이학석사 학위논문

산성 종양 미세환경 조절을 통한 항암면역 증진에 대한 연구

Modulation of tumor microenvironment acidity enhances anti-tumor immunity

울산대학교대학원

의 과 학 과

최 다 솜

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이 논문을 이학석사 학위 논문으로 제출함

2021 년 8 월

울산대학교대학원

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최 다 솜

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2021 년 8 월

ABSTRACT

The tumor microenvironment (TME) is a complex environment where cancer cells reside and interact with different types of cells, including immune cells. The TME has been known to be a critical facilitator of immune escape and cancer progression. Clinical data from diverse cancer types shows that the increased T cell infiltration in tumors correlates with improved patient prognosis. Acidic extracellular pH is a major attribute of TME, mainly due to the high aerobic and glycolytic rate of tumor cells. The acidic TME has been reported to promote immune evasion and tumor progression. Therefore, antagonizing tumor acidity can be a powerful approach in cancer immunotherapy. Herein, the effect of tumor acidity on the efficacy of immunotherapy was investigated. An acidic pH environment suppressed antigen-specific memory $CD8^+$ T cell responses *in vitro*. Extracellular acidity was shown to upregulate co-inhibitory immune checkpoint receptors and inhibit mTOR signaling pathways in memory $CD8^+$ T cells, which impaired effector functions. Furthermore, an acidic pH environment increased the expression and engagement of TIGIT and its ligand CD155, which suggested that the extracellular pH can regulate the suppressive function of TIGIT pathway. A $NaHCO_3$ loaded thermosensitive hydrogel (pH modulating injectable gel (pH_e-MIG)) was employed to focally neutralize extracellular pH of the TME. Normalization of the acidic TME by intratumoral pH_e-MIG injection transformed the immune-suppressive TME into an immune-favorable condition, as evidenced by the decrease of immune-suppressive cells and increase of tumor infiltrating $CD8^+$ T cells. Furthermore, the intratumoral $CD8^+$ T cells exhibited enhanced cytolytic activity with a less exhausted profile. The combination of pH_e-MIG with a low dose of immune checkpoint inhibitors, anti-PD-1 and anti-TIGIT antibodies, improved intratumoral cytotoxic T cell function and tumor clearance. Collectively, these findings suggest that pH_e-MIG holds potential as a new TME modulator for effective immune checkpoint inhibitor therapies.

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LIST OF ABBREVIATIONS

APC: Antigen presenting cell

CEF: Cytomegalovirus, Epstein-Barr Virus and Flu virus

ELISA: Enzyme-Linked Immunosorbent Assay

GrzB: Granzyme B

ICI: Immune checkpoint inhibitor

IFN-γ: Interferon-gamma

IgG: Immunoglobulin G

IP: Intraperitoneal injection

IV: Intravenous injection

LAG-3: Lymphocyte activation gene-3

MDSC: Myeloid-derived suppressor cell

MFI: Median fluorescence intensities

PBMC: Peripheral blood mononuclear cell

PD-1: Programmed cell death receptor-1

pH_e-MIG: pH modulating injectable gel

TAM1: Tumor-associated macrophages 1

TAM2: Tumor-associated macrophages 2

TGI: Tumor growth inhibition

TIGIT: T cell immunoglobulin and ITIM domain

TIL: Tumor-infiltrating lymphocyte

TIM-3: T cell immunoglobulin-3

TNF-α: Tumor necrosis factor-alpha

TME: Tumor microenvironment

Treg: Regulatory T cell

INTRODUCTION

Tumor microenvironment (TME) is the cellular environment containing a wide range of cells such as tumor cells, stromal cells, and immune cells like T, NK, B and myeloid cells [1]. All these cells create a heterogenous complex network that supports tumor growth and metastasis. The TME is characterized by acidity, hypoxia, nutrient deficiency and immune-suppression. It has been known that the TME has a great impact on the treatment of tumors [2].

Crosstalk between tumor, immune cells and different environment cues is a complex process that will dictate the fate for anti-tumor or pro-tumor immunity. The immune system not only can protect the host against tumor growth, but also can actively participate in promoting its progression. T cells are key players of tumor immunity, since they selectively can recognize and kill cancer cells. Dendritic cells (DCs) clear the antigen-containing cell debris released into the TME. Then DCs migrate to regional lymph nodes, where they present the antigens to $CD8^+$ T lymphocytes bearing the cognate T cell receptors (TCR). Upon priming from naïve T cells, activated $CD8^+$ T cells are driven to clonal expansion and to enter the blood, for eventually homing to the tumor site. Activated $CD8^+$ T cells sense tumor cells through the interaction of the cognate TCR with the antigen/MHC complex expressed on the tumor cells. The activated $CD8^+$ T cells release of perforin-containing granules, forming pores on the tumor cell membrane and causing its death by osmotic unbalance and apoptosis via DNA-damaging molecules (i.e. granzyme) [3].

The tumor can avoid T cell-mediated attack through diverse mechanisms, such as loss of tumor antigens, downregulation of MHC class I and the upregulation of inhibitory surface proteins (e.g. PD-L1). As a consequence, these escape mechanisms could render tumor cells insensitive to the effector mechanisms of the immune system.

Immune checkpoint inhibitor therapy (ICI) reinvigorates antitumor immune responses by blocking inhibitory receptors such as programmed death-1 (PD-1), which restrict T cell responses [4]. ICI has emerged as an effective treatment for numerous types of cancer, resulting in remarkable clinical responses. To date, six immune checkpoint inhibitors (ICIs) targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), PD-1, and programmed death-ligand 1 (PD-L1) have been approved by the U.S. Food and Drug Administration. However, despite the clinical success, only a limited fraction of total treated patients responds to the current ICIs [5]. Therefore, there are high demands for developing new immune-modulating agents, which can alter the immunosuppressive TME and allow more efficient antitumor immunity for broadening clinical benefits of ICIs.

The tumor infiltrating lymphocytes (TILs) are often dysfunctional or exhausted in the tumor environment, possibly due to continuous tumor antigen stimulation and the presence of immunosuppressive receptor. Exhausted $CD8^+$ T cells exhibit impaired proliferation, altered metabolism, decreased cytokine production, and high expression of inhibitory receptors, including CTLA-4, PD-1, T cell immunoglobulin and mucin-domain containing protein-3 (TIM-3), lymphocyte activation gene-3 (LAG-3), and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) [6]. ICI treatment may confer anti-tumor effects by reinvigorating tumor-specific exhausted T cells. A subset of exhausted $CD8^+$ T cells are responsive to anti-PD-1/PD-L1 therapy. TIGIT is a co-inhibitory receptor that binds PVR (poliovirus receptor, CD155) and Nectin-2 (CD112) [7]. TIGIT is upregulated and co-expressed with PD-1 on exhausted T cells, and antibody-mediated blockade of TIGIT restores the anti-tumor immunity of $CD8^+$ T cells [8]. Combined treatment of anti-TIGIT with ICIs such as PD-1, PD-L1, and Tim-3 resulted in enhanced anti-tumor immunity in mouse tumor models [9]. It is becoming increasingly clear that exhausted T cells are the main target point for immunological checkpoint inhibitors (ICIs) therapy [10-11].

The acidosis of TME has been reported to be associated with immune evasion [12]. Acidic extracellular pH is mainly due to the high aerobic and glycolytic rate of tumor cells. Tumor cells undergo a metabolic reprogramming characterized by the switch of their energy metabolism into glycolysis, even under aerobic conditions. In contrast to mitochondrial oxidative phosphorylation (OXPHOS), the glycolytic metabolism of cancer cells employs lactate dehydrogenase (LDH) to convert pyruvate into lactate. The accumulation of lactate and protons, products of cancer metabolism, causes a decrease of intracellular pH. To neutralize metabolic acid loading, cancer cells exploit several proton pumps and transporters whose activity is tightly controlled by intra-cytoplasmatic sensors.

The acidic TME has been reported to antagonize the efficacy of ICIs by promoting TME-mediated immune suppression [13-14]. Recent evidence suggests that acidic pH in TME affects the effector functions of T and NK cells, protumor macrophage polarization, and the suppressive functions of myeloid-derived suppressor cells (MDSCs) [15]. Therefore, alleviating acidity within the TME back to the normal pH could be a successful strategy to maximize the efficacy of ICIs [16].

There are two main approaches that have been employed for acidity reconstruction of TME: preventing acidification and buffering the microenvironment. The first technique, such as utilizing proton pump inhibitors [17] and the knockdown of lactate dehydrogenase (LDHA) [18], clearly showed betterments in survival of tumor-bearing mice due to the retrenchment of immunosuppressive cytokines, and pH stability of the TME. However, the consequences of these approaches may adversely affect not only the tumors but also the immune cells, which inhibits effector function and viability of $CD8^+$ T cells. Additional technique is targeting method, such as using nanoparticle as a carrier, could avoid these issues, but the delivery efficiency still stays as a challenge [19]. Potentially, the buffering approach is a secure alternative to directly increasing the extracellular acidity pH of TME [20-21]. Robey et al. was able to enhance efficacy of immunotherapy by stabilizing pH through an oral

administration of sodium bicarbonate to mice [22]. Despite the simple procedure, this sodium bicarbonate therapy can induce metabolic alkalosis. Gu and his colleagues synthesized anti-CD47 antibody loaded CaCO3 nanoparticles which was then incorporated in a spray-able fibrin gel for post-surgical treatment [23]. Although the sprayed gel was able to increase pH and reduce immunosuppressive TME, the spraying approach limits the application only towards the post-surgical treatment.

In the study, I employed a $NaHCO_3$ loaded thermosensitive hydrogel as a novel method to focally neutralize extracellular pH of the TME. Intratumoral injection of extracellular pH modulating injectable gel (pH_e-MIG) increased pH value of the TME and cytotoxic immune cell infiltration, which lead to a substantial improvement in anti-PD-1 and anti-TIGIT blockade therapies. The extracellular acidity led to upregulation of co-inhibitory immune checkpoint receptors and inhibition of mTOR (mechanistic target of rapamycin) signaling pathways, resulting in impaired memory $CD8^+$ T cell responses.

MATERIALS AND METHODS

Human samples

This study was approved by the Institutional Review Board (#2018-1525) of Asan Medical Center. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors via density gradient centrifugation. $CD8^+$ T cells were separated by negative selection with a magnetic isolation kit (STEMCELL Technologies) and cultured in RPMI 1640 medium (pH 7.4) containing 25 mM HEPES, 0.5% penicillin/streptomycin, 2 mM l-glutamine and 10% heat inactivated FBS. To achieve lower pH, 20 mM L-lactic acid was added to the culture medium resulting in pH values of 6.6 ± 0.1 [24]. To remodulate pH, 40mM sodium bicarbonate ($NaHCO_3$) was added to the culture medium resulting in pH values of 7.4 ± 0.1. Reagents were purchased from Sigma-Aldrich.

Human T cells assay

For activation, $CD8^+$ T cells were stimulated with anti-CD3 (clone UCHT1,5 μg/mL) and anti-CD28 (clone CD28.2, 2 μg/ml) antibodies in a plate bound form. Neutralizing monoclonal antibody against human PD-1 (MK-3475, Pembrolizumab) and TIGIT (MBSA43) or isotype controls were used as indicated. The proliferation of CellTrace Violet (CTV; Invitrogen)-labeled $CD8^+$ T cells was evaluated by quantification of CTV dilution. To measure antigen-specific memory T cell response, PBMC were stimulated for 5–6 days with CEF pooled peptides (JPT Peptide Technologies) under different pH conditions. To generate monocyte-derived DCs, $CD14^+$ monocytes were isolated from PBMCs using the MojoSort™ Human Pan Monocyte Isolation Kit (BioLegend) and cultured in complete RPMI1640 medium containing GM-CSF (2 ng/ml) and IL-4 (10 ng/ml). Cell supernatants were collected and IFN-γ production was measured by ELISA (BioLegend).

Mixed lymphocyte reaction (MLR) assay

Monocytes were isolated from PBMCs using Mojosort Human Pan monocyte isolation kit (Biolegend). Dendritic cells (DC) were generated by culturing monocytes with 500 U/mL IL-4 (Peprotech) and 800 U/mL GM-CSF (Peprotech). Allogeneic T cells were labeled with CTV and incubated with DCs under different pH conditions. After 5 days, IFN-γ secretion in culture supernatants was analyzed by ELISA (BioLegend), and proliferation of T cells was measured by dilution of CTV.

Western blot

Human $CD8^+$ T cells were cultured under normal or acidic conditions for 3 days after pre-activation with anti-CD3/CD28 antibodies. The cells were lysed in SDS sample buffer containing 60 mmol/L Tris HCl, 2% SDS, 10% glycerol, and 100 mmol/L dithiothreitol. Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies [25]. For immunoblotting, primary antibodies against phospho-ERK1/2 (#4370), phospho-pS6 (#2211), phospho-p70 S6 kinase (#9205), phospho-AKT (#4060), and β-actin (#8457) were purchased from Cell Signaling Technology.

Cell lines

JE6.1, E.G7-OVA and A375 cells were obtained from the American Type Culture Collection (TIB-152; CRL-2113; CRL-1619). MC38 was purchased from Kerafast Inc (ENH204-FP). The cells for *in vivo* mouse experiments were tested and negative for mycoplasma contamination before transplantation.

Cell conjugation assay

EL4 cells stably expressing human CD155 were labeled with the red fluorescent dye PKH26 (Sigma), and JE6.1 cells stably expressing human TIGIT were labeled with the green

fluorescent dye PKH67 (Sigma). Both cells were incubated in RPMI 1640 containing 2% BSA for 30 min at 37 °C. Conjugate formation was analyzed immediately by flow cytometry.

Mice

The animal study described was approved and supervised by Asan Medical Center Institutional Animal Care and Use Committee. Male C57BL/6 mice at 6 weeks of age were purchased from Oriental Bio. All mice were kept in pathogen free conditions.

Mouse tumor studies

For tumor challenges, age-matched mice were subcutaneously implanted with 1×10^6 MC38 cells in 1× HBSS (Welgene). After the formation of a solid tumor with a volume of about 100 mm^3 (±25 mm^3), the tumor-bearing mice were randomly divided into four groups: (1) IG; (2) pH_e-MIG; (3) IG plus anti-TIGIT/anti-PD-1 antibodies; (4) pH_e-MIG plus anti-TIGIT/anti-PD-1 antibodies. Mice were given three treatments every 72 hrs. IG or pH_e-MIG was injected into the tumor center via insulin syringes (28.5 gauge, 0.5-inch needles; BD). To test the therapeutic efficacy of pH_e-MIG, 25 μg anti-TIGIT (clone 1G9, BioXcell) and 25 μg anti-PD-1 (clone J43, BioXcell) antibodies were administered via intraperitoneal injection on days 7, 10 and 13. Tumor growth was measured twice weekly by caliper and tumor volume was calculated as (length × $width^2$)/2. Mice were euthanized if tumors exceeded 2,000 mm^3. Tumor growth inhibition rate (TGI %) was calculated as described elsewhere [26]

Analysis of tumor infiltrating lymphocytes by flow cytometry

Tumor tissues were dissected and digested with collagenase IV (250 unit/ml, Worthington Biochemical Cooperation), DNase I (100 μg/ml, Roche), FBS (5%, Welgene) and $CaCl_2$ (5 mM, biosesang) in 1X HBSS using a gentleMACS dissociator (Miltenyi Biotec). Tumor

infiltrating lymphocytes were enriched by density separation on Lymphopure™ (BioLegend), and single cells were recovered for analysis by flow cytometry and *in vitro* assay.

Antibodies and flow cytometry

The prepared single cells were blocked with anti–mouse CD16/32 (Biolegend) or human BD Fc Block™ (BD) and surface stained in FACS buffer (PBS containing 2% FBS and 0.1% sodium azide) for 20 min at 4 °C. Dead cells were excluded using the Zombie NIR Fixable viability kit (Biolegend). Cytokine intracellular staining was conducted after restimulation with PMA/ionomycin in the presence of Golgi-Plug for 5 hrs. Flow cytometric analysis was performed using a CytoFLEX flow cytometer (Beckman Coulter) and performed using FlowJo software (v.10.5.3, TreeStar). The following fluorescent dye-conjugated anti-human antibodies were used for staining: anti-CD8 (SK1), anti-CD45RA (HI100), anti-CD45RO (UCHL1), anti-PD-1 (EH12.2H7), anti-LAG3 (11C3C65), anti-TIM3 (F38-2E2), anti-TIGIT (MBSA43), anti-IFNγ (4S.B3), anti-TNF (MAb11), anti-Granzyme B (GB11), anti-CD14 (63D3), anti-CD11c (3.9), anti-CD69 (FN50), anti-CD155 (SKII.4) (Biolegend); Antibodies to mouse proteins were as follows: anti-CD45 (30-F11), anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD11b (M1/70), anti-PD-1 (29 F.1A12), anti-LAG3 (C9B7W), anti-TIM3 (B8.2C12) anti-Ly6C (HK1.4), anti-MHC-II (AF6-120.1), anti-F4/80 (BM8), anti-CD24 (M1/69), anti-CD11c (N418), anti-IFN-γ (XMG1.2) and anti-TNF (C92-605) were all purchased from Biolegend. Anti-Foxp3 (FJK-16 s) was obtained from eBioscience.

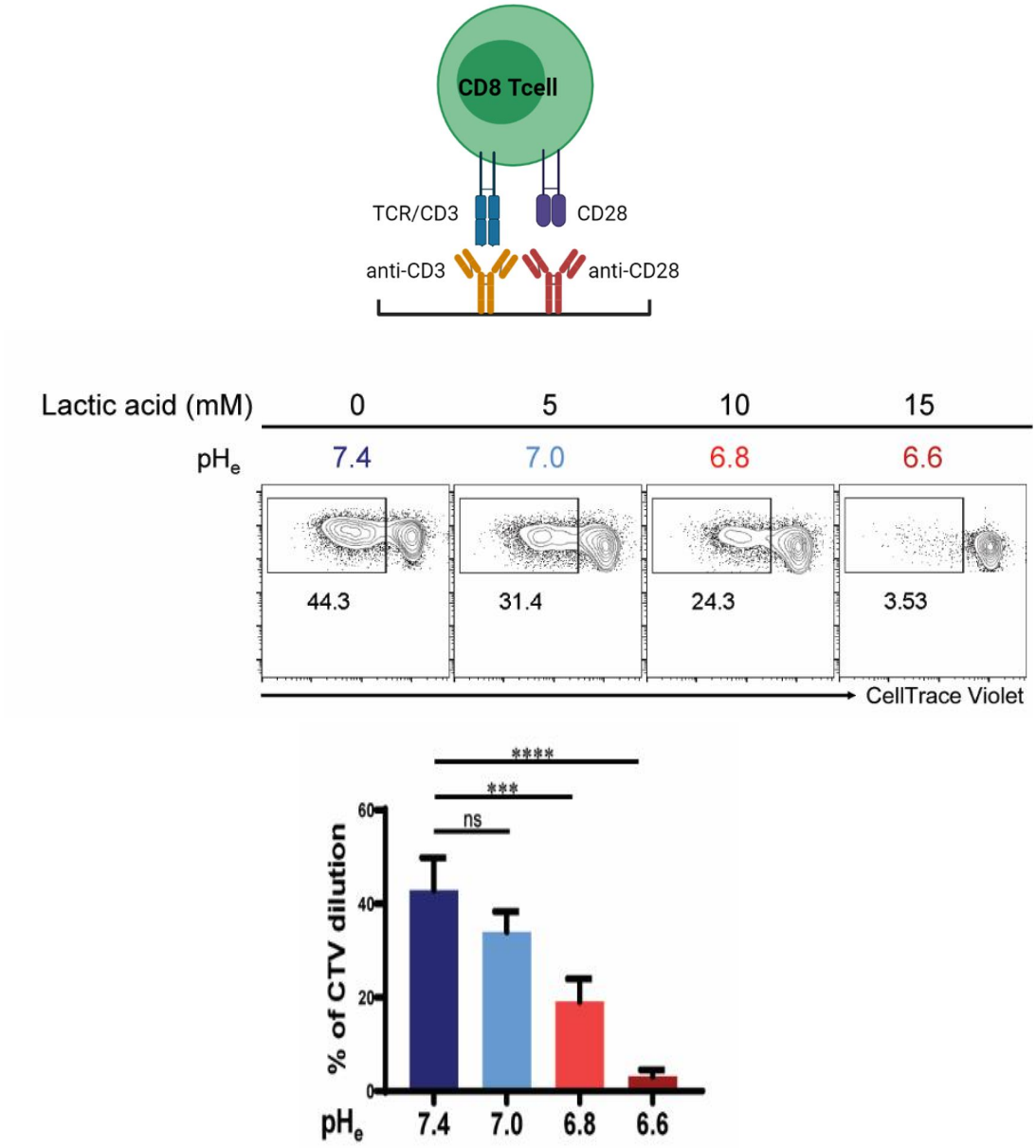
RESULTS

1. Extracellular acidity suppresses activity of immune cells.

To test the effect of pH alterations on the activation of $CD8^+$ T cells, human $CD8^+$ T cells were isolated from PBMCs and *in vitro* stimulated with anti-CD3/CD28 antibodies under different pH conditions ranging from pH 6.6 to pH 7.4. Lactic acid was used to adjust the pH value. FACS analysis of cell division showed that the proliferation of human $CD8^+$ T cells was significantly decreased at pH lower than physiologic levels. IFN-γ production of human $CD8^+$ T cells was also significantly declined at an acidic pH (pH 6.6) **(Figure 1A and 1B)**. However, it is yet unclear how acidic pH affects the memory $CD8^+$ T cell responses. Since tumor infiltrating T cells are mostly effector or memory cells that are primed in tumor draining lymph nodes prior to the migration into tumor sites, the effect of extracellular acidity on antigen-specific memory $CD8^+$ T cells responses was examined to minutely identify the mechanism of extracellular acidosis-induced immune evasion. Human PBMCs were stimulated by CEF peptide pool, which consists of 32 well-defined $CD8^+$ T cells specific peptides derived from Cytomegalovirus, Epstein-Barr Virus and Flu virus, to measure the recall responses of memory $CD8^+$ T cells against CEF antigens [27] The proliferation and IFN-γ production of memory $CD8^+$ T cells upon CEF antigen challenge were significantly abrogated in acidified culture medium **(Figure 2A and 2B)**. To further elucidate the mechanism underlying intrinsic regulation of memory T cell responses by extracellular acidosis, expression of immune checkpoint receptors in re-stimulated memory $CD8^+$ T cells that were cultured under acidic (pH 6.6) and normal (pH 7.4) pH conditions after pre-activation with anti-CD3/CD28 antibodies were analyzed. Memory $CD8^+$ T cells expressed higher levels of co-inhibitory immune checkpoint receptors including programmed cell death protein-1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte-activation gene 3 (Lag-3) and T-cell immunoglobulin and mucin-domain

containing-3 (Tim-3) at the acidic pH environment, while co-stimulatory immune checkpoint receptor of DNAX accessory molecule-1 (DNAM-1; CD226) expression was reduced, which may represent the state of T cell dysfunction and exhaustion **(Figure 3)**.

(A)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TCR/CD3
CD28
anti-CD3
anti-CD28
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5
10
15
pHe
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7.0
6.8
6.6
44.3
31.4
24.3
3.53
CellTrace Violet
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% of CTV dilution
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40
20
0
pHe
7.4
7.0
6.8
6.6

(B)

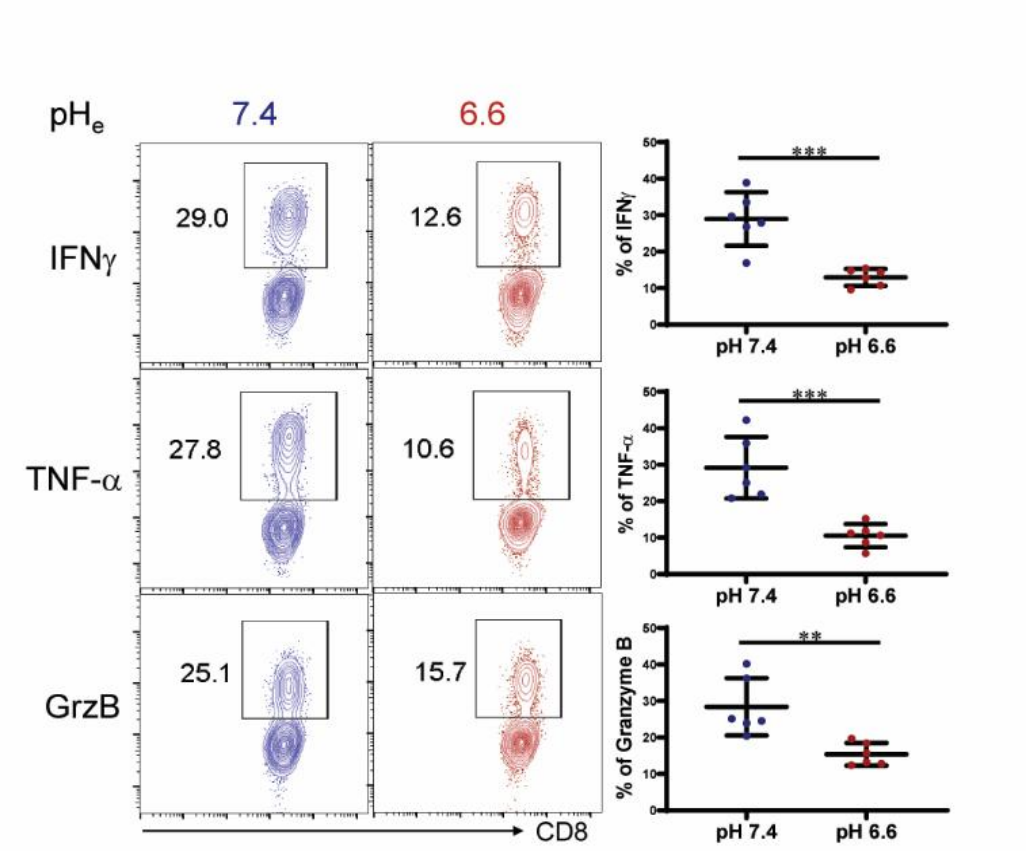
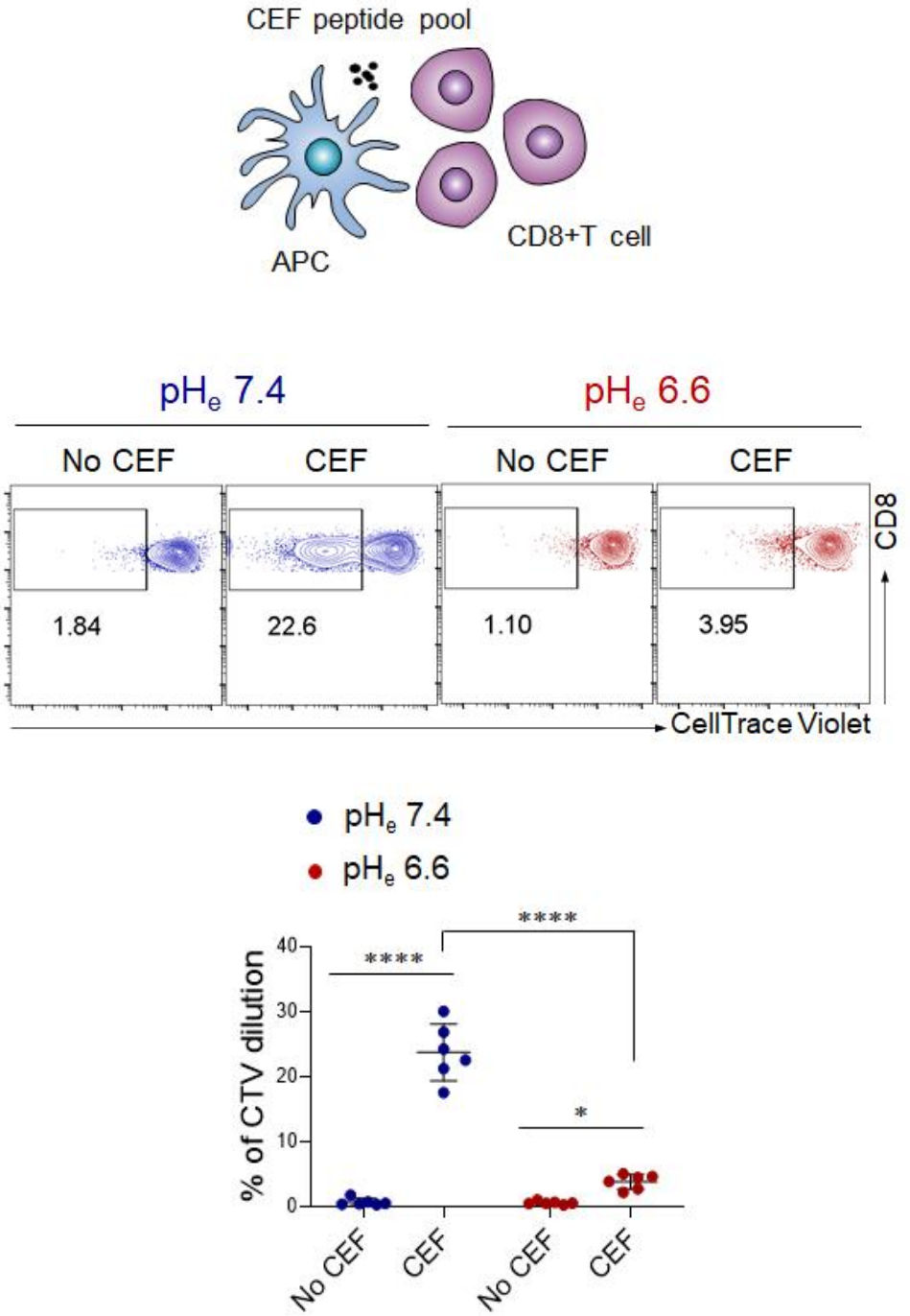


Figure 1. Effect of pH on activation of human primary T cells.

(A) Flow cytometry analyzing the proliferation of human $CD8^+$ T cells were labelled with CTV (CellTrace Violet). Data are pooled from or representative of four independent experiments.

(B) Flow cytometry analyzing the IFN-γ, TNF-α or Granzyme B (GrzB) production of human $CD8^+$ T cells stimulated with PMA (Phorbol 12-myristate 13-acetate)/Ionomycin under normal (pH7.4) or acidic (pH 6.6) conditions for 6 hrs. T cell proliferation and was measured by CTV dilution and IFN-γ secretion quantified in supernatants by ELISA. Data are pooled from or representative of two independent experiments; n=6 per each condition. Each symbol represents an individual donor. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <$ 0.0001 (two-tailed unpaired t-test).

(A)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CD8+T cell
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pH$_e$ 6.6
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CEF
No CEF
CEF
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1.10
3.95
CD8
CellTrace Violet
pH$_e$ 7.4
pH$_e$ 6.6
****
****
*
% of CTV dilution
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30
20
10
0
No CEF
CEF
No CEF
CEF

(B)

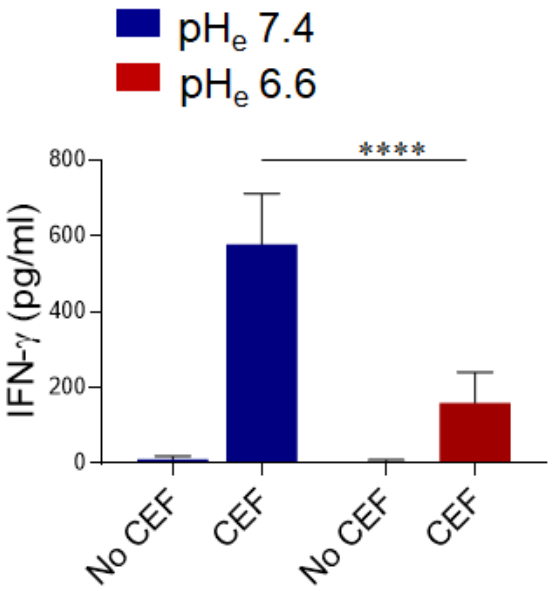


Figure 2. Effect of pH on memory recall responses of $CD8^+$ T cells.

(A) Flow cytometry analyzing the proliferation, of human $CD8^+$ T cells were labelled with CTV.

(B) IFN-γ production of CEF-specific human memory $CD8^+$ T cells after CEF peptide pool stimulation cultured under normal (pH7.4) or acidic (pH 6.6) conditions for 5 days. T cell proliferation and was measured by CTV dilution and IFN-γ secretion quantified in supernatants by ELISA. Data are pooled from or representative of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired *t*-test)

(A)

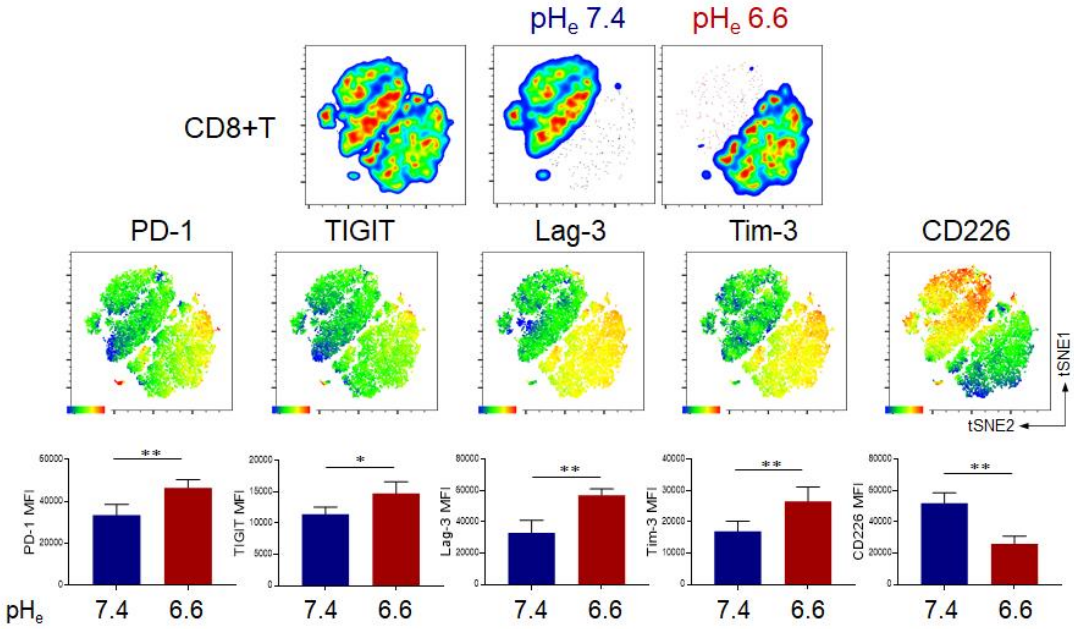


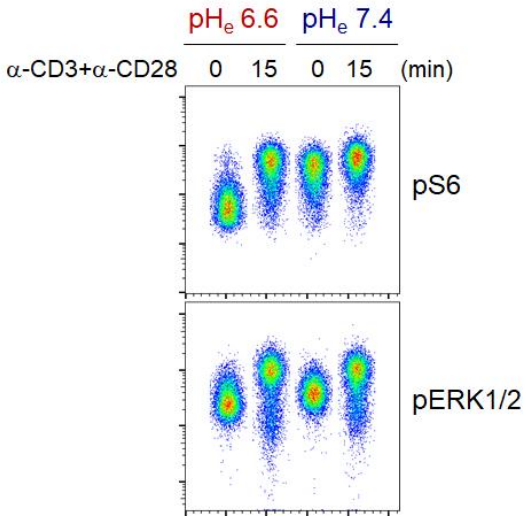
Figure 3. Effect of pH on expression of ICRs in memory CD8$^+$ T cells.

Immune checkpoint receptor expression was measured in CD8$^+$ T cells by flow cytometry. *t*-SNE plot (above) and relative MFI (below) of human memory CD8$^+$ T cells overlaid with the expression of indicated surface receptors. Human CD8$^+$ T cells were cultured under normal (pH7.4) or acidic (pH 6.6) conditions for 3 days after pre-activation with anti-CD3/CD28 antibodies. Data are representative of three independent experiments.

2. Acidic pH inhibits mTOR signaling pathways in memory $CD8^+$ T cells.

Once activated through a T-cell receptor, metabolic reprogramming of T cells to glycolysis and fatty acid oxidation (FAO) is demanded to apply growth, proliferation, activation and function. Recent studies have shown that immune checkpoint receptors can directly regulate cellular metabolism of T cells, which relies on activation of distinct signaling pathways. PD-1 binding to PD-L1 inhibits Akt/mTOR activation, which leads to reduction of glycolysis and enhancement of fatty acid oxidation (FAO) in T cells [28]. Therefore, we hypothesized that the upregulation of co-inhibitory immune checkpoint receptor expressions at an acidic pH provokes the alteration of mTOR activation. Intracellular phospho-flow cytometry and immuno-blotting analysis of $CD8^+$ T cells revealed that both basal- and TCR/CD28 stimulation induced notably lower levels of S6 phosphorylation under acidic condition compared to that of the normal pH, while the level of ERK1/2 was comparable regardless of different pH conditions **(Figure 4A and 4B)**. These data suggest that extracellular acidity has an intrinsic inhibitory effect on memory $CD8^+$ T cells by negatively regulating the mTOR signaling pathway. The generation and maintenance of memory $CD8^+$ T cells is critical to elicit sustainable antitumor immunity [29], which is tightly associated with mTOR activity. Reduced mTOR activity has been known to prevent secondary. immune responses of memory $CD8^+$ T cells upon antigen challenge [30]. Therefore, relieving the tumor acidity can contribute to forge stable and functional memory $CD8^+$ T cell pools that are capable of protecting from tumor recurrence after immunotherapy.

(A)



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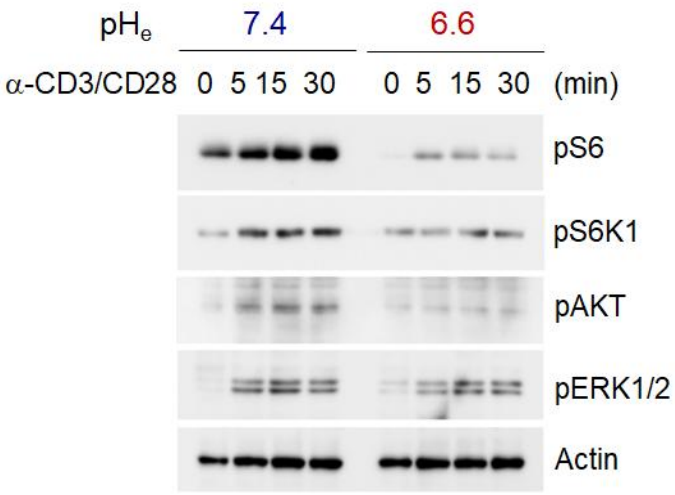


Figure 4. Extracellular acidity impaired the effector functions of memory CD8$^+$ T cell.

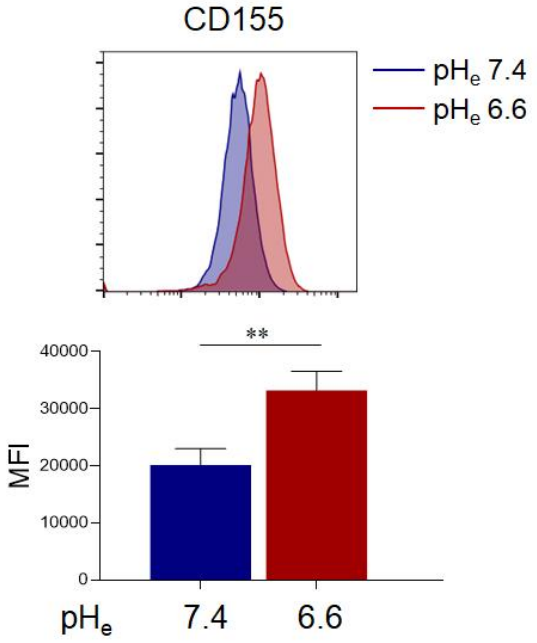
(A) Phospho-flow cytometry analyzing the phosphorylation of S6 and ERK1/2 in human CD8$^+$ T cells incubated under normal or acidic conditions for 24 hrs. Data are representative of three independent experiments.

(B) Immunoblot analysis of CD8$^+$ T cells as in A. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired *t*-test).

3. Acidic extracellular pH regulates the suppressive function of TIGIT-CD155 axis.

One of the major immune evasion mechanism in tumor is to lose immunogenicity by expressing immunosuppressive molecules such as PD-L1 and CD155 [31]. TIGIT ligand CD155 expression is upregulated in several human cancers while the expression in most normal tissues is low [32]. As a biomarker, CD155 overexpression correlates positively with poor prognosis [33]. TIGIT is also highly expressed on tumor-infiltrating lymphocytes (TILs) from many different tumor types. Engagement between CD155 and TIGIT induced suppression of the $CD8^+$ T cells effector function of tumor clearance [34,35]. Since we observed increased expression of TIGIT on $CD8^+$ T cells under acidic environment **(Figure 3)**, I reasoned that extracellular acidosis might contribute to facilitate the loss of immunogenicity of tumor. Indeed, an increased expression of CD155 was observed on human melanoma cell line (A375) cultured under acidic condition **(Figure 5A)**. To examine whether extracellular acidity could affect the interaction between CD155 and TIGIT, a cell conjugation assay was performed. Human TIGIT-overexpressing JE6.1 cells and human CD155-overexpressing EL-4 cells were co-incubated under different pH conditions. FACS analysis revealed that extracellular acidity promoted TIGIT engagement with CD155 **(Figure 5B)**. These results suggest TIGIT-CD155 axis could exert more suppressive function in acidic TME.

(A)


CD155
pHe 7.4
pHe 6.6
**
40000
30000
20000
10000
0
MFI
pHe
7.4
6.6

(B)

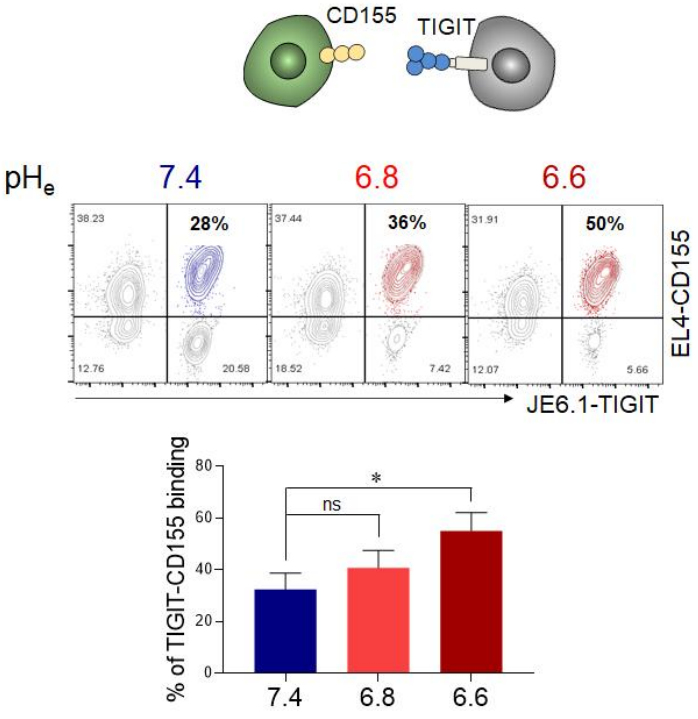

CD155
TIGIT
pHe
7.4
6.8
6.6
38.23
28%
37.44
36%
31.91
50%
12.76
20.58
18.52
7.42
12.07
5.66
EL4-CD155
JE6.1-TIGIT
% of TIGIT-CD155 binding
80
60
40
20
0
*
ns
7.4
6.8
6.6

Figure 5. Acidic pH increased expression of CD115 (PVR) and conjugation of TIGIT-CD155.

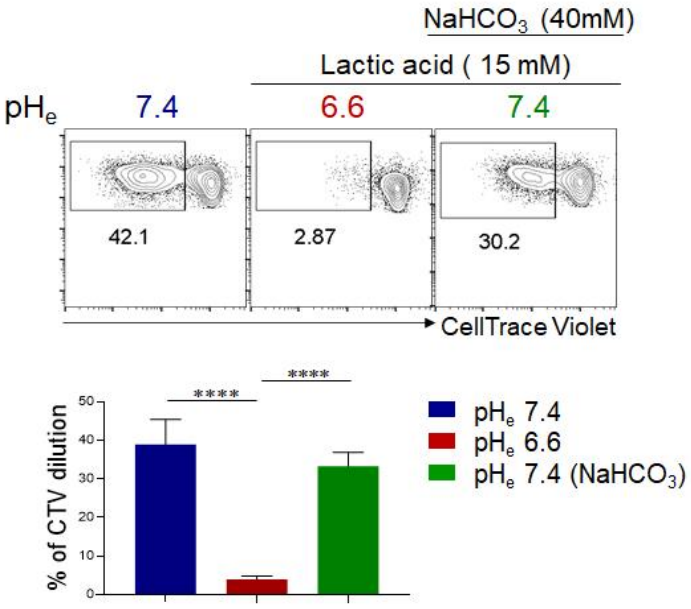
(A) Flow cytometry analyzing the expression of CD155 (PVR) on A375 cells cultured under normal or acidic conditions. Data pooled from or representative of three independent experiments.

(B) Flow cytometry assessing the binding of the red dye-labeled human CD155 expressing EL4 cells and the green dye-labeled human TIGIT expressing JE6.1 cells. Data pooled from or representative of three independent experiments. *$p < 0.05$, **$p < 0.01$ (two-tailed unpaired *t*-test).

4. $NaHCO_3$ increases the efficacy of immune checkpoint inhibitors.

Regarding to our notion of reverting defective T cell from the extracellular acidosis through pH buffering, the effects of $NaHCO_3$ to human $CD8^+$ T cells pre-incubated with acidified culture medium was examined. Lactic acid was used to adjust the pH of culture medium to 6.6, which is considered as an intratumoral pH value. The introduction of $NaHCO_3$ was sufficient to relieve the effector responses of human primary $CD8^+$ T cells from the acidic pH-mediated inhibition, as evidenced by increased proliferation and IFN-γ production **(Figure 6A, 6B)**. Then, the effect of different pH conditions on the potency of immune checkpoint inhibitors was investigated. Anti-PD-1 and anti-TIGIT antibodies were added in the culture of CEF-stimulated $CD8^+$ T cells. Anti-PD-1 and/or anti-TIGIT antibodies and CEF antigen-specific $CD8^+$ T cells at pH 6.6 were significantly compromised by extracellular acidity. However, $NaHCO_3$ -mediated pH buffering restored the potency of anti-PD-1 and anti-TIGIT therapy, which suggest that the neutralization of tumor acidity could be a prerequisite for more effective ICIs by not only augmenting intratumoral infiltration of $CD8^+$ T cells, but also weakening the immune checkpoint mediated immune suppression in the TME **(Figure 7A, 7B)**.

(A)



(B)

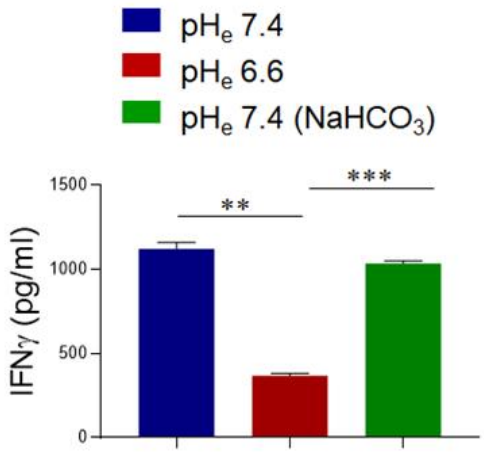
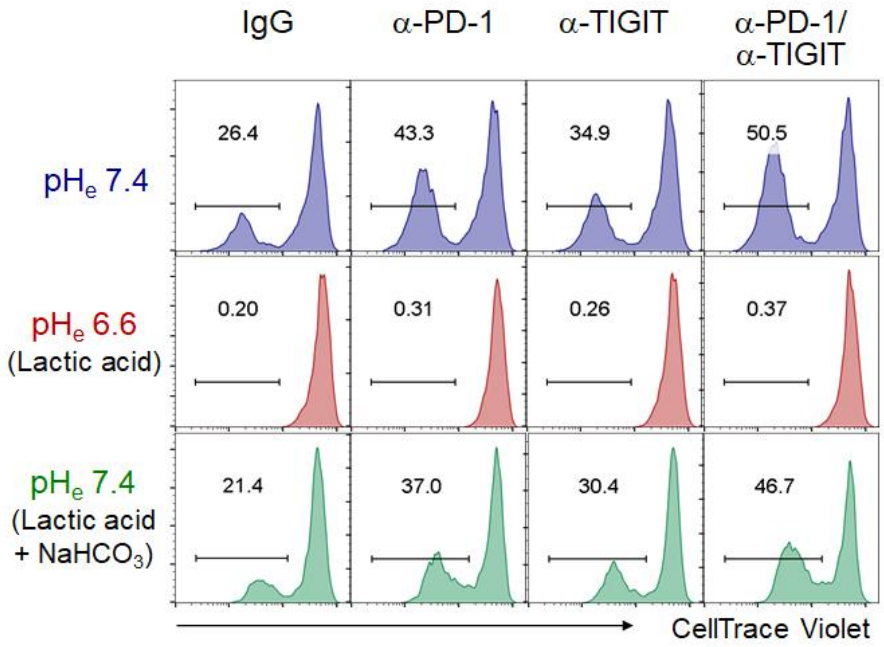


Figure 6. pH modulating culture condition with $NaHCO_3$ promoted the proliferation and activation of $CD8^+$ T cells.

(A) Flow cytometry analyzing the proliferation of human $CD8^+$ T cells were labelled with CTV.

(B) IFN-γ production of human $CD8^+$ T cells cultured under normal (pH7.4), acidic (pH6.6) or 40mM $NaHCO_3$ buffered (pH7.4) conditions for 3 days after pre-activation with anti-CD3/CD28 antibodies. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (two-tailed unpaired *t*-test).

(A)


IgG
α-PD-1
α-TIGIT
α-PD-1/
α-TIGIT
pHe 7.4
26.4
43.3
34.9
50.5
pHe 6.6
(Lactic acid)
0.20
0.31
0.26
0.37
pHe 7.4
(Lactic acid
+ NaHCO3)
21.4
37.0
30.4
46.7
CellTrace Violet

(B)

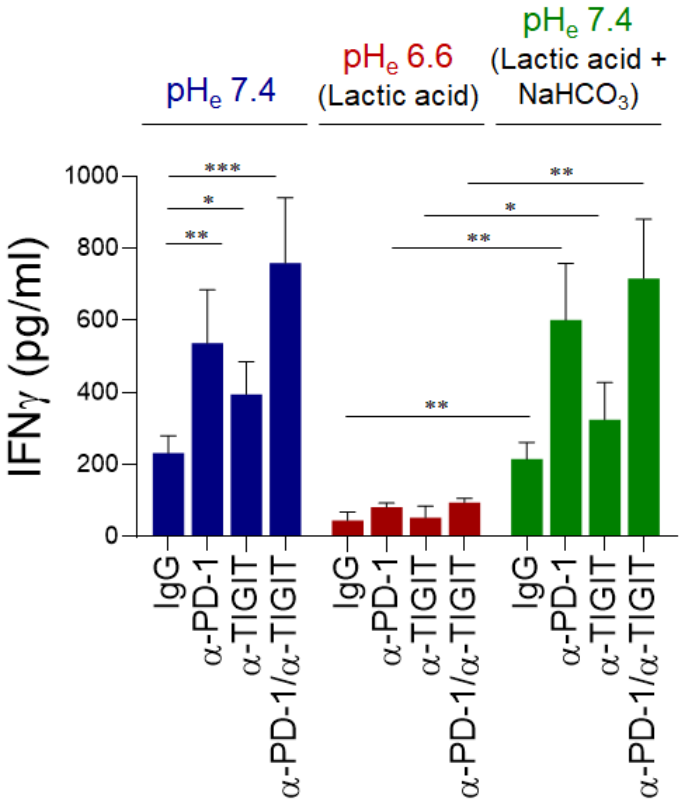

pHe 7.4
pHe 6.6
(Lactic acid)
pHe 7.4
(Lactic acid +
NaHCO3)
IFNγ (pg/ml)
1000
800
600
400
200
0
***
*
**
**
*
**
**
IgG
α-PD-1
α-TIGIT
α-PD-1/α-TIGIT
IgG
α-PD-1
α-TIGIT
α-PD-1/α-TIGIT
IgG
α-PD-1
α-TIGIT
α-PD-1/α-TIGIT

Figure 7. $NaHCO_3$ -mediated pH buffering restored the potency of immune checkpoint inhibitor therapy.

(A) Flow cytometry analyzing the proliferation of human $CD8^+$ T cells were labelled with CTV.

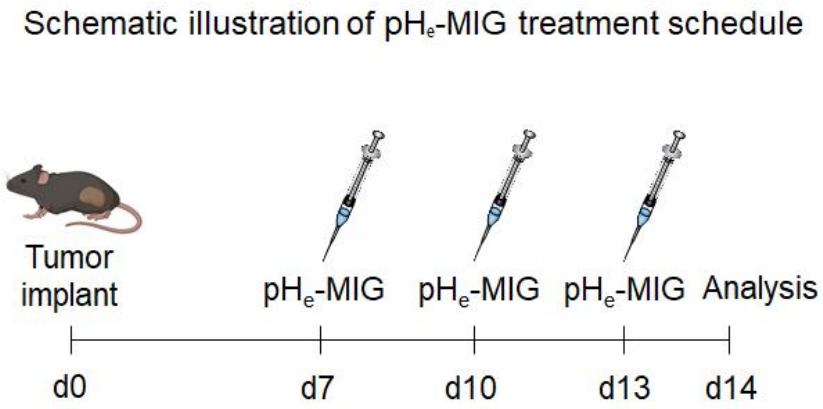
(B) IFN-γ production of CEF-specific memory human $CD8^+$ T cells after CEF peptide pool stimulation with or without anti-PD-1 and/or anti-TIGIT antibodies cultured under normal (pH7.4), acidic (pH6.6) or 40mM $NaHCO_3$ buffered (pH7.4). T cell proliferation was evaluated by CTV dilution and IFN-γ secretion concentration was measured by ELISA assay. Data are pooled from or representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (two-tailed unpaired *t*-test).

5. pH_e-MIG treatment renders acidic tumor microenvironment more immunologically favorable.

In order to deliver $NaHCO_3$ towards the acidic TME, $NaHCO_3$ containing F-127 gel was synthesized with 100 mM $NaHCO_3$ concentration and these pH modulating injectable gels were referred to as pH_e-MIGs. Next, the abilities of pH_e-MIG to facilitate reconditioning of the immunosuppressive TME were examined. Based on the pH values and kinetics of pH_e-MIGs *in vivo*, intratumoral injection of 60 ul pH_e-MIG with 100 mM $NaHCO_3$ to MC38 bearing mice was conducted three times every 72 hrs **(Figure. 8A and 8B)**. A day after the third treatment of pH_e-MIG, we analyzed the immune microenvironment in the blank F-127 gel (IG) and pH_e-MIG injected MC38 tumors from wildtype mice. The MC38 tumor was excised and prepared as a single cell suspension. A multi-color flow cytometry analysis and progressive gating strategy revealed that the injection of pH_e-MIG led to an increase of $CD45^+$ immune cell, and $CD3^+$ and $CD8^+$ T cells infiltration into tumor **(Figure. 9A, and 9B)**. Importantly, the frequency of tumor-infiltrating $Foxp3^+$ regulatory T (Treg) cells, which are involved in suppression of antitumor immunity in TME, was decreased in the pH_e-MIG injected tumors, as revealed by the increased $CD8^+$ T cell/Treg ratios **(Figure. 9C)**. Tumor acidity neutralization with pH_e-MIG increased the accumulation of cytotoxic $CD8^+$ T cells. Various types of myeloid cells, including myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages 2 (TAM2) have also been known to generate an immuno-suppressive environment. TAM1 is known to promote tumor regression while TAM2 appears to be associate with tumor promotion [36]. The proportion of immunosuppressive myeloid populations including $CD11b^+$ $Ly6C^+$ MDSC and $F4/80^{hi}CD24^{lo}CD11b^{lo}CD11c^{hi}Ly6C^-$ TAM2 was decreased, but there was an increase of $F4/80^{hi}CD24^{lo}CD11b^{hi}CD11c^{lo}Ly6C^-$ TAM1 in the pH_e-MIG treated tumors as compared with the IG injected ones **(Figure. 10A and 10B)**. These results show that the neutralization of acidic TME by pH_e-MIG can generate immune favorable TME by selectively supporting cytotoxic immune cell

accumulation. Since the absolute number of immunosuppressive populations including Treg, MDSC and TAM2 was not altered in the pH_e-MIG treated tumors (data not shown), the increased proportion of $CD8^+$ T cells or TAM1 indicates that pH_e-MIG generates immune-favorable TME for $CD8^+$ T cell activation without any harmful effect on other immune cell populations.

(A)



(B)

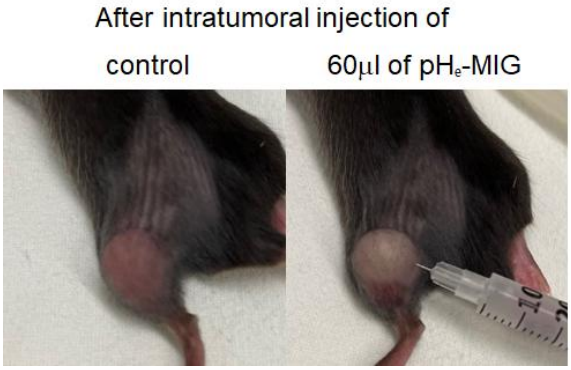
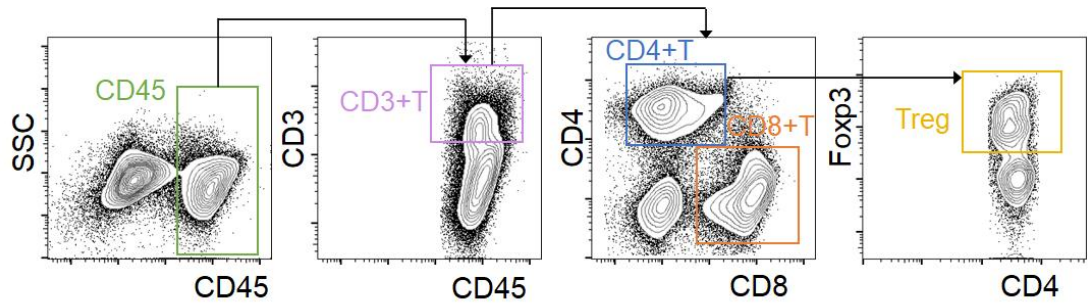


Figure 8. Schematic illustration of tumor implant of pH_e-MIG.

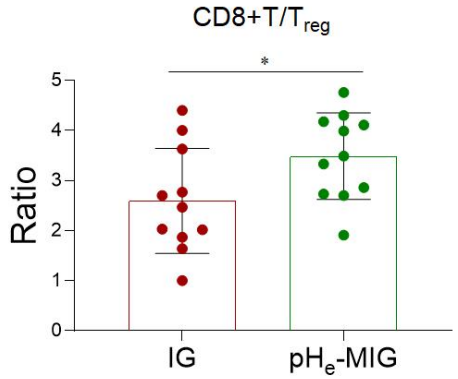
(A) pH_e-MIG treatment schedule. pH_e-MIG is $NaHCO_3$ containing F-127 hydrogel.

(B) pH_e-MIG intratumoral injection up to 60ul. MC38-bearing C57BL/6 mice treated with F-127 gel (IG) and pH_e-MIG. Representation image of MC38 tumors from each treatment group at day7.

(A)


CD45
SSC
CD45
CD3+T
CD3
CD45
CD4+T
CD8+T
CD4
CD8
Treg
Foxp3
CD4

(B)


CD8+T/Treg
*
Ratio
5
4
3
2
1
0
IG
pHe-MIG

(C)

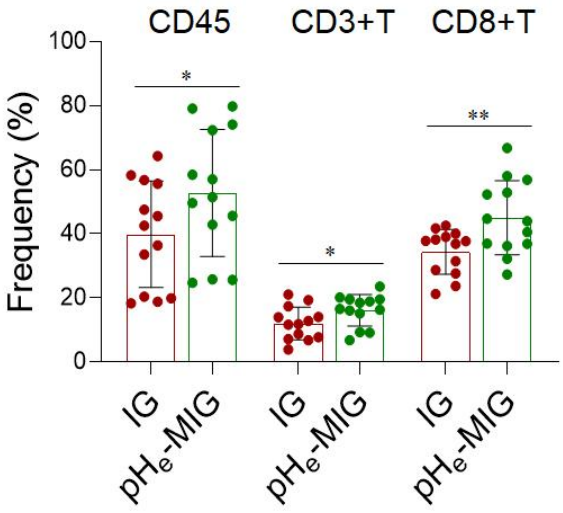

CD45
CD3+T
CD8+T
Frequency (%)
100
80
60
40
20
0
*
*
**
IG
pHe-MIG
IG
pHe-MIG
IG
pHe-MIG

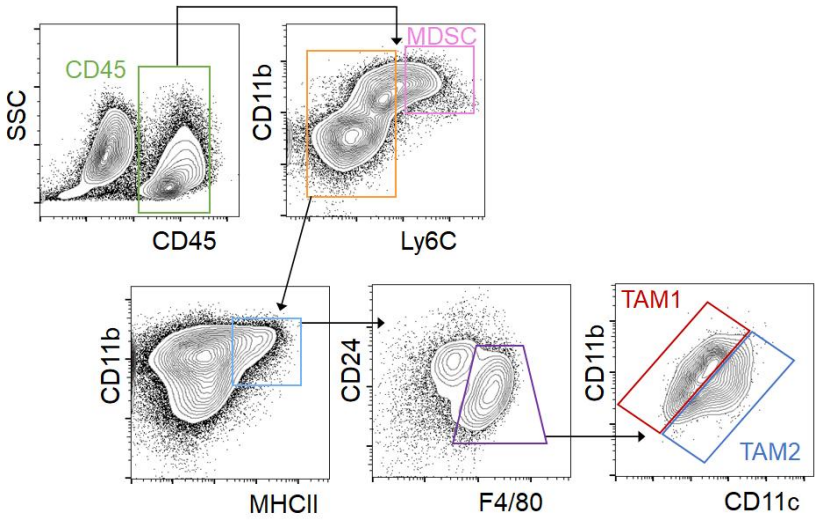
Figure 9. Tumor acidity neutralization with pH_e-MIG increased the accumulation of cytotoxic $CD8^+$ T cells.

(A) Flow cytometry gating strategy to analyze tumor-infiltrating T cell subsets.

(B) Flow cytometry analyzing tumor infiltrating T cells from MC38-bearing C57BL/6 mice treated with F-127 gel (IG) and PH_e-MIG. Data are pooled from three independent experiments; $n = 13$ mice per each treatment group.

(C) The ratio of cytotoxic $CD8^+$ T cells to $Foxp3^+$ Treg cells by flow cytometry. Data are pooled from three independent experiments; $n = 11$ mice per each treatment group. $^*p < 0.05$, $^{**}p < 0.01$ (two-tailed unpaired *t*-test)

(A)



(B)

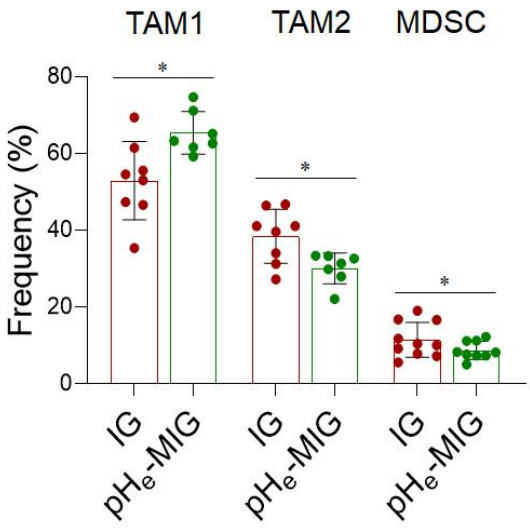


Figure 10. Tumor acidity neutralization with pH_e-MIG increased the frequency of TAM1.

(A) The Flow cytometric gating strategy to analyze myeloid cells subsets in tumor.

(B) Frequency of myeloid cells analyzed by flow cytometry. Data are pooled from three independent experiments; group IG (n = 8) mice, group pH_e-MIG (n = 7). *$p < 0.05$, **$p < 0.01$ (two-tailed unpaired t-test)

6. Combination of immune checkpoint blockades with pH_e-MIG for cancer immunotherapy.

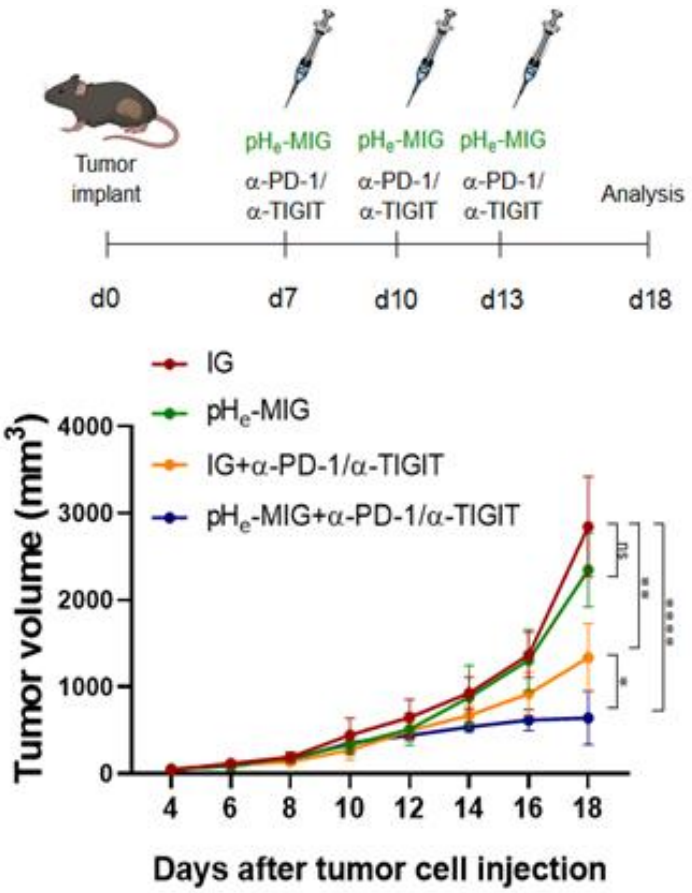
Following up to the enrichment of tumor-infiltrating T cells after intratumoral pH_e-MIG injection, anti-tumor efficacy of immune checkpoint inhibitors was investigated. Low dose administration of anti-PD-1 (1.25 mg/kg) and anti-TIGIT antibodies (1.25 mg/kg) via intraperitoneal (i.p.) injection was performed after intratumoral pH_e-MIG injection to MC38-bearing mice. Combined treatment of anti-PD-1 and TIGIT antibodies after pH_e-MIG injection resulted in 75% of tumor growth inhibition (TGI), while IG with the combined treatment only produced ~47% TGI **(figure. 11A and 11B)**. These results are precisely elucidated as chance of developing new immune-modulating agents. Immune monitoring of tumor infiltrating lymphocytes showed that the co-treatment of anti-PD-1/TIGIT antibodies with pH_e-MIG considerably enhanced cytotoxic $CD8^+$ T cell responses, as evaluated by higher levels of TNF-α, IFN-γ and 4-1BB expressions **(Figure. 12A and 12B)**.

Tumor infiltrating $CD8^+$ T cells were analyzed to examine the expression of co-inhibitory immune checkpoint receptors (PD-1, Lag-3 and Tim-3) that mark the exhausted state of $CD8^+$ T cells. The mark skewing towards lower expression of immune checkpoint receptors in $CD8^+$ T cell population was observed in response to co-treatment of pH_e-MIG and anti-PD-1/TIGIT antibodies, which is an indicative phenotype of less exhausted T cells **(Figure. 13A)**. Also, co-treatment of pH_e-MIG with anti-PD-1/TIGIT antibodies more significantly decreased the expression of a percentage of PD-1, Lag-3 and Tim-3 T cells in TME than the other three condition **(Figure. 13B)**.

These data suggest that pH_e-MIG synergistically potentiates the anti-tumor effects of PD-1 and TIGIT inhibitor therapies by preventing T cell exhaustion. Intratumoral $CD8^+$ T cells exhibit abroad spectrum of dysfunctional states, termed as T cell exhaustion which serves as a principle barrier in effective tumor destruction [37]. Exhausted $CD8^+$ T cells can be subcategorized on the basis of their activation status and functional differences that is

characterized by immune checkpoint expressions and epigenetic features [38]. Recent studies reported that progenitor (or less) exhausted $CD8^+$ T cells are the responding population to anti-PD-1 therapy, while terminally exhausted $CD8^+$ T cells are not effected by anti-PD-1 [39]. Herein this study revealed that the pH modulation of TME accumulates less exhausted $CD8^+$ T cells that could make $CD8^+$ T cells more susceptible to ICIs. Importantly, combination of ICIs with intratumoral pH-modulator could be a novel method to design more effective cancer immunotherapies.

(A)


Tumor implant
pHe-MIG
α-PD-1/ α-TIGIT
pHe-MIG
α-PD-1/ α-TIGIT
pHe-MIG
α-PD-1/ α-TIGIT
Analysis
d0
d7
d10
d13
d18
IG
pHe-MIG
IG+α-PD-1/α-TIGIT
pHe-MIG+α-PD-1/α-TIGIT
Tumor volume (mm3)
4000
3000
2000
1000
0
4
6
8
10
12
14
16
18
Days after tumor cell injection
ns
**
****
*

(B)

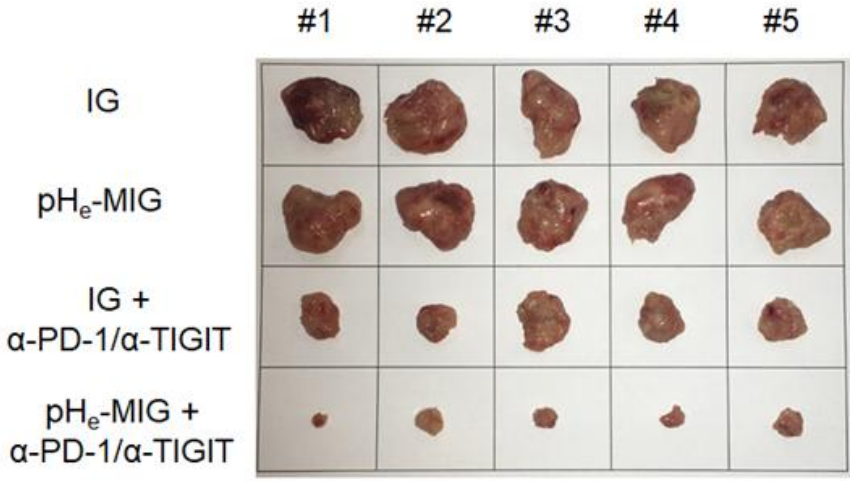
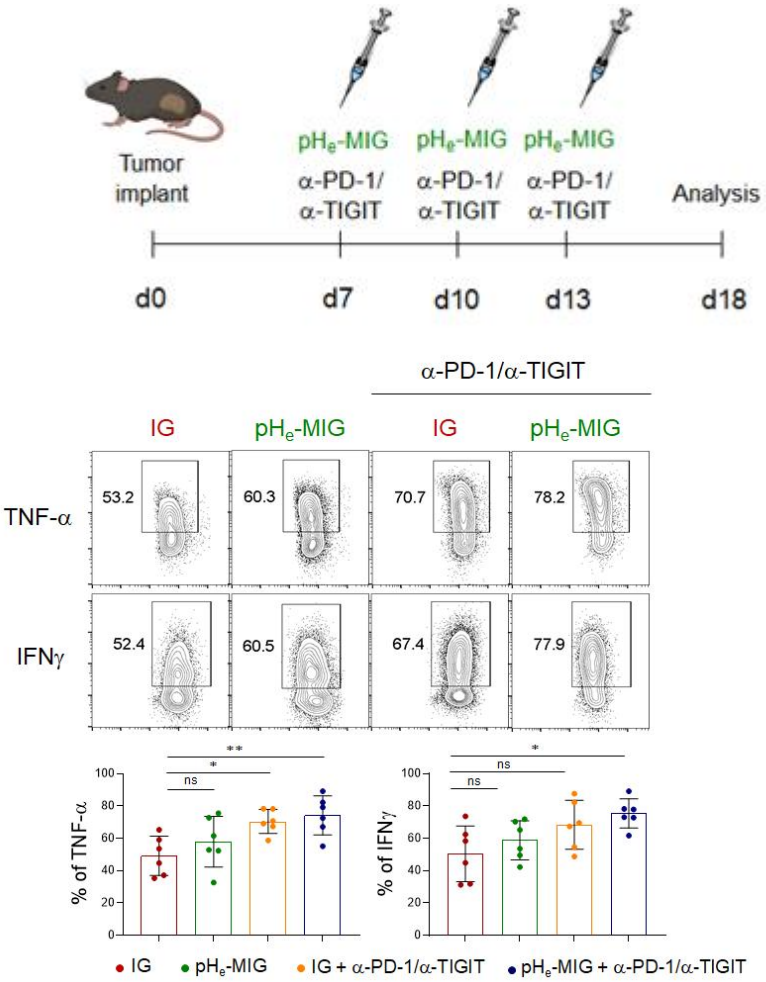

#1
#2
#3
#4
#5
IG
pHe-MIG
IG + α-PD-1/α-TIGIT
pHe-MIG + α-PD-1/α-TIGIT

Figure 11. Combination of immune checkpoint blockades with pH_e-MIG for cancer immunotherapy.

(A) Schematic representation of the combinatorial treatment schedule (above). MC38 tumor growth curves in each treatment group (below). Data are pooled from two independent experiments; n = 10 mice per each treatment group.

(B) Representation image of MC38 tumors from each treatment group at day18 of tumor growth are shown; n = 5 mice per each treatment group. Data are pooled from two independent experiments; n = 6 mice per each treatment group.

(A)


Tumor implant
pHe-MIG
α-PD-1/ α-TIGIT
Analysis
d0
d7
d10
d13
d18
α-PD-1/α-TIGIT
IG
pHe-MIG
IG
pHe-MIG
TNF-α
IFNγ
53.2
60.3
70.7
78.2
52.4
60.5
67.4
77.9
% of TNF-α
% of IFNγ
ns
*
**
IG
pHe-MIG
IG + α-PD-1/α-TIGIT
pHe-MIG + α-PD-1/α-TIGIT

(B)

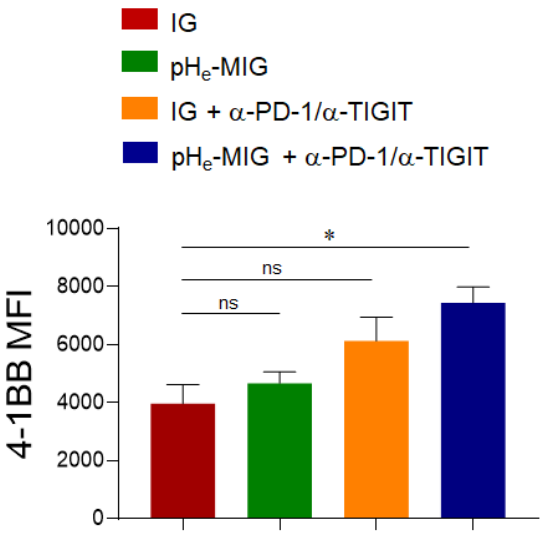
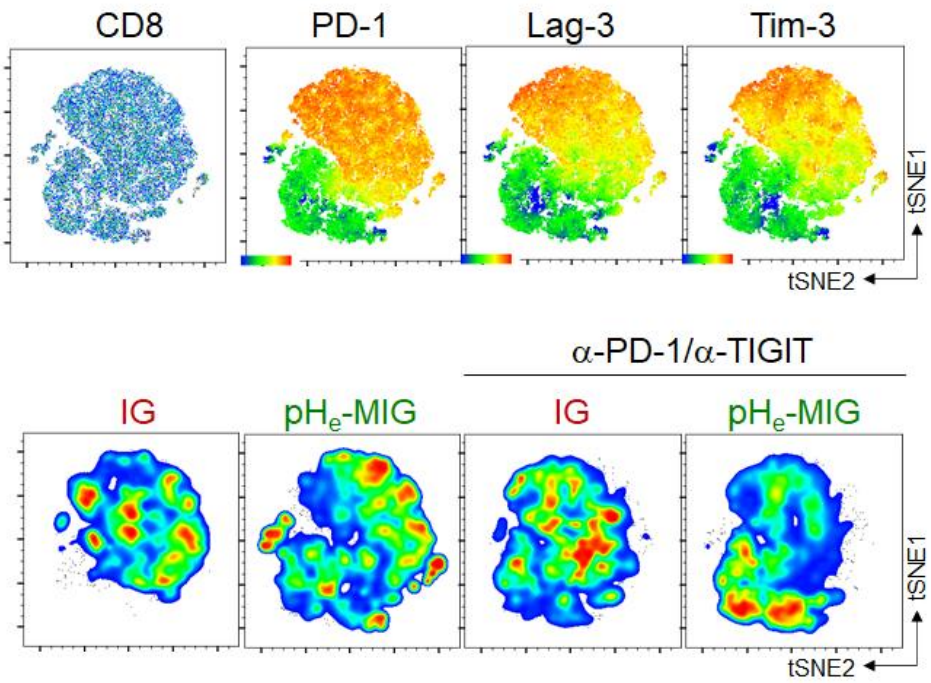

IG
pHe-MIG
IG + α-PD-1/α-TIGIT
pHe-MIG + α-PD-1/α-TIGIT
4-1BB MFI
ns
ns
*

Figure 12. Co-treatment of pH_e-MIG with anti-PD-1/TIGIT antibodies enhanced the activation of $CD8^+$ T cells.

(A) Flow cytometry analyzing TNF-α or IFN-γ expression in MC38 tumor infiltrating $CD8^+$ T cells from each treatment group. Representative flow cytometric plot (above) and summary (below). Data are pooled from or representative of two independent experiments; *n* = 6 mice per each treatment group.

(B) MFI of 4-1BB surface expression on tumor infiltrating $CD8^+$ T cells from each treatment group. Data are pooled from or representative of two independent experiments; *n* = 6 mice per each treatment group. $*p < 0.05$, $**p < 0.01$ (two-tailed unpaired *t*-test).

(A)


CD8
PD-1
Lag-3
Tim-3
tSNE1
tSNE2
α-PD-1/α-TIGIT
IG
pHe-MIG
IG
pHe-MIG
tSNE1
tSNE2

(B)

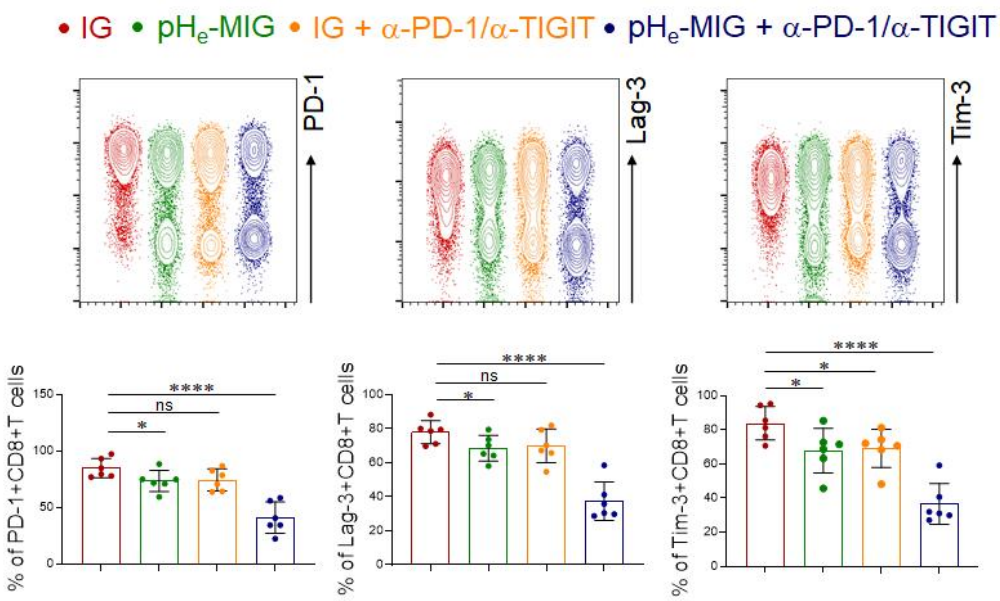

• IG • pHe-MIG • IG + α-PD-1/α-TIGIT • pHe-MIG + α-PD-1/α-TIGIT
PD-1
Lag-3
Tim-3
% of PD-1+CD8+T cells
% of Lag-3+CD8+T cells
% of Tim-3+CD8+T cells
****
ns
*

Figure 13. pH modulation of TME accumulated less exhausted $CD8^+$ T cells in TME.

(A) Measurement of immune checkpoint receptor expression in tumor infiltrating $CD8^+$ T cells. Density *t*-SNE plot of tumor infiltrating $CD8^+$ T cells overlaid with the expression of indicated surface receptor (above). *t*-SNE plots of an equal number of tumor-infiltrating $CD8^+$ T cells from each treatment group (below). Data are pooled from or representative of two independent experiments; $n = 6$ mice per each treatment group.

(B) Flow cytometry analyzing the expression of PD-1, Lag-3, or Tim-3 in tumor infiltrating $CD8^+$ T cells from each treatment group. Data are pooled from or representative of two independent experiments; $n = 6$ mice per each treatment group. $*p < 0.05$, $**p < 0.01$ (two-tailed unpaired *t*-test)

7. pH_e-MIG potentiates new approach of immune checkpoint inhibitor therapies.

A $NaHCO_3$ loaded thermosensitive hydrogel was utilized as a novel method to focally neutralize extracellular pH of the TME. Intratumoral injection of extracellular pH modulating injectable gel (pH_e-MIG) increased pH value of the TME and cytotoxic immune cell infiltration, which lead to a substantial improvement in anti-PD-1 and anti-TIGIT blockade therapies. Furthermore, this study systematically proved that the acidic extracellular pH depletes the immune responses and induce exhaustion of T cells. The extracellular acidity led to upregulation of co-inhibitory immune checkpoint receptors and inhibition of mTOR (mechanistic target of rapamycin) signaling pathways, resulting in impaired memory $CD8^+$ T cell responses **(Figure 14)**.

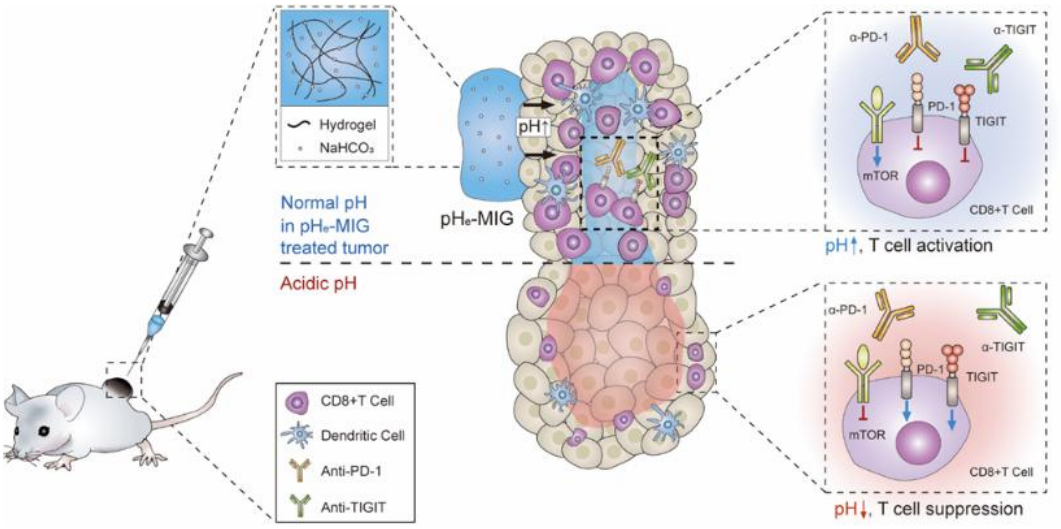


Figure 14. pH_e-MIG maximized synergy effect of immune checkpoint inhibitor therapies.

Extracellular acidity impairs the effector functions of T cells and avoids immune checkpoint inhibitor. MC38-bearing C57BL/6 mice treated with F-127 PH_e-MIG in order to accurately deliver $NaHCO_3$. pH_e-MIG mediated reversion of tumor acidity synergizes with checkpoint blockades to affect enhancing the intratumoral $CD8^+$ T cell activation and tumor killing mechanisms.

DISCUSSION

The study provides evidence that tumor acidity negatively regulates anti-tumor immune responses. Acidic pH inhibits the activation of $CD8^+$ tumor specific effector T cells in both human and murine settings [40]. FACS analysis revealed that the extracellular acidity increased the expression of co-inhibitory immune checkpoint receptors in memory $CD8^+$ T cells. Particularly, extracellular pH regulated the suppressive function of TIGIT pathway. As revealed by a cell conjugation assay, conjugation of TIGIT-CD155 was promoted at extracellular acidity condition [26]. In the light of recent excitement on anti-TIGIT therapy as the next generation checkpoint inhibitor, this study may provide an effective strategy for the clinical application of TIGIT inhibitors.

Tumor mouse model experiments showed that pH_e-MIG is an efficient modulator for focally neutralizing extracellular pH of TME. The pH_e-MIG can reversibly change its sol-gel phases responding to temperature; sol phase at 4 °C to gel phase above room temperature, which allows facile introduction of $NaHCO_3$ to the gel matrix and curing ability in TME. The biocompatibility and biodegradability of pH_e-MIG enabled to gradually neutralize pH without any cytotoxic effects to the surrounding tissues and T cells. Normalization of the acidic TME by intratumoral pH_e-MIG injection transformed the immune-suppressive TME into an immune-favorable condition, as evidenced by the decrease of immune-suppressive cells and increase of tumor infiltrating $CD8^+$ T cells. In addition, Enhanced cytolytic activity and a less exhausted profile were observed in the intratumoral $CD8^+$ T cells.

The pH_e-MIG therapy combination with a low dose of immune checkpoint inhibitors, anti-PD-1 and anti-TIGIT antibodies improved intratumoral cytotoxic T cell function and tumor clearance **(Figure 14)**. However, tumor immunosuppressive microenvironments are described as highly complex environments such as malnutrition, acidosis, hypoxia, and immune escape. Therefore, single pH_e-MIG therapy of pH modulation may have limitations

that remain to be overcome. Collectively, pH_e-MIG has a high potential to be used as a pre-conditioning regimen for T cell-directed cancer therapies, such as immune checkpoint blockade and chimeric antigen receptor (CAR) T cells. Reconditioning TME through modulation of extracellular acidity contributes not only to convert non-responders into responders, but also to reduce immune-related adverse events (IrAE) by allowing a low-dose administration of immunotherapeutics.

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국문 초록

암은 면역세포, 기질세포 및 다양한 세포와 세포 외 요소들로 이루어진 종양 미세환경 (TME)을 구성한다. 종양 미세환경은 면역 회피와 암 진행의 중요한 촉진자로 알려져 있다. 다양한 임상 데이터 분석 결과는 종양 침윤 T 세포의 증가가 환자 예후 개선과 관련이 있음을 보여주고 있다. 종양 세포의 높은 호기성(aerobic)과 활발한 해당작용율(glycolytic rate)의 원인으로, 종양 미세환경 (TME)은 세포 외 pH 가 산성인 주요한 특징을 가진다. TME 의 산성 환경은 종양내 T 세포의 침투와 T 세포의 cytotoxicity 활성을 방해하고 억제하는 면역 회피를 유도하고, 반면에 종양의 생장을 촉진하는 것으로 보고되었다. 따라서, T 세포의 cytotoxicity 활성 기능을 회복시키고 향상시키기 위해 종양의 세포 외 산도를 조절하는 것은 항암 면역 요법에서 강력한 접근법이 될 수 있다.

본 연구에서는 종양의 세포 외 산도가 면역 치료(immunotherapy)의 효능에 미치는 영향을 연구를 하였다. 먼저 생체 외(*in vitro*) 실험에서 산성 pH 환경은 항원 특이적인 기억 $CD8^+$ T 세포의 면역 반응을 억제하였다. 세포 외 산도는 co-억제 면역 체크포인트 수용체를 상향 조절하고 기억 $CD8^+$ T 세포에서 mTOR 신호 경로를 억제하는 것을 발견하였다. 이 결과는 산성 pH 환경은 기억 $CD8^+$ T 세포의 활성 작용기능(effector functions)을 저해한다는 것을 나타낸다.

또한 산성 pH 환경에서 종양 세포의 리간드 CD155 발현이 증가함을 확인하였고, T 세포 계열의 리간드 TIGIT 과 종양 세포의 리간드 CD155 결합이 증가함을 확인하였다. 이 결과는 세포외 pH 가 TIGIT pathway 의 억제하는 기능을 조절할 수 있음을 보여주고, 종양 세포에서 CD155 발현 증가는 면역 회피에 중요한 역할을 할 가능성을 나타낸다.

생체 내(*in vivo*) 실험에서 TME 의 세포 외 pH 를 중점적으로 중화시키기 위해 $NaHCO_3$ 를 지닌 하이드로겔(pH modulating injectable gel; pH_e-MIG)을 사용하였다.

하이드로겔(pH_e-MIG)은 4°C 에서 액체 상태이며 체온에서 굳는 열에 민감한 (thermosensitive) 성질에 기초하여 종양 내 주입 (intratumoral injection)이 가능하다. 종양 내 pH_e-MIG 주입에 의한 산성 TME 의 정상화는 면역 억제적인 환경을 면역 호의적인 환경으로 전환시켰다. 그 결과 종양에 침투하는 세포 독성 T 세포 (cytotoxic $CD8^+$ T cells)의 증가를 확인하였다. 또한 종양 내에 침투하는 세포를 분석한 결과 소진 T 세포 (exhausted $CD8^+$ T cells)의 특징인 억제 면역 체크포인트가 감소하는 것을 확인하였다. 이러한 결과들은 종양 내 침투된 T 세포가 직접적으로 종양세포의 세포 용해 및 세포자살을 유도하여 항암 작용이 강화됨을 보여준다. 또한 낮은 용량의 면역 관문 억제제 (항 PD-1 및 항 TIGIT 항체)와 pH_e-MIG 의 동시 치료는 종양 내 세포 독성 T 세포의 기능을 더욱 개선시키고 종양 제거에 효과적임을 확인하였다.

종합적으로, 본 연구의 결과는 pH_e-MIG 을 이용한 종양세포 외 산도의 중화는 면역 관문 억제제, 즉 면역항암제의 효과적인 치료를 위한 새로운 TME 조절인자로서 잠재력을 지니고 있음을 보여준다.