Analyzing Jurkat T Cell Viability and T Cell Receptor Signaling in Tumor Microenvironments

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The immune system is responsible not only for protection against foreign invaders (e.g., viruses and bacteria) but also against abnormal cell growth (e.g., cancer). Although the initiating causes of cancer are wide-ranging, one universal characteristic common to all is that healthy cells mutate in ways that dysregulate their growth and survival. Unchecked, these cells may grow and mutate uncontrollably, making immunosurveillance vital to maintaining a healthy organism. T cells recognize foreign antigens via their T Cell Receptors (TCRs). A sampling of all proteins made by the cell is presented to T cells in the context of Major Histocompatibility Complexes (MHCs) present on all nucleated cells. A successful interaction between a T cell and an MHC bearing a non-self peptide will initiate an immune response including TCR signaling and proliferation. Cells that avoid this recognition can grow into tumors. Interestingly, these tumors have been observed to exist in acidic microenvironments. One concern is that the acidic nature of the tumor microenvironment could negatively impact the interaction between the T cell and MHC molecule thereby reducing the efficacy of an immune response. We used Jurkat T cells as a model T cell line to evaluate the survival and immune signaling of T cells under low pH environments. We observed less total phosphorylation in the acidic conditions, however, the relative increase in phosphorylation above background was much greater under acidic conditions. These results indicate a qualitative change in T cells activation signals when exposed to an acidic environment.

The immune system is responsible for protecting the body from foreign invaders such as viruses and bacteria, as well as endogenous abnormal cell growth.[1,2] One way that the immune system recognizes and destroys abnormal cells and invaders is through recognition by T cells[3] which recognize foreign/non-self peptides via randomly generated (and negatively selected against self) TCRs.[3,4] Throughout the body, all nucleated cells present a sampling of their cellular proteins within MHCs on the surface of the cell for interrogation.[5] There are two classes of MHC molecules, each responsible for presenting peptides, from either exogenous or endogenous sources to T cells. This interaction between T cells and MHC molecules occurs throughout the body via MHC class I antigen presentation by every nucleated cell or via MHC class II antigen presentation of exogenous material taken up and processed by antigen-presenting cells (APCs). A successful interaction between the TCR on a T Cell and an MHC bearing foreign/non-self peptide will initiate an immune response resulting in a cascade of phosphorylation events constituting a signal from the TCR.[1,6,7] In the case of CD8 T Cells, this signal leads to cell proliferation and the secretion of cytolytic substances resulting in the destruction of the target cell.[1,8,9]

Endogenously arising neoplasms that should be destroyed by the immune system sometimes go unrecognized. This insufficient immune response leads to the development of cancer and has become a major source of morbidity and mortality in an otherwise healthy, aging population.[10] In recent years, the view of cancer has shifted from an autonomous cellular disease to a complex system of interactions between cancer cells and the tumor microenvironment (TME).[10] This shift has led scientists to believe that to truly understand and treat cancer more effectively, it is vital to understand the interaction of the tumor with the immune system in the context of these TMEs.

When healthy cells mutate in a way that dysregulates their growth and survival, it leads to the loss of healthy cellular checkpoints and inhibitory signals.[11] These mutations, along with the promotion of growth signals, loss of anti-growth signals, evasion of apoptosis, unlimited replicative potential (immortalization), sustained angiogenesis (formation of new blood vessels), tissue invasion, and metastasis are characteristic hallmarks of cancer.[12] In most cases, immune cells recognize these hallmarks, however, because cancer cells emerge from normal healthy cells, the antigens they present are unseen by a lymphocyte repertoire that has been cleared of self-reactive cells. If T (and B) cells are unable to recognize these cancer cells, they will begin to grow uncontrollably resulting in the formation of tumors.

As these tumors form, they create a unique TME which consists of immune cells, stromal cells, blood vessels, and extracellular matrix.[13] Interestingly, these TMEs have been observed to be acidic, with a pH as low as 5.5,[13,14] which is drastically different from the normal physiological pH of blood and tissues, pH 7.4.[15] As T cells survey the body to check for abnormal cell growth, T cells will inevitably sample cells within a tumor.[13] At this time, the T cell is exposed to the acidic TME, where the acidic environment can negatively impact proteins, potentially causing them to denature.[16,17] It

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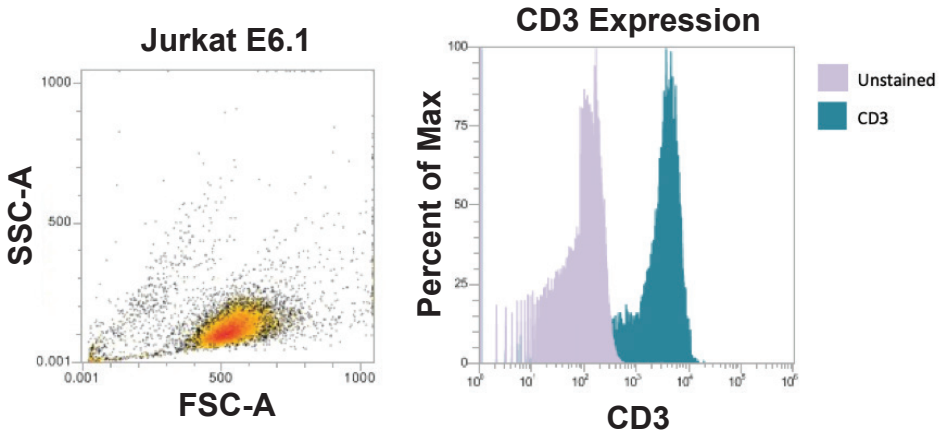

Figure 1 | Jurkat E6.1 cells express surface receptor expression of CD3. The surface expression of the CD3 receptor on Jurkat E6.1 T cells was assessed by flow cytometry. Representative plots of CD3 expression of Jurkat cells stained for CD3 vs unstained cells are shown.

is possible that the acidic TME could negatively impact the interaction between the T Cell's TCR and the cancer cell's MHC, thereby reducing the efficacy of the immune response.

Indeed, it has been suggested that acidic environments do inhibit T cell signaling. Gillies et al. demonstrated that T cells may produce "acidic niches" in lymph nodes which suppress their effector functions and act as negative regulators of T cell responses.[18] They also showed that "low pHe [extracellular pH] does not block the process of activation by antigen, [but] it will suppress the production and release of many (but not all) cytokines."[18] This suggests that the acidic environment within the lymph node acts as a mechanism to protect the lymph node tissue from an overactive T cell response. While T cells were still able to become activated within this acidic environment, evaluation of the downstream TCR signaling pathway under acidic environments was not shown, and the question remained whether the suppressive nature of an acidic TME could be hindering the T cell response to cancer.

Intriguingly, it has also been shown that proteins such as ZAP70, a signaling protein in the TCR pathway, may experience greater phosphorylation following exposure to an acidic microenvironment.[19] This would seem to suggest that the T cell does, in fact, respond differently in various pH environments, leaving open whether this difference leads to a qualitative change in outcome for the responding cell. A review of recent literature on this topic is summarized by Hwang et al.[6] Briefly, in CD4 T Cells, a strong TCR stimulation is associated with T helper type 1 (Th1) differentiation, while a relatively weaker stimulation drives cells to a Th2 fate.[20] This fate decision is critical to the success of the response as Th1 responses are required to support immune reactions against intracellular infections and, of critical interest here, cancer; while Th2 responses support reactions targeting extracellular parasites.

In this research, we sought to investigate how the acidic TME affects T cell signaling in the context of tumor recognition using Jurkat T cells in vitro. This was done by verifying the surface expression of the TCR on this model T cell line, establishing TCR expression and viability of these cells following acidic exposures, and ultimately evaluating the signaling of CD3-activated Jurkat T cells (via phosphorylation of tyrosine residues) following incubation and stimulation under acidic conditions.

Materials and Methods

Cell Line

The Jurkat E6.1 T cells were kindly provided by Dr. Jon Houtman and Dr. Gary Weisman (previously purchased from ATCC: TIB-152TM). Jurkat E6.1 T cells were grown at 37°C in 5% CO_2 using complete RPMI 1640 media (Gibco, Cat. No. 11875119) supplemented with 10% FBS (HyClone, Cat. No. SH30541.03), 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco, Cat. No. 15140-122). Cultures were maintained at a concentration of 1-5x10^5 cells/mL.

Preparation of Acidic Buffers

Acidic buffers were prepared at a concentration of 1 M (pH 5.5 MES Sodium Salt [Cat. No. M-091-50], pH 6.5 Bis-Tris [FisherBiotech, Cat. No. 6976-37-0]) and adjusted to the appropriate pH by titrating with 6 M HCl. The pH was measured by an electronic pH meter (Mettler Toledo, FiveEasy Plus). Buffers were then sterile filtered and stored at 4°C.

Confirmation of CD3 on Jurkat E6.1 T Cells

TCR expression on Jurkat E6.1 cells was assessed by staining with 5 µL anti-CD3•FITC (Invitrogen, Cat. No. 11-0037-42), and incubating in dark for 20 minutes. Samples were analyzed using the Life Technologies Attune NxT Flow Cytometer.

Timepoint Viability and CD3 Assay

To determine Jurkat E6.1 viability and maintained expression of the TCR under acidic conditions, cells were plated, treated with 100 mM buffer (pH 5.5 MES, pH 6.5 Bis-Tris, or pH 7.4 DI water), and incubated at 37°C in 5% CO_2 for 15, 30, 45, or 60 minutes. Samples were then collected, washed, resuspended in 1 mL 1% BSA in PBS (Fisher Bioreagents, Ca. No. BP1605-100), and stained with SYTOX AADvanced Ready Flow (Invitrogen, Cat. No. R37173). Samples were then analyzed using the Attune NxT Flow Cytometer. The percentage of live and dead cells were determined. Once a timepoint for viability was established, the expression of CD3 was confirmed following a 60 min incubation at the indicated pHs. Cells were treated with the appropriate buffer as above and incubated at 37°C for 60 min. Samples were collected, washed, and resuspended in 1 mL 1% BSA in PBS, stained with 5 µL CD3•FITC and SYTOX AADvanced Ready Flow, and incubated on ice in the dark for 20 min. Samples were then analyzed using the Attune NxT Flow Cytometer.

Cell Signaling Assay

Jurkat E6.1 T cells were grown at a concentration of 1-5x10^5 cells/mL. 2x10^6 cells were treated with 100 mM buffer (pH 5.5 MES, pH 6.5 Bis-Tris, and pH 7.4 DI water). Samples were collected, washed, and resuspended in 200 µL RPMI. Stimulated samples were treated with 20 µg/mL of azide-free anti-CD3 (OKT3) antibody (Biolegend, Cat. No. 317325) for 2 min in a 37°C water bath. Samples were washed with RPMI, lysed with RIPA buffer (150 mM Sodium Chloride (Fisher Science Ed-

ucation, Cat. No. S25541A), 50 mM Tris-HCl (Fisher Scientific, Cat. No. BP153-1) pH 8.0, 1% Triton X (Fisher Scientific, Cat. No. BP151-100), 0.5% Sodium Deoxycholate (Sigma Aldrich, Cat. No. 302-95-4), 0.1% SDS (MP Biomedicals, Cat. No. 190522) + 1 mM Sodium Orthovanadate (MP Biomedicals, Cat. No. 159664), run through a 20G needle to reduce viscosity, and then spun at 21.1 RCF for 15 min to remove nuclear and membrane material.[21] The protein concentration of the cell lysate was determined using Pierce BCA Kit (Thermo Scientific, Cat. No. 23225).

Western blotting

The protein components, 35 µg per sample, were prepared using 6x Laemmli SDS sample buffer (Alfa Aesar, Cat. No. J61337) supplemented with 5% ß-mercaptoethanol (MP Biomedicals, Cat. No. 190242), boiled at 95°C for 5 min, and separated by SDS-PAGE using an 8% polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane (BIO-RAD, Cat. No. 1620112) and blocked with 5% milk (LabScientific, Cat. No. M0841) in TBST (50 mM Tris Base (Fisher Bioreagents, Cat. No. BP152-1), 150 mM Sodium Chloride, 1% Tween 20 (Thermo Scientific, Cat. No. J20605-AP), adjusted to pH 7.5) for 1 hour at room temperature. The membrane was then incubated at room temperature with primary antibody, mouse anti-phosphotyrosine (1:1000) (Sigma-Aldrich Ref. No. 05-321), in 1% milk in TBST for 1 hour, washed 3x for 10 min in TBST, followed by secondary antibody incubation, horse anti-mouse•HRP (1:1000) (Cell Signaling, Cat. No. 7076S), for 1 hour at room temperature, and washed 3x for 10 min in TBST. 1 mL ECL was added to the membrane (Thermo Scientific, Cat. No. 32209) and visualized by chemiluminescence using a Li-Cor C-Digit imager. The membrane was then stripped (200 mM glycine (Invitrogen, Cat. No. 15527-013), 3.5 mM SDS, 1% Tween 20, adjusted to pH 2.2) and blocked with 5% milk in TBST for 1 hour at room temperature. The stripped membrane was incubated at room temperature with primary antibody, rabbit anti-ß-actin (1:1000) (Cell Signaling, Cat. No. 4970S), in 1% milk in TBST for 1 hour, washed 3x for 10 min in TBST, followed by secondary antibody incubation, goat anti-rabbit•HRP (1:1000) (Cell Signaling, Cat. No. 7074S), for 1 hour at room temperature, and washed 3x for 10 min in TBST. 1 mL ECL was added to the mem-

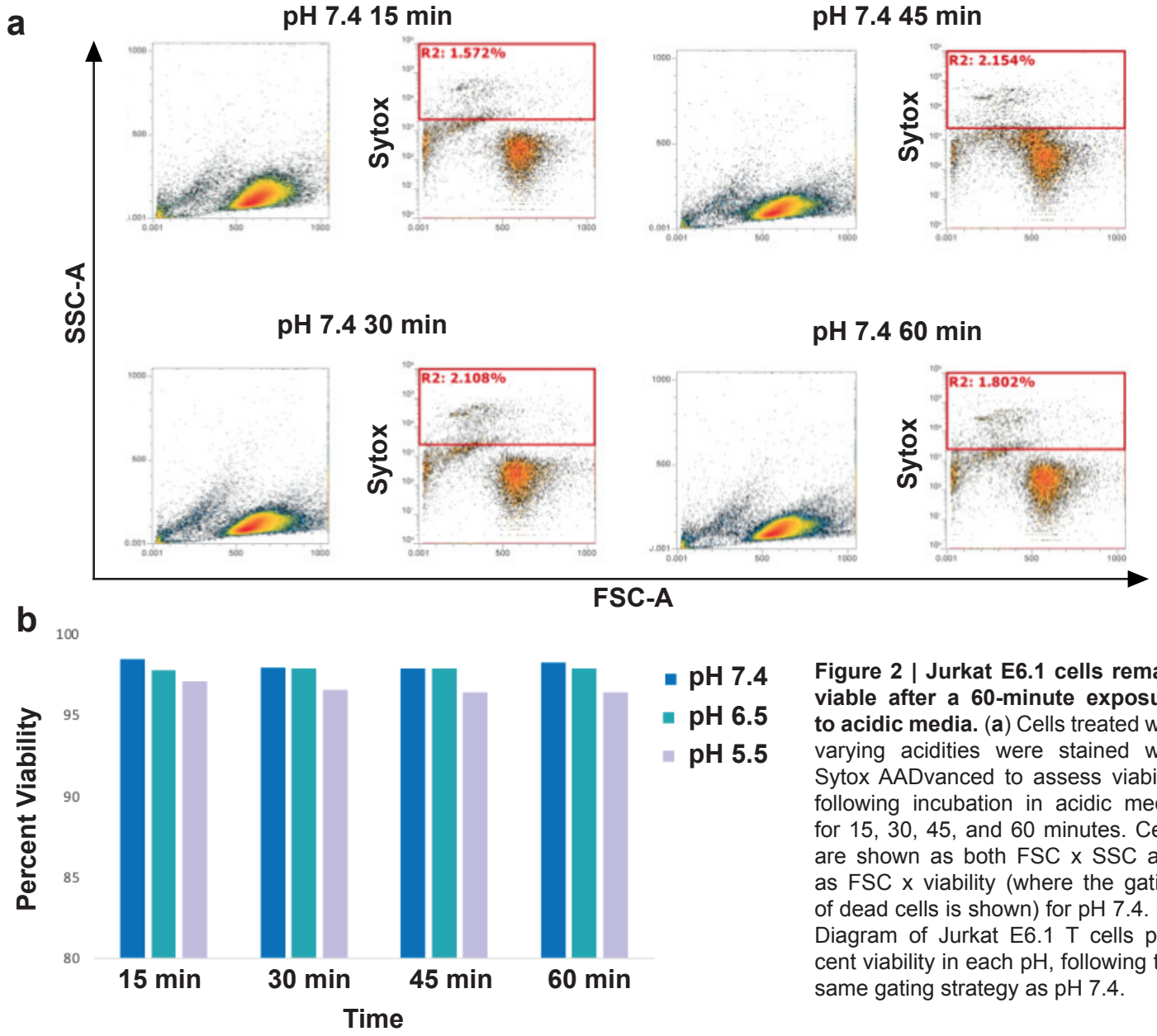

Figure 2 | Jurkat E6.1 cells remain viable after a 60-minute exposure to acidic media. (**a**) Cells treated with varying acidities were stained with Sytox AADvanced to assess viability following incubation in acidic media for 15, 30, 45, and 60 minutes. Cells are shown as both FSC x SSC and as FSC x viability (where the gating of dead cells is shown) for pH 7.4. (**b**) Diagram of Jurkat E6.1 T cells percent viability in each pH, following the same gating strategy as pH 7.4.

brane (Thermo Scientific, Cat. No. 32209) and visualized by chemiluminescence using a Li-Cor C-Digit imager.

Analysis of Western blotting

Immunoblots were analyzed using NIH Image J. The ratio of phosphotyrosine expression was determined for stimulated and unstimulated samples by first normalizing each protein to ß-actin expression and then normalized to the appropriate unstimulated condition.

Results

Jurkat E6.1 T cells contain the necessary receptors needed to induce TCR-mediated signaling.

The TCR complex consists of many components, that upon stimulation, result in intracellular signaling events, leading to an immune response. One component of this complex is the CD3 receptor. CD3 is a surface receptor on T cells that when engaged, initiates immune cell signaling. Jurkat E6.1 T cells have been shown to express this receptor, however over time in culture, these cells have been known to down-regulate their CD3 receptor from the surface (Jon Houtman, personal communication). Due to the potential downregulation of CD3, it was first necessary to establish CD3 expression on the Jurkat E6.1 T cells. To establish this, the surface expression of the CD3 receptor was examined by flow cytometry (**Figure 1**). When compared to the unstained control, a shift in the fluorescence intensity was observed, indicating high expression of CD3 on the surface of these cells.

Having established surface expression of the CD3 receptor on our initial population of Jurkat E6.1 T cells, we next wanted to model signaling during a short-term exposure of a T cell in a TME. To do so, we evaluated the viability of these cells exposed to the acidic environment for variable amounts of time. Time points were evaluated by plating Jurkat E6.1 T cells in media and exposing them to neutral or acidic conditions by adding high concentration buffers, (MES, Bis-Tris, and deionized water) to adjust the pH of the media to 5.5, 6.5, and 7.4, respectively. Cells were then incubated at 37°C and 5% CO_2 for 15, 30, 45, or 60 minutes. Once the incubation was complete, cells were collected, and viability was assessed by flow cytometry. Jurkat E6.1 T cell viability remained strong, 96%-98% viable, at each time point and pH (**Figure 2a and 2b**). Since it was established that viability did not decrease when exposed to an acidic environment for 60 minutes, the 60-minute exposure to the acidic media was chosen for the following experiments.

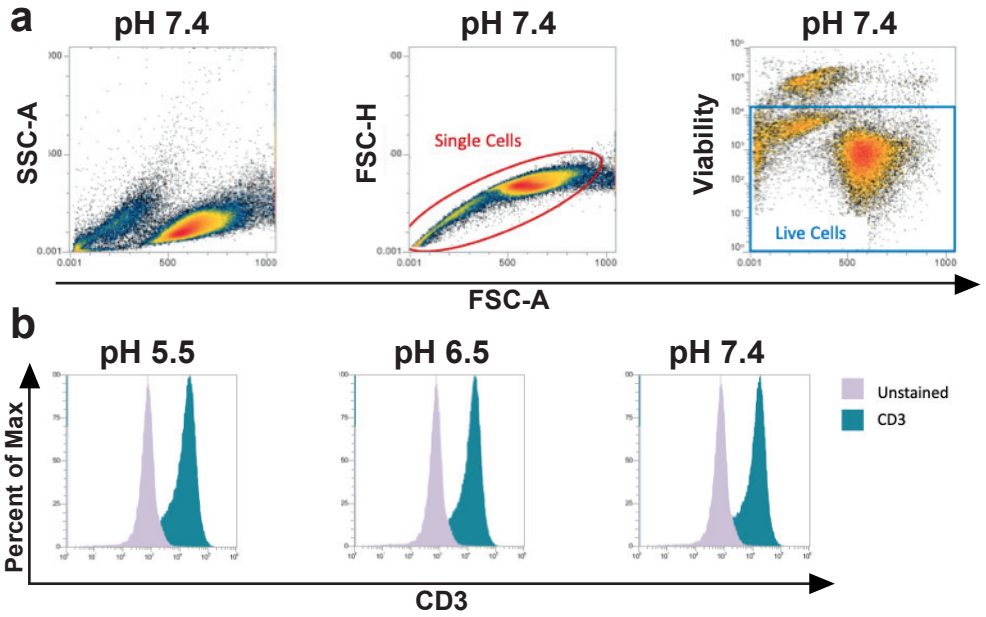

Figure 3 | Jurkat E6.1 cells maintain surface receptor expression of CD3 following 60-minute exposure to acidic media. (**a**) Cells incubated in media of varying acidities were stained with both anti-CD3-FITC and Sytox AADvanced to assess the surface expression of CD3 and viability following a 60-minute incubation in acidic or control (pH7.4) media. Surface expression of CD3 was evaluated using the gating strategy of single, live cells as exemplified by the representative culture at pH 7.4. (**b**) Following the gating strategy shown in **3a**, the CD3 expression of Jurkat E6.1 T cells stained for CD3 vs unstained cells is shown for each pH.

It has been previously shown that Jurkat E6.1 cells can decrease their surface expression of the CD3 receptor when exposed to acid, therefore it was again necessary to establish this expression at each pH condition following the 60-minute exposure. Cells were again incubated in their appropriate buffers for 60 minutes at 37°C and 5% CO_2. Following a 60-minute incubation, surface expression of the CD3 receptor was assessed by flow cytometry. We again observed a consistent shift in the fluorescent intensity, demonstrating that the CD3 receptor remained on the cell surface under all the conditions tested (**Figure 3**). Further, these receptors remain in a condition able to be bound by the antibody, suggesting, that CD3 can be used to stimulate and activate the Jurkat E6.1 T cells to evaluate the signaling pathway of activated T cells.

Jurkat E6.1 T cells experience quantitatively different signaling after exposure to acidic environments

Once the surface expression of the CD3 receptor was established after a 60-minute exposure to acidic media, we wanted to examine the signaling pathway of activated Jurkat E6.1 T cells following exposure to differing acidic environments. It is well appreciated that antigen receptor signaling is mediated by the phosphorylation of intermediate proteins through the generation of phosphotyrosines by kinase enzymes. This phosphorylation directly correlates with the strength of the signal activating these cells.[22] We were able to activate the Jurkat E6.1 T cells through exposure to anti-CD3 antibodies and evaluated the amount of protein phosphorylation as a measure of the strength of signaling. We observed an increase in tyrosine phosphorylation in each pH condition compared to the unstimulated condition (**Figure 4a**), which is to be expected.

It appears that one protein is only present after stimulation and is around 35 kDa in weight. While we cannot be sure of the exact identity of this protein, it is reasonable to assume that this protein is consistent with the protein Linker for the Activation of T Cells (LAT). LAT is a 36 kDa scaffolding

protein that is essential for the formation of a multiprotein signaling complex that will transmit signals from the TCR complex to downstream effectors.[23] Upon the formation of this multiprotein complex, LAT becomes heavily phosphorylated,[24] which is consistent with the increase in phosphorylation of the protein in **Figure 4a**.

To investigate the phosphorylation of this protein further, we performed densitometry on the putative LAT protein. To do this, we first normalized this protein to its beta-actin band to account for differences in protein loading. Following this normalization, we further normalized each stimulated condition to its pH-matched unstimulated condition. As seen in **Figure 4b**, following stimulation with anti-CD3, an increase in phosphorylation of putative LAT over pH-matched unstimulated cells was observed. We observed an approximately 100-fold increase in the amount of phosphorylation of this protein at pH 5.5, an approximately 41-fold increase in pH 6.5, and an approximately 17-fold increase in phosphorylation in the neutral condition. This increase in relative phosphorylation under acidic conditions compared to the neutral condition suggests a more nuanced measure of signal strength may be warranted.

Discussion

T cells remain the body's best defense against abnormal cell growth and cancer. However, many therapies are ineffective due to T cells' inability to respond to antigens with a high degree of similarity to 'self'. Effects of the TME on T cells remain a necessary area of research. Since the recent discovery that the lymph nodes contain acidic niches which have been speculated to act as negative regulators of T cells, it was important to consider the implications of this environment in the context of a TME and evaluate the effects of T Cell stimulation in an acidic environment. Here, we used tyrosine phosphorylation on what we expect is the LAT scaffolding protein to investigate the responses of cells in various pH environments.

To evaluate the T cell response after stimulation in an acidic TME we first established that Jurkat E6.1 T cells expressed a key component of the TCR, specifically the CD3 receptor. Once we established the expression of CD3, we modeled a short-term exposure of the T cell in a simulated TME by exposing the cells to both acidic and neutral pH conditions. There are a number of modulations that can happen to the TCR when exposed to acid. It could be that the TCR is denatured and can no longer recognize the presentation of peptide via MHC, or perhaps the surface expression could be downregulated. To directly study this, we stained the Jurkat E6.1 T cells for viability and CD3 expression following an acidic exposure. Viability and TCR expression were established, confirming that the Jurkat E6.1 TCR remained in a condition that could be stimulated by cross-linking antibody and remained an appropriate model to evaluate the signaling pathway of stimulated T cells. Once viability and TCR expression were confirmed, we evaluated the signaling pathway of activated T cells after being exposed to acidic environments. We found that the Jurkat E6.1 T cells were able to be stimulated in each pH condition following a 60-minute exposure through western blot analysis. When comparing the activation status of these cells, we evaluated the western blot both qualitatively and quantitatively. The first interpretation was to examine the relative total amount of phosphorylation present in the cells. It appears that qualita-

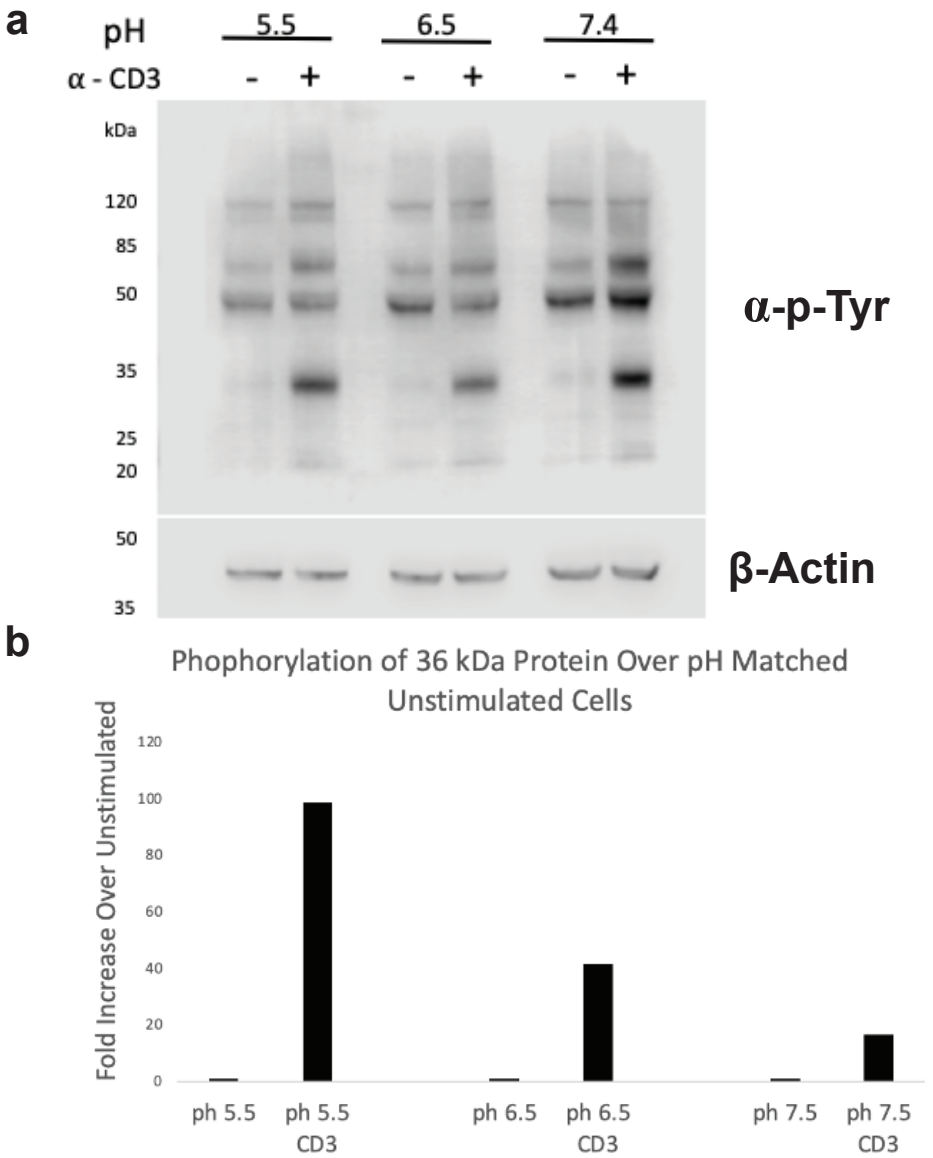

Figure 4 | Jurkat E6.1 cells experience stronger relative signaling after stimulation in acidic media. (**a**) Jurkat E6.1 T cells were incubated in varying pH conditions for 60 minutes. Following incubations, the cells were stimulated via anti-CD3 (OKT3) for 2 minutes and the protein extracts were separated using SDS-PAGE. Activation via anti-phosphotyrosine (4G10) was assessed by western blot analysis. Unstimulated (-) and stimulated (+) conditions for each pH are shown, along with the loading control, ß-actin. (**b**) Diagram of 35 kDa protein normalized to appropriate ß-actin and normalized to pH matched unstimulated control.

tively, the acidic conditions experience less total phosphorylation compared to the neutral condition which would indicate that the acidic environment is weakening the signal propagation of T cells.

The second interpretation was obtained quantitatively by determining the fold increase of phosphorylation in the stimulated conditions compared to the unstimulated using densitometry. After examining the increase of phosphorylation following TCR stimulation of putative LAT, we found that there is a drastic increase in the activation of putative LAT in an acidic environment compared to a neutral one. This would indicate that the Jurkat E6.1 T cells experience stronger activation signals when exposed to an acidic environment.

Future Directions

This study suggests a new way to measure antigen receptor signal strength in T cells that may be inconsistent with prior measures. Of interest is the question of how this signaling leads to the fate decisions of T cells engaging in an immune response, and whether this response is appropriate to meet the challenge of a growing tumor. While increased relative activation following TCR stimulation was observed in the acidic environment compared to the unstimulated condition, the total level of phosphorylation was lower than that seen in the neutral pH. It remains possible that this may not be sufficient to induce proliferation and cytokine release. To evaluate this hypothesis, a functional assay assessing T cell proliferation and cytokine release should be performed under each of the various pH conditions. Further, it would be interesting to repeat these experiments using freshly isolated naive T cells from peripheral blood to distinguish if the differences observed in Jurkat E6.1 T cells were recapitulated in primary cells, as these would be more representative of native function. Using primary cells would also allow for the examination of differences between $CD4^+$ and $CD8^+$ T cell responses in acidic versus neutral environments. This raises the question as to whether the more physiologically relevant $CD8^+$ T cells would respond similarly and allow for greater investigation of the functional outcomes these signals impart.

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Author's Biography

Rylee is an undergraduate in the biotechnology program at the University of Kansas, Edwards Campus. She has a passion for immunology, specifically lymphocyte signaling in response to cancer. She plans to gain more research experience in a position at the Mayo Clinic where she will continue to develop her skills before pursuing a Ph.D. in immunology.

Author Contributions

R.M.H contributed to the experimental work, design, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.

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