Death and Proliferation of Lymphocytes in Immune Response and Tumor Cells Are Controlled by pH Balance

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Abstract

Many biological processes are controlled by cell death and proliferation. Previous evidence suggests that cell proliferation and death by apoptosis are regulated by separate pathways. The present study found that cellular pH was positively correlated with proliferation but negatively with cell death. Alkaline treatments enhanced lymphocyte proliferation in response to antigen challenge in vivo and in in vitro cultures, whereas acid treatments induced cell death. Low pH was incompatible with the survival of highly proliferating cells, and the susceptibility to the acid-induced death was determined in part by the proliferative status of the lymphocytes. Likewise, alkaline treatments maintained tumor cell proliferation whereas acid treatments induced death. These data support a unified theory for the regulation of cell death and proliferation where a cellular pH balance controls both events, and the mitochondria as proton generators act as pH-stats. Thus, the Warburg effect is viewed as necessary for proliferating cells to have a high cellular pH environment to both survive and accelerate proliferation.

Introduction

Cell death and proliferation play fundamental roles in numerous biological processes throughout the life cycle of an organism. In humans and animals, cell death and proliferation are key to understanding immune response and tumorigenesis, two biological processes that are of particular importance for the health of an individual. An immune response commences with clonal expansion of antigen specific lymphocytes and ends with the death of most of the expanded antigen specific lymphocytes by mitochondrion- and death receptor-initiated apoptosis. (*1-4*). Likewise, the basic features of tumor cells are unrestricted proliferation and resistance to cell death. Indeed, in tumor cells, the genes for apoptosis inhibitors Bcl-2, Bcl-x Mcl-1 are over expressed or amplified whereas genes for apoptosis activators Bim, Puma, Bad and Bax are suppressed or deleted. (*5*). While the death receptor-initiated apoptosis is sufficient for the cell death under certain circumstances, it often needs to be linked to the mitochondrial process to complete cell killing such that mitochondria play a central role in apoptosis. (*6-8*).

On the other hand, mammalian cell proliferation is primarily controlled at the "restriction point" by retinoblastoma family proteins (pRB). (*9, 10*). Mitogenic signals generated by external stimulants at the cell membrane are transduced to the nucleus to induce the transcription of D and E type cyclin genes, which lead to the activation of cyclin-dependent kinases CDK4/6 and CDK2, respectively. The activated CDKs phosphorylate the pRB proteins. (*11-14*). Phosphorylation of pRB proteins dissociate with E2F family transcription factors to allow the latter to activate target gene expression, which causes the cell to pass the restriction point, transition from G1 to S phase and commit to completing the cell cycle. (*15, 16*).

Although apoptosis and G1/S transition appear to be controlled by independent pathways, the increased demand of energy and substrates for anabolic metabolism of the proliferating cells suggests that mitochondria may regulate both cell death and proliferation. On the contrary, almost a century ago Otto Warburg observed that cell malignant transformation includes a precancerous phase, in which the cell must incur irreversible "injury of respiration" or "uncoupling of respiration and phosphorylation" and switch energy production to glycolysis followed by pyruvate fermentation and excretion of the fermentation waste product lactate (herein referred to as the glucose/lactate pathway). (*17, 18*). This phenomenon has come to be known as the "Warburg Effect", and later found to be shared by both tumor and normal proliferating cells. (*19-21*). However, the glucose/lactate pathway in the cytosol is not only inefficient for energy production, but also unlikely to contribute to anabolic metabolism. This is because in this pathway only 2 ATPs per glucose are produced as opposed to a net production of 32 ATPs by glycolysis followed by TCA cycle and oxidative phosphorylation in the mitochondria; all atoms in the glucose are converted to lactate that cannot be further used by the cell and is excreted; and there is no net production of NADH or FADH as potential reducing equivalents for biomass synthesis. (*22*). Intriguingly, unlike originally suggested by Warburg, mitochondria in most tumor cells and normal proliferating cells are not "injured" but functional. (*21, 23*). Given that most tumor microenvironments are poor in nutrients (*24*), it is puzzling why the inefficient glucose/lactate pathway predominates in the tumor cells.

In this article, it was found that cell proliferation positively whereas apoptosis negatively correlates with pH. Alkaline treatment enhanced cell proliferation whereas

acid treatment preferentially induced death of proliferating cells. These findings support a unified theory of cell fate determination that a cellular pH balance dictates whether a cell dies or proliferates, and the mitochondria as proton generators via carbohydrate catablolism act as "pH-stats". Thus, the Warburg effect or the tuning down of mitochondrial energy production is viewed as necessary for highly proliferating cells to avoid low pH-induced death and create a high pH environment for proliferation.

Results

The current study stemmed from an unexpected finding of thymic atrophy in animals that received a test compound prepared in an acid solution. (Manuscript in preparation). Since thymus is one of a few organs in adults that maintain robust cell proliferation, this observation led to the investigation of whether pH regulates immune responses by affecting lymphocyte survival and proliferation in the draining lymph nodes (DLN) where immune responses to local antigen exposure take place.

Positive relationship between pH value and lymphocyte proliferation

Experiments were performed to determine whether there is a natural correlation between cellular pH and proliferation. Peripheral lymphocytes were labeled with CFSE, and cultured either with IL-2 alone or IL-2 and anti-CD3 antibodies for the study of T cell proliferation, or alternatively with either IL-4 alone or IL-4 and the B cell mitogen LPS for the study of B cell proliferation. Lymphocyte proliferation was measured by the serial dilution of the CFSE signals after each cell division, whereas the intracellular pH was measured by the fluorescence intensity of the pH indicator pHrodo™ Red AM that inversely correlates with intracellular pH. Proliferating lymphocytes were detected even

in cultures with IL-2 or IL-4 alone, indicating the pre-existence of proliferating cells prior to mitogenic stimulation. (Figure 1a and b). As expected, proliferating cells ($CFSE^{lo}$) increased with concurrent decrease of non-proliferating cells ($CFSE^{hi}$) after stimulation by anti-CD3 antibodies or LPS; and even the pre-existent proliferating cells underwent further divisions as evidenced by the decrease of the CFSE signals. (Figure 1a and b). Importantly, the highly proliferating cells that had undergone more than 3 divisions were almost exclusively in the cell population with high intracellular pH, whereas cells that had fewer than 3 divisions were in cell populations of low intracellular pH. (Figure 3.1a and b). Within the highly proliferating cells, the higher numbers of cell divisions correlate with higher intracellular pH. (Figure 1a and b).

Effects of treatments with pH modifiers on the total number of MLN cells

To investigate whether lymphocyte survival and proliferation in an active immune response in vivo could be affected by acid and alkaline treatments, ovalbumin (OVA)-sensitized mice were intra-tracheally challenged with OVA, and the lung-draining lymph nodes mediastinal lymph nodes (MLN) were analyzed. In the first set of experiments, MLNs were collected from mice 3 days after the first OVA challenge and 1 day after a single treatment with saline or saline containing HOAc or HCl. In the second set of experiment, MLNs were collected 7 days after the initial challenge and 3 days after 3 treatments with saline or saline containing HOAc, HCl or NaOH. (Figure 2a and b, upper panels). The average numbers of total MLN were greatly reduced in HOAc-treated mice as compared with those of the saline-treated mice (0.75×10^7 vs. 4.5×10^7, or 84% reduction) in the fist set of experiments. In the second set of experiments in which MLNs were collected at a later time, the reduction of total MLN cells in the HOAc-treated mice

as compared with the saline-treated mice was also dramatic but less severe than in the first set of experiments (4.23 x 10^7 vs. 5.96 x 10^7, or 29% reduction), which suggested on-going replenishment of the MLNs with lymphocytes after the HOAc treatment. The total MLN cells were also significantly reduced in HCl-treated mice but less dramatic than in the HOAc-treated mice in either the first (4.0 x 10^7, or 12% reduction) or second (5.6 x 10^7, or 5.5% reduction) set of experiments. In contrast, treatment with NaOH increased total MLN cells (7.9 x 10^7, or 32% increase). (Figure 2a and b, lower panels). The differences in the total MLN cells were not due to differences in Treg cell populations because there were similar percentages of Treg cells in CD4 T cells among the different treatment groups. (Manuscript in preparation).

Effects of treatments with pH modifiers on different lymphocyte populations in MLNs

Total lymphocytes, CD4, CD8 T cells and B cells in MLNs were further analyzed. The majority of the total lymphocytes in the MLNs in the first set of experiments had relatively small sizes and low granularities, and were referred to as Lym1. In addition, some lymphocytes were of larger sizes and higher granularities, and were referred to as Lym2, which were likely lymphoblasts. (Figure 3a, upper right). The percentages of both the Lym1 and Lym2 in MLN cells were greatly reduced in the HOAc-treated mice as compared with those in the saline-treated mice (32.4% vs. 73.7% and 0.35% vs. 2.26% for Lym1 and Lym2, respectively). The percentage of Lym1 was also somewhat lower in the HCl-treated mice (68.3%), but the Lym2 percentage was similar 2.83%) to saline-treated mice. (Figure 3a, upper right). Within the Lym1 population, the percentages of CD4, CD8 T cells and B cells in the HOAc-treated mice were all lower than those of the saline-treated mice (8.17% vs. 39.6%, 1.75% vs. 13.9%, and 22.2% vs. 42.5%,

respectively), and similar reductions were also observed in the Lym2 lymphocytes (3.23% vs. 14.2%, 2.7% vs. 9.69%, and 46.9% vs. 62.6%). To lesser degrees, the percentages of CD4, CD8 T cells and B cells (13.4%, 8.2% and 44.8%, respectively) in Lym2 of the HCl-treated mice were also reduced. In contrast, within the Lym1 only the percentage of B cells (34.4%) of the HCl-treated mice was reduced. (Figure 3a, lower).

In the second set of experiments or at a later time of MLN collection, the Lym2 populations had expanded as compared with the first set of experiments, and the combined percentages of Lym1 and Lym2 were similar in all treatment groups, even in the HOAc-treated mice, again indicating replenishment of MLNs with lymphocytes. (Figure 3c, upper right). The relative proportions of Lym1 and Lym2 were similar among the saline, HCl and NaOH treatment groups. However, the proportion of the Lym2, which as shown in Figure 3c lower right panels consisted mostly of B lymphoblasts, in the HOAc-treated mice was greatly increased as compared with the saline-treated mice (38.7% vs. 8.10%). (Figure 3c, upper and lower right). This result shows that B lymphoblasts were less susceptible to depletion by HOAc than T lymphoblasts, therefore were selectively retained in the MLNs.

Within the Lym1 in the second set of experiments, the percentages of CD4 and CD8 T cells in the HOAc-treated mice were lower than those of the saline-treated mice (44.3% vs. 54.9% or 19.3% reduction, and 7.39% vs. 13.9% or 46.8% reduction), whereas the percentage of B cells were higher (43.8% vs. 27.3%), once again showing that B cells were less susceptible than T cells to depletion. The percentages of CD4, CD8 T cells and B cells in Lym1 of the HCl-treated mice were similar to those of the saline-treated mice. In the NaOH-treated mice percentages of CD4 (45.2%) and CD8 (10.2%)

were slightly reduced, whereas that of B cells (38.4%) was increased. (Figure 3c, lower left). Within the Lym2 populations, the reductions of the percentages of CD4 and CD8 T cells in the HOAc-treated mice were even greater than in Lym1 (0.8% vs. 6.91% or 88% reduction, and 0.35% vs. 4.21% or 91.7% reduction), consequently the percentage of B cells proportionally increased (93% vs. 84%). In contrast, the percentage distributions of CD4, CD8 T cells and B cells in Lym2 of the HCl- and NaOH-treated mice were similar to those of the saline-treated mice. (Figure 3c, lower right).

Thus, acid treatments reduced, whereas alkaline treatment increased, the number of MLNs cells during active immune responses. HOAc treatment appeared to exert stronger effects than HCl treatment. T cells were more susceptible than B cells to the acid-mediated depletion.

Effects of treatments with pH modifiers on cell proliferation in MLNs

The proliferative statuses of the lymphocytes in the MLNs were determined by their expression of Ki-67. Ki-67 is a widely used, dependable marker of proliferating cells; its level of expression positively correlates with rRNA and DNA synthesis. (*25*).

In the first set of experiments, the majority of the Lym1 cells expressed only low levels of Ki-67 but small percentages of the Lym1 cells expressed high levels of Ki-67. In the HOAc-treated mice, the percentage of Ki-67^{hi} Lym1 cells was less than half of that of the saline-treated mice (1.65% vs. 3.31%), the percentage of the Ki-67^{hi} Lym1 cells in the HCl-treated mice (2.92%) was also reduced. In the HOAc-treaed mice, even the percentage of Ki-67^{lo} cells in Lym1 was also reduced (32.9% vs. 80.4%), whereas the percentage of Ki-67^{lo} Lym1 cells in HCl-treated mice was similar to that of the saline-treated mice. (Figure 3b). The Lym2 cells were overall more proliferative than the Lym1

cells. In the HOAc-treated mice the percentage of Ki-67^{hi} Lym2 cells was less than half of that of the saline-treated mice (27.2% vs. 54.5%), the percentage of Ki-67^{hi} Lym2 cells in HCl-treated mice (49.1%) was also reduced. (Figure 3b).

Thus, proliferating lymphocytes in the MLNs were preferentially depleted by acid treatments. Lymphocytes of high proliferative status were more susceptible to depletion than those of low proliferative status, which in turn were more susceptible than the non-proliferating cells.

Since B cells in MLNs were less susceptible to depletion by acid treatments, experiments were conducted to determine whether proliferative status of B cells nonetheless also correlates with susceptibility to depletion by acids. In both the Lym1 and Lym2, the percentages of Ki-67^{hi} B cells were the lowest in HOAc-treated mice (1.58% and 37.9%, respectively), followed by those in HCl-treated mice (2.79% and 51.1%), and highest in the saline-treated mice. (3.91% and 62.6%). (Figure 3b).

In the second set of experiments, the percentages of Ki-67^{hi} Lym1 cells were lower in HCl- or HOAc-treated mice (2.42% and 2.67%), but higher in NaOH-treated mice (3.48%), than that of saline-treated mice (2.73%). The percentages of Ki-67^{hi} B cells in Lym1 were also lower in the HCl- or HOAc-treated mice (3.62% and 2.38%) than, but similar in the NaOH-treated mice (4.23%) to, that of the saline-treated mice (4.29%). (Figure 3d, upper panels). The percentage of Ki-67^{hi} Lym2 cells in HOAc-treated mice (25.3%) was lower, whereas that of NaOH-treated mice (42.2%) was higher, than that of saline-treated mice (34.9%). The percentages of Ki-67^{hi} B cells in Lym2 followed the same pattern, with those of saline-, HOAc- and NaOH-treated mice being 35.2%, 24.9% and 44.7%, respectively. The percentages of Ki-67^{hi} Lym2 and Lym2 B

cells in the HCl-treated mice (34.9% and 35.5%) were similar to those of the saline-treated mice. (Figure 3d, lower). However, the mean fluorescence intensities of the Ki-67^{hi} Lym2 and Lym2 B cells of the HCl-treated mice, like those of the HOAc-treated mice, were much lower, whereas they were higher in NaOH-treated mice, than those of the saline-treated mice. (Figure 3e). Thus, acid treatments not only preferentially depleted Ki-67^{hi} cells but also lower their proliferative profiles, whereas alkaline treatment had the opposite effects.

Effects *of in vitro treatments with pH modifiers on proliferating lymphocytes*

Due to the complexity of the in vivo environment, it cannot be definitively determined whether the effects of in vivo treatments with the pH modifiers on MLN lymphocytes were direct or indirect. Further, the fluidity of the in vivo environment could mask the full effects of the pH modifiers. For example, circulating and/or newly activated lymphocytes could replenish the MLNs after lymphocyte depletion by acid treatments. Moreover, the kinetics of absorption and the subsequent transportation of the pH modifiers into and out of the MLNs are unknown. As such, the actual duration and concentrations of pH modifiers at which the pH modifiers acted in the MLNs could not be determined.

To by pass these complexities and better assess the full potentials of the pH modifiers, in vitro experiments were carried out, in which primary MLN cells were treated with saline or saline containing the pH modifiers in 100% FBS in a 37 ^{o}C water bath. Such in vitro cultures were designed to be free of interference by pH buffering agents in common tissue culture system. As shown in Figure 4, the in vitro treatments with HCl or HOAc eliminated almost all Ki-67^{+} (Ki-$67^{hi\ and\ lo}$) cells in total lymphocytes,

the CD4, CD8 T cells and B cells. In contrast, in vitro NaOH treatment more than tripled the percentages of Ki-67^{+} cells in total lymphocytes and CD8 T cells, and nearly tripled in the CD4 T cells and B cells. Thus, the in vitro results collaborated the in vivo observation that acid treatments preferentially depleted, whereas alkaline treatment increased, the proliferating lymphocytes. The in vitro treatments showed stronger effects than the in vivo treatments, which was likely due to higher concentration and longer time of action of the pH modifiers in the in vitro cultures than in the MLNs in vivo.

Acid treatment induced lymphocyte apoptosis

Lymphocytes were treated with saline or saline containing HOAc then analyzed by annexin V and 7-AAD staining. In the saline-treated cells, 16.4% were early apoptotic (Annexin V^{+} 7AAD$^{neg\text{-}lo}$), and 0.36% were dead (Annex V^{+} 7AAD^{hi}) cells. In HOAc-treated cells, 44.7% were early apoptotic, and 54.3% were dead cells. (Figure 5). Therefore, the acid treatment induced cell death by apoptosis.

Effects of treatments with pH modifiers on tumor cell viability and proliferation

Cell proliferation is a common basic feature of immune response and tumorigenesis. To determine weather the pH modifiers would have effects on tumor cells similar to those on proliferating lymphocytes, the human T and B cell leukemia cell lines Jurkat and Raji, respectively, were treated with saline or saline containing HCl, HOAc or NaOH. Compared with saline treatment, HCl and HOAc treatments greatly reduced the viabilities of the Jurkat (22.1% and 34.3% vs. 73/4%) and Raji (49.2% and 48.9% vs. 89.4%) cells. In contrast, NaOH treatment only slightly decreased the viabilities of Jurkat and Raji cells (70.4% and 76.2%, respectively). (Figure 6a). The Jurkat cells were further analyzed for Ki-67 expression. After saline treatments, the majorities of live

Jurkat cells remained Ki-67$^+$ (71.7%). However, few, if any, (<2%) live Jurkat cells after the HCl or HOAc treatments expressed Ki-67. In contrast, the percentage of Ki-67$^+$ live Jurkat cells after NaOH treatment was similar to that after saline treatment. (Figure 6b).

Discussion

This article studies the impact of pH on the survival and proliferation of normal lymphocytes in an active immune response and in tumor cells. It was found that cell proliferation is positively whereas cell death by apoptosis is negatively correlated with pH. Therefore, cellular pH balance determines whether a cell proliferates or dies. When the balance tips towards higher pH, cell proliferation increases, when it tips lower the cell dies. In agreement, alkaline treatments enhanced cell proliferation whereas acid treatments induced cell death. Highly proliferative cells were more susceptible than modestly proliferating cells, which in turn were more susceptible than non-proliferating cells, to acid-induced cell death. Thus, a high pH environment is not only favorable for proliferation but also necessary for the survival of the proliferating cells.

Mitochondria are not only energy but also proton generators. Catabolizing 1 glucose molecule through glycolysis and mitochondrial pathways produce 6 CO_2, 10 NADH and 2 FADH. CO_2 is converted to carbonic acids in the cell by carbonic anhydrase, which produces protons. (*26, 27*). NADH and FADH are fed to the electron transport chain to create mitochondrial membrane potential and proton gradient to drive ATP synthesis. However, up to 20 to 25% of mitochondrial respiration is uncoupled from ATP synthesis, and instead is expended to drive "futile" proton leak across mitochondrial membranes, which can also cause net increases of protons in the cell. (*28,*

29). Consequently, if mitochondrial respiration were to increase to meet the heightened energy demand of proliferating cell, it would be incompatible with a high cellular pH environment for proliferation, but instead tip the cellular pH balance lower to trigger apoptosis. In contrast, the glucose/lactate pathway produces no CO_2 and no net NADH and FADH, therefore is pH neutral after the lactate is excreted from the cell.

Thus, the Warburg effect could be the strategy of the proliferating, especially highly proliferating, cells to satisfy the requirement of a high cellular pH environment for proliferation and survival. This provides an alternative explanation for the Warburg effect to the theory that Warburg effect is required for satisfying increased demand for substrates of biomass synthesis. (*20, 30*). The biomass theory suggests that the use of the mitochondrial pathways for energy production might cause over production of ATP, which could inhibit glycolysis resulting in insufficient supply of glycolytic intermediates for biomass synthesis. While both the pH balance and the biomass theories need to be validated by further experimental evidence, logically they are not mutually exclusive.

The Warburg effect is evolutionary conserved as similar phenomena are observed across species. For example, yeast growth accelerates as glucose concentration increases. However, when the yeast growth and glucose concentration reach critical points, the yeasts switch energy production from oxidative respiration to fermentation. This phenomenon is known as Crabtree effect. (*31, 32*). It is conceivable that the switch is necessary for the yeasts' survival because if oxidative respiration continues to increases, it could lower cellular pH to induce cell death. In bacteria, the Entner-Doudoroff glycolytic pathway is low efficient for energy production as it produces only 1 ATP per glucose, but counter intuitively it is much more common among aerobic and facultative

than anaerobic bacteria, suggesting this pathway is needed to substitute for aerobic respiration. (*33*). Indeed, like the yeasts, bacteria switch to energetically inefficient metabolism when growth rate increases to a critical level. (*34*). Similarly, parasites that cause major parasitic diseases such as malaria, schistosomiasis, trypanosomiasis and leishmaniasis switch to glycolysis during the stages of their life cycles in mammalian hosts, likely to avoid cell death. (*35-37*).

The evolutionary conservation suggests that pH modifiers can be used to control not only immune response and tumorigenesis as shown in this study but also the survival and proliferation of pathogens. This is particularly important considering that the increase of antimicrobial resistance has become a major health threat. For example, the prevalence of vancomycin-resistant *Staph. aureus* (VRSA) has increased from 2% before 2006 to 7% in 2015-2020. (*38*). Based on the present study, new therapies can be developed to use pH modifiers to tilt pH balance to induce cell death or to diminish propagation of pathogens regardless of their drug resistant abilities.

The present study provides potentially new ways to either enhance or suppress immune and/or inflammatory responses. In situations such as cancers and infectious diseases where there is insufficient protective immunity, pH modifiers that increase or resist the fall of pH could be used to promote lymphocyte proliferation therefore enhance immune and inflammatory responses against the infectious agents or cancer cells. In other situations where overzealous immune or inflammatory responses impair normal tissue structure and functions, pH modifiers that decrease pH or resist the rise of pH may be used to dampen the overzealous immune or inflammatory responses. Overzealous immune and inflammatory responses occur in various clinical conditions, for examples,

allergic and autoimmune diseases, and infectious disease where immune and inflammatory responses to infections cause collateral damages to tissues. Such treatments could also simultaneously suppress tissue cell hyperplasia that often accompanies inflammation.

Further, the present study provides new insights into the role of pH in tumorigenesis. Previous study has found that acidic tumor microenvironment (TME) promotes tumor invasion of local tissue. (*39*). Therefore, it has been believed that acidic environment is favorable for tumorigenesis, and neutralizing the acidic TME by alkaline treatment has been proposed as anti-tumor therapy. (*40*). However, according to the present study, low pH is unfavorable to tumor cell proliferation and could induce apoptosis. Thus, contrary to the previous view, the present study shows that lowering rather than raising pH could be a potentially new anti-tumor therapy by directly inducing tumor cell apoptosis. Lowering pH could be achieved by inducing accumulation of protons in the tumor cells through over active mitochondrial respiration or inhibiting the export of protons or acids to the extracellular space or the import of bicarbonate or other alkaline substances to the cells. Alternatively, lowering pH could be achieved by exogenous reduction of pH in tumor extracellular space, for example by acid treatment as in this study, that over powers the tumor cells' ability to maintain high intracellular pH. Since small molecular pH modifiers such as acids can permeate all tissues, they could be particularly anti-tumor agents at the early or metastatic phases of tumorigenesis when the tumor cells are difficult to locate. In contrast, the present study shows that alkaline treatment carries an inherent risk of increasing tumor cell proliferation, nonetheless,

patients could benefit from its enhancement of proliferation of anti-tumor lymphocytes or other inflammatory cells.

A related aspect of the findings in the present study is that different cells varied in their responses to pH modifiers. For example, B cells in the MLNs were less susceptible to acid-induced depletion than T cells. The exact mechanisms for the difference is unknown, but could be attributable to their different expression and/or activities of the enzymes and membrane transporters responsible for pH homeostasis, or different anatomical distribution in the lymph nodes. In addition, different pH modifiers of the same class could have varied effects on the same cells. For example, HOAc was found to have stronger effects than HCl in inducing lymphocyte death in the MLNs. Furthermore, the stronger effects of in vitro treatments than in vivo treatments showed that the concentration or dose of pH modifiers and the duration of treatment are also important factors that determine the effects of pH modifiers. These features can be exploited to selectively target pathological cells or protective normal cells for the treatment and prevention of diseases such as inflammatory, neoplastic and infectious diseases where cell proliferation contributes to pathogenesis. Thus, by selecting pH modifiers, their doses and dosing regimens, it is possible to achieve selective inhibition and stimulation of pathological cells and protective normal cells, respectively. Such strategy may also be applied to vaccine design to enhance or shape the types of immune responses to the vaccines by selectively promoting or suppressing certain lymphocyte subsets.

As indicated by the foregoing discussion, it should be pointed out that pH modifiers for disease treatment and prevention should include not only acids, bases and pH buffering agents, but also ionophores, and inhibitors and stimulators of the expression

and activities of enzymes (e.g., carbonic anhydrases), membrane transporters (e.g., monocarboxylate transporters, Na^+/H^+ exchangers) and ion channels that that are responsible for the maintenance and change of pH in and around a cell.

Finally, although it is generally believed that the pH in a human or animal is maintained at a stable level of about 7.45, humans can experience transient severe acidosis without serious pathological consequences in the aftermath. (*41-43*). In the present study, local administration of pH modifiers in the lungs or abdomen was well tolerated by the animals. Therefore, disease treatments and prevention with pH modifiers can be reasonably expected to be well tolerated by human and animal subjects.

Materials and Methods

CFSE labeling and activation of lymph node cells

Lymph node cells were washed 3 times with plain PBS. After the wash, the cells (3-5 x 10^6/ml) were incubated in 1μm Carboxyfluorescein succinimidyl ester (CFSE) (Fluka/Sigma-Aldrich, Burlington, VT) in plain PBS at room temperature for 7 minutes. After the incubation, 1/4 volume of FBS was added to stop the labeling, and cells were washed 4 times with PBS plus 1% FBS. The labeled cells were cultured with IL-2 (20 units/ml) or IL-2 plus anti-CD3 antibody (1μg/ml) (BD Pharmingen (San Diego, CA). Alternatively, the labeled cells were cultured with IL-4 (4ng/ml) or IL-4 plus lipopolysaccharide (LPS) (10μg/ml) (Sigma-Aldrich, St. Louis, MO). Two and half days later, cells were harvested for further experiments.

Measuring intracellular pH

Intracelluar pH of the CFSE labeled and active cells was measured using the Intracellular pH detection pack according to the manufacture's instruction. (ThermoFisher Scientific, Waltham, MA). containing the pH indicators pHrodo™ Green AM sd and the PowerLoad was purchased from ThermoFisher Scientific (Waltham, MA). Briefly, cells were washed once with Live Cell Image Solution (LCIS) (Life Technoology/ThermoFisher Scientific, Grand Island, NY). Immediately prior to use, the pH indicator pHrodo™ Red AM and the PowerLoad were mixed then diluted in the LCIS to produce working solution containing 0.5-1μM pH indicator. The cells (1-2 x 10^7/ml) were suspended in the working solution and incubated in a 37°C water bath for 30 minutes. After the incubation, the cells were washed once in LCIS containing 1% FBS, and stained with Zombie-Violet viability dye. (Biolegend, San Diego, CA). Fluorescence emitted by the pH indicators was detected by flow cytometry.

OVA sensitization, challenge and treatments of mice

Animal studies were performed according to the protocols approved by the Charles River Accelerator and Development Lab (CRADL) (Cambridge, MA, USA) Institutional Animal Care and Use Committee (IACUC), and essentially as previously described. (Rangasamy T, Guo J, 2005; Zeng WP, McFarland MM, 2017). Briefly, Balb/c mice were sensitized by i.p. injection of 20μg OVA (Sigma-Aldrich, St. Louis, MO) plus Alum adjuvant (Thermo Scientific, Rockford, IL). Two weeks later, the sensitization is repeated with 500μg OVA. About 2.5 weeks later, mice were challenged with 100μg OVA in saline by intratracheal instillation, and challenges were repeated as described in each experiment. For acid treatments, 60μl saline containing 175mM HOAc or HCl alone with OVA challenge. Since mice were less tolerant to NaOH by

intratracheal instillation, NaOH treatments were carried out by i.p. injection of 200μl of saline containing 87.5mM NaOH. After the challenge and treatment, mediatinal lymph nodes were collected at the times specified in the different experiments.

In vitro treatments of lymphocytes and tumor cells

Lymph node cells (4 x 10^6 cells/ml) were incubated in FBS containing 10% of saline or 87.5mM HCl, HOAc or NaOH prepared in saline in 37°C water bath for 5 hours before analyses. The Jurkat and Raji cells (2 x 10^6 cells/ml) were incubated in FBS containing 10% saline or 87.5mM HCl, HOAc or NaOH prepared in saline in 37°C water bath for 3-5 hours.

Flow cytometry

Fluorochrome-conjugated anti-mouse antibodies against CD4, CD8, CD19 and Ki-67 (PE), Zombie-Green and Zombie-Violet fixable viability dyes and Foxp3 buffer set were purchased from Biolegend (San Diego, CA). Lymphocytes were first stained with Zombie viability dye, followed by staining for cell surface marker CD4, CD8 and CD19. The cells were then fixed with CytoFix/CytoPerm buffer, washed with PBS plus 1% FBS and 1x CytoPerm buffer, and stained for Ki-67. Human Jurkat cells were stained with Zombie dye followed by fixation, permealization and staining with anti-human Ki-67 (PE). (Biolegend, San Diego, CA). For apoptosis assay, cells were washed twice with plain PBS and once with Annexin V binging buffer (10mM HEPES, pH7.4, 140mM NaCl, 2.5mM $CaCl_2$) and incubated with fluorochrome-conjugated Annexin V in Annexin V binding buffer for 15min at room temperature. The cells were washed twice in Annexin V binding buffer, and resuspended in Annexin V binding buffer. 7-AAD was added to the cells before analyzed by flow cytometry.

References

1. M. Adachi, R. Watanabe-Fukunaga, S. Nagata, Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* **90**, 1756-1760 (1993).
2. F. Burnet, *The Clonal Selection Theory of Acquired Immunity*. (Cambridge University Press, London, 1959).
3. D. A. Hildeman, Y. Zhu, T. C. Mitchell, J. Kappler, P. Marrack, Molecular mechanisms of activated T cell death in vivo. *Curr Opin Immunol* **14**, 354-359 (2002).
4. X. Liu, C. N. Kim, J. Yang, R. Jemmerson, X. Wang, Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147-157 (1996).
5. P. E. Czabotar, G. Lessene, A. Strasser, J. M. Adams, Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* **15**, 49-63 (2014).
6. P. J. Jost *et al.*, XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature* **460**, 1035-1039 (2009).
7. H. Li, H. Zhu, C. J. Xu, J. Yuan, Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491-501 (1998).
8. X. Luo, I. Budihardjo, H. Zou, C. Slaughter, X. Wang, Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**, 481-490 (1998).

9. S. H. Friend *et al.*, A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**, 643-646 (1986).

10. R. A. Weinberg, The retinoblastoma protein and cell cycle control. *Cell* **81**, 323-330 (1995).

11. T. Akiyama, T. Ohuchi, S. Sumida, K. Matsumoto, K. Toyoshima, Phosphorylation of the retinoblastoma protein by cdk2. *Proc Natl Acad Sci U S A* **89**, 7900-7904 (1992).

12. M. E. Ewen *et al.*, Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**, 487-497 (1993).

13. P. W. Hinds *et al.*, Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**, 993-1006 (1992).

14. J. Kato, H. Matsushime, S. W. Hiebert, M. E. Ewen, C. J. Sherr, Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev* **7**, 331-342 (1993).

15. S. P. Chellappan, S. Hiebert, M. Mudryj, J. M. Horowitz, J. R. Nevins, The E2F transcription factor is a cellular target for the RB protein. *Cell* **65**, 1053-1061 (1991).

16. N. Dyson *et al.*, Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRB-associated E2F-1. *J Virol* **67**, 7641-7647 (1993).

17. O. Warburg, Versuche an überlebendem Carcinomgewebe. *Biochem Zschr* **142**, 317–333 (1923).

18. O. Warburg, On the origin of cancer cells. *Science* **123**, 309-314 (1956).

19. I. Martinez-Reyes, N. S. Chandel, Cancer metabolism: looking forward. *Nat Rev Cancer* **21**, 669-680 (2021).

20. M. G. Vander Heiden, L. C. Cantley, C. B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033 (2009).

21. P. Vaupel, G. Multhoff, The Warburg Effect: Historical Dogma Versus Current Rationale. *Adv Exp Med Biol* **1269**, 169-177 (2021).

22. L. Stryer, *Biochemistry*. (W. H. Freeman and Company, New York – Basingstoke, ed. 4th, 1995).

23. M. Potter, E. Newport, K. J. Morten, The Warburg effect: 80 years on. *Biochem Soc Trans* **44**, 1499-1505 (2016).

24. G. Grasmann, E. Smolle, H. Olschewski, K. Leithner, Gluconeogenesis in cancer cells - Repurposing of a starvation-induced metabolic pathway? *Biochim Biophys Acta Rev Cancer* **1872**, 24-36 (2019).

25. Z. Darzynkiewicz *et al.*, Initiation and termination of DNA replication during S phase in relation to cyclins D1, E and A, p21WAF1, Cdt1 and the p12 subunit of DNA polymerase delta revealed in individual cells by cytometry. *Oncotarget* **6**, 11735-11750 (2015).

26. N. U. Meldrum, F. J. Roughton, Carbonic anhydrase. Its preparation and properties. *J Physiol* **80**, 113-142 (1933).

27. B. C. Tripp, K. Smith, J. G. Ferry, Carbonic anhydrase: new insights for an ancient enzyme. *J Biol Chem* **276**, 48615-48618 (2001).

28. V. R. Fantin, J. St-Pierre, P. Leder, Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **9**, 425-434 (2006).

29. D. F. Rolfe, G. C. Brown, Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* **77**, 731-758 (1997).

30. N. N. Pavlova, C. B. Thompson, The Emerging Hallmarks of Cancer Metabolism. *Cell Metab* **23**, 27-47 (2016).

31. T. Pfeiffer, A. Morley, An evolutionary perspective on the Crabtree effect. *Front Mol Biosci* **1**, 17 (2014).

32. N. Ziv, N. J. Brandt, D. Gresham, The use of chemostats in microbial systems biology. *J Vis Exp*, (2013).

33. A. Flamholz, E. Noor, A. Bar-Even, W. Liebermeister, R. Milo, Glycolytic strategy as a tradeoff between energy yield and protein cost. *Proc Natl Acad Sci U S A* **110**, 10039-10044 (2013).

34. G. N. Vemuri, E. Altman, D. P. Sangurdekar, A. B. Khodursky, M. A. Eiteman, Overflow metabolism in Escherichia coli during steady-state growth: transcriptional regulation and effect of the redox ratio. *Appl Environ Microbiol* **72**, 3653-3661 (2006).

35. C. A. Homewood, Carbohydrate metabolism of malarial parasites. *Bull World Health Organ* **55**, 229-235 (1977).

36. J. Moyersoen, J. Choe, E. Fan, W. G. Hol, P. A. Michels, Biogenesis of peroxisomes and glycosomes: trypanosomatid glycosome assembly is a promising new drug target. *FEMS Microbiol Rev* **28**, 603-643 (2004).

37. H. You, R. J. Stephenson, G. N. Gobert, D. P. McManus, Revisiting glucose uptake and metabolism in schistosomes: new molecular insights for improved schistosomiasis therapies. *Front Genet* **5**, 176 (2014).

38. Q. Wu *et al.*, Systematic review and meta-analysis of the epidemiology of vancomycin-resistance Staphylococcus aureus isolates. *Antimicrob Resist Infect Control* **10**, 101 (2021).

39. V. Estrella *et al.*, Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res* **73**, 1524-1535 (2013).

40. C. Corbet, O. Feron, Tumour acidosis: from the passenger to the driver's seat. *Nat Rev Cancer* **17**, 577-593 (2017).

41. J. Bangsbo, L. Johansen, T. Graham, B. Saltin, Lactate and H+ effluxes from human skeletal muscles during intense, dynamic exercise. *J Physiol* **462**, 115-133 (1993).

42. E. Hopkins, S. Sharma, in *Physiology*. (StatPearls Publishing, NCBI Bookshelf, 2019).

43. C. E. Orringer, J. C. Eustace, C. D. Wunsch, L. B. Gardner, Natural history of lactic acidosis after grand-mal seizures. A model for the study of an anion-gap acidosis not associated with hyperkalemia. *N Engl J Med* **297**, 796-799 (1977).

Figure Legends

Figure 1. Positive correlation between pH and proliferation rate of lymph node cells. Total lymph node cells were labeled with CFSE and (a) cultured with IL-2 only (left panels), or IL-2 and anti-CD3 antibodies; and alternatively (b) cultured with IL-4 or

IL-4 and LPS. The cultured cells were sequentially stained with the intracellular pH pHrodo™ Red AM and Zombie-Violet. Pseudocolor plots and histograms of flow cytometric analyses of live cells are shown. Numbers in the pseudocolor plots are the percentages of cells in their respective quadrants.

Figure 2. Total cell numbers of MLNs. The top panels in (a) and (b) are is a schematics illustrating the schedules for OVA challenges and treatments with saline, or saline plus HCl, or HOAc of OVA-sensitized mice for the first (a) and second (b) sets of experiments. Arrows indicate the time points for the challenges or treatments. Lower panels are graphs showing the average total numbers of MLN cells of mice in the different treatment groups in the first (a) and second (b) set of experiments. Data were pooled from 3 experiments. Statistical significance between saline and the other groups were determined by Student t test; * $p < 0.05$, ** $p < 0.01$.

Figure 3. Lymphocyte populations and proliferation in the MLNs. (a and c) Lymphocyte populations in the MLNs of representative mice of the different treatment groups in the first (a) and second (c) sets of experiments. Top left panels are the same schedules for the challenges and treatments of the mice as in Figure 2. The top right panels are flow cytometry pseudocolor plots showing the gates of two lymphocyte populations (Lym1 and Lym2). Lower panels are pseudocolor plots showing CD4 T cells (Q3), CD8 T cells (Q1) and B cells ($CD19^+$) (Q5) in Lym1 (left) and Lym2 (right). Numbers in the plots are the percentages of each cell populations. (b and d) Ki-67 expression in the different lymphocyte populations in the representative mice of the different treatment groups in the first (b) and second (d) sets of experiments. Left panels are histograms showing the expression of Ki-67 in Lym1 and Lym2 and their respective

B cell populations. Numbers are the percentages of Ki-67 high, low or negative cells. Right panels are graphic presentation of the percentages of Ki-67^{hi} and Ki-67^{lo} cells shown in the histograms. (e) Graph showing the mean fluorescence intensities of Ki-67^{hi} cells in the Lym2 populations and its B cells of the different treatment groups in the second set of experiments.

Figure 4. Ki-67 expression by different lymphocyte populations of lymph node cells treated in vitro. Primary lymph node cells were incubated in FBS containing 10% saline or saline containing HCl, HOAc or NaOH. Live cells were analyzed by flow cytometry determine Ki-67 expression in total lymphocytes, CD4, CD8 T cells and B cells (CD19^{+}). Left panels are histograms of Ki-67 staining. Numbers in the histograms are percentages of Ki-67^{+} cells in the different lymphocyte populations. Right panel is graphic presentation of the percentages of Ki-67^{+} cells in the different lymphocyte populations shown in the histograms.

Figure 5. Induction of lymphocyte apoptosis by HOAc treatment. Lymphocytes treated with saline or saline containing HOAc were stained with Annexin V and 7AAD, and analyzed by flow cytometry. Pseudocolor plots are shown. Populations of early apoptotic and dead cells are indicated. Numbers in the plots are percentages of cells in each quadrants.

Figure 6. Effects of pH modifiers on viability and Ki-67 expression of tumor cells. (a) Comparison of average viabilities of Jurkat and Raji cells by Tryplan Blue staining in the different treatment groups as indicated. Statistical significance of differences between saline and other treatment groups was determined by Student t test, * $p < 0.05$, ** $p < 0.01$. (b) Histograms of Ki-67 staining in live Jurkat cells in the different

treatments as indicated. The percentages of Ki-67^{+} cells are shown in the histograms. Representatives of 2 experiments are shown.

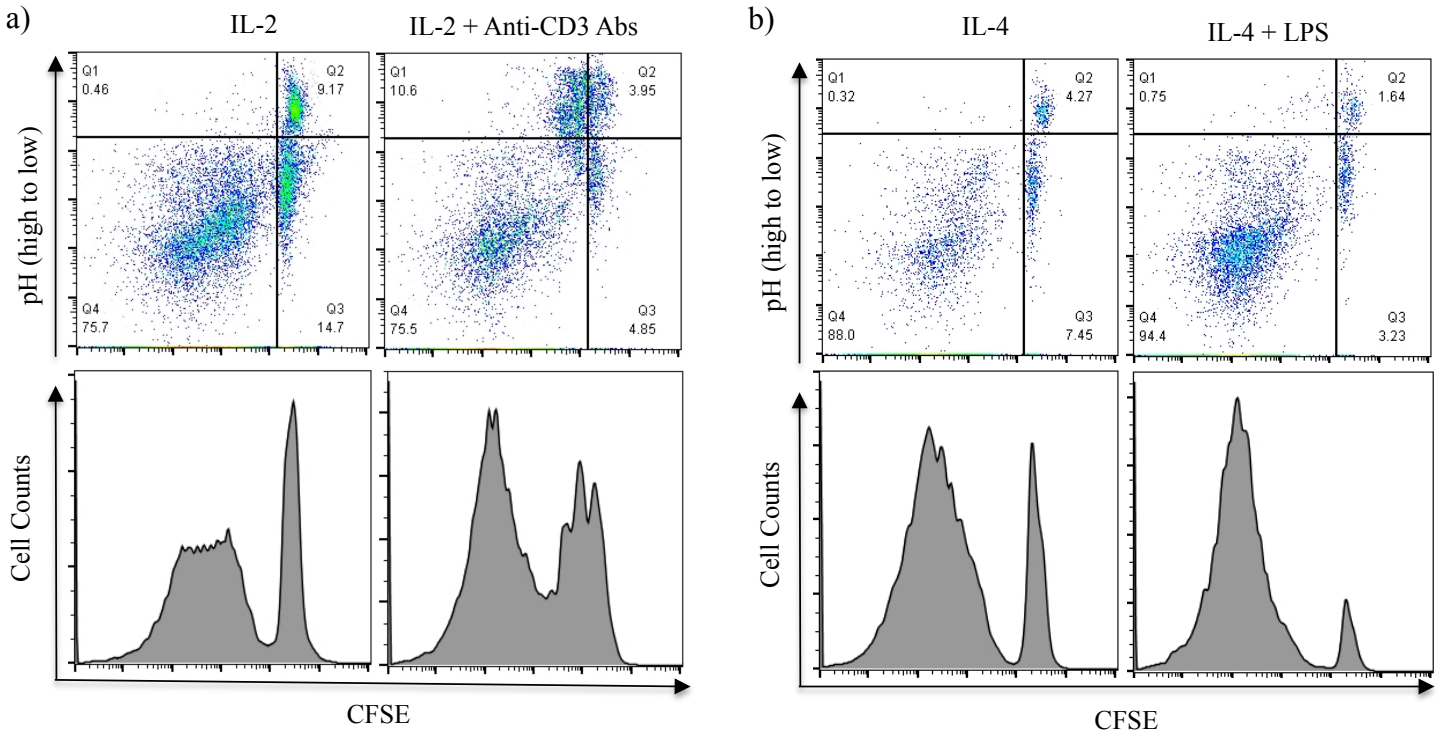

a)
IL-2
IL-2 + Anti-CD3 Abs
b)
IL-4
IL-4 + LPS
pH (high to low)
Cell Counts
CFSE
Q1
0.46
Q2
9.17
Q4
75.7
Q3
14.7
Q1
10.6
Q2
3.95
Q4
75.5
Q3
4.85
Q1
0.32
Q2
4.27
Q4
88.0
Q3
7.45
Q1
0.75
Q2
1.64
Q4
94.4
Q3
3.23

Figure 1

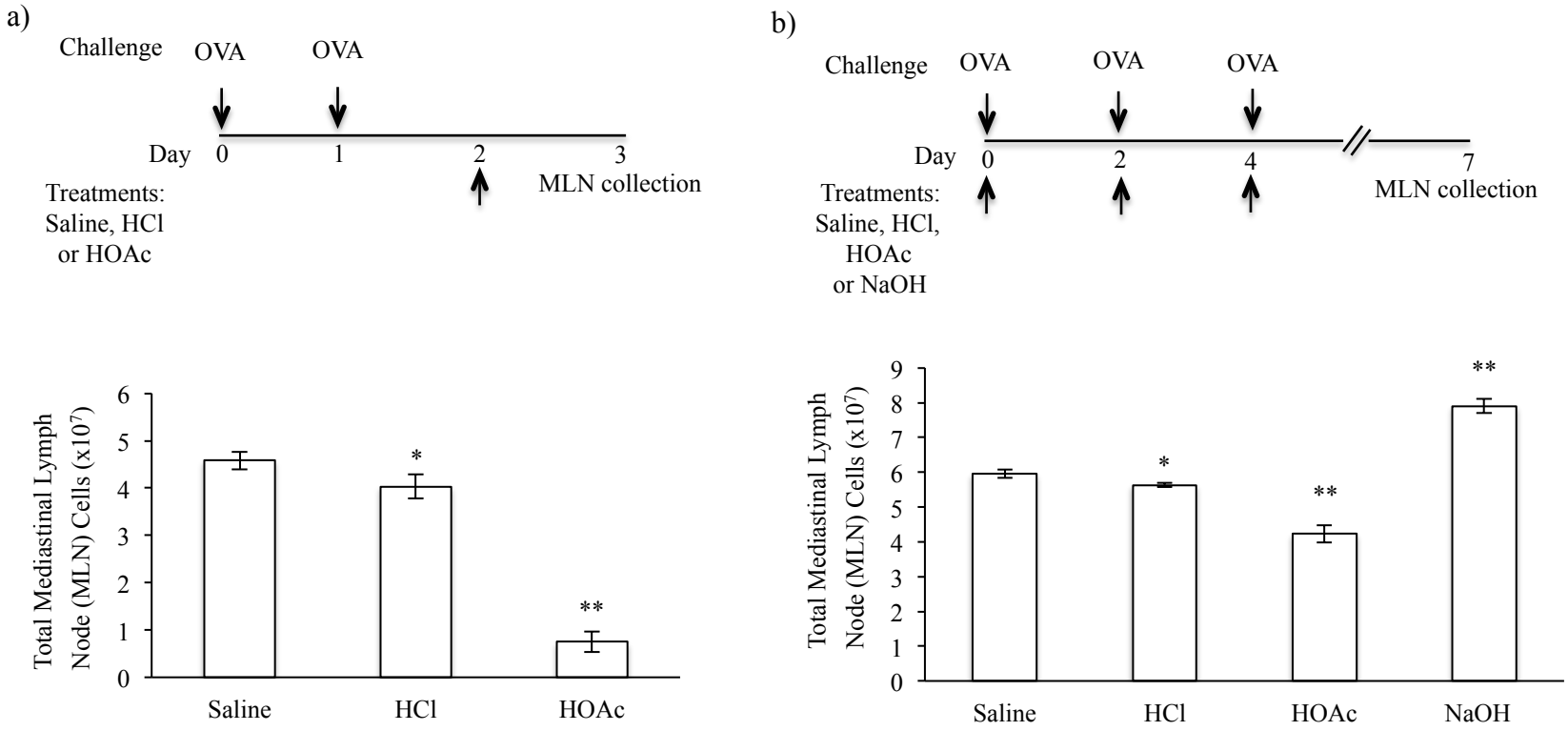

a)
Challenge
OVA
OVA
Day
0
1
2
3
MLN collection
Treatments:
Saline, HCl
or HOAc
Total Mediastinal Lymph
Node (MLN) Cells (x10^7)
6
5
4
3
2
1
0
*
**
Saline
HCl
HOAc
b)
Challenge
OVA
OVA
OVA
Day
0
2
4
7
MLN collection
Treatments:
Saline, HCl,
HOAc
or NaOH
Total Mediastinal Lymph
Node (MLN) Cells (x10^7)
9
8
7
6
5
4
3
2
1
0
*
**
**
Saline
HCl
HOAc
NaOH

Figure 2

a)

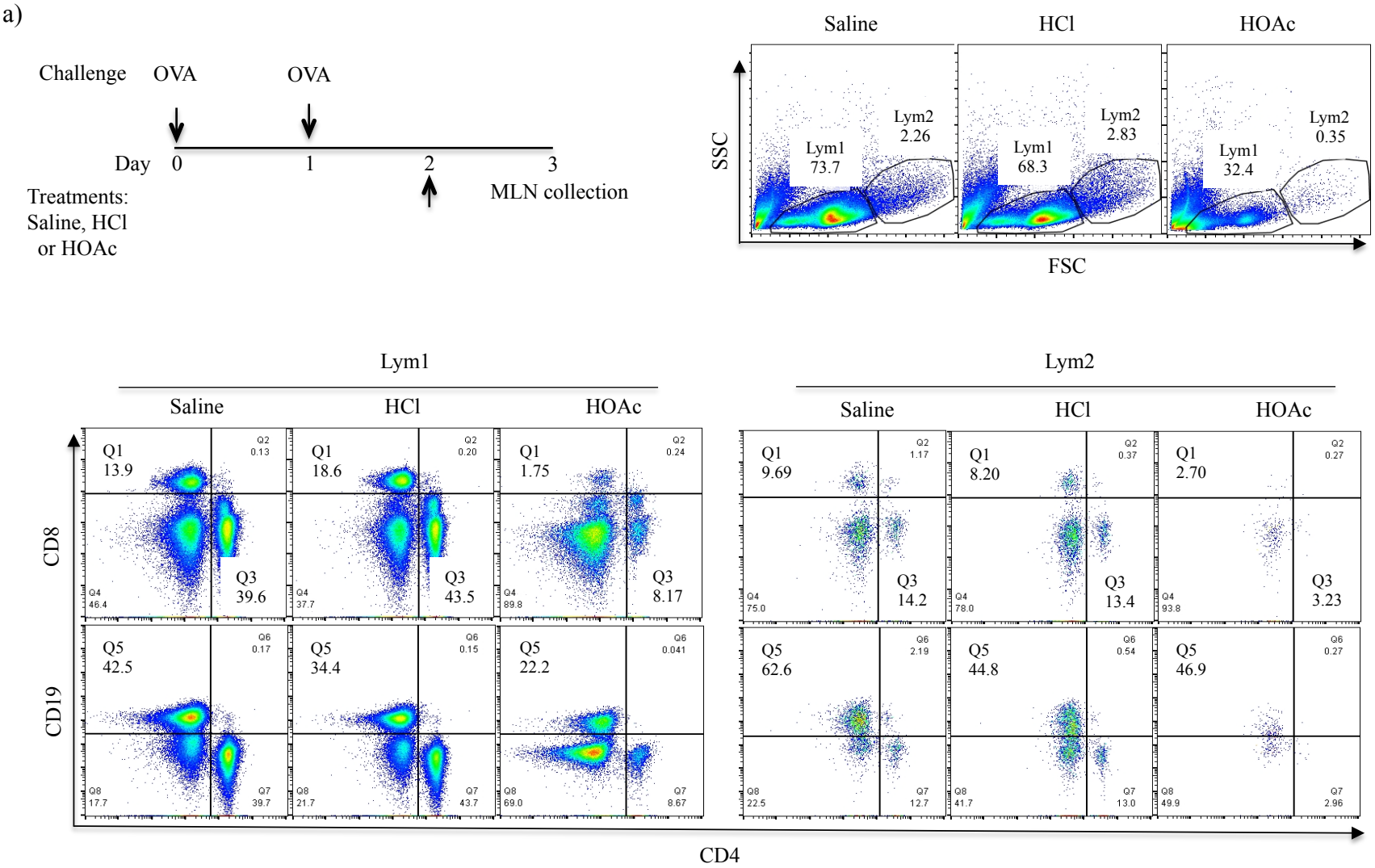
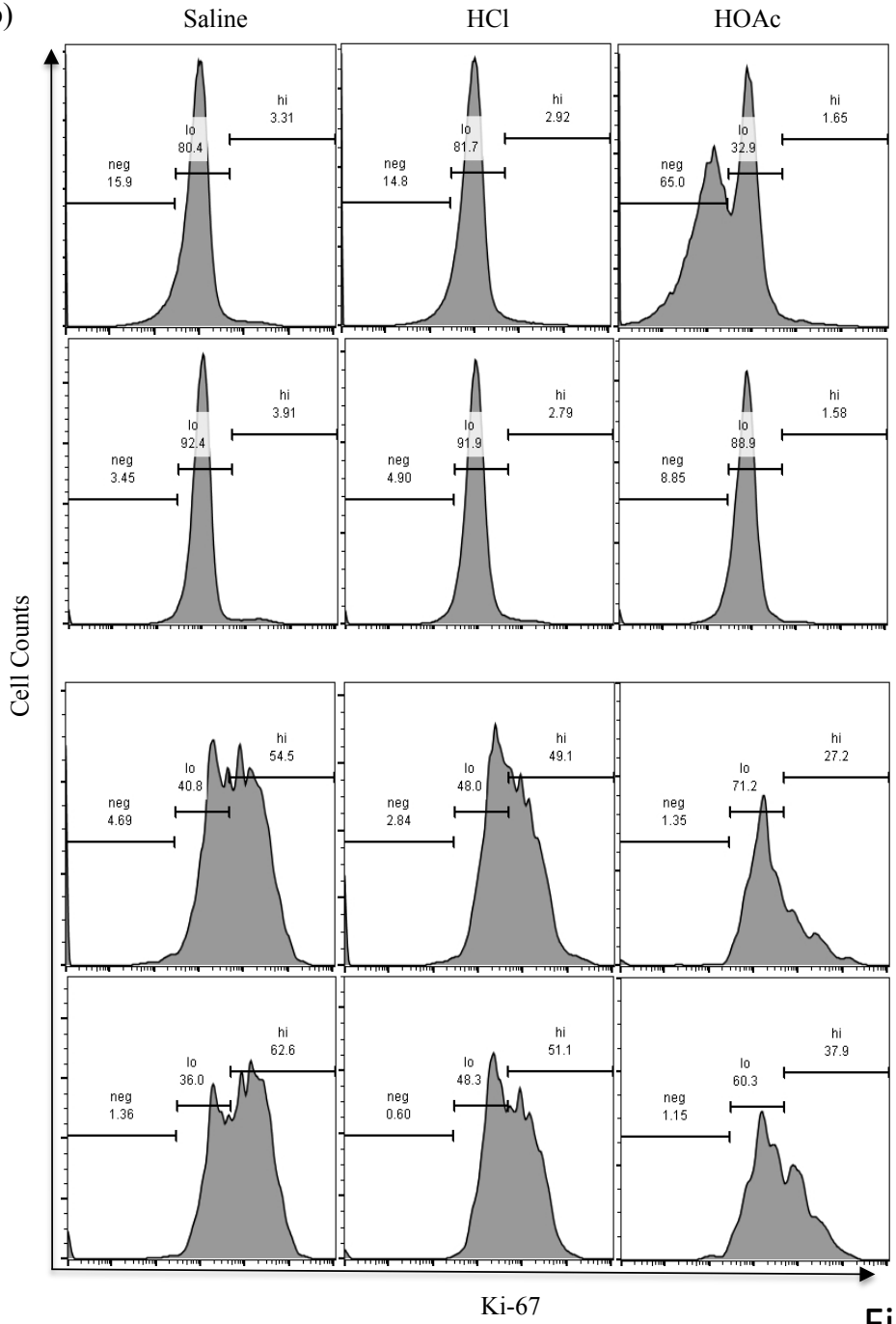

Challenge
OVA
OVA
Day
0
1
2
3
MLN collection
Treatments:
Saline, HCl
or HOAc
Saline
HCl
HOAc
SSC
FSC
Lym1
73.7
Lym2
2.26
Lym1
68.3
Lym2
2.83
Lym1
32.4
Lym2
0.35
Lym1
Lym2
Saline
HCl
HOAc
CD8
CD19
CD4
Q1
13.9
Q2
0.13
Q3
39.6
Q4
46.4
Q5
42.5
Q6
0.17
Q7
39.7
Q8
17.7
Q1
18.6
Q2
0.20
Q3
43.5
Q4
37.7
Q5
34.4
Q6
0.15
Q7
43.7
Q8
21.7
Q1
1.75
Q2
0.24
Q3
8.17
Q4
89.8
Q5
22.2
Q6
0.041
Q7
8.67
Q8
69.0
Q1
9.69
Q2
1.17
Q3
14.2
Q4
75.0
Q5
62.6
Q6
2.19
Q7
12.7
Q8
22.5
Q1
8.20
Q2
0.37
Q3
13.4
Q4
78.0
Q5
44.8
Q6
0.54
Q7
13.0
Q8
41.7
Q1
2.70
Q2
0.27
Q3
3.23
Q4
93.8
Q5
46.9
Q6
0.27
Q7
2.96
Q8
49.9

Figure 3a

b)


Saline
HCl
HOAc
Cell Counts
Ki-67
neg 15.9
lo 80.4
hi 3.31
neg 14.8
lo 81.7
hi 2.92
neg 65.0
lo 32.9
hi 1.65
neg 3.45
lo 92.4
hi 3.91
neg 4.90
lo 91.9
hi 2.79
neg 8.85
lo 88.9
hi 1.58
neg 4.69
lo 40.8
hi 54.5
neg 2.84
lo 48.0
hi 49.1
neg 1.35
lo 71.2
hi 27.2
neg 1.36
lo 36.0
hi 62.6
neg 0.60
lo 48.3
hi 51.1
neg 1.15
lo 60.3
hi 37.9

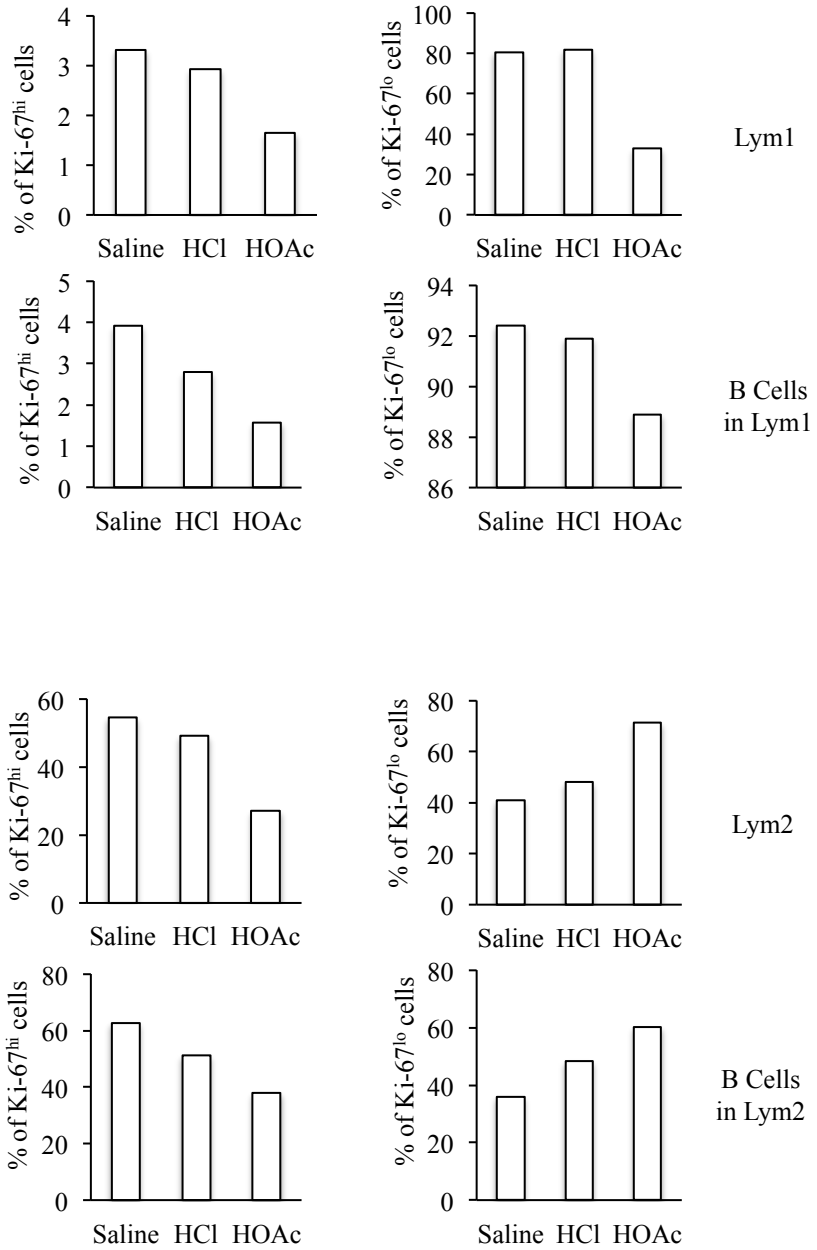
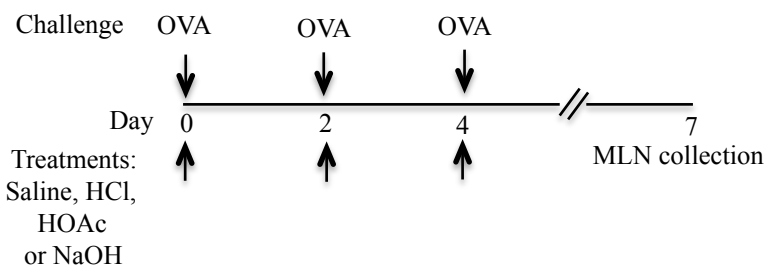
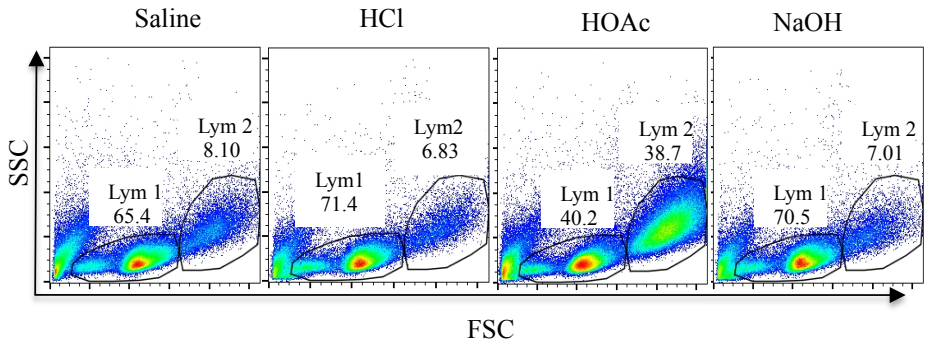

% of Ki-67hi cells
% of Ki-67lo cells
Saline HCl HOAc
Lym1
B Cells in Lym1
Lym2
B Cells in Lym2

Figure 3b

c)


Challenge
OVA
OVA
OVA
Day
0
2
4
7
MLN collection
Treatments:
Saline, HCl,
HOAc
or NaOH


Saline
HCl
HOAc
NaOH
SSC
FSC
Lym 1
65.4
Lym 2
8.10
Lym1
71.4
Lym2
6.83
Lym 1
40.2
Lym 2
38.7
Lym 1
70.5
Lym 2
7.01

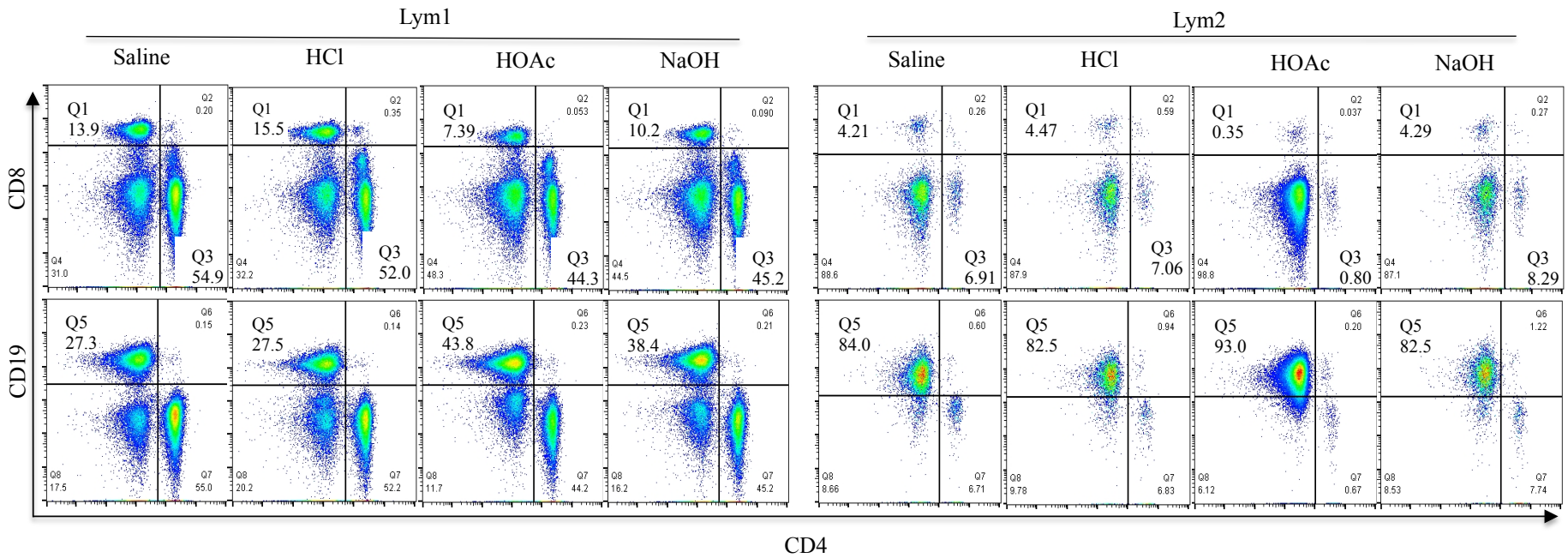

Lym1
Saline
HCl
HOAc
NaOH
Lym2
Saline
HCl
HOAc
NaOH
CD8
CD19
CD4
Q1 13.9
Q2 0.20
Q3 54.9
Q4 31.0
Q5 27.3
Q6 0.15
Q7 55.0
Q8 17.5
Q1 15.5
Q2 0.35
Q3 52.0
Q4 32.2
Q5 27.5
Q6 0.14
Q7 52.2
Q8 20.2
Q1 7.39
Q2 0.053
Q3 44.3
Q4 48.3
Q5 43.8
Q6 0.23
Q7 44.2
Q8 11.7
Q1 10.2
Q2 0.090
Q3 45.2
Q4 44.5
Q5 38.4
Q6 0.21
Q7 45.2
Q8 16.2
Q1 4.21
Q2 0.26
Q3 6.91
Q4 88.6
Q5 84.0
Q6 0.60
Q7 6.71
Q8 8.66
Q1 4.47
Q2 0.59
Q3 7.06
Q4 87.9
Q5 82.5
Q6 0.94
Q7 6.83
Q8 9.78
Q1 0.35
Q2 0.037
Q3 0.80
Q4 98.8
Q5 93.0
Q6 0.20
Q7 0.67
Q8 6.12
Q1 4.29
Q2 0.27
Q3 8.29
Q4 87.1
Q5 82.5
Q6 1.22
Q7 7.74
Q8 8.53

Figure 3c

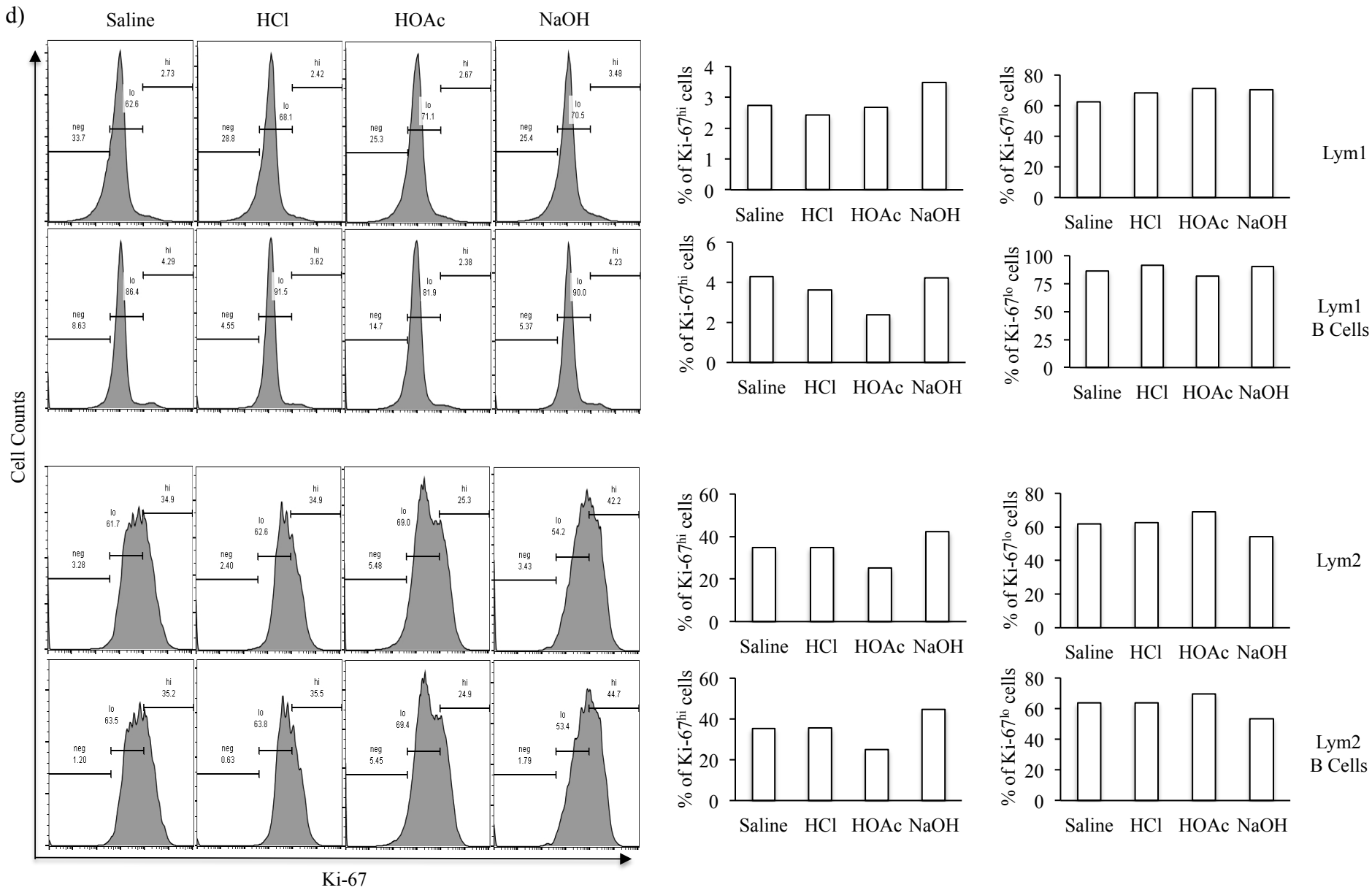

d)
Saline
HCl
HOAc
NaOH
Cell Counts
Ki-67
neg 33.7
lo 62.6
hi 2.73
neg 28.8
lo 68.1
hi 2.42
neg 25.3
lo 71.1
hi 2.67
neg 25.4
lo 70.5
hi 3.48
neg 8.63
lo 86.4
hi 4.29
neg 4.55
lo 91.5
hi 3.62
neg 14.7
lo 81.9
hi 2.38
neg 5.37
lo 90.0
hi 4.23
neg 3.28
lo 61.7
hi 34.9
neg 2.40
lo 62.6
hi 34.9
neg 5.48
lo 69.0
hi 25.3
neg 3.43
lo 54.2
hi 42.2
neg 1.20
lo 63.5
hi 35.2
neg 0.63
lo 63.8
hi 35.5
neg 5.45
lo 69.4
hi 24.9
neg 1.79
lo 53.4
hi 44.7
% of Ki-67hi cells
% of Ki-67lo cells
Lym1
Lym1 B Cells
Lym2
Lym2 B Cells
Saline HCl HOAc NaOH

Figure 3d

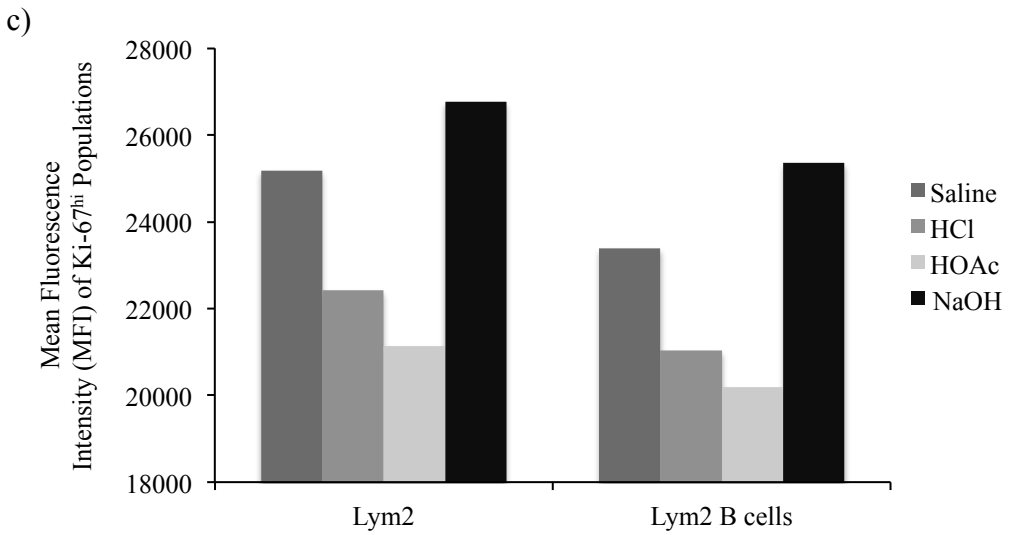
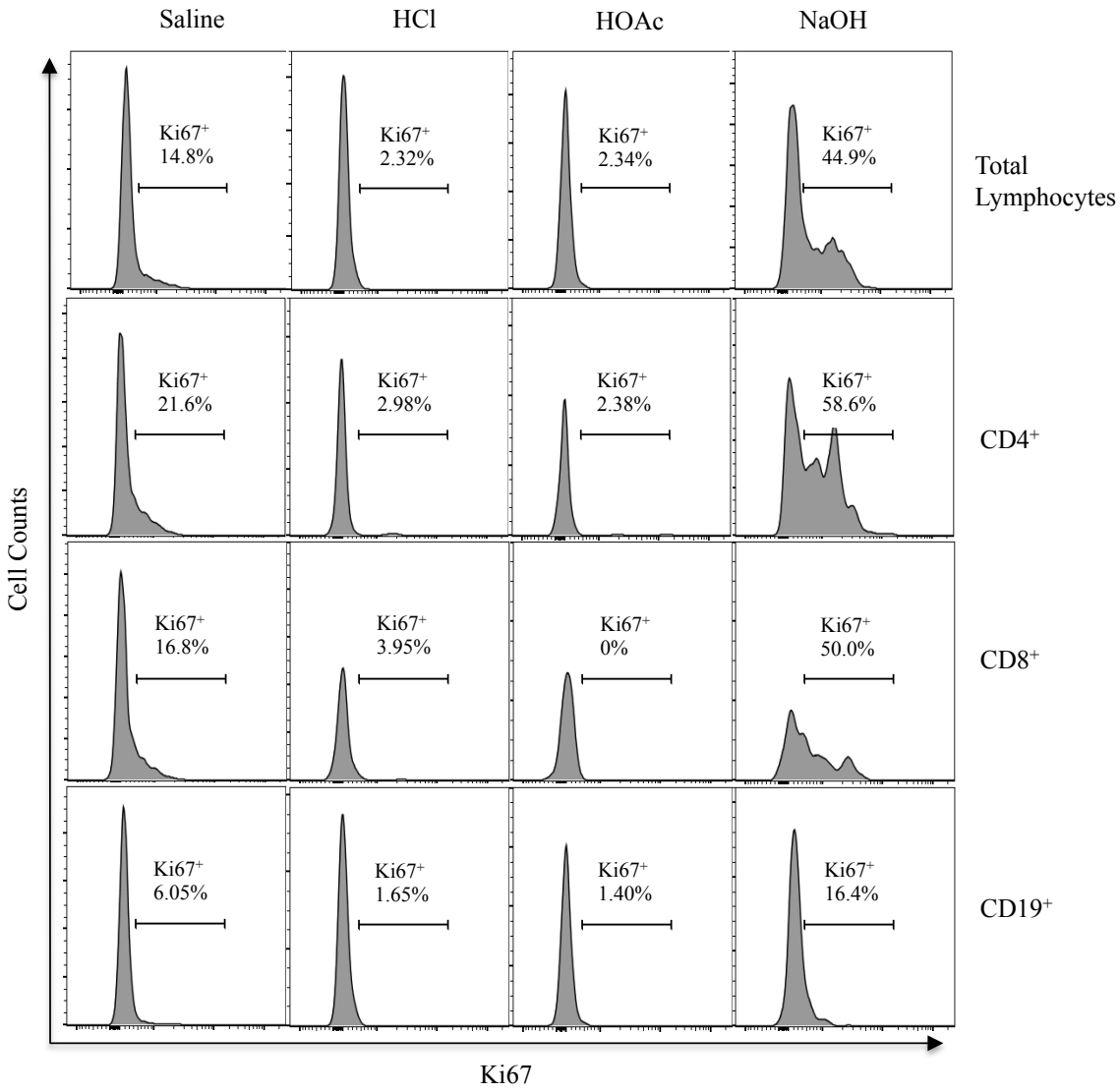

c)
Mean Fluorescence Intensity (MFI) of Ki-67hi Populations
28000
26000
24000
22000
20000
18000
Lym2
Lym2 B cells
Saline
HCl
HOAc
NaOH

Figure 3e


Saline
HCl
HOAc
NaOH
Ki67+ 14.8%
Ki67+ 2.32%
Ki67+ 2.34%
Ki67+ 44.9%
Total Lymphocytes
Ki67+ 21.6%
Ki67+ 2.98%
Ki67+ 2.38%
Ki67+ 58.6%
CD4+
Ki67+ 16.8%
Ki67+ 3.95%
Ki67+ 0%
Ki67+ 50.0%
CD8+
Ki67+ 6.05%
Ki67+ 1.65%
Ki67+ 1.40%
Ki67+ 16.4%
CD19+
Cell Counts
Ki67

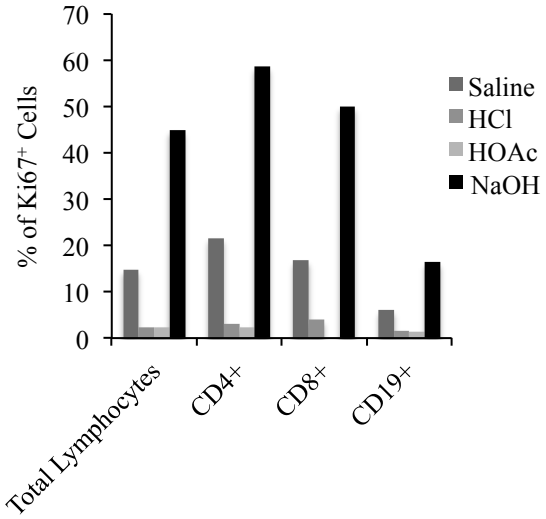

% of Ki67+ Cells
70
60
50
40
30
20
10
0
Saline
HCl
HOAc
NaOH
Total Lymphocytes
CD4+
CD8+
CD19+

Figure 4

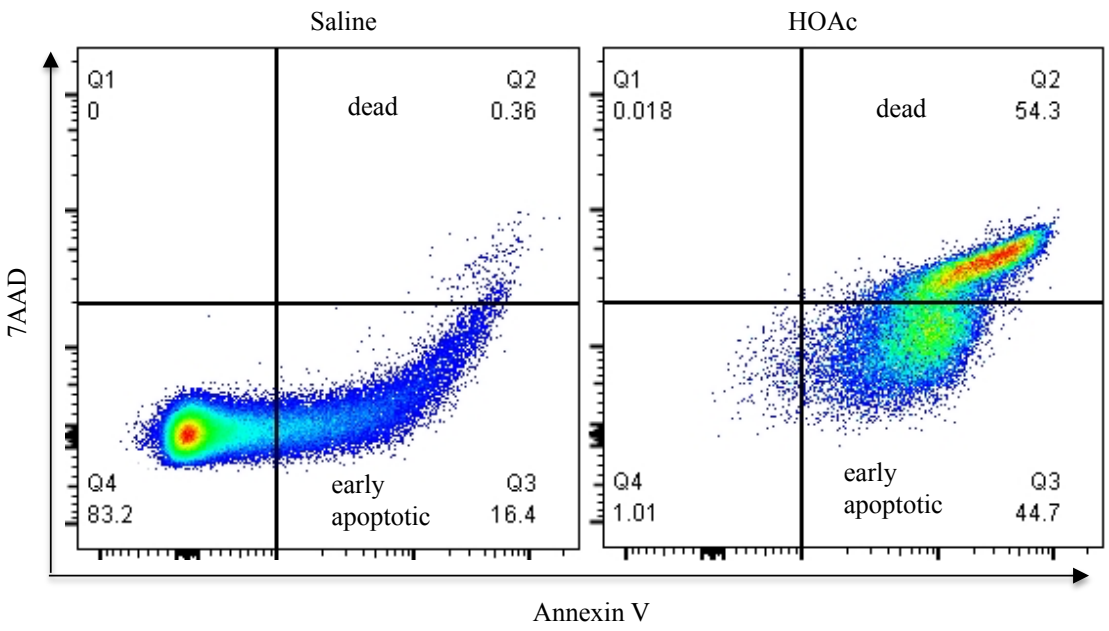

Saline
HOAc
7AAD
Annexin V
Q1
0
dead
Q2
0.36
Q4
83.2
early
apoptotic
Q3
16.4
Q1
0.018
dead
Q2
54.3
Q4
1.01
early
apoptotic
Q3
44.7

Figure 5

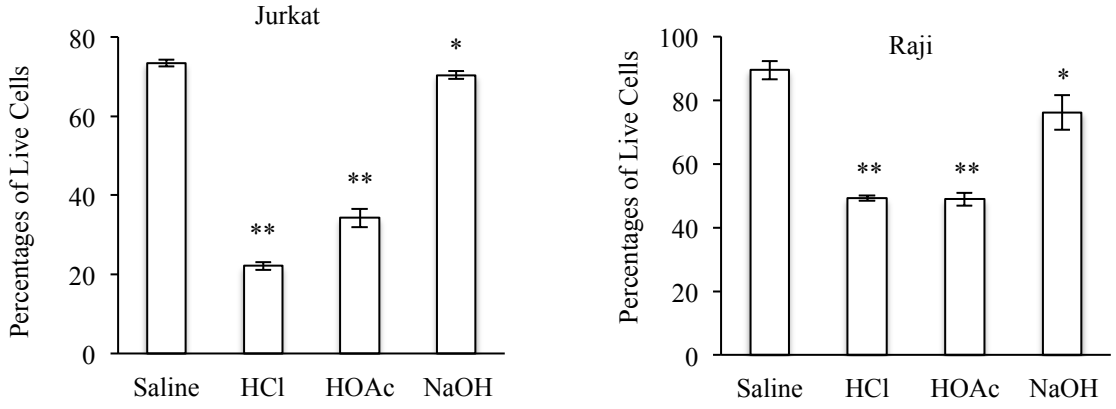

Jurkat
Percentages of Live Cells
80
60
40
20
0
**
**
*
Saline
HCl
HOAc
NaOH
Raji
Percentages of Live Cells
100
80
60
40
20
0
**
**
*
Saline
HCl
HOAc
NaOH

Figure 6a

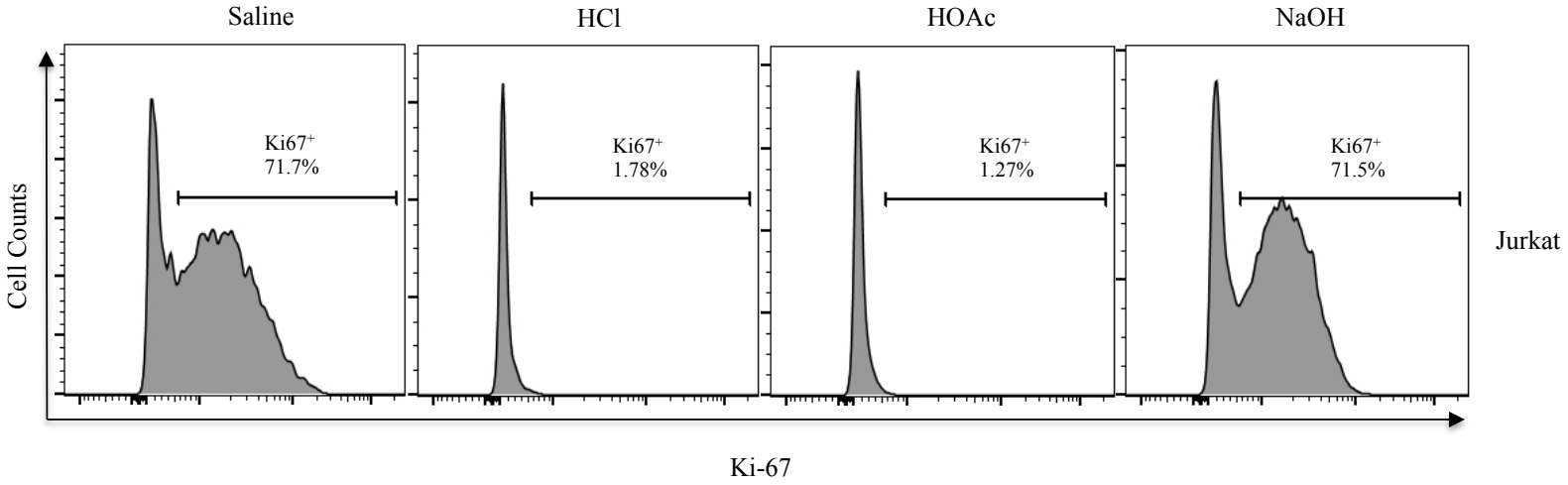

Saline
HCl
HOAc
NaOH
Ki67+
71.7%
Ki67+
1.78%
Ki67+
1.27%
Ki67+
71.5%
Jurkat
Cell Counts
Ki-67

Figure 6b