (pH 7.2; open circles). (D) LINE-OVA cells were incubated in normal CTM (pH 7) or acidic CTM (LA-CTM, pH 6) for 4 h at 37 °C. Cells were then washed once and incubated further with ^{51}Cr-labeled EG7-OVA cells in normal CTM or acidic CTM for 4 h, followed by ^{51}Cr-release assay. *$p < 0.05$, ***$p < 0.005$ compared with cultures in CTM (open circles). (E) LINE-IIIB cells were incubated with ^{51}Cr-labeled f15-12 target cells in CTM at different pH values (open circles, pH 7.2; closed circles, pH 6.5; closed squares, pH 6.1) as in (C). *$p < 0.05$, ***$p < 0.005$ compared with cultures in CTM (pH 7.2; open circles). The results shown are representative of three independent experiments.


(A)
EG7
LINE-OVA
1.9
4.8
pH 7
0.03
3.8
3.1
4.6
pH 6
0.02
3.6
PI
Annexin V
(B)
EG7-OVA
H-2Kb
PD-L1
PD-L2
counts
(C)
LINE-OVA
CD3
CD8
TcR
CD25
CD69
PD-1
counts
(D)
Granzyme B (ng/ml)
pH 7
pH 6
2h
4h
Granzyme B (ng/ml)
pH 7
pH 6
(E)
IFN-γ (pg/ml)
pH 7
pH 6
2h
4h

2.13. Detection of apoptosis

CTL apoptosis was detected with the Annexin V-FITC Apoptosis Detection Kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's instructions.

2.14. Immunohistological staining of HIF-1α

Cultured cells were plated on micro-slide glass and fixed with 4% paraformaldehyde (PFA)-PBS for 30 min at room temperature. After washing with PBS, blocking solution (PBS containing 1% BSA and 0.3% normal goat serum) was added and incubated for 1 h. After washing with PBS, rabbit polyclonal anti-HIF-1α antibody (100×) (Novus Biologicals, Littleton, CO, USA) was added, and the mixture was incubated for an additional 1 h at room temperature. Peroxidase-conjugated goat anti-rabbit antibody was then added and incubated for 1 h. After washing with PBS, diaminobenzidine (DAB; Vector Laboratories) was added, and the reaction was stopped with distilled water.

2.15. Statistical analysis

Data were analyzed using Student's *t*-test and are presented as the mean value ± SD. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. Effects of culture medium pH on the cytotoxic activity of the OVA-specific CD8$^+$ CTL line

Previous studies reported that tumor cells have the ability to not only secrete lactic acid to establish acidic conditions with low pH but also create a hypoxic environment *in vivo* [28,29], which may inhibit the cytotoxicity of CD8$^+$ CTLs. To examine CTL-based immune responses under *in vivo*-like conditions, we herein investigated the effects of low pH and hypoxia on CTL activity using murine antigen-specific CTL lines.

Cytotoxicity was significantly decreased in the OVA-specific CD8$^+$ CTL line when incubated in the supernatant of 3-day-cultured EG7-OVA with OVA-antigen-expressing syngeneic thymoma cells (EG7-OVA) for 4 h at various E/T ratios compared with incubation in normal culture medium (CTM). This reduction was almost completely abrogated by the addition of 1 N sodium hydroxide solution, which raised the acidic low pH to a normal pH (7.3) (Fig. 1A). The pH of the culture medium gradually became acidic, and the concentration of lactic acid in the medium increased. When EG7-OVA cells were cultured in high-glucose R-10 medium for 4 days, a larger amount of lactic acid was produced, and the pH value was closer to 6.0 (Fig. 1B). Several studies reported that the cytolytic activity of CD8$^+$ CTLs was lower when the cells were incubated in an acidic culture medium with target cells than when they were incubated in neutral pH medium [12,16,17]. Thus, we determined whether CTL activity was inhibited by the extracellular acidic conditions used in our experimental system. When the OVA-specific CD8$^+$ CTL line LINE-OVA was incubated with EG7-OVA cells in culture media with various pH values, cytotoxic activity decreased in a pH-dependent manner (Fig. 1C). Specific lysis by CTLs was approximately 50% lower in medium with pH 6.1 than in normal CTM at E/T ratios of 5/1 and 2.5/1. Furthermore, when LINE-OVA cells were preincubated in acidic medium for 4 h and then further incubated with EG7-OVA for an additional 4 h in normal CTM, cytotoxic activity was almost completely restored (Fig. 1D). These results clearly demonstrated that the observed reduction in CTL activity in the acidic environment was a transient phenomenon that was not based on cell death or the permanent inhibition of activity. The weakened CTL activity in acidic medium was also observed in another distinct HIV-1 *env* gp160-specific CTL line, LINE-IIIB (Fig. 1E).

Taken together, these results demonstrate that the inhibitory effects of low extracellular pH on the function of antigen-specific CTLs could also be induced in our experimental system.

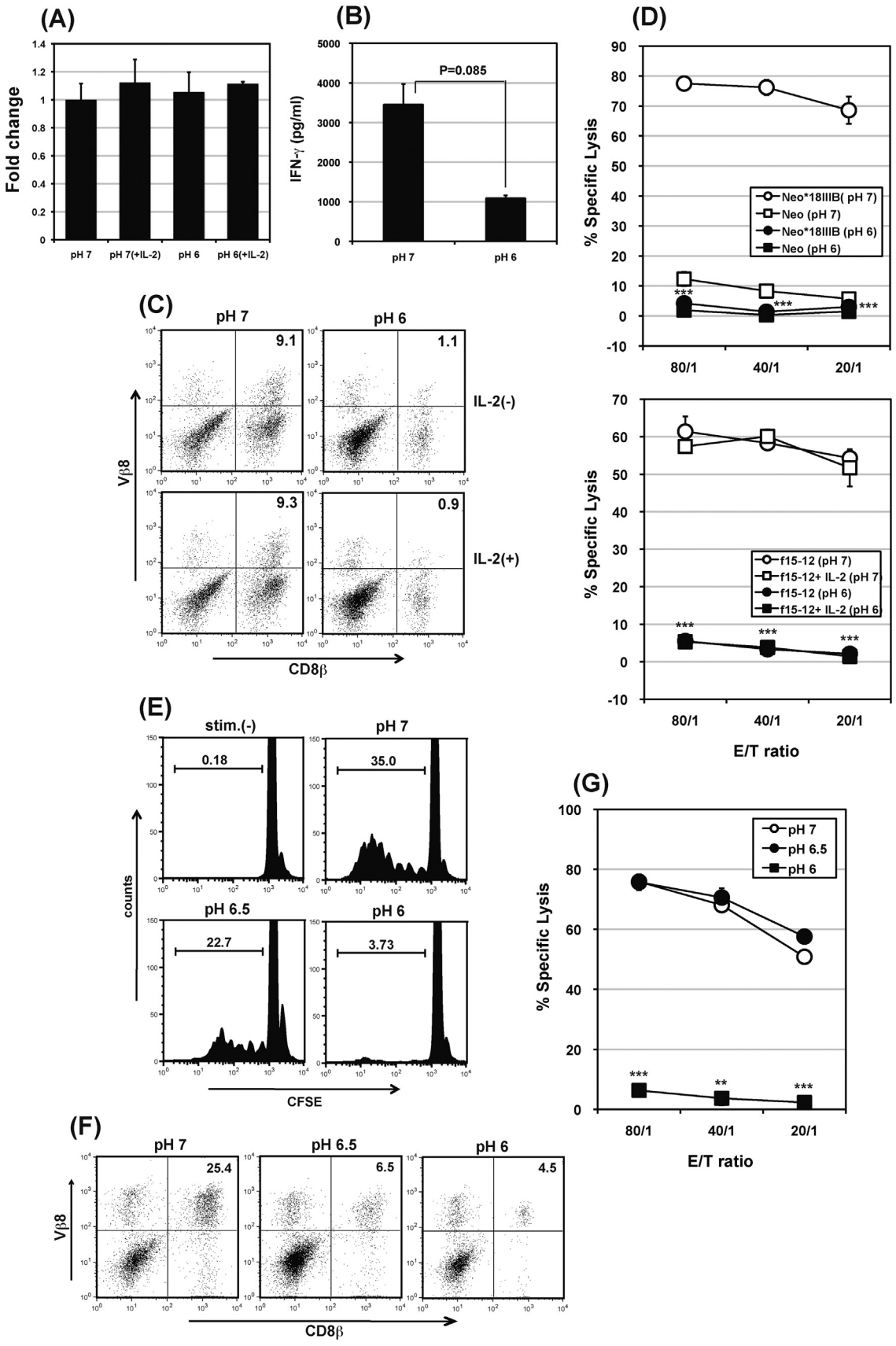
3.2. Mechanism by which acidic conditions inhibit cytolytic activity of CTLs

To analyze the mechanism underlying the diminished cytotoxicity of CTLs in acidic medium, we first analyzed whether apoptosis or necrosis occurred in CTLs and target cells under conditions of varying pH. As shown in Fig. 2A, although a small number of Annexin V-stained early apoptotic cells and propidium iodide (PI)-stained late apoptotic or necrotic cells were present in both LINE-OVA and EG7 target cells, no significant difference was observed at these two pH conditions. Moreover, LINE-OVA and EG7-OVA cells were incubated in CTM with different pH values and were stained with mAbs against cell surface molecules known to be involved in the lysis of target cells. However, no significant differences for any of these antigen profiles were detected for either LINE-OVA or EG7 cells (Fig. 2B and C). We next examined the release of the effector molecule GrB from CTLs in culture with target cells under different pH conditions. As shown in Fig. 2D, when LINE-OVA cells were incubated with EG7 in CTM with pH 6, GrB content in the supernatant significantly decreased compared with that in CTM with pH 7. In contrast, the intracellular content of GrB was enhanced after incubation in CTM with pH 6. Similarly, IFN-γ content in the supernatant also decreased in CTM with pH 6 (Fig. 2E). These results clearly indicated that the release of important effector molecules for the cytolysis of target cells was inhibited in an acidic environment. Mendler et al. previously reported that degranulation and cytokine production were inhibited in an acidic environment using human alloantigen-specific CTLs [17], and our results are consistent with theirs.

3.3. Effects of low extracellular pH on the induction of antigen-specific CTLs in vitro

We next examined the effects of culture medium pH on the induction of functional CD8$^+$ CTLs from primed memory T cells *in vitro*. Although OVA-specific CTLs could be induced from mice immunized with OVA-peptide-pulsed bone marrow-derived dendritic cells (DCs), a large number of non-specific CTLs were co-induced in this system. Therefore, to examine the effects of extracellular pH on the induction of antigen-specific CTLs, we examined HIV-1 *env*-specific CTL induction in our system (Supplementary Fig. S1). The spleen cells of mice immunized with the HIV-1 *env* gp120 protein mixed with the AbISCO adjuvant were restimulated with the MMC-treated f15-12 transfectants expressing

Fig. 2. Mechanism by which acidic medium inhibits the cytotoxic activity of CTLs. (A) EG7-OVA cells and LINE-OVA cells were incubated in normal CTM (pH 7) or acidic CTM (pH 6) for 4 h at 37 °C. After incubation, apoptotic and necrotic cells were detected by FITC-labeled Annexin V and propidium iodide staining. (B and C) EG7-OVA cells (B) and LINE-OVA cells (C) were incubated as in (A), and cell surface molecules were analyzed by flow cytometry (thin line, isotype control of cells incubated in normal CTM; grey, cells incubated in normal CTM; thick line, cells incubated in acidic CTM). (D) LINE-OVA cells were incubated with EG7-OVA target cells in CTM with varying pH values for 4 h at 37 °C. Supernatants (left) or the remaining cells (right) were then harvested, and GrB activity was determined as described in the Section 2. (E) LINE-OVA cells were incubated with EG7-OVA target cells in CTM with varying pH values as in (D). Supernatants were isolated, and IFN-γ content was determined by ELISA. The results shown are representative of three independent experiments.


(A)
Fold change
pH 7
pH 7(+IL-2)
pH 6
pH 6(+IL-2)
(B)
IFN-γ (pg/ml)
P=0.085
pH 7
pH 6
(C)
pH 7
pH 6
9.1
1.1
9.3
0.9
IL-2(-)
IL-2(+)
Vβ8
CD8β
(D)
% Specific Lysis
Neo*18IIIB(pH 7)
Neo (pH 7)
Neo*18IIIB (pH 6)
Neo (pH 6)
80/1
40/1
20/1
f15-12 (pH 7)
f15-12+ IL-2 (pH 7
f15-12 (pH 6)
f15-12+ IL-2 (pH 6
E/T ratio
(E)
stim.(-)
pH 7
0.18
35.0
pH 6.5
pH 6
22.7
3.73
counts
CFSE
(F)
pH 7
pH 6.5
pH 6
25.4
6.5
4.5
Vβ8
CD8β
(G)
pH 7
pH 6.5
pH 6
% Specific Lysis
E/T ratio

HIV-1 *env* gp160 of the IIIB isolate in CTM at various pH values for 5 days. Because the pH of the medium in the culture plates slightly shifted to a higher pH in the CO_2 incubator during the 5-day incubation period, we expressed the medium pH as the start and end values in the. Significant differences were not observed in the number of viable cells after 5 days of culture in medium with a pH of approximately 6 compared with cells in normal CTM with a pH of 7 (Fig. 3A). However, the percentage of CD8β$^+$, Vβ8$^+$, antigen-specific CTLs present and the interferon-γ (IFN-γ) content in the culture supernatant after a 5-day culture in acidic medium both decreased (Fig. 3B and C). Moreover, the cytolytic activity of cells generated from spleen cells in acidic medium was also strongly inhibited (Fig. 3D). To determine whether this decline in CTL induction was due to the lack of interleukin-2 (IL-2), which is required for efficient induction, we exogenously added IL-2 to the culture Plates 1 day after the addition of stimulator cells. However, as shown in Fig. 3C and D, functional CTLs were not induced in acidic medium even in the presence of IL-2. These results indicated that the acidification of CTM directly inhibited the induction of antigen-specific CTLs *in vitro*.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2015.07.003

In general, 5–6 days are needed to induce functional antigen-specific CTLs from primed spleen cells *in vitro*. However, as demonstrated previously, epitope-specific CTLs were rapidly induced within 3–4 days [23] when spleen cells from Tg-RT-1 mice expressing the TCR αβ genes of CTL clone RT-1 [22] were re-stimulated *in vitro*. Therefore, to maintain the pH of the medium during the induction period and to examine the pH dependence of CTL induction in more detail, we used Tg-RT-1 instead of antigen-primed mice. As shown in Fig. 3E, when CFSE-labeled splenic CD8$^+$ T cells from Tg-RT-1 mice were co-cultured with f15-12 cells for 4 days in CTM at various pH conditions, cell proliferation was inhibited in a pH-dependent manner. Similarly, the percentage of CD8β$^+$, Vβ8$^+$, antigen-specific CTLs after the 4-day incubation also decreased in acidic conditions (Fig. 3F). However, the cytotoxic activity of the generated cells in the medium with a pH 6.5 was similar to that of cells generated in normal CTM with a pH 7 (Fig. 3G). Taken together, these results clearly indicate that the induction of antigen-specific CTLs *in vitro* was inhibited in the acidic environment.

3.4. *Return to neutral pH during* in vitro *re-stimulation of CTLs completely reversed the inhibitory effects induced by a low pH*

We investigated whether the inhibitory effects of extracellular acidic conditions on CTL induction were reversed by neutralization of the acidic medium. When we cultured primed spleen cells with MMC-treated f15-12 stimulator cells in acidic medium buffered with sodium hydroxide solution for 5 days, the percentage of CD8β$^+$, Vβ8$^+$, antigen-specific CTLs, the cytotoxic activity of the generated cells and the IFN-γ content in the culture supernatant almost completely recovered (Fig. 4B–D). No significant differences were observed in the number of viable cells after the 5-day culture among cells cultured in normal CTM (pH 7), acidic CTM (pH 6), and acidic CTM buffered with sodium hydroxide (pH 7) (Fig. 4A).

3.5. *Effects of hypoxia on the cytotoxic activity of LINE-OVA cells*

In addition to low pH, the concentration of oxygen is another important *in vivo* microenvironmental factor for various tissues including local sites of inflammation and growing tumors. Therefore, we examined the effects of hypoxia on the function and induction of antigen-specific CTLs using the BIONIX-1 hypoxic cell culture kit system as described in the Section 2. The concentration of oxygen in lymphoid tissues, such as the murine spleen and lymph nodes is between 2 and 5% [1,30]. However, hypoxia inducible factor-1α, the main transcription factor mediating many cellular adaptive responses to hypoxia, was mostly found to be activated at an oxygen concentration of less than 1%. Therefore, we selected two oxygen concentration levels, 2–4% and <1%, in the present study.

LINE-OVA cells were incubated with ^{51}Cr-labeled EG7-OVA target cells at various E/T ratios under an oxygen concentration of 20%, 2–3%, or <1%. As shown in Fig. 5A, no significant differences were observed in cytolytic activity between normoxic (20% O_2) and hypoxic conditions (2–3% O_2, <1% O_2). Similar results were also noted in another CTL line, LINE-IIIB (Fig. 5B). The pH of the medium did not change under normoxic and hypoxic conditions during the 4-h incubation. By determining the uptake of PI, we also confirmed that cell viability after the incubation with target cells under hypoxia was similar to that under normoxia (data not shown).

Because we demonstrated that CTL activity was decreased under acidic conditions, the additive effects of low pH and hypoxia on CTL activity were also examined. LINE-OVA cells were incubated with EG7-OVA in CTM at pH 7 or 6 (LA-CTM) under normoxic or hypoxic conditions for 4 h. The results revealed a slight synergistic effect when CTLs were incubated in medium at pH 6 under hypoxic conditions (2–3% O_2), but no statistical difference was observed (Fig. 5C).

3.6. *Effects of hypoxia on the induction of antigen-specific CTLs* in vitro

We investigated the effects of hypoxia on the induction of functional CD8$^+$ CTLs from antigen-primed memory T cells *in vitro*. According to the manufacturer's instructions, the BIONIX-1 hypoxic cell culture kit creates a low oxygen concentration and approximately 5% CO_2 within 1 h. However, because the open side of the pouch containing the culture plates and oxygen absorber is sealed with a clip in this kit, the concentration of CO_2 could not be modulated during the 4–5-day culture period. Therefore, we first tested whether the pH of the medium was changed during the culture period. As shown in Supplementary Fig. S2, no significant difference

Fig. 3. Effects of medium pH on the induction of antigen-specific CTLs *in vitro* from primed spleen cells. (A) Spleen cells from mice immunized with the HIV-1 env gp120 protein mixed with the AbISCO adjuvant were re-stimulated *in vitro* with MMC-treated f15-12 cells in 24-well culture plates in normal CTM (pH 7.1–7.2) or acidic CTM (pH 6.1–6.7) at 37 °C in the presence or absence of exogenous IL-2. After a 5-day culture, cells were harvested, and viable cells were counted using a hemocytometer. Fold changes were calculated as the ratio of the number of live cells after the 5-day culture under various conditions to the number of live cells after the 5-day culture in CTM with a pH of 7 in the absence of IL-2. (B) The IFN-γ content in 5-day culture supernatants was analyzed by ELISA. (C and D) Primed spleen cells were re-stimulated in vitro with MMC-treated f15-12 cells in CTM with varying pH values for 5 days at 37 °C as in (A). Cells were harvested, and the percentage of CD8β$^+$, TCR-Vβ8$^+$ cells was analyzed by flow cytometry. The cytolytic activity of the generated cells was determined by a ^{51}Cr-release assay in normal CTM (pH 7) using f15-12 cells or epitope peptide P18IIIB-pulsed syngeneic fibroblast cells (Neo*18IIIB) as target cells. $^{***}p < 0.005$ compared with the cytotoxicity of generated cells in CTM with pH 7 (open circles). (E) Splenic CD8$^+$ T cells from Tg-RT-1 mice were labeled with 2.5 μM of CFSE and re-stimulated *in vitro* with MMC-treated f15-12 cells in 96-well round-bottomed culture plates in CTM with various pH values for 4 days at 37 °C. The generated cells were then harvested and analyzed by flow cytometry. (F and G) Spleen cells from Tg-RT-1 mice were re-stimulated *in vitro* with MMC-treated f15-12 cells in 24-well culture plates in normal CTM (pH 7.2–7.0) or acidic CTM (pH 6.5–6.7, 6.1–6.3) for 4 days at 37 °C as in (A). Cells were harvested, and the percentage of CD8β$^+$, TCR-Vβ8$^+$ cells was analyzed by flow cytometry. The cytolytic activity of the generated cells was determined by a ^{51}Cr-release assay in normal CTM (pH 7) using f15-12 cells as target cells. $^{**}p < 0.01$, $^{***}p < 0.005$ compared with the cytotoxicity of generated cells in CTM with pH 7 (open circles). The results shown are representative of three independent experiments.

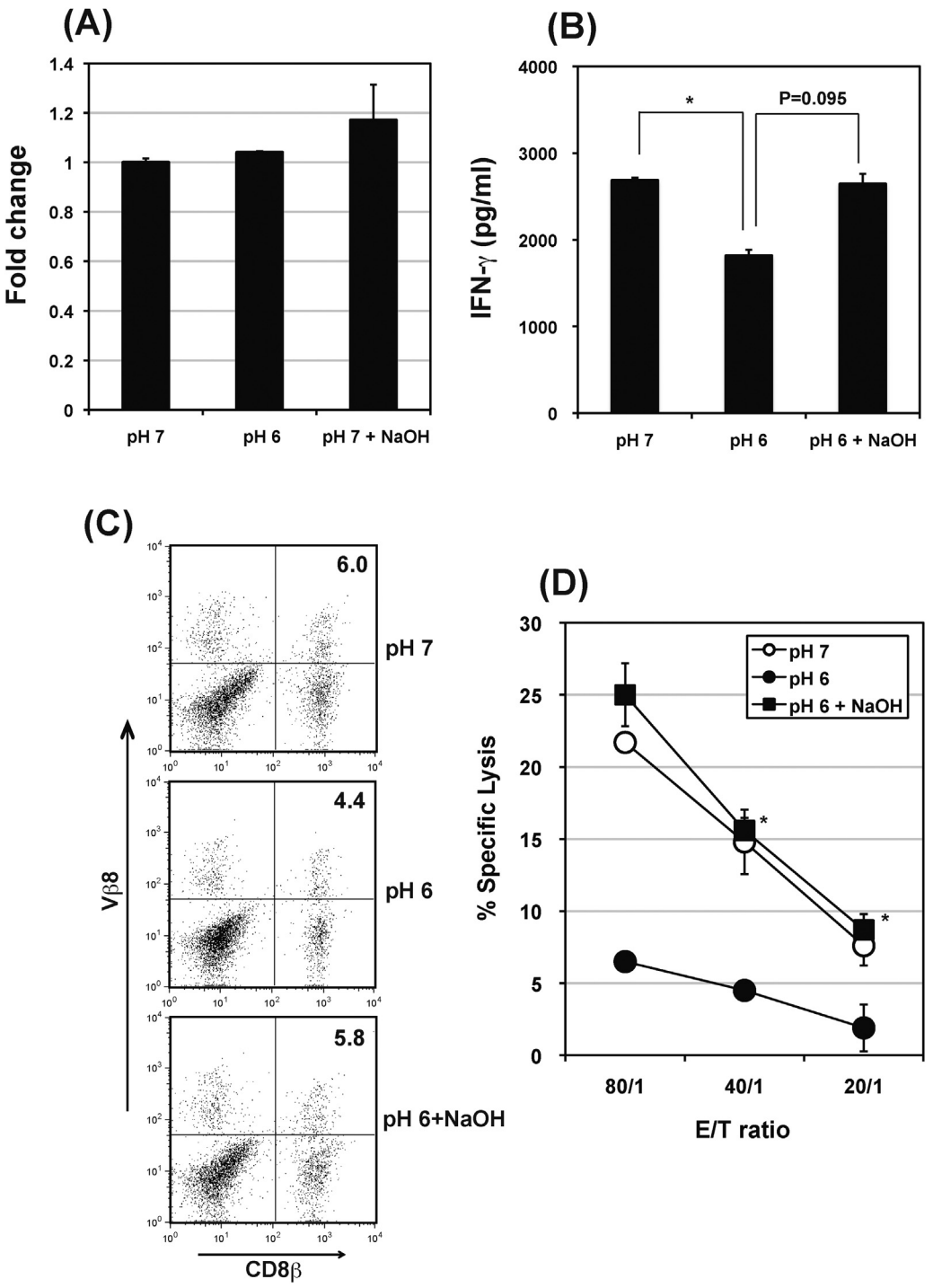


Fig. 4. Return to neutral pH of acidic medium during re-stimulation of CTLs *in vitro* by sodium hydroxide completely reversed the inhibitory effects. (A) Spleen cells from mice immunized with the HIV-1 env gp120 protein mixed with the AbISCO adjuvant were re-stimulated *in vitro* with MMC-treated f15-12 cells in 24-well culture plates in normal CTM (pH 7.0–7.1), acidic CTM (pH 6.0–6.5), or acidic CTM buffered with 2 N sodium hydroxide solution (pH 7.0–7.1) at 37 °C for 5 days. After the 5-day culture, the cells were harvested, and the number of viable cells was counted using a hemocytometer. Fold changes were calculated as the ratio of the number of live cells after the 5-day culture under various conditions to the number of live cells after the 5-day culture in CTM with a pH 7. (B) The IFN-γ content in 5-day culture supernatants was analyzed by ELISA. $^{*}p < 0.05$. (C and D) Primed spleen cells were re-stimulated *in vitro* with MMC-treated f15-12 cells for 5 days at 37 °C as in (A). Cells were harvested, and the percentage of CD8β^{+}, TCR-Vβ8^{+} cells was analyzed by flow cytometry. The cytolytic activity of the generated cells was determined by a ^{51}Cr-release assay in normal CTM (pH 7) using f15-12 cells as target cells. $^{*}p < 0.05$ compared with the cytotoxicity of generated cells in acidic CTM (pH 6; closed circles). The results shown are representative of three independent experiments.

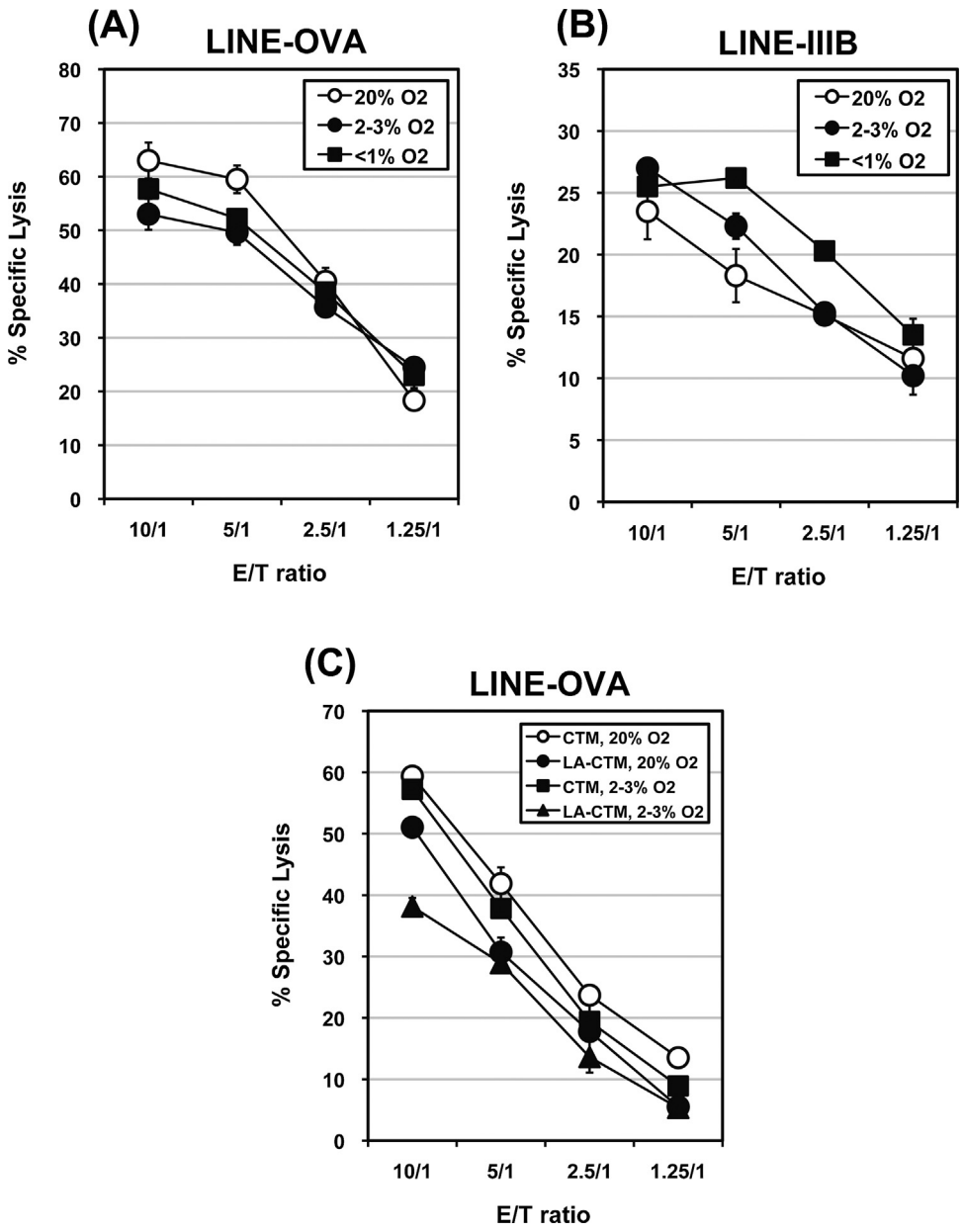


Fig. 5. Effects of hypoxia on the cytotoxic activity of LINE-OVA cells. (A) LINE-OVA cells were incubated with ^{51}Cr-labeled EG7-OVA target cells in normal CTM (pH 7) for 4 h at the indicated effector-target ratios under various oxygen concentrations (open circles, 20% O_2; closed circles, 2–3% O_2; closed squares, <1% O_2) at 37 °C for a standard ^{51}Cr-release assay. (B) LINE-IIIB cells were incubated with ^{51}Cr-labeled f15-12 target cells in CTM (pH 7) under various oxygen concentrations as in (A). (C) LINE-OVA cells were incubated with ^{51}Cr-labeled EG7-OVA target cells in normal CTM (pH 7) or acidic CTM (LA-CTM, pH 6) at the indicated effector-target ratios under an oxygen concentration of 20% or 2–3%, respectively, for a standard ^{51}Cr-release assay. The results shown are representative of three independent experiments.

was observed in the pH of the medium between oxygen concentrations of 20% and 3–4% during the 4-day culture period. Accordingly, we were able to avoid the effects of pH changes in our experiment.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2015.07.003

Spleen cells from mice previously immunized with the HIV-1 *env* gp120 protein mixed with the AbISCO adjuvant or infected with vSC25 were re-stimulated *in vitro* with MMC-treated f15-12 cells for 5 days at 37 °C under normoxic (20%) or hypoxic (3–4% O_2 and <1% O_2) conditions. As shown in Fig. 6A, the number of viable cells after the 5-day culture was slightly higher under hypoxia, especially in the 3–4% O_2 condition, than under normoxia, but the difference was not statistically significant. Cell proliferation; the percentage of CD8β$^+$, Vβ8$^+$, antigen-specific CTLs; and IFN-γ content in the culture supernatant after the 5-day culture were all enhanced under an O_2 concentration of 3–4% and were slightly increased even under <1% O_2 (Fig. 6B–D). Furthermore, the cytotoxic activity of the CTLs generated from spleen cells under hypoxia was also markedly enhanced (Fig. 6E). Similar results were also obtained for spleen cells from Tg-RT1 mice (Fig. 6D and E). These results indicated that a low oxygen concentration, which may reflect an *in vivo* local environment, influenced the induction of antigen-specific CTLs.

3.7. *Synergistic effects of acidosis and hypoxia on the induction of antigen-specific CTLs* in vitro

Helmlinger et al. found an inverse correlation between local pH profiles and corresponding pO_2 profiles but a strong correlation between mean pH and pO_2 profiles in solid tumors [5]. Moreover, Rotstein et al. and Simchowitz et al. reported the additive effects of

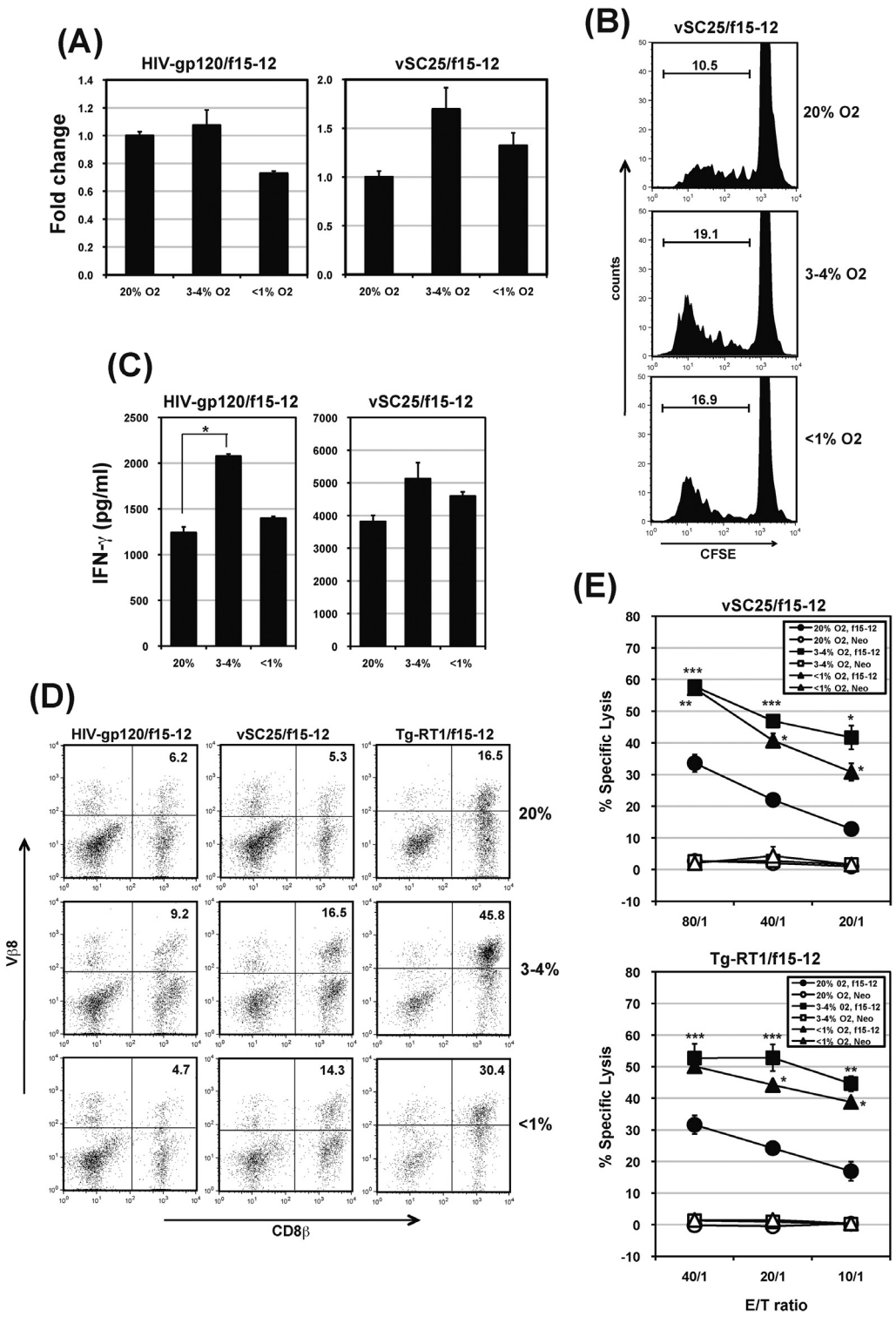


Fig. 6. Effects of hypoxia on the induction of antigen-specific CTLs in vitro from primed spleen cells. (A) Spleen cells from mice previously immunized with 5 μg of recombinant HIV-1 IIIB envelope glycoprotein gp120 mixed with the AbISCO adjuvant or 1×10^7 PFU/mouse of vSC25 were re-stimulated *in vitro* with MMC-treated f15-12 cells in 24-well culture plates under various oxygen concentrations (20%, 3–4%, <1%) at 37 °C. After a 5-day culture, cells were harvested, and viable cells were counted using a hemocytometer. Fold changes were calculated as the ratio of the number of live cells after the 5-day culture under various oxygen concentrations to the number of live cells after the 5-day culture in CTM under atmospheric conditions. (B) Splenic $CD8^+$ T cells from mice infected with 1×10^7 PFU/mouse of vSC25 were labeled with 2.5 μM of CFSE and re-stimulated *in vitro* with MMC-treated f15-12 cells in 96-well round-bottomed culture plates in CTM under various oxygen concentrations (20%, 3–4%, <1%) at 37 °C. After a 5-day culture, the generated cells were harvested and analyzed by flow cytometry. (C) Primed spleen cells were re-stimulated *in vitro* with MMC-treated f15-12 cells for 5 days at 37 °C as in (A). The IFN-γ content in the 5-day culture supernatant was analyzed by ELISA. $^*p < 0.05$. (D and E) Primed spleen cells or spleen cells from Tg-RT-1 mice were re-stimulated in vitro with MMC-treated f15-12 cells for 4-5 days at 37 °C as in (A). Cells were harvested, and the percentage of $CD8\beta^+$, TCR-$V\beta8^+$ cells was analyzed by flow cytometry. The cytolytic activity of the generated cells was determined by ^{51}Cr-release assay under 20% O_2 using f15-12 cells or control fibroblast cells (Neo) as target cells. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared with the cytotoxicity of generated cells at 20% O_2 (closed circles). The results shown are representative of three independent experiments.

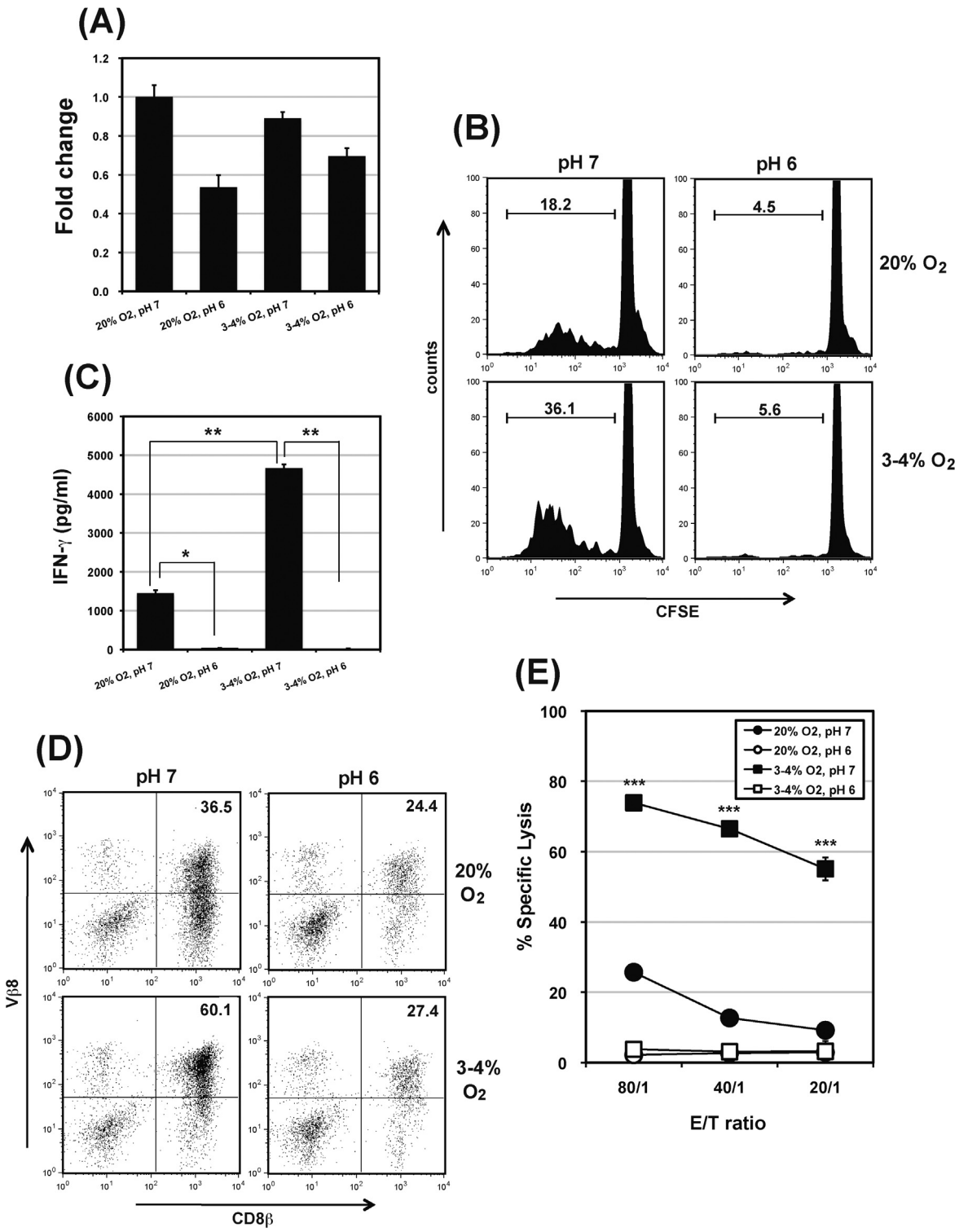


Fig. 7. Synergistic effects of low pH and hypoxia on the induction of antigen-specific CTLs *in vitro*. (A) Spleen cells from Tg-RT-1 mice were re-stimulated *in vitro* with MMC-treated f15-12 cells in 24-well culture plates in normal CTM (pH 7.1–7.3) or acidic CTM (pH 6.1–6.7) under an oxygen concentration of 20% or 3–4% at 37 °C. After a 4-day culture, cells were harvested, and viable cells were counted using a hemocytometer. Fold changes were calculated as the ratio of the number of live cells after the 4-day culture under various oxygen concentrations to the number of live cells after the 4-day culture in CTM under atmospheric conditions. (B) Splenic $CD8^+$ T cells from Tg-RT-1 mice were labeled with 2.5 μM of CFSE and re-stimulated in vitro with MMC-treated f15-12 cells in 96-well round-bottomed culture plates at 37 °C as in (A). After a 4-day culture, the generated cells were harvested and analyzed by flow cytometry. (C) The IFN-γ content in 4-day culture supernatants was analyzed by ELISA. $^*p < 0.05$, $^{**}p < 0.01$. (D and E) Spleen cells from Tg-RT-1 mice were re-stimulated in vitro with MMC-treated f15-12 cells for 4 days at 37 °C as in (A). Cells were harvested, and the percentage of $CD8\beta^+$, TCR-$V\beta8^+$ cells was analyzed by flow cytometry. The cytolytic activity of the generated cells was determined by ^{51}Cr-release assay under 20% O_2 using f15-12 cells as target cells. $^{***}p < 0.005$ compared with the cytotoxicity of generated cells in CTM (pH 7) at 20% O_2 (closed circles). The results shown are representative of three independent experiments.

hypoxia combined with a lower pH on the inhibition of neutrophil chemotaxis [31,32]. Therefore, we examined the synergistic effects of both low pH and hypoxia on the induction of antigen-specific CTLs *in vitro*. To minimize changes in medium pH during the induction period, we used the spleen cells of Tg-RT-1 mice in a manner similar to Fig. 3. Spleen cells were re-stimulated *in vitro* with MMC-treated f15-12 cells in CTM (pH 7) or LA-CTM (pH 6) for 4 days under normoxic or hypoxic (3–4% O_2) conditions. As shown in Fig. 7A, although the number of viable cells after the 4-day culture was slightly changed under low pH and hypoxic conditions, there was no significant difference. Cell proliferation was enhanced under 3–4% O_2 in CTM in a manner similar to that of spleen cells from antigen-primed mice; however, this enhancement was abolished in acidic medium even under hypoxic conditions (Fig. 7B). The percentage of $CD8\beta^+$, $V\beta8^+$, antigen-specific CTLs; the cytolytic activity of the generated cells; and IFN-γ content in the culture supernatant were all strongly enhanced under hypoxic conditions, but these changes were abrogated by an acidic environment (Fig. 7C–E). These results indicate that the induction of antigen-specific CTLs might be more susceptible to low pH rather than to oxygen concentration *in vivo*.

3.8. The mechanism by which low oxygen concentration enhanced the induction of antigen-specific CTLs.

To analyze the mechanism by which low oxygen concentration enhanced the induction of antigen-specific CTLs, we analyzed the expression of the cell surface H-$2D^d$ class I MHC molecule on f15-12 stimulator cells after overnight incubation under varying oxygen concentrations. However, as shown in Fig. 8A, no significant difference was detected between the 20% and 3–4% oxygen concentrations. Co-stimulatory molecules such as CD80 and CD86 were not detected on f15-12 cells under normoxic or hypoxic conditions (data not shown).

One of the important mechanisms responsible for the adaptation of cells to low oxygen tension is mediated by the transcription factor HIF-1α, which is essential for switching to glycolysis and promoting angiogenesis [33,34]. Therefore, we examined HIF-1α expression in CTLs induced under various oxygen concentrations. Several HIF-1α(+) cells were detected among the cells induced under 3–4% oxygen, and the number of positive cells was increased when induced under a 1% concentration (Fig. 8B). However, because the activation of primed spleen cells *in vitro* was markedly enhanced under 3–4% oxygen, no direct correlation was observed between the enhanced induction of functional CTLs and HIF-1α expression levels. Therefore, this enhancement might not occur through a HIF-1α-mediated signaling pathway.

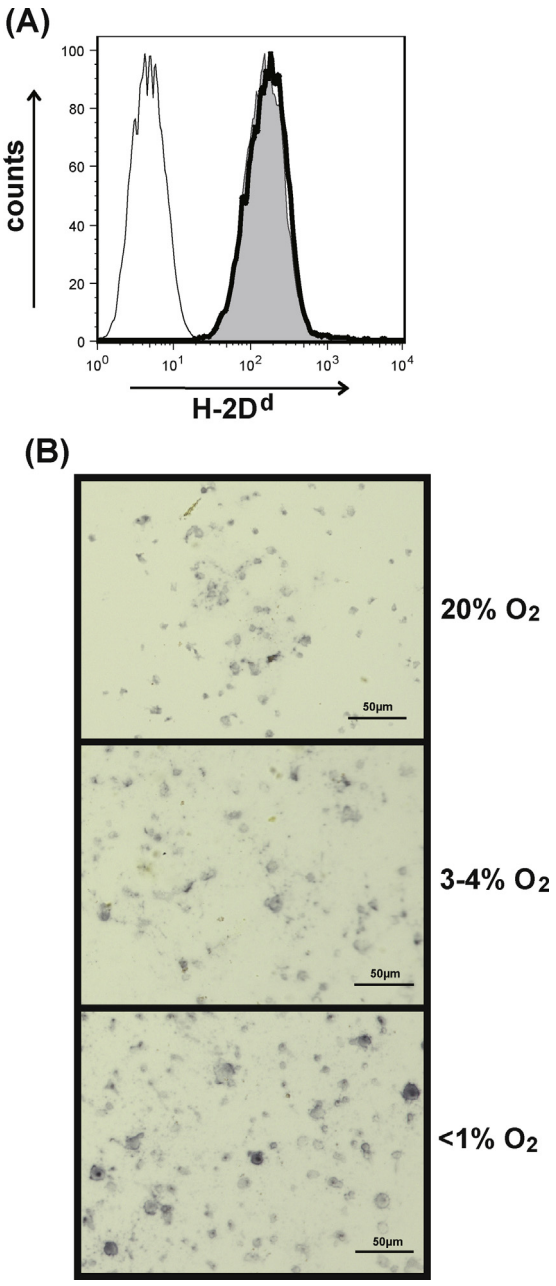


Fig. 8. (A) f15-12 cells were incubated in normal CTM (pH 7) under an oxygen concentration of 20% or 3–4% overnight at 37 °C. After incubation, cells were harvested and analyzed by flow cytometry (thin line, isotype control of cells incubated under 20% O_2; grey, cells incubated under 20% O_2; thick line, cells incubated under 3–4% O_2). (B) Spleen cells from Tg-RT-1 mice were re-stimulated in vitro with MMC-treated f15-12 cells in 24-well culture plates under various oxygen concentrations (20%, 3–4%, <1%) at 37 °C. After a 4-day culture, cells were harvested, and purified $CD8^+$ T cells were analyzed by immunohistological staining using anti-HIF-1α antibody. HIF-1α (+) cells were not detected in the anti- HIF-1α antibody (−) or secondary antibody-alone control samples. (Magnification, 200×).

4. Discussion

In the present study, we examined the function and induction of antigen-specific CTLs under patho-physiological conditions with either low pH or hypoxia, which differ from *in vitro* experimental conditions. Previous studies using established CTL lines demonstrated that the cytotoxic activity of CTLs against target cells was inhibited in an acidic environment [16,17]. Similar results were obtained in our antigen-specific CTL lines. We also showed here that the induction of functional CTLs from memory T cells was strongly inhibited in acidic medium using murine antigen-primed spleen cells. Although Fischer et al. previously reported that the proliferation of and cytokine production by CTLs stimulated with PHA/ionomycin were both inhibited in an acidic medium [12], these authors used a previously established human CTL line; therefore, we originally speculated that our results may differ from their findings. However, the results shown in the present study clearly demonstrated that both mature and newly induced CTLs were affected by physico-chemical environmental conditions such as low pH.

We also examined the function and induction of CTLs under low oxygen conditions using the BIONIX-1 hypoxic cell culture kit. Experiments performed under hypoxic conditions are generally conducted in a special workstation using a fixed percentage of oxygen, with 5% CO_2 containing mixed gases. However, in

our cell culture kit, the oxygen concentration could not be regulated during the 4–5-day culture period because the open side of the pouch containing the culture plates and the oxygen absorber was sealed with clips. Therefore, although the oxygen concentration changed (by approximately 0.5%) between the start and end times, pH changes were not detected in the medium during the culture period, as shown in Supplementary Fig. S2. Thus, both cytolytic activity of the established CTLs and induction of CTLs from memory cells could be determined under hypoxic conditions.

Unexpectedly, although their cytolytic activity against target cells in a ^{51}Cr-release assay was almost unchanged, the induction of maturated functional CTLs *in vitro* was somewhat enhanced under a low oxygen concentration. Recent *in vitro* studies demonstrated that T cell proliferation was significantly lower when primed cells were stimulated under lower oxygen concentrations than under room air oxygen conditions [35–37]. In contrast, using a murine mixed lymphocyte reaction experiment, Caldwell and colleagues found that although CTL development was delayed, the cytolytic activity of the cells generated was markedly stronger at 2.5% oxygen tension [18]. The reason for the discrepancy between our results and theirs remains unknown. However, because most studies have used human peripheral blood mononuclear cells (PBMCs) or unprimed murine lymphocytes as effector cells and a non-specific stimulus such as Concanavalin-A, anti-CD3 plus anti-CD28, or high-dose IL-2, these experiments clearly differ from our antigen-primed T cell-induction system. In this study, we also showed that the enhancement of the induction of CTLs *in vitro* by hypoxia was completely abrogated when CTLs were induced under hypoxic conditions with low pH (Fig. 7). Therefore, antigen-specific primed-CTL functions may be more vulnerable to low pH than oxygen concentration *in vivo*.

Adoptive immunotherapy using *ex vivo*-activated CTLs has been used for various tumors, and the appearance of functional $CD8^+$ effector T cells *in vivo* is considered for critical treatment [38–40]. However, based on the above findings, it is important to activate CTLs *in vivo* at local sites using various means to maintain their ability to eliminate target cells.

One of the important mechanisms responsible for the adaptation of cells to low oxygen concentrations is mediated by the transcription factor hypoxia inducible factor-1α (HIF-1α), which is essential for cells to switch to glycolysis and for promoting angiogenesis [33,34]. Therefore, we examined HIF-1α expression in CTLs induced under various oxygen concentrations. However, as shown in Fig. 8B, no direct correlation was observed between enhanced induction of functional CTLs and HIF-1α expression. Recently, it has been shown that there are several HIF-independent hypoxic signaling pathways, such as the mammalian target of rapamycin (mTOR) pathway [41] and the Akt/PI3K pathway [42]. Therefore, CTL activation by hypoxia as shown here might be mediated through these HIF-independent signaling pathways. However, the true mechanism underlying this enhancement in CTL induction remains to be elucidated.

In conclusion, we studied the effects of low pH and hypoxia on the function and induction of antigen-specific CTLs. The results obtained in the present study indicated that the cytotoxic activity of CTLs against targets and the induction of functional CTLs *in vitro* were both inhibited in low pH medium. In contrast, in hypoxic conditions, although cytotoxic activity was mostly unchanged, the induction of CTLs was enhanced *in vitro* and completely abrogated in low pH conditions. An analysis of the precise mechanisms responsible for functional alterations in CTLs *in vivo* and the development of procedures to recover their impaired activity will provide a new strategy for controlling tumor growth by maintaining a CTL-based immune-surveillance system.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

We thank Ms. Atsuko Owaki for the detection of TCR expression in Tg-RT-1 mice.

This study was supported in part by grants from the Ministry of Education, Science, Sport, and Culture from the Ministry of Health and Labor and Welfare, Japan (25461715 to H.T.); from the Japanese Health Sciences Foundation; from the Promotion and Mutual Aid Corporation for Private Schools of Japan; and from a MEXT-supported Program for the Strategic Research Foundation at Private Universities, Japan.

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