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Original Article

Colorectal cancer extracellular acidosis decreases immune cell killing and is partially ameliorated by pH-modulating agents that modify tumor cell cytokine profiles

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Abstract: Tumor cells upregulate myriad proteins that are important for pH regulation, resulting in the acidification of the extracellular tumor microenvironment (TME). Abnormal pH is known to dampen immune function, resulting in a worsened anti-tumor immune response. Understanding how extrinsic alterations in pH modulate the interactions between immune cells and tumors cells will help elucidate opportunities for new therapeutic approaches. We observed that pH impacts the function of immune cells, both natural killer (NK) and T cells, which is relevant in the context of a highly acidic TME. Decreased NK and T cell activity was correlated with decreasing pH in a co-culture immune cell-mediated tumor cell-killing assay. The addition of pH-modulating drugs cariporide, lansoprazole, and acetazolamide to the co-culture assay was able to partially mitigate this dampened immune cell function. Treatment of colorectal cancer (CRC) cells with NHE1 inhibitor cariporide increased CRC cell-secreted cytokines involved in immune cell recruitment and activation and decreased cytokines involved in epithelial-mesenchymal transition (EMT). Cariporide treatment also decreased CRC cell shed TRAIL-R2, TRAIL-R3, and PD-L1 which is relevant in the context of immunotherapy. These experiments can help inform future investigations into how the pH of the tumor microenvironment may be extrinsically modulated to improve anti-tumor immune response in solid tumors such as colorectal cancer.

Keywords: Tumor pH, immune microenvironment, NHE1, cariporide, lansoprazole, acetazolamide, NK cell, T cell, TRAIL-R2, PD-L1

Introduction

Tumor cells upregulate proteins that regulate pH resulting in acidification of the extracellular tumor microenvironment. This acidosis of the TME contributes to invasion, progression, and therapeutic resistance in cancer [1-3]. Tissue acidosis is commonly observed in solid tumors and results in an extracellular pH range of 6.0 to 7.0. Extracellular acidosis disrupts immune cell activation and dampens immune function which can result in a worsened anti-tumor immune response [4]. This deteriorated anti-tumor immune response is partially due to decreased cytotoxic ability of CD8+ cytotoxic T lymphocytes [5, 6] as well as natural killer (NK) cells [7-10]. By contrast, TME pH modulation has been shown to reverse anergy in tumor-infiltrating T lymphocytes [4, 11, 12]. Understanding how alterations in pH modulate the interactions between immune cells and tumors cells could help elucidate opportunities for new

therapeutic approaches. This is particularly relevant in the context of checkpoint blockade therapy, as it has been shown that manipulation of pH in combination with immune checkpoint blockade may improve anti-tumor immune response [13].

There exist several mechanisms for pH regulation within the cell including monocarboxylate, bicarbonate, and proton transporters [14]. Here, we examined several therapeutics that have the ability to regulate pH including cariporide, lansoprazole, and acetazolamide. Cariporide is a potent Na+/H+ exchanger isoform 1 (NHE1) inhibitor [15]. The Na+/H+ exchanger is a ubiquitously expressed plasma membrane protein that exchanges Na+ for H+ to regulate pH homeostasis, as has been shown to play a role in transformation and cancer progression [12]. Although there are ten identified isoforms of NHE, the NHE1 isoform is the main isoform of the exchanger. NHE1 activity is primarily stimulated by intracellular acidosis [16] and hypoxia [17, 18]. Increased NHE1 activity results in increased intracellular pH and decreased extracellular pH, therefore, pharmacologic inhibition of NHE1 is an emerging anticancer strategy. Preclinical activity of cariporide has been demonstrated across multiple cancer types [15, 19, 20]. In doxorubicin-resistant colon cancer cells NHE activity is increased, and inhibition of NHE1 was able to reverse doxorubicin resistance [21]. Moreover, biopsies of colonic adenocarcinomas and colon tissues from patients with colorectal cancer showed that net acid extrusion is increased in colon cancer crypts, and that treatment with cariporide significantly decreased net acid extrusion [22].

Proton pump inhibitors (PPIs) like lansoprazole are also being considered in the context of cancer treatment to target TME acidosis, and have shown significant preclinical activity in regards to the prevention of metastasis [23], chemosensitization [24, 25], and induction of apoptosis [26]. Lansoprazole selectively inhibits the membrane enzyme H+/K+ ATPase [27] and has been shown to prevent cancer cell binding to extracellular matrix (ECM) components including laminin, fibronectin, and type IV collagen [28]. Furthermore, lansoprazole was able to prevent the formation of lung metastases by murine colon cancer cells [12]. PPIs also sensitize cancer cells to chemotherapeutic agents 5-fluorouracil, cisplatin, and vinblastine and result in a significant increase in cytoplasmic retention of these drugs [29]. Moreover, because PPIs like lansoprazole are weak bases, the activated, protonated form of these cytotoxic agents preferentially accumulates in the acidic TME [30].

A third class of pH-modulating drugs that may be used to target hypoxic or acidic tumors include carbonic anhydrase inhibitors such as acetazolamide [31]. Carbonic anhydrase IX (CAIX) is a metalloenzyme that catalyzes the reversible reaction and interconversion of carbon dioxide and water to carbonic acid, protons, and bicarbonate ions [32]. CAIX expression is correlated with poor prognosis in multiple tumor types including breast cancer [33], ovarian cancer [34], and bevacizumab-treated patients with metastatic colorectal cancer [35].

Materials and methods

Cell culture and reagents

Human colorectal cancer cell lines SW480, HCT-116, HT-29, and KM12C were used in this study. SW480 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% Penicillin-Streptomycin. HCT-116 and HT-29 were cultured in McCoy's 5A (modified) Medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. KM12C cells were cultured in Eagle's Minimal Essential Medium Supplemented with 10% FBS and 1% Penicillin-Streptomycin. TALL-104 cells (CD2+; CD3+; CD7+; CD8+; CD56+; CD4-; and CD16-) were purchased from ATCC and were cultured in RPMI-1640 containing 20% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Recombinant human IL-2 (Miltenyi cat# 130-097744) with a final concentration of 100 units/mL was added to the TALL-104 culture media. NK-92 cells were cultured in Alpha Minimum Essential Medium without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate supplemented with 0.2 mM inositol; 0.1 mM 2-mercaptoethanol; 0.02 mM folic acid; 100 U/mL recombinant IL-2, 12.5% horse serum, and 12.5% FBS. All cell lines were incubated at 37°C in humidified atmosphere containing 5% CO_2. Cell lines were

authenticated and tested to ensure the cultures were free of mycoplasma infection.

Measurement of cell viability

Cells were plated at a density of 3×10^3 cells per well in a 96-well plate (Greiner Bio-One, Monroe, NC, USA). Cell viability was assessed using the CellTiter-Glo assay (Promega, Madison, WI, USA). Cells were mixed with 25 µL of CellTiter-Glo reagents in 100 µL of culture volume, and bioluminescence imaging was measured using the Xenogen IVIS imager (Caliper Life Sciences, Waltham, MA).

Cytokine, chemokine, and growth factor profiling

Colorectal cancer cells (HCT-116, HT-29, KM12C) were treated with cariporide at indicated doses and cancer cell culture supernatants were collected and analyzed using a Luminex 200 multiplexing instrument. An R&D systems Human Premixed Multi-Analyte Kit (R&D Systems, Inc., Minneapolis, MN) was run on a Luminex 200 Instrument (LX200-XPONRUO, Luminex Corporation, Austin, TX) according to the manufacturer's instructions. Cell culture supernatant levels of TNF-alpha, IL-6, IL-8/CXCL8, Ferritin, IFN-beta, IL-10, CCL2/JE/MCP-1, VEGF, CXCL13/BLC/BCA-1, IFN-gamma, CCL20/MIP-3 alpha, CCL3/MIP-1 alpha, CCL22/MDC, CCL4/MIP-1 beta, IL-4, IL-17/IL-17a, TRAIL R2/TNFRSF10B, GM-CSF, CXCL5/ENA-78, CXCL9/MIG, G-CSF, CXCL11/I-TAC, Granzyme B, CCL5/RANTES, Prolactin, IFN-alpha, CXCL14/BRAK, IL-12/IL-23 p40, CXCL10/IP-10/CRG2, CCL7/MCP-3/MARC, IL-7, CCL8/MCP-2, TRANCE/TNFSF11/RANK L, IL-15, TRAIL R3/TNFRSF10C, CCL11/Eotaxin, IL-18/IL-1F4, TRAIL/TNFSF10, IL-21, and C-Reactive Protein/CRP were measured.

Immune cell co-culture experiments

Co-culture experiments were conducted with target GFP+ SW480 or HCT-116 colorectal cancer cells and either NK-92 natural killer or TALL-104 T effector cells at various pHs in a 48-well plate. Manipulation of pH occurred using the compounds lactic acid and sodium bicarbonate. Ethidium homodimer was used as a marker of cell death. Target or effector cells were labeled using CellTracker™ Green CMFDA or CellTracker™ Blue CMAC Dyes (Thermo Fisher Scientific, Waltham, MA, USA). Images were quantified using Fiji Image J software [36].

Statistics

A one-way Anova was used to determine statistical significance of groups of three or more and a post-hoc Tukey's multiple comparisons test was used for multiple comparisons. Significance is reported as follows: *P≤0.05, **P≤0.01, and ***P≤0.001.

Results

Immune cell function decreases with decreasing pH of culture media

To determine how pH modification impacts immune cell-mediated killing of tumor cells, co-culture experiments were conducted with GFP+ SW480 colorectal cancer cells and either natural killer (NK-92) cells or T (TALL-104) cells at pH 7.7, 7.0, and 6.0. We observed that both NK-92 cells and TALL-104 cells exhibited decreasing amounts of cell killing in response to decreasing pH regardless of treatment condition (**Figure 1**).

Cariporide, lansoprazole, and acetazolamide treatment increases immune-cell mediated tumor cell killing at pH 6.0

Next, to determine if therapeutic manipulation of pH via alteration of pH pump activity could overcome the decrease in immune cell activity associated with pH, we added cariporide, lansoprazole, and acetazolamide monotherapy or combination therapy to the co-culture experiments. Treatment with all three of the drugs increased natural killer cell-mediated tumor cell-killing when the cell culture media was adjusted to a pH of 6.0 (**Figure 2**). We observed the most significant single-agent activity when acetazolamide was added to the co-culture experiments. In contrast, cariporide showed the least significant increase over baseline natural killer cell-mediated tumor cell killing. Two doses of each drug were used, and interestingly, we consistently observed improved tumor cell death across most of the treatment groups using the 10 µM low-dose, as compared to the 50 µM high-dose, in both single and combination treatment groups. Combinations of therapeutics differentially impacted immune cell killing of tumor cells with

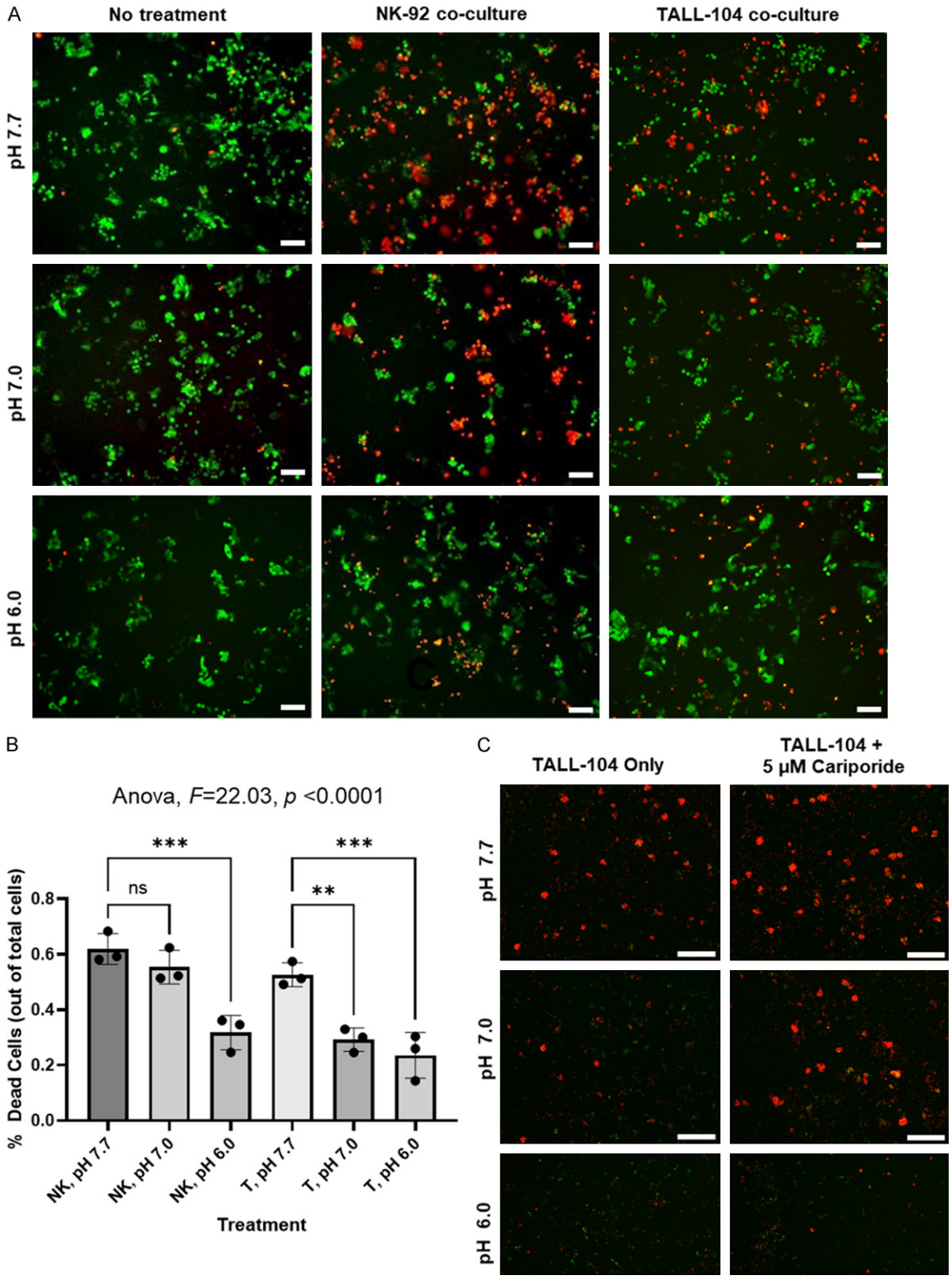

Figure 1. Natural killer and T cell killing of SW480 colon cancer cells decreases with reduced cell culture media pH. (A) Co-culture of GFP+ SW480 colon cancer cells and either NK-92 or TALL-104 cells with or without 5 μM cariporide treatment. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 μm. (B) Quantification of (A). (C) 4× magnification shows T cell clustering with and without 5 μM cariporide treatment. 24-hour timepoint. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 500 μm.

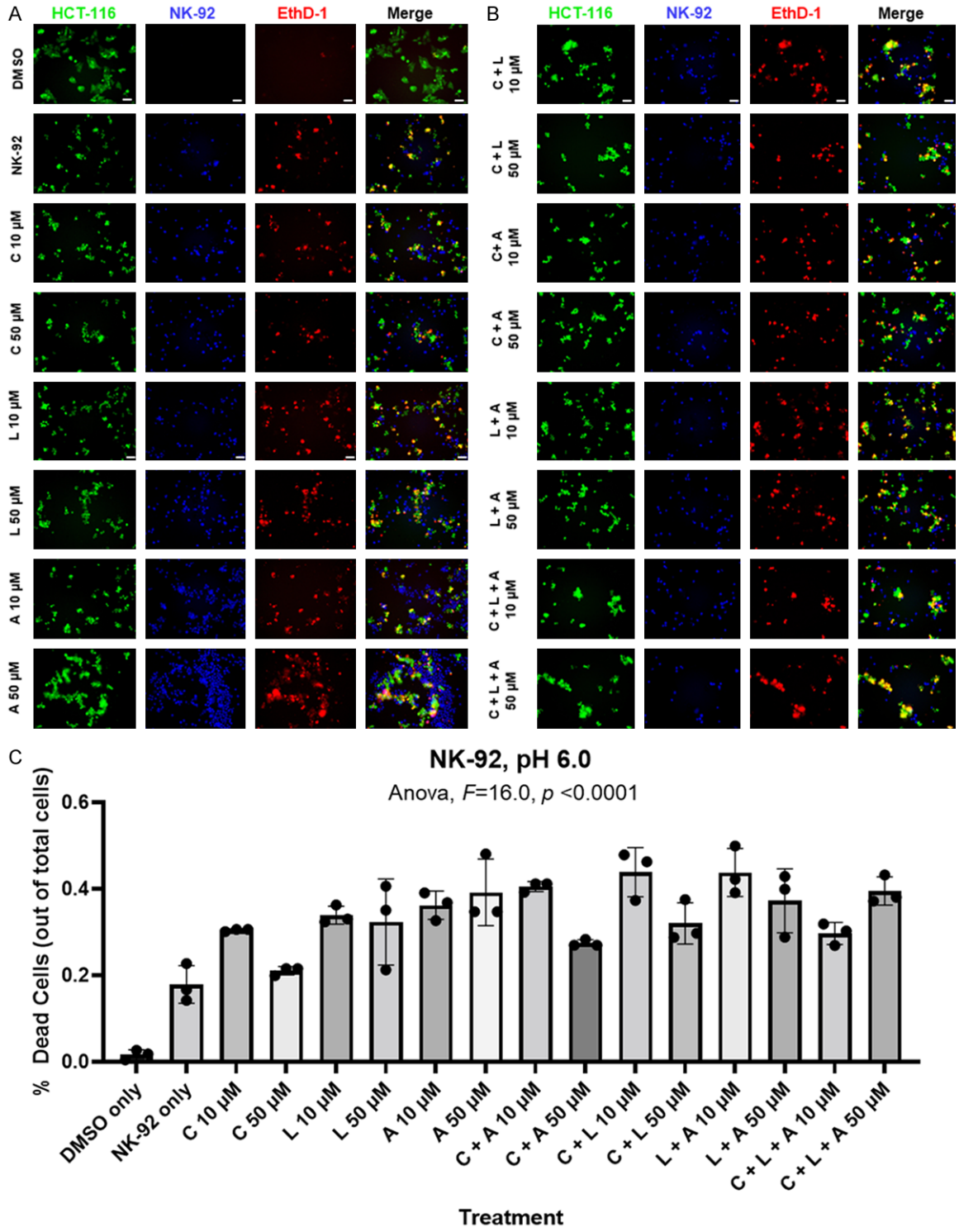

Figure 2. Treatment of HCT-116 colorectal cancer cells with pH-modulating agents increases natural killer cell-mediated killing. Co-culture of HCT-116 colon cancer cells and NK-92 cells with designated drugs using a cell culture media pH of 6.0. A 1:1 effector: target cell ratio was used. "C": Cariporide. "L": Lansoprazole, "A": Acetazolamide. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 µm. (A) Single treatment results and (B) combination treatment results. (C) Quantification of images in (A and B).

the most significant upregulation of tumor cell death observed with the cariporide plus lansoprazole combination low-dose treatment and the lansoprazole plus acetazolamide low-dose treatment. The percentage of dead cells out of total cells did not significantly increase in the combination therapy groups as compared to the single-treatment groups. Treatment doses up to 100 μM did not impact tumor cell viability (Supplementary Figure 1).

Next, we observed similar trends when we co-cultured T cells and colorectal cancer cells using a culture media with an adjusted pH of 6.0 (**Figure 3**). Again, we observed the most significant single agent activity with acetazolamide. Interestingly, combination treatment groups did not provide an advantage over single treatment with cariporide, lansoprazole, or acetazolamide at low concentrations.

Cariporide, lansoprazole, and acetazolamide treatment differentially modulates immune-cell mediated tumor cell killing at pH 7.0 and 7.7

We observed similar trends for natural killer cell-mediated tumor cell killing using a culture media pH of 7.0 (Supplementary Figure 2) and 7.7 (Supplementary Figure 3). At pH 7.7, we once again observed the greatest single agent activity with lansoprazole treatment. We observed the highest overall percentage of dead cells in the triple therapy combination group at the low dose. Once again, we observed the lowest single agent activity from the cariporide only treatment group. At pH 7.0, we observed the highest single agent activity with the acetazolamide only group and the lowest with the cariporide only group. The most effective combinatorial group was the cariporide plus lansoprazole group.

Moreover, we observed similar trends for T cell-mediated tumor cell killing using a culture media of 7.0 (Supplementary Figure 4) and 7.7 (Supplementary Figure 5). Again, the acetazolamide only treatment group had the highest single agent activity and the cariporide only group had the least amount of activity in terms of the promotion of immune cell-mediated tumor cell death at pH 7.7. Interestingly, the high concentration, triple therapy group showed the largest amount of tumor cell death. At pH 7.0, acetazolamide again showed the largest single-agent activity. Lansoprazole treatment alone had the lowest amount of tumor cell death. We again observed that the high-dose, triple therapy group showed the highest amount of tumor cell death.

Cariporide treatment modifies cytokine, chemokine, and growth factor profiles; results are heterogeneous across cell lines

Given the importance of cell signaling on immune function, and because we saw significant changes in immune killing with the pH-modulating agents tested, we then evaluated how cariporide treatment impacts the colorectal cancer cell secretome (**Figure 4**). Our custom cytokine, chemokine, and growth factor profiling panel was designed specifically to monitor analytes involved in immunomodulation in the context of colorectal cancer (**Figure 5**) [37-41]. We utilized HCT-116 cells, as used in the co-culture experiments, and also analyzed two other colorectal cancer cell lines (HT-29 and KM12C) in order to evaluate potential heterogeneity of response based on different cell line genetic backgrounds (**Figure 4**). Interestingly, in HCT-116 cells treated with cariporide, Interleukin-6 (IL-6) was the only analyte that increased. Analytes that decreased post-treatment included CCL3/MIP-1 alpha, vascular endothelial growth factor (VEGF), CXCL9/MIG, macrophage-colony stimulating factor (M-CSF), Prolactin, Interleukin-8 (IL-8/CXCL8), soluble tumor necrosis factor-related apoptosis inducing ligands receptor 2 (sTRAIL-R2), CXCL13/BLC/BCA-1, soluble tumor necrosis factor-related apoptosis inducing ligand receptor 2 (sTRAIL-R3), granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin-2 (IL-2), soluble programmed death-ligand 1 (sPD-L1), C-reactive protein (CRP), and interferon-beta (IFN-beta). In HT-29 cells we saw increases in VEGF, prolactin, CXCL13/LBC/BCA-1, CXCL11/I-TAC, tumor necrosis factor alpha (TNF alpha), and IL-6. In contrast, we observed decreases in IL-8/CXCL8, Interferon gamma-induced protein 10 (CXCL10/IP-10), sTRAIL-R2, IL-2, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (CCL5/RANTES), TNF-related activation-induced cytokine (TRANCE), sPD-L1, interferon-alpha (IFN-alpha), sTRAIL-R3, CRP, IFN-beta, and GM-CSF. In the third cell line we tested, KM12C, we observed increases in VEGF,

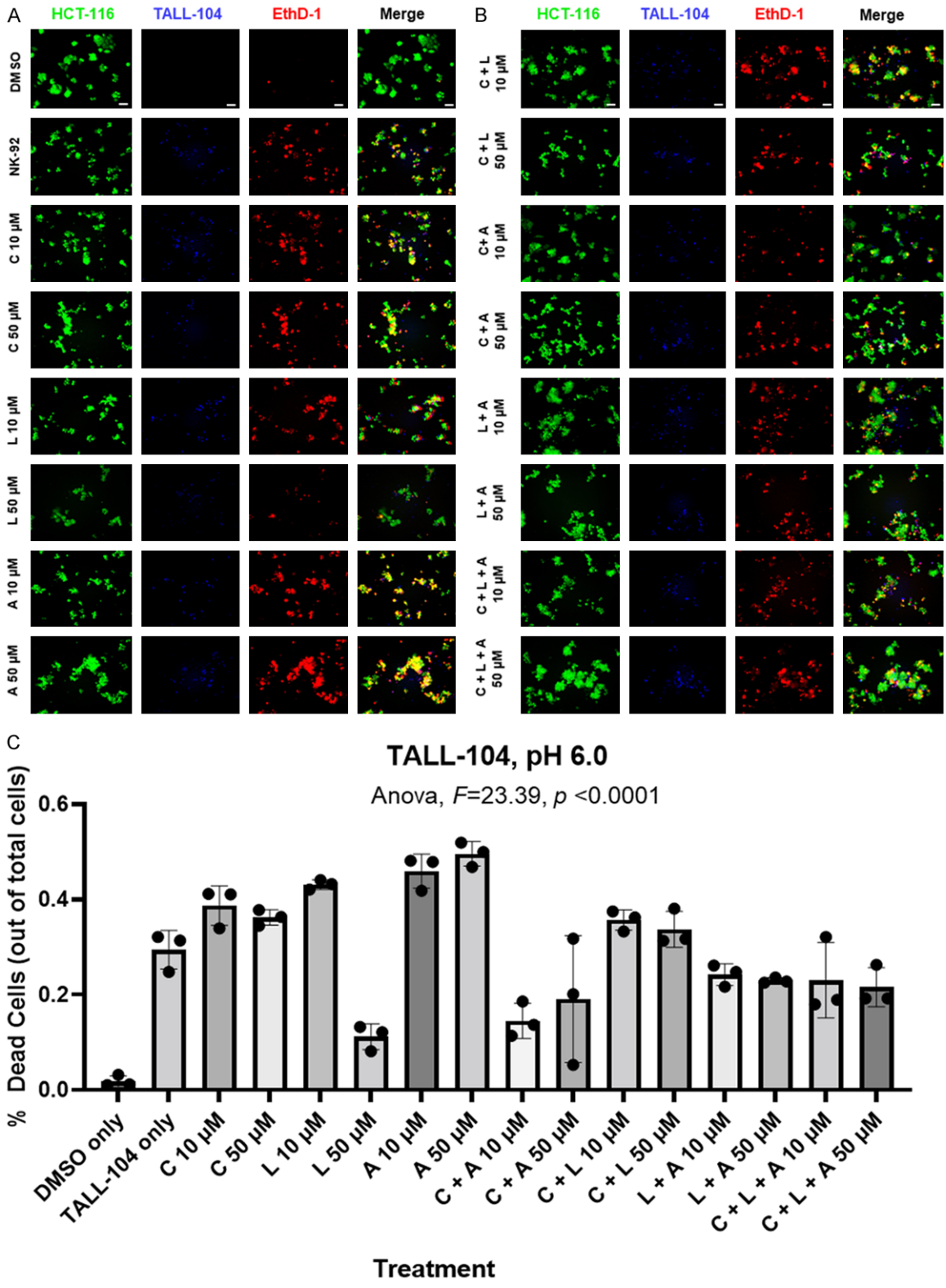

Figure 3. Treatment of HCT-116 colorectal cancer cells with pH-modulating agents increases T cell-mediated killing. Co-culture of HCT-116 colon cancer cells and TALL-104 cells with designated drugs using a cell culture media pH of 6.0. A 1:1 effector: target cell ratio was used. "C": Cariporide. "L": Lansoprazole, "A": Acetazolamide. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 µm. (A) Single treatment results and (B) combination treatment results. (C) Quantification of images in (A and B).

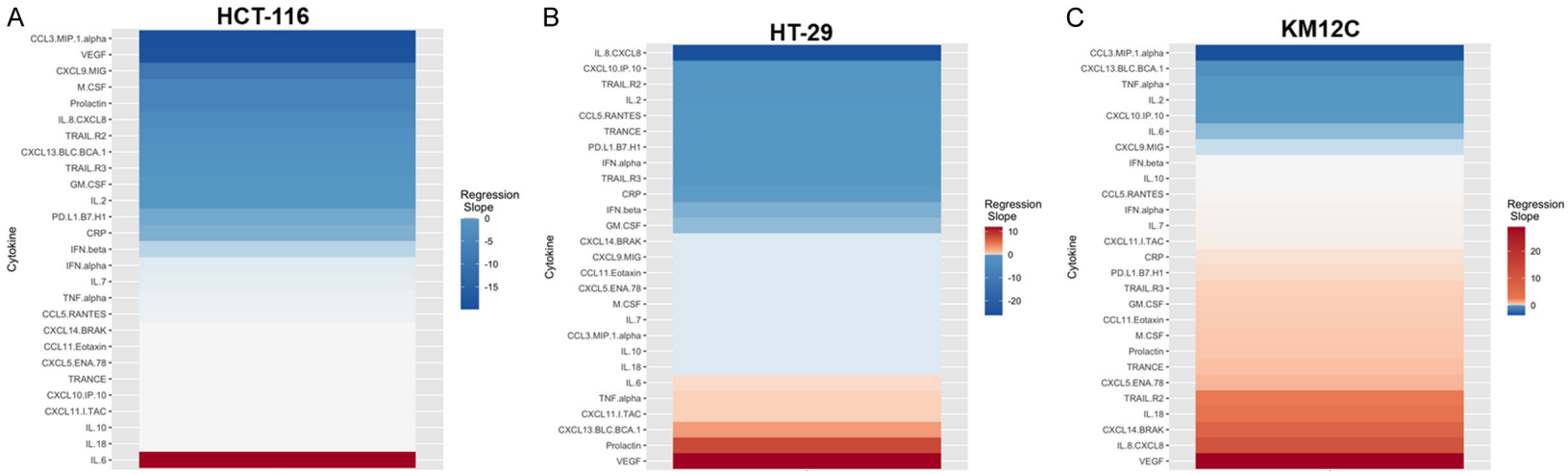

Figure 4. Cytokine, chemokine, and growth factor profiles of colorectal cancer cell lines treated with cariporide show heterogeneous responses. (A) HCT-116 (B) HT-29 and (C) KM12C cells were treated with cariporide for 48 hours and cell culture supernatant was analyzed. Slopes of the dose-response linear regression were used to create heat maps.

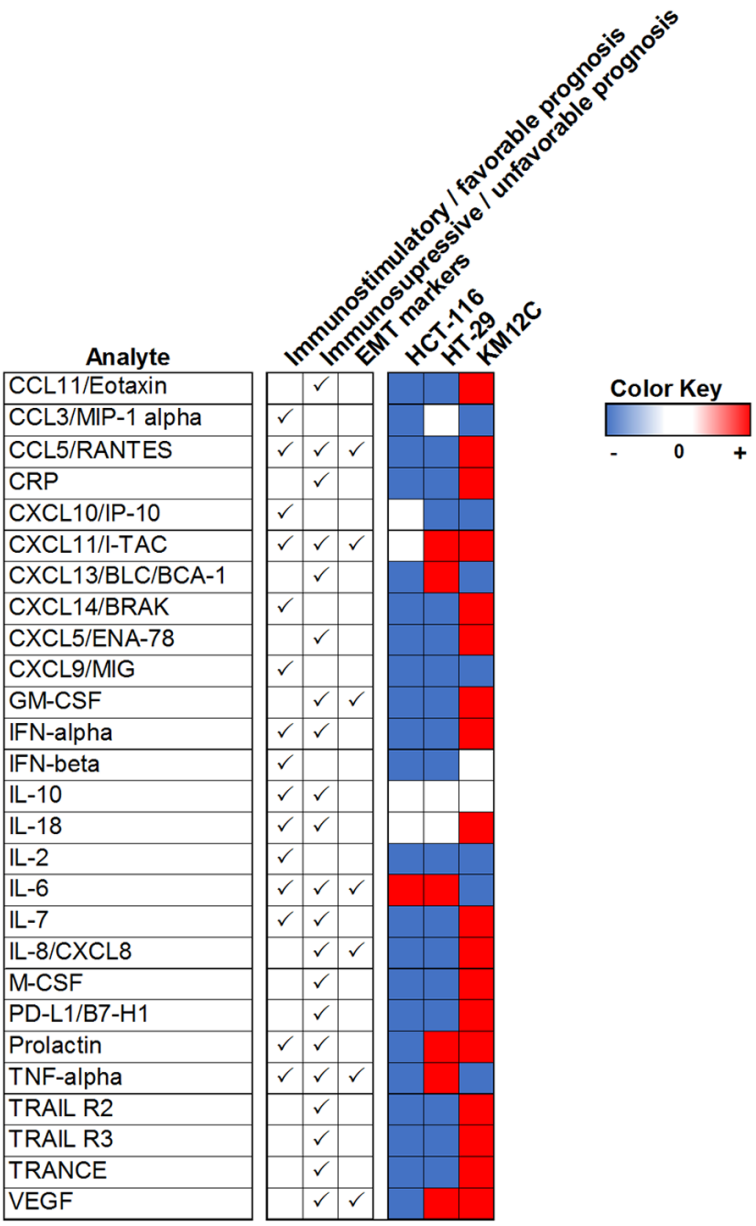

Figure 5. Cytokine, chemokine, and growth factors modified post-cariporide treatment are involved in immunomodulation and epithelial mesenchymal transition. Analytes were indicated as belonging to the following categories: (1) immunostimulatory/favorable colorectal cancer (CRC) prognosis, (2) Immunosuppressive/unfavorable CRC prognosis, and (3) markers of epithelial mesenchymal transition (EMT). The heatmap indicates the dose-response linear regression slope of each cell line (HCT-116, HT-29, and KM12C) post-cariporide treatment. Color Key: Red indicates a positive slope, white indicates a slope of zero, and blue indicates a negative slope.

amide treatment impacts the colorectal cancer cell cytokinome.

Discussion

We observed that pH impacts the function of immune cells which is relevant in the context of a highly acidic TME. We observed that treatment of colon cancer and immune cell co-cultures with several pH-modulating agents increased immune-cell mediated tumor cell-killing. We observed heterogenous responses in tumor cell death in the double- and triple-combination treatment groups. Therapeutic modification of TME pH using pH-modulating agents such as cariporide, lansoprazole, or acetazolamide may be a way to improve anti-tumor immune response in solid tumors. These types of pH-modulating therapies may also have applicability in the context of immunotherapies such as immune checkpoint blockade with anti-PD-1 or anti-PD-L1. Further *in vitro* and *in vivo* studies are needed to support these hypotheses.

We observed that cariporide treatment increases colorectal cancer cell-secreted cytokines that are involved in immune cell recruitment and activation. Analytes that increased post-cariporide treatment in at least two out of the three cell lines that are known to be immunostimulatory include CXCL11, TNF-alpha, IL-6, and prolactin. The CXCL11-CXCR3 axis is important for the migration [42] and activation [43] of CD8+ T lymphocytes as well as the recruitment of natural killer cells [44]. Moreover, CXCL11 is an independent prognostic biomarker and high CXCL11 expression is correlated with antitumor

IL-8/CXCL8, CXCL14/BRAK, Interleukin-18 (IL-18), sTRAIL-R2, CXCL5/ENA-78, TRANCE, prolactin, M-CSF, CCL11/Eotaxin, GM-CSF, sTRAIL-R3, sPD-L1, and CRP. Future experiments will characterize how lansoprazole and acetazol-

immunity in patients with colon adenocarcinoma [45]. Furthermore, TNF-alpha enhances NK cell cytotoxicity [46], T cell proliferation [47], and T cell activation [48]. Next, IL-6, a pleotropic cytokine, has been shown to promote T cell activation and proliferation [49], but has been shown to inhibit NK cell cytotoxicity [50]. Lastly, prolactin is an immunomodulatory hormone which increases NK cell cytotoxicity [51] and promotes T cell proliferation and activation [52]. The increases we noted in these immunostimulatory cytokines may help explain the results we observed in the co-culture system. However, it should be noted that many of these cytokines, chemokines, and growth factors have pleotropic roles in the tumor microenvironment and changes in their individual expression levels may not be predictive of immunoregulation or EMT in a biological system.

Cariporide treatment also decreased CRC cell-secreted cytokines that are commonly involved in immunosuppression and epithelial mesenchymal transition. Analytes that decreased post-cariporide treatment in at least two out of the three cell lines tested that have previously characterized immunosuppressive roles in the context of colorectal cancer included M-CSF, TNF-alpha, IL-8/CXCL8, CXCL13/BLC/BCA-1, GM-CSF, IL-10, and CRP. M-CSF is a hematopoietic growth factor that is frequently elevated in cancer patients. Interestingly, in patients with colorectal cancer, elevated serum M-CSF levels are correlated with increased lymph node metastasis and poor prognosis [53]. Overexpression of TNF-alpha, an inflammatory cytokine, is correlated with advanced colorectal cancer stages and is well known to activate transcription factors that induce EMT [39, 54]. Additionally, the chemoattractant cytokine IL-8 is upregulated in colon cancer tissue compared with normal adjacent colonic tissue and IL-8 expression in CRC cancer stem cells is regulated by the EMT activator protein SNAIL [55]. Moreover, CXCL13/BLC/BCA-1 is an inflammatory factor that plays a role in colorectal cancer growth and invasion via the PI3K/AKT pathway [56]. The CXCL13/CXCR5 axis has also been shown to regulate the epithelial to mesenchymal transition in breast cancer [57], prostate cancer [58], and non-small cell lung carcinoma [59]. Next, GM-CSF is a hematopoietic cytokine that has been shown to facilitate the development of inflammation-associated colorectal carcinoma [60]. Similarly, elevated serum levels of IL-10 are also corelated with advanced colorectal cancer [61]. Finally, CRP is a liver-secreted protein that is associated with an inflammatory response and plasma CRP concentration is a biomarker of colorectal cancer [62].

We also observed a significant decrease in VEGF secretion when HCT-116 colorectal cells were treated with NHE1 inhibitor cariporide. VEGF is a potent angiogenic factor upregulated by hypoxia and other conditions, and VEGF downregulation with NHE1 inhibitors has also been observed in other cancer cells [63]. Moreover, NHE1 regulation has been implicated in the metastatic potential of many cancer cell types including breast [64], acute lymphoblastic leukemia [65], gastric, liver, esophageal, and cervical cancer [66]. In contrast, VEGF increased in HT-29 and KM12C cells post-cariporide, indicating a heterogenous cytokine profile response of colorectal cancer cells to treatment and emphasizing the complexity and interrelatedness of pathways involved in regulating the cellular response to hypoxia and pH. Therapeutic modification of TME pH using pH-modulating agents such as cariporide, may be a way to help prevent EMT in solid tumors with certain genetic backgrounds by modifying the tumor cell secretome. Future experiments are needed to determine which genetic markers may be used as biomarkers of response to pH-modulating therapies in colorectal cancer.

Cariporide treatment decreased sTRAIL-R2, sTRAIL-R3, and sPD-L1 in both HCT-116 and HT-29 cell lines post-cariporide treatment. Interestingly, these analytes all increased in the KM12C cell line, again emphasizing the heterogeneity in cytokine profile response to cariporide treatment. TRAIL-R2 is a tumor cell-expressed surface receptor involved in the apoptotic response upon binding with this cognate ligand, TNF-related apoptosis-inducing ligand (TRAIL). The soluble version of TRAIL-R2 is thought to function as a decoy receptor for TRAIL, meaning an increase in sTRAIL-R2 might lead to a decrease in the amount of TRAIL that binds to the cell surface-bound version of TRAIL-R2, thus reducing tumor cell apoptosis. Moreover, TRAIL-R3 is a decoy receptor that also functions by binding TRAIL, and increased

concentrations of sTRAIL-R3 might similarly function to decrease the amount of TRAIL that binds to tumor cells to mediate apoptosis. The soluble versions of these receptors may have relevance in the context of TRAIL receptor-targeting monoclonal antibodies which could be bound by soluble TRAIL receptors, thus reducing efficacy of these therapies. Lastly, PD-L1, also known as B7 homolog 1 (B7-H1), is a protein that binds to PD-1 on the surface of lymphocytes to inhibit cell activation and proliferation. The soluble version of PD-L1 presumably functions as a decoy receptor in a similar manner that may be predicted to decrease efficacy of immunotherapy.

These experiments can help inform future investigations into how pH may be extrinsically modulated to improve immune cell response, possibly in conjunction with conventional chemotherapeutics, immunotherapies, or other treatments for colorectal cancer. Future experiments could also determine if therapeutically-induced cytokine modulation is pH-dependent or independent. Additionally, we are planning future experiments to test these pH-modulating agents in the context of hepatocellular carcinoma, a cancer type often treated with local therapies such as embolization-based procedures that rely on hypoxia and immune cell infiltration for therapeutic effect.

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Disclosure of conflict of interest

None.

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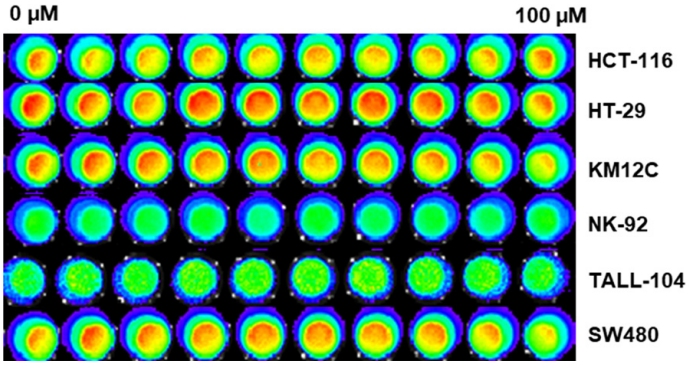
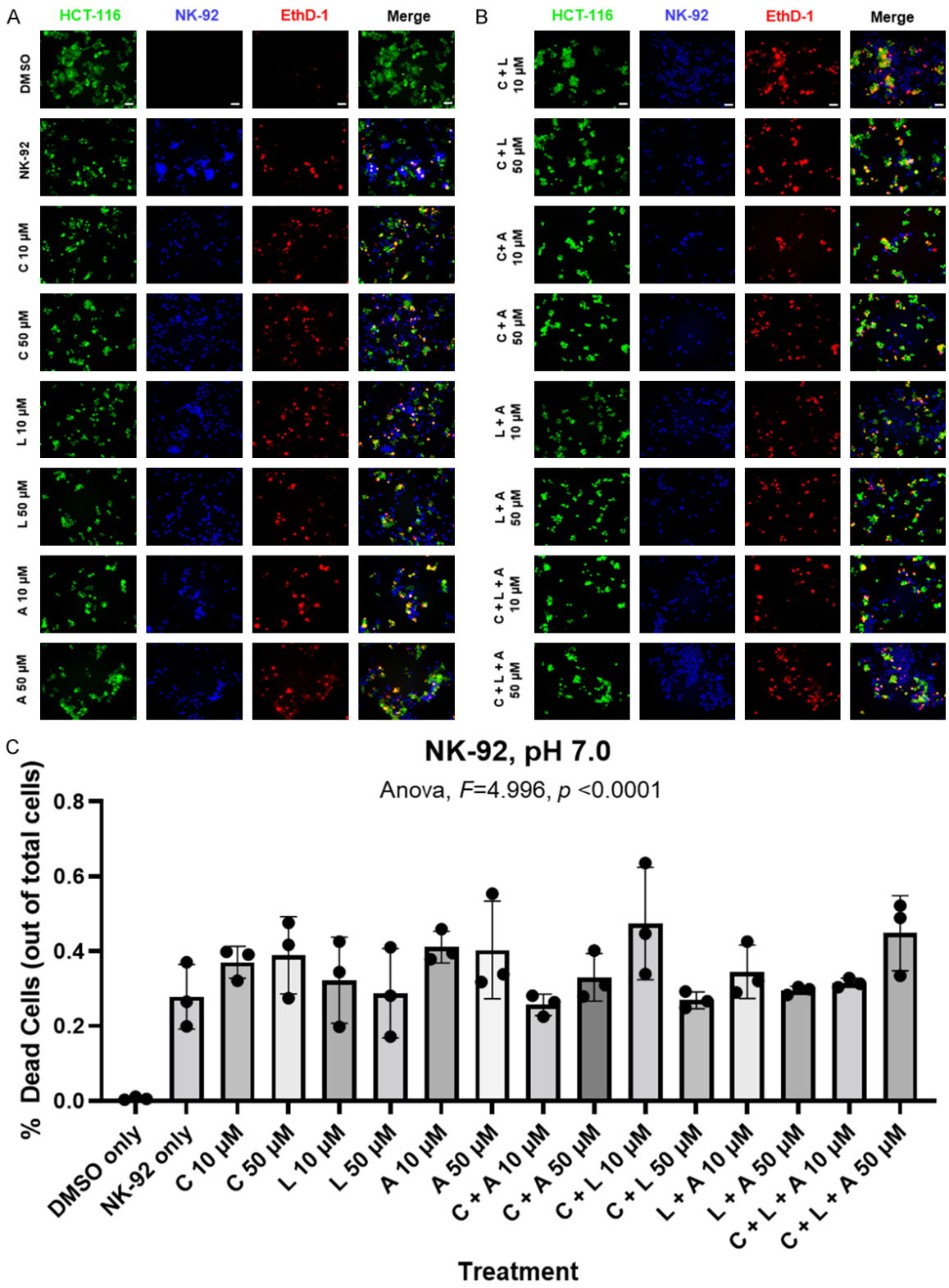
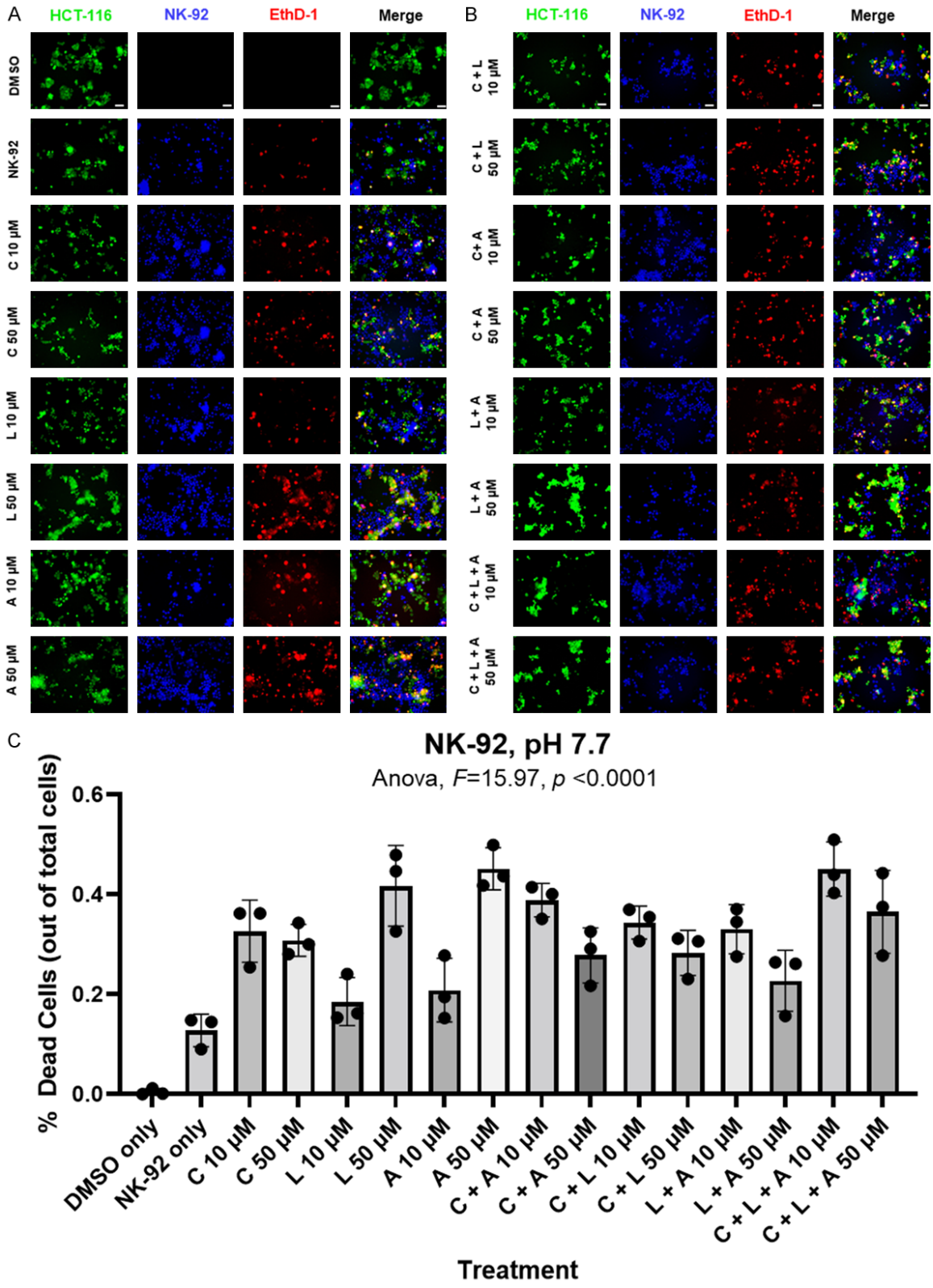


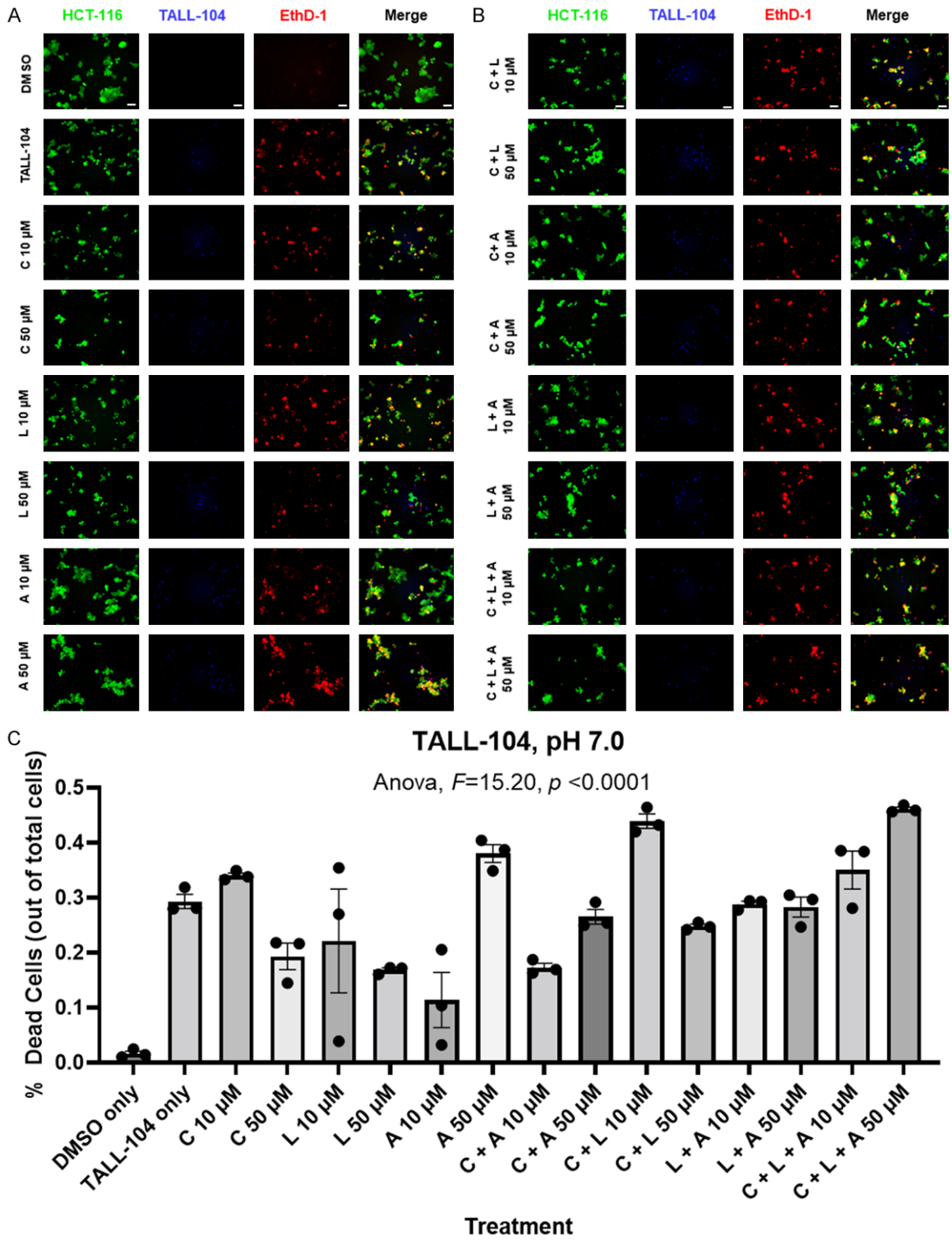
Supplementary Figure 1. Cell viability results show that NHE1 inhibitor cariporide is non-toxic to immune cell and tumor cell lines selected for analysis at the concentrations used. Colorectal cancer cell lines HCT-116, HT-29, KM12C, and SW480 and immune cell lines NK-92 and TALL-104 were treated with doses up to 100 µM of cariporide for 48 hours.



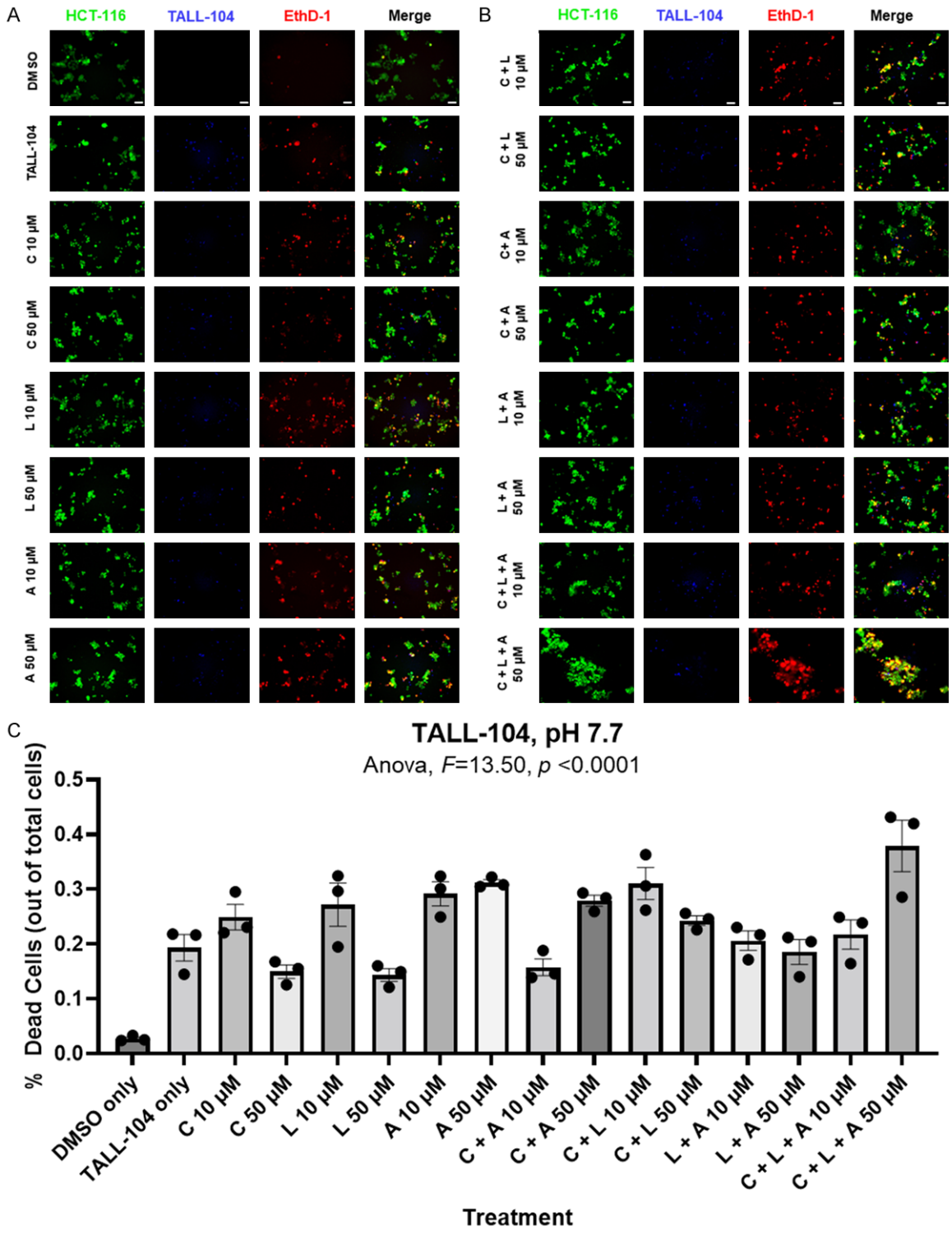
Supplementary Figure 2. Treatment of HCT-116 colorectal cancer cells with pH-modulating agents increases natural killer cell killing at pH 7.0. Co-culture of HCT-116 colon cancer cells and NK-92 cells with designated drugs using a cell culture media pH of 7.0. A 1:1 effector: target cell ratio was used. "C": Cariporide. "L": Lansoprazole, "A": Acetazolamide. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 µm. (A) Single treatment results and (B) combination treatment results. (C) Quantification of images in (A and B).



Supplementary Figure 3. Treatment of HCT-116 colorectal cancer cells with pH-modulating agents increases natural killer cell killing at pH 7.7. Co-culture of HCT-116 colon cancer cells and NK-92 cells with designated drugs using a cell culture media pH of 7.7. A 1:1 effector: target cell ratio was used. “C”: Cariporide. “L”: Lansoprazole, “A”: Acetazolamide. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 µm. (A) Single treatment results and (B) combination treatment results. (C) Quantification of images in (A and B).



Supplementary Figure 4. Treatment of HCT-116 colorectal cancer cells with pH-modulating agents increases T cell killing at pH 7.0. Co-culture of HCT-116 colon cancer cells and TALL-104 cells with designated drugs using a cell culture media pH of 7.0. A 1:1 effector: target cell ratio was used. "C": Cariporide. "L": Lansoprazole, "A": Acetazolamide. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 µm. (A) Single treatment results and (B) combination treatment results. (C) Quantification of images in (A and B).



Supplementary Figure 5. Treatment of HCT-116 colorectal cancer cells with pH-modulating agents increases T cell killing at pH 7.7. Co-culture of HCT-116 colon cancer cells and TALL-104 cells with designated drugs using a cell culture media pH of 7.7. A 1:1 effector: target cell ratio was used. “C”: Cariporide. “L”: Lansoprazole, “A”: Acetazolamide. 24-hour timepoint and 10× magnification. Ethidium homodimer was used to visualize dead cells. Scale bar indicates 100 µm. (A) Single treatment results and (B) combination treatment results. (C) Quantification of images in (A and B).