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Kwame Armah April 12, 2022

Hygiene Hypothesis in Acute Lymphoblastic Leukemia

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2022

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Abstract

Hygiene Hypothesis in Acute Lymphoblastic Leukemia
By Kwame Armah

The Hygiene Hypothesis (HH) posits that we live in a society that is becoming devoid of adequate pathogen exposure relative to our past ancestors without 21st-century medical care. Furthermore, this hypothesis suggests that our immune system becomes limited in identifying and responding to insults, including pathogens and cancer cells, which leads to higher mortality rates in these contexts. Cancer is driven by cell-intrinsic (e.g., mutations) and extrinsic (e.g., tumor microenvironment) parameters.

The immune system governs the development or elimination of cancer cells, with T-cells (cell-mediated immunity) (TCs) playing a role in the destruction of malignant cells. Cell-mediated immunity relies on potent naive and memory TC responses to foreign antigens. As a result, research and clinical efforts are focused on optimizing their efficacy as new forms of cancer treatments, immunotherapy. In this study, we hypothesized that pathogenic exposure enhances the ability of TCs to eliminate cancer cells. We predict that this is due to components of the HH where memory TCs generated in response to pathogenic exposure, direct infections, or vaccinations eliminate cancer cells in a process called “cross protection/cross recognition.” This property relies on the potent killing capacity of memory TCs mediated by the death-receptor CD95 expressed on the surface of these cells and the inherent property of some cancer cells expressing high surface levels of the receptor for CD95 known as CD95 ligand (CD95L). This study will provide mechanistic insight into the extent of cross-protection in B-cell acute lymphoblastic leukemia (B-ALL) models and provide public health information regarding the potentially pleiotropic health benefits of vaccinating pediatric populations.

Methods: To define the surface expression (SE) of CD95 on human T-cells and CD95L on human B-ALL cell lines, non-malignant primary B-cells, and malignant B-cells from pediatric patients with B-cell acute lymphoblastic leukemia (B-ALL), we fluorescently detected CD95/CD95L via flow cytometry (FC). The SE of the proteins was determined by using Flowjo software. To define if the “cross recognition/cross protection” of malignant B-cells by antigen-stimulated TCs is mediated through the CD95/CD95L pathway, FC, and live-cell imaging to determine malignant B-ALL death when co-cultured with unstimulated and stimulated TCs.

Results: We have confirmed human B-ALL SE of CD95L. We have also confirmed that TCs can cross-recognize and kill malignant B-ALL cells. Next, we will determine if cytotoxicity depends on the CD95/CD95L pathway and if primary B-ALL cells from patients are killed by TCs using this mechanism.

Hygiene Hypothesis in Acute Lymphoblastic Leukemia

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Chapter I: Introduction

A. Cancer

Cancer encompasses an umbrella of over 100 diseases that are characterized by abnormal cell growth and have the potential to spread into additional parts of the body.[3] In 2020, cancer accounted for an estimated 19.3 million cases and caused over 10 million deaths globally.[3] The World Health Organization (WHO) classified cancer as either number one or two in worldwide causes of death before the age of 70.[3] As a result of its pathogenicity, global cancer burden poses an obstacle to enhanced life expectancy.[3]

Cancer is a byproduct of a malfunctioning cell cycle (CC). Progression through the CC is essential for multicellular organisms to grow, reproduce, repair, or regenerate tissues. This ordered series of events encompasses cell growth, DNA replication, and cell division.[1] The CC is heavily regulated to prevent unchecked advancement.[1] Regulation of the cell cycle is generally maintained by four types of genes.[2] These genes are proto-oncogenes, tumor suppressor genes, DNA repair genes, and apoptotic genes.[2] Proto-oncogenes are normal genes that become cancerous when mutated or epigenetically altered. Tumor suppressor genes halt the cell cycle when a problem is identified. DNA repair genes fix errors within the genetic code that arise during DNA replication or external manipulation. Apoptotic genes signal a cell to self-destruct if it has become old or damaged beyond repair. Accumulation of DNA damage in these genes can facilitate unfettered cell growth and replication.[2] Understanding the cell cycle and how we can play a role in lowering risk factors for malignant development is vital to decreasing cancer burden and extending global life expectancy.

I. Solid vs. Blood Cancer (Hematological Malignancies)

Cancer fall into two categories, solid and blood-derived. Solid cancers are abnormal masses of cells that grow within organ systems. This type of malignancy does not contain large amounts of soluble or cyst elements. Examples of common solid cancers include breast, lung, prostate, colorectal, melanoma, and bladder cancer.[4,5] Solid cancers account for 94% of global incidences of cancer and 93% of cancer-related deaths.[22] In contrast, hematologic cancers originate within the blood, bone marrow, and lymph. Examples of common blood cancers include Leukemia, Lymphoma, and Myeloma.[4,5] Hematological cancers generally do not form solid tumors. Blood cancers account for 6% of global incidences of cancer and 7% of cancer-related deaths.[22]

II. High Risk Factors for Oncogenesis

Cancer is a disease that arises from changes within our genetic code.[6] Carcinogens exacerbate the likelihood for our cells to generate errors, fail to repair errors, and facilitate oncogenesis.[6] This occurs through changes in cellular metabolism, DNA damage, DNA repair mechanism damage, level of oxidative stress, levels and duration of inflammation, cell immortalization, immunosuppression, alteration in cell proliferation, alteration in cell death, and alteration in cell nutrient supply.[6] These modifications influence the cell cycle and prompt malignant cell division.[6] While the International Agency for Research on Cancer identified over 500 carcinogens, the WHO defines three carcinogenic categories: physical, chemical, and biological carcinogens: physical factors such as ultraviolet radiation and ionizing radiation; chemical factors such as asbestos, alcohol, and arsenic; biological carcinogens include infections from certain viruses, bacteria, or parasites.[6,7] External factors are influenced by lifestyle choice and enhanced with age due to maintenance mechanisms degradation.[8] Understanding risk factors and promoting lifestyle changes may be necessary for lowering cancer incidence.

III. Immunosuppression

The immune system is a critical barrier in preventing disease and plays a role in cancer development. Higher rates of carcinogenesis are correlated with immune disorders.[9,10] If the immune response does not recognize damaged cells, abnormal cellular proliferation, the mis-expression of surface proteins marking cells for death, oncogenic gene expression, and other markers of carcinogenesis, cancer development is initiated.[9,10] TCs mediate cell-mediated immunity. The main players of this type of immunity are naive and memory TC who responses to foreign antigens (e.g., bacterial or viral) and aberrantly expressed "self-antigens." However, cancers do not evolve from exogenous sources; they manifest via mutations in our genome.[10] Therefore, the lack of "foreign" antigens creates opportunities for immune evasion. Furthermore, through cancer immunoediting, cancer cells evade immunosurveillance.[12] Our immune system places selection pressure on malignant cells to have a non-immunogenic phenotype, a phenotype that does not produce a robust immune response. Our immune system unintentionally does this by eliminating cancer cells that rapidly divide, produce inflammatory cytokine, and are genetically unstable. Therefore, the cancer cells that survive exhibit genetic mutations that augment their ability to evade immune-mediated destruction.[12,13] This process is called cancer immunoediting.[12,13] In conclusion, research efforts to enhance our immune system's ability to recognize and destroy cancer cells will lower the incidence of disease and global cancer burden.

IV. Adiposity and Carcinogenesis

High body mass index (BMI) is a risk factor for all-cause mortality to its comorbidities such as hypertension, cancers, stroke, osteoarthritis, diabetes, dyslipidemia, and cardiovascular

disease.[38] In 2016, 44% of adults worldwide were overweight or obese.[11,23-26] For adults, a BMI between 25.0 – 30 is classified as overweight, while greater than 30 is classified as obese by the CDC.[27]

Our immune system is modulated by environmental factors such as high adipose tissue levels. A key feature of our innate immune system is the production of inflammatory cytokines, chemokines, and other macromolecules.[11,14] The inflammatory response is activated and intensified in high BMI individuals.[14] A cancerous feedback loop is promoted in high adipose environments: inflammatory response mediators, such as cytokines, chemokines, and free radicals, cause tissue damage and oxidative stress. These states induce chronic inflammation which increases the risk of carcinogenesis.[9] Other mechanisms of carcinogenesis related to high levels of adipose tissue arise via hormonal secretion of estrogen, insulin, and adipokines.[11] Likewise, a recent study observed how the tumor microenvironment (**TME**) is modified in obese settings (Figure 1). The obese TME impairs infiltrating $CD8^+$ T-cells by limiting PHD3 expression on tumor cells. Simultaneously, the increased fat uptake accelerates tumor growth.[36] In conclusion, obesity is a risk factor for carcinogenesis and should be studied closely to determine its impact of carcinogenesis, immunity, and developing immune-based therapies for cancer treatment.

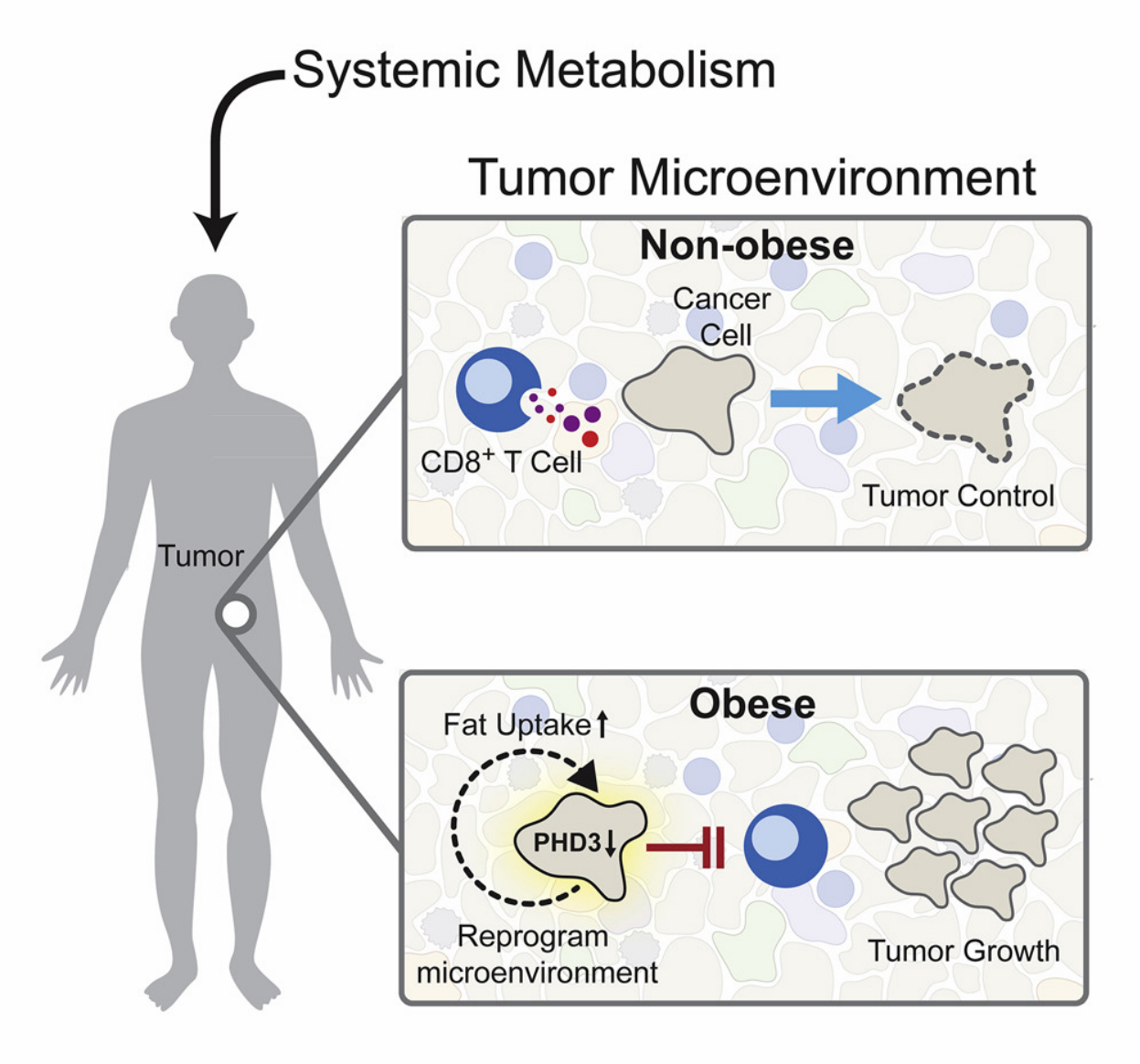

Figure 1. Graphical Abstract of Obesity Shapes Metabolism in the Tumor Microenvironment to Suppress Anti-Tumor Immunity. *The obese environment impair infiltrating CD8+ T-cells by limiting PHD3 expression on tumor cells. PHD3 Loss Correlates with Reduced Anti-Tumor CD8+ T Cell Function across Multiple Human Cancers.*

Note: Adapted from Ringel, A. E., Drijvers, J. M., Baker, G. J., Catozzi, A., García-Cañaveras, J. C., Gassaway, B. M., Miller, B. C., Juneja, V. R., Nguyen, T. H., Joshi, S., Yao, C.-H., Yoon, H., Sage, P. T., LaFleur, M. W., Trombley, J. D., Jacobson, C. A., Maliga, Z., Gygi, S. P., Sorger, P. K., … Haigis, M. C. (2020). Obesity shapes metabolism in the tumor microenvironment to suppress anti-tumor immunity. Cell, 183(7). https://doi.org/10.1016/j.cell.2020.11.009

B. Vaccination

Vaccines strive to prevent illness, hospitalization, and death by developing a defense against specific pathogens through a stimulated infection. Our adaptive immune system is broken down into humoral and cell-mediated immunity. B-cells are responsible for the humoral response, while TCs are responsible for the cell-mediated response. These lymphocytes work in tandem to modulate our adaptive immunity. B-cells produce antibodies.[39] When antibodies bind to a foreign antigen, our immune system mounts a response: plasma cells proliferate and generate antibodies, antibodies are secreted to attract phagocytic cells, antibodies agglutinate pathogens, antibodies block pathogens from entering cell tissue, and memory B-cells are made in preparation for another infection.[39] T-cells use their T-cell receptor to conduct immunosurveillance.[39] Effector TCs are sentinels which recognize and kill target cells expressing foreign antigens or those expressing surface-bound death receptors, such as such as CD95L ligand, granzyme-B, and tumor necrosis factors.[39,42-43] Death receptors are responsible for apoptosis induction due to their ~80 amino acid cytoplasmic sequence, the death domain.[42] Helper T-cells recruit other immune cells, including cytotoxic T-cells to eliminate “marked” cells, and regulatory T-cells modulate the immune response from getting too aggressive, which protects the host.[39] Memory T-cells are generated in preparation for another infection.[39] Through global vaccination efforts to build our adaptive immunity, we have increased life expectancy and eliminated the threat of some debilitating diseases.[15]

I. Vaccines and Cancer

Vaccines have been employed to lessen cancer incidence or treat cancer. There are two types of vaccines related to the prevention or treatment of cancer: Vaccines against biological carcinogens and Treatment Vaccines/Immunotherapies.[16] As stated earlier, biological

carcinogens promote carcinogenesis. Examples of biological carcinogens include Hepatitis papillomavirus (HPV), Hepatitis C virus (HCV), and Hepatitis B Virus (HBV).[16,17] Vaccination against biological carcinogens is against cancer development by training our immune system to target and eliminate the biological carcinogen. However, when a patient is diagnosed with cancer, immunotherapies are employed. Immunotherapies are different from traditional vaccines that work against pathogens (bacterium, virus, or other microorganism) because immunotherapies are given to promote an immune response against already present cancer cells, a preexisting condition.[44] These vaccines work by training the immune system to target specific antigens only found on the malignancy.[16]

C. The Hygiene Hypothesis

The Hygiene Hypothesis is an idea sprouted by David P. Strachan in 1989. He studied the incidence of hay fever and asthma in varying household sizes. He found that larger households had lower infection rates of hay fever compared to smaller households.[18] This idea later evolved to state that exposure to pathogens and microorganisms allows us to have a proper immune response to stimuli. When our immune system is suboptimal, it may be hyperactive towards non-harmful stimuli, develop autoimmunity, generate chronic inflammation, or have a lower capability to recognize a variety of pathogens. Without proper exposure to pathogens, poor immunity in conjunction with chronic inflammation play a role in cancer development.[12-13,19-20] A case-control study observed the incidence of Acute Lymphoblastic Leukemia (ALL) and other pediatric cancers in children and demonstrated that social contact within the first year of life was protective against developing ALL and other pediatric cancers (Table 1-3).[19,21] The Hygiene Hypothesis and this study have highlighted the importance of early exposure in lowering the risk of oncogenesis.

Activity level	Controls No (%)	ALL No (%)	ALL Odds ratio* (95% CI)	cALL No (%)	cALL Odds ratio* (95% CI)	TEL-AML1 No (%)	TEL-AML1 Odds ratio* (95% CI)	Hyperdiploid ALL No (%)	Hyperdiploid ALL Odds ratio* (95% CI)	Non-ALL malignancies No (%)	Non-ALL malignancies Odds ratio* (95% CI)	ALL *v* non-ALL malignancy: odds ratio* (95% CI)
Aged over 2 years												
Total No†	6238	1272		791		138		417		1825		
Any social activity	5343 (85.7)	1020 (80.2)	0.66 (0.56 to 0.77)	640 (80.9)	0.67 (0.55 to 0.82)	110 (79.7)	0.59 (0.38 to 0.90)	335 (80.3)	0.64 (0.50 to 0.83)	1496 (82.0)	0.78 (0.68 to 0.90)	0.88 (0.73 to 1.06)
No social activity	895 (14.4)	252 (19.8)	1.00	151 (19.1)	1.00	28 (20.3)	1.00	82 (19.7)	1.00	329 (18.0)	1.00	1.00
Social activity, but no day care	2840 (45.5)	587 (46.1)	0.73 (0.62 to 0.87)	358 (45.3)	0.74 (0.60 to 0.91)	60 (43.5)	0.61 (0.38 to 0.97)	199 (47.7)	0.76 (0.58 to 1.00)	880 (48.2)	0.83 (0.71 to 0.96)	0.91 (0.74 to 1.11)
Informal day care only	1768 (28.3)	325 (25.6)	0.62 (0.51 to 0.75)	218 (27.6)	0.67 (0.53 to 0.84)	38 (27.5)	0.60 (0.36 to 1.00)	105 (25.2)	0.57 (0.42 to 0.78)	435 (23.8)	0.72 (0.61 to 0.85)	0.90 (0.71 to 1.13)
Formal day care	735 (11.8)	108 (8.5)	0.48 (0.37 to 0.62)	64 (8.1)	0.44 (0.32 to 0.60)	12 (8.7)	0.47 (0.24 to 0.94)	31 (7.4)	0.38 (0.24 to 0.59)	181 (9.9)	0.73 (0.59 to 0.90)	0.69 (0.51 to 0.93)
P for trend‡			<0.001		<0.001		0.04		<0.001		<0.001	0.04
Aged 2-5 years												
Total No†	2475	671		471		81		248		546		
Any social activity	2156 (87.1)	545 (81.2)	0.63 (0.50 to 0.79)	387 (82.2)	0.67 (0.51 to 0.88)	68 (84.0)	0.73 (0.39 to 1.35)	198 (79.8)	0.57 (0.40 to 0.80)	447 (81.9)	0.66 (0.51 to 0.85)	0.98 (0.73 to 1.31)
No social activity	319 (12.9)	126 (18.8)	1.00	84 (17.8)	1.00	13 (16.0)	1.00	50 (20.2)	1.00	99 (18.1)	1.00	1.00
Social activity, but no day care	1023 (41.3)	294 (43.8)	0.70 (0.54 to 0.89)	195 (41.4)	0.69 (0.52 to 0.93)	35 (43.2)	0.74 (0.38 to 1.43)	115 (46.4)	0.69 (0.48 to 0.99)	234 (42.9)	0.70 (0.53 to 0.91)	1.03 (0.75 to 1.42)
Informal day care only	778 (31.4)	182 (27.1)	0.59 (0.45 to 0.77)	142 (30.1)	0.71 (0.52 to 0.96)	25 (30.9)	0.76 (0.38 to 1.53)	63 (25.4)	0.51 (0.34 to 0.76)	139 (25.5)	0.59 (0.44 to 0.79)	1.01 (0.71 to 1.43)
Formal day care	355 (14.3)	69 (10.3)	0.49 (0.35 to 0.68)	50 (10.6)	0.52 (0.35 to 0.77)	8 (9.9)	0.62 (0.25 to 1.53)	20 (8.1)	0.33 (0.19 to 0.58)	74 (13.6)	0.68 (0.48 to 0.96)	0.73 (0.48 to 1.13)
P for trend‡			<0.001		0.004		0.4		<0.001		0.01	0.2

cALL=B cell precursor common ALL; TEL-AML1=ALL with fusion of the *TEL* and *AML1* genes.
*Odds ratio for cases compared with all controls, or with non-ALL malignancies where stated, adjusted for age at diagnosis/pseudodiagnosis, sex, region, maternal age, mother working at time of birth, and deprivation.
†Excluding missing values.
‡Trend test across categories none through to formal day care.

Table 1. Levels of social activity in the first year of life for ALL subgroups and non-ALL malignancies. Reduced exposure to infection in the first few months of life increases the risk of developing acute lymphoblastic leukemia. Red asterisk "*" placed by a column or row denote data pertaining to the hygiene hypothesis.

Note: Adapted from Gilham, C., Peto, J., Simpson, J., Roman, E., Eden, T. O., Greaves, M. F., Alexander, F. E., & UKCCS Investigators (2005). Day care in infancy and risk of childhood acute lymphoblastic leukaemia: findings from UK case-control study. BMJ (Clinical research ed.), 330(7503), 1294. https://doi.org/10.1136/bmj.38428.521042.8F

No of siblings	Controls No (%)	ALL * No (%)	ALL * Odds ratio* (95% CI)	cALL * No (%)	cALL * Odds ratio* (95% CI)	TEL-AML1 * No (%)	TEL-AML1 * Odds ratio* (95% CI)	Hyperdiploid ALL * No (%)	Hyperdiploid ALL * Odds ratio* (95% CI)	Non-ALL malignancies * No (%)	Non-ALL malignancies * Odds ratio* (95% CI)
Cases aged over 2 years											
Total No†	6197	1270		789		138		416		1830	
None*	2690 (43.4)	565 (44.5)	1.00	365 (46.3)	1.00	62 (44.9)	1.00	189 (45.4)	1.00	799 (43.7)	1.00
1	2216 (35.8)	443 (34.9)	0.96 (0.84 to 1.11)	266 (33.7)	0.88 (0.74 to 1.05)	49 (35.5)	0.89 (0.60 to 1.32)	149 (35.8)	0.97 (0.77 to 1.22)	667 (36.5)	1.03 (0.91 to 1.16)
2	899 (14.5)	182 (14.3)	0.99 (0.82 to 1.21)	115 (14.6)	0.94 (0.74 to 1.20)	20 (14.5)	0.86 (0.50 to 1.48)	57 (13.7)	0.94 (0.68 to 1.30)	281 (15.4)	1.11 (0.94 to 1.31)
≥3	392 (6.3)	80 (6.3)	0.99 (0.74 to 1.30)	43 (5.4)	0.78 (0.54 to 1.12)	7 (5.1)	0.70 (0.30 to 1.62)	21 (5.0)	0.75 (0.46 to 1.25)	83 (4.5)	0.76 (0.59 to 1.00)
P for trend‡			0.9		0.2		0.4		0.4		0.7
Cases aged 2-5 years											
Total†	2463	673		473		82		248		548	
None*	1071 (43.5)	308 (45.8)	1.00	221 (46.7)	1.00	40 (48.8)	1.00	114 (46.0)	1.00	256 (46.7)	1.00
1	873 (35.4)	233 (34.6)	0.95 (0.78 to 1.16)	164 (34.7)	0.93 (0.74 to 1.17)	29 (35.4)	0.81 (0.49 to 1.36)	89 (35.9)	0.98 (0.72 to 1.33)	186 (33.9)	0.95 (0.77 to 1.19)
2	366 (14.9)	96 (14.3)	0.95 (0.72 to 1.25)	65 (13.7)	0.89 (0.65 to 1.22)	8 (9.8)	0.52 (0.23 to 1.16)	35 (14.1)	0.97 (0.64 to 1.48)	81 (14.8)	1.05 (0.78 to 1.40)
≥3	153 (6.2)	36 (5.3)	0.85 (0.56 to 1.29)	23 (4.9)	0.72 (0.44 to 1.19)	5 (6.1)	0.81 (0.29 to 2.25)	10 (4.0)	0.61 (0.29 to 1.27)	25 (4.6)	0.80 (0.50 to 1.28)
P for trend‡			0.5		0.2		0.2		0.4		0.7

cALL=B cell precursor common ALL; TEL-AML1=ALL with fusion of the *TEL* and *AML1* genes.
*Odds ratio for cases compared with all controls, adjusted for age at diagnosis/pseudo diagnosis, sex, region, maternal age, mother working at time of birth, and deprivation.
†Excluding missing values.
‡Trend test across categories none through to ≥3.

Table 2. Number of older children in a household ("siblings") at time of index birth for ALL subgroups and non-ALL malignancies. Reduced exposure to infection in the first few months of life increases the risk of developing acute lymphoblastic leukemia. Red asterisk "*" placed by a column or row denote data pertaining to the hygiene hypothesis.

Note: Adapted from Gilham, C., Peto, J., Simpson, J., Roman, E., Eden, T. O., Greaves, M. F., Alexander, F. E., & UKCCS Investigators (2005). Day care in infancy and risk of childhood acute lymphoblastic leukaemia: findings from UK case-control study. BMJ (Clinical research ed.), 330(7503), 1294. https://doi.org/10.1136/bmj.38428.521042.8F

Age first attended (months)	Day care						Formal day care					
	No (%)			Odds ratio* (95%CI) *			No (%)			Odds ratio* (95%CI) *		
	Controls (n=6250)†	ALL (n=1274)†	Non-ALL (n=1830)†	ALL *v* controls	Non-ALL *v* controls	ALL *v* non-ALL	Controls (n=6269)†	ALL (n=1278)†	Non-ALL (n=1833)†	ALL *v* controls	Non-ALL *v* controls	ALL *v* non-ALL
None	3736 (59.8)	839 (65.9)	1210 (66.1)	1.00	1.00	1.00	5534 (88.3)	1170 (91.5)	1652 (90.1)	1.00	1.00	1.00
<3	1091 (17.5)	185 (14.5)	289 (15.8)	0.71 (0.60 to 0.85)	0.88 (0.76 to 1.02)	0.82 (0.66 to 1.02)	231 (3.7)	27 (2.1)	71 (3.9)	0.56 (0.37 to 0.83)	1.07 (0.81 to 1.40)	0.52 (0.32 to 0.83)
3-5	548 (8.8)	94 (7.4)	130 (7.1)	0.71 (0.56 to 0.90)	0.83 (0.68 to 1.02)	0.91 (0.68 to 1.22)	204 (3.3)	35 (2.7)	42 (2.3)	0.71 (0.49 to 1.03)	0.77 (0.55 to 1.08)	1.01 (0.62 to 1.64)
6-11	875 (14.0)	156 (12.2)	201 (11.0)	0.76 (0.63 to 0.92)	0.76 (0.64 to 0.91)	1.00 (0.79 to 1.27)	300 (4.8)	46 (3.6)	68 (3.7)	0.69 (0.50 to 0.96)	0.82 (0.62 to 1.08)	0.81 (0.54 to 1.21)

*Odds ratio for cases compared with all controls or with non-ALL malignancies where stated, adjusted for age at diagnosis or pseudodiagnosis, sex, region, maternal age, mother working at time of birth, and deprivation.
†Excluding missing values.

Table 3. Effect of age at first day care during the first year of life for ALL and non-ALL malignancies. Reduced exposure to infection in the first few months of life increases the risk of developing acute lymphoblastic leukemia. Red asterisk "*" placed by a column or row denote data pertaining to the hygiene hypothesis.

Note: Adapted from Gilham, C., Peto, J., Simpson, J., Roman, E., Eden, T. O., Greaves, M. F., Alexander, F. E., & UKCCS Investigators (2005). Day care in infancy and risk of childhood acute lymphoblastic leukaemia: findings from UK case-control study. BMJ (Clinical research ed.), 330(7503), 1294. https://doi.org/10.1136/bmj.38428.521042.8F

D. The Death Receptor CD95 and its Natural Ligand CD95L

CD95 (FasR/APO-1/APT/TNFRSF6) is a transmembrane death receptor found in brain, cardiac, renal, liver, pancreas, thymus, and lymphoid tissues. Membrane bound CD95L (CD178) is a transmembrane protein that facilitates cytotoxic activity when bound to CD95.[31] CD95 and its natural ligand CD95L are one of the few death receptor/death ligand mechanisms involved in apoptosis to maintain proper immune homeostasis.[30] CD95 and CD95L are either expressed singularly or consecutively in cancers, especially human B-cell leukemia.[31] Although CD95/L is expressed by all cell types, it is tightly regulated by a variety of mechanisms to inhibit accidental apoptosis. In cancers, CD95/L is up-regulated due to high levels of oxidative stress, genome instability, and increased cellular proliferation. This pathway should be studied in greater detail to deduce its role in immunosurveillance.

E. Experimental Goals and Significance

Despite having extensive knowledge of pathogen-induced immunity our understanding of cancer immunology is still being developed. In this study, we hypothesize that pathogenic exposure and lower adiposity is protective against leukemia development resulting from the non-specific killing of cancer cells by pathogen-specific TCs. Mechanistically, we posit that this "cross recognition/cross protection" phenomena is mediated by the pro-apoptotic CD95/CD95L pathway (Figure 2). We will examine the CD95/CD95L apoptotic mechanism to define its role against malignancy development and informing the public health conversation surrounding the increased benefit of vaccination use in relation to this pro-apoptotic pathway.

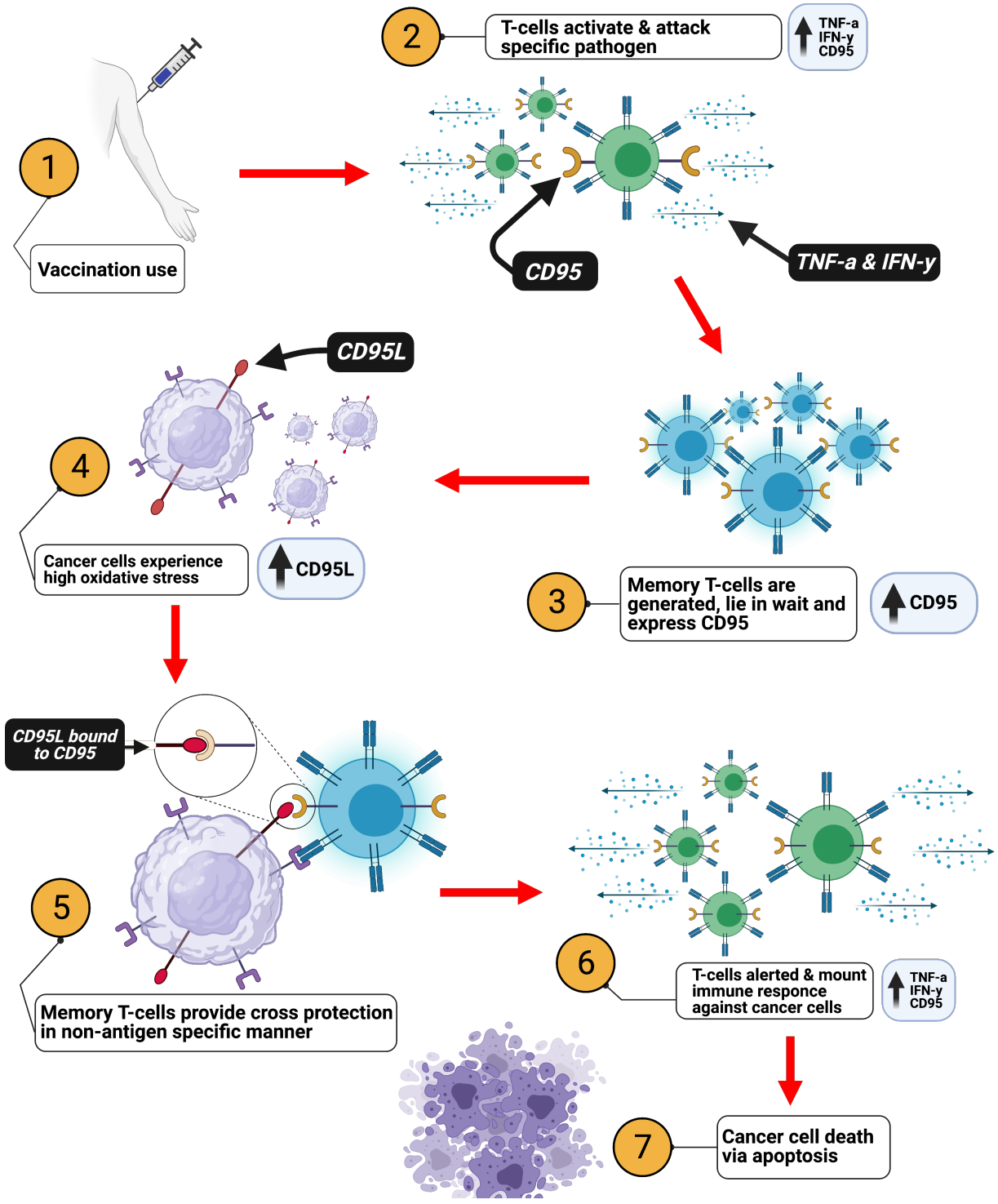

Figure 2. Graphical Representation of T-cell Activation and B-ALL Cells Using CD95L Pathway Hypothesis. This figure demonstrates how cross protection may arise through use of the CD95/CD95L pathway. Robust vaccination efforts may increase CD95-expressing memory T-cells. The memory T-cells are potent eliminators of pathogen-infected and cancer cells.

Chapter II: Methods

Cell Lines

As described previously in within the Henry Laboratory, human B-cell acute lymphoblastic leukemia (**B-ALL**) cell lines were gifted from Dr. Graham and Dr. Porter Laboratories (Department of Pediatrics at Emory School of Medicine).[28] Nalm6 cell lines are grown in RPMI1640 (cat# 10-040-CV corning) supplemented with 10% fetal bovine serum (FBS, cat# S11550 Atlanta Biologicals).[28] REH, SEM, RCH-AcV cell lines were grown in RPMI1640 supplemented with 20% FBS. OP-9 bone marrow stromal cells were grown in Alpha-Minimum Essential Medium (αMEM, cat# 15-012-CV, Corning) supplemented with 20% FBS.[28]

Flow Cytometry Analysis of CD95L & CD95 Surface Stains

To assay the surface phenotype of B-ALL cell lines, we plated 82% viable RCH-ACV cells at $5x10^5$ in 2 mL of three different growth medias for 48 hours: Adipocyte Conditioned Media (**ACM**), Stromal Conditioned Media (**SCM**), and RPIMI1640 media supplanted with 10% FBS (**UCM**). After 48 hours, we placed the cells in a round bottom 96 well plate at 10^5 in triplicates. Each well, except for the unstained and isotype control, was treated with 1:100 dilution of CD95L fluorescent antibodies. The stain sat for one hour. The cells were then assessed with flow cytometry.

Cytotoxicity assay with Co-culture Experiments

TCs were plated at 2 x 10^6 into a 12 well plate. One well was stimulated with phorbol 12-myristate 13-acetate (**PMA**) and ionomycin cocktail in UCM for 72 hours. One well was unstimulated in UCM for 72 hours. TC were then split into four groups: labeled with cell tracker orange (**CTO**) and unstimulated, labeled with CTO and stimulated in UCM, labeled with CTO and

stimulated in ACM, labeled with CTO and stimulated in SCM. BC were labeled with CTO and placed in UCM for 24 hours on day two of TC stimulation. After 24hr, we plated 10^4 B-ALL cells and 10^4 jurkat T-cells in a 96 well plate in alone and combined conditions (Table 4). We conducted LionheartFx live cell imaging analysis for 72 hours of imaging. We measured for loss of CTO. Quantitative analysis was done using GEN-5 software.

Adipocyte Differentiation

Confluent cells were trypsinized and plated at 10^5 cells per well in 6-well plates.[28] Cells were cultured in DMEM/F-12 media (Bio- Whittaker, cat#12-719 F) media supplemented with 10% FBS.[28] On the following day, media was changed with either fresh Dulbecco's Modified Eagle Medium (**DMEM**) media (BioWhittaker, cat#12-719 F) supplemented with 10% FBS for stromal cell culture or insulin-oleate media (IOM, 1.8 mM Oleate bound to BSA with the molar ratio of 5.5:1) for adipocyte differentiation.[28] Supernatants from bone marrow stromal cells and adipocytes were harvested on days 1, 2, and 3 of culture for use in leukemia experiments.[28]

Chapter III: Results

Surface Stain of CD95L on RCH-ACV B-ALL

To assay if B-ALL cell lines express CD95L or CD95, we assayed for the surface expression of these proteins on human B-ALL cell lines using flow cytometry. Additionally, to determine if growth environment influenced the expression of CD95 and CD95L, we cultured the B-ALL cell lines in different growth media: Unconditioned media (UCM) (RPMI1640 supplemented with 10% of FBS), ACM, and SCM. We assayed RCH-ACV B-ALL cells for the expression of CD19, CD95, and CD95L. CD19 is a positive control marker of B-ALL.[29]

The percentage of B-ALL cells expressing the surface markers of interest is as follows: **UCM group**- 79.43% expressed CD19, 54.9% expressed only CD95L, 1.27% expressed CD95 only, and 22.26% expressed both CD95L and CD95. Of the **SCM group**, 76.33% expressed CD19, 54.26% of the SCM group expressed only CD95L, 1.95% expressed CD95 only, and 20.7% of the SCM group expressed both CD95L and CD95. Of the **ACM group**, 77.16% expressed CD19, 62.93% expressed only CD95L, .75% expressed CD95 only, and 13.06% expressed both CD95L and CD95. There was not a significant difference in expression between growth conditions (Figure 3 and 10).

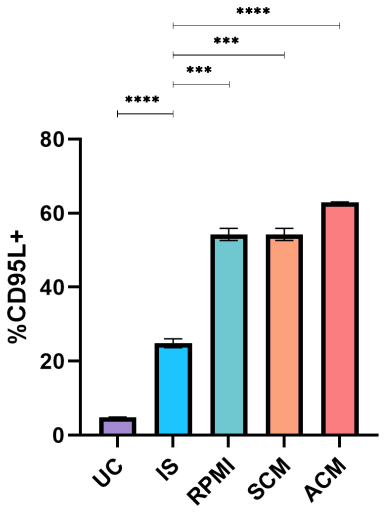

Figure 3. Percent of B-ALL cells that express CD95L in Different Growth Conditions. CD95L expression in unstained control (UC), isotype control (IS), ACM, UCM, and SCM was measured by quantifying CD95L fluorescent antibody illuminance using flow cytometry. Means ± s.d. are shown (***p < 0.01, **p<.01, *p<.05 n = 5 independent experiments, unpaired t-test).

The mean fluorescent intensity (MFI) of CD19 in our isotype and unstained control was 1599.5 and 1467 respectively. The MFI of CD95 in our isotope and unstained control was 1382.5 and 381 respectively. The MFI of CD95L in our isotope and unstained control was 391 and 136 respectively. Of the UCM group, the MFI of CD19, CD95, and CD95L was 17681.66, 1571, and 1128.66 respectively. Of the SCM group the MFI of CD19, CD95, and CD95L was 13965.66, 1523, and 890 respectively. Of the ACM group the MFI of CD19, CD95, and CD95L was 16750.33, 1361.66, 934.66 respectively. There was not a

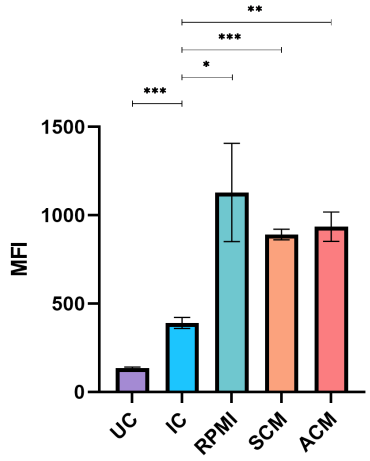

Figure 4. Mean Fluorescent Intensity of CD95L on B-ALL cells in Different Growth Conditions. CD95L fluorescence intensity was measured using anti CD95L antibodies and flow cytometry techniques. Conditions are unstained control (UC), isotype control (IC), ACM, UCM, and SCM. Means ± s.d. are shown (***p < 0.01, **p<.01, *p<.05 n = 5 independent experiments, unpaired t-test).

significant difference in expression between growth conditions (Figure 4). Overall, these results demonstrate that CD19 and CD95L surface expression is stable and expressed at high levels on human B-ALL cells.

Co-culture of Jurkat T-cells with B-ALL

We assayed if human TCs eliminated human B-ALL cells when co-cultured for 72 hours at varying levels of T-cell: B-ALL ratios. We conducted this assay with a human T-cell line (Jurkat) co-cultured with a human B-ALL cell line (REH). Prior to co-culture, human B-ALL cells were labeled with cell trace yellow, which would only be released from the cells if they were lysed (killed; dye-negative cells are dead leukemia cells). The following percentages are averages exhibited by each experimental group. When B-ALL were not co-cultured, 89% were viable and roughly 11% underwent spontaneous cell death. When plated at a 2:1 T-cell:B-ALL cell ratio ,the degree of T-cell stimulation did not matter and TCs killed between 25-30% of leukemia cells over a 3 day period. Furthermore, the TC:B-ALL ratio did not impact the degree of cross-recognition mediated killing (Figure 5). Additionally, previously demonstrated within Henry Lab, 72 hour TC co-cultures exhibit negligible cell death. Therefore, we can deduce that B-ALL cell death is not attributed to overcrowding.[28] In summary, these data demonstrate that human T-cells can kill human B-ALL cells when T-cells recognize malignant cells in a non-antigen specific fashion. Therefore, my results support that cross-protection indeed plays a role in TC-mediated recognition and killing of malignant B-cells.

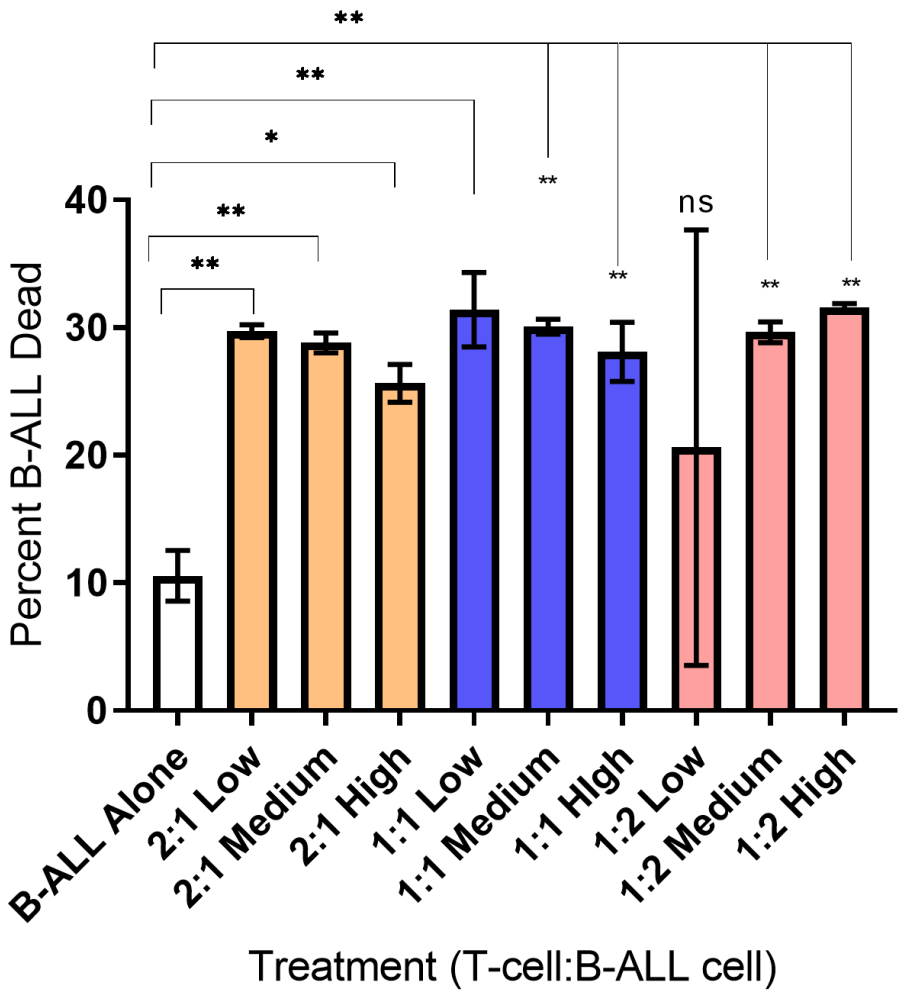

Figure 5. Cell Survival of B-ALL cells when Co-cultured with T-cells for 72 Hours. B-ALL cells were stained with cell trace orange 24 hours after T-cell were stimulated with PMA/ionomycin. Both populations of cells were co-cultured at 10^4 and observed via live cell imaging and flow cytometry techniques. High, medium, and low denotes level of stimulation given to T-cells. Means ± s.d. are shown (***p < 0.01, **p<.01, *p<.05 n = 10 independent experiments, one-way ANOVA).

Chapter IV. Discussion:

Acute Lymphoblastic Leukemia (ALL) cell lines are important to study because they represent a range of genetic or epigenetic changes that may exhibit different functionality. In this present study, we assayed the B-ALL cell line RCH-ACV for the expression of CD95 and CD95L. The RCH-ACV cell line is derived from a child's bone marrow who had ALL. The cell line lacks the Epstein-

Barr virus (EPV) nuclear antigen and non-random chromosome translocation 1;19.[33] We found that membrane bound CD95L was significantly expressed on RCH-ACV B-ALL cell surface but not CD95. Mechanistically, we posit that TC mediate “cross-protective” cell death through this pro-apoptotic CD95/CD95L pathway. As a result, oncogenesis may be limited.

T-cells represent the adaptive immune component of our immune system, and their name is derived from the fact that these cells are trained to recognize specific foreign or abnormal antigens, and respond accordingly.[40] By identifying antigens with the T-cell receptor (TCR), TC can conduct immunosurveillance.[40] This identification process modulates the immune response in conjunction with stimulatory or inhibitory signals from other cell types depending if the epitope is foreign or local to the body.[40] Jurkat T-cells are an immortalized cell line derived from a 14-year-old who exhibited T-Cell Acute Lymphoblastic Leukemia (T-ALL).[41] These cells have been instrumental in dissecting T-cell biology and the role of TCs in various pathologies.

To determine whether non-specific stimulated TC target B-ALL cells, we conducted a 72 hour co-culture experiment. The TCs used in this experiment have never encounter B-ALL cells; the immortal TC cell line is derived from a patient who suffered from T-ALL with no documented report of other blood cancers. Additionally, the T-cells used in my experiments have never been cultuered or exposed to B-ALLs. Therefore, the TC do not recognize antigens expressed by these leukemia cells. In comparison to the B-ALL alone group, stimulated TCs, despite never encountering B-ALL antigens, killed up to 30% of leukemia cells over a period of 3 days of *in vitro* co-cultures. This experiment served as a benchmark for our hypothesis and the creation of this assay can be used by our lab and others to delineate this cross-recognition mechanism. Now that we have the premise, supporting data, and this assay establish we will next determine

if TC-mediated killing of human B-ALL cells is induced by the pro-apoptotic CD95/CD95L pathway.

Chapter V. Future Objectives & Conclusion

We found that RCH-ACV B-ALL cells express CD95L. In future studies, I would like to assay more B-ALL cell lines to validate this finding.

In this study, we found that human TC can kill human B-ALL cells in a non-antigen specific manner. To elucidate whether the CD95/CD95L pathway mediates this process, I would like to conduct knockdown experiments using viral methodologies to eliminate CD95 from TCs to determine how the elimination of this protein impacts TC-mediated cytotoxicity of human B-ALL cells. Such methods include small interfering RNAs, micro RNAs, or CRISPR/Cas9 techniques, which silence or inhibit CD95 expression.

If it is found that the pathway mediates killing *in vitro*, I would conduct murine experiments. Mice would be binned into three groups and receive three vaccines: PBS (control), Lymphocytic choriomeningitis virus (LCMV) Clone 13 to elicit and poor TC response, and LCMV Armstrong to elicit a strong TC response indicative by large numbers of highly functional memory TCs.[45] Clone 13's suboptimal TC response is due to the chronic infection it inflects.[45-47] This infection is never cleared by the host and results in clonal exhaustion.[46-47] Clonal exhaustion is characterized by poor TC effector function, chronic inhibition, and distinct transcriptional state of effector TCs not typical of their functional version.[46-47] After vaccination, the mice will then be challenged with B-ALL. We expect that the mice who received LCMV Armstrong will show significantly higher cell survival compared to LCMV Clone 13 and PBS groups. We posit that the memory TC population

produced by the vaccination utilize the CD95/CD95L pathway to enhance leukemia cell elimination and protection from death.

Chapter VI. Supplementary Data:

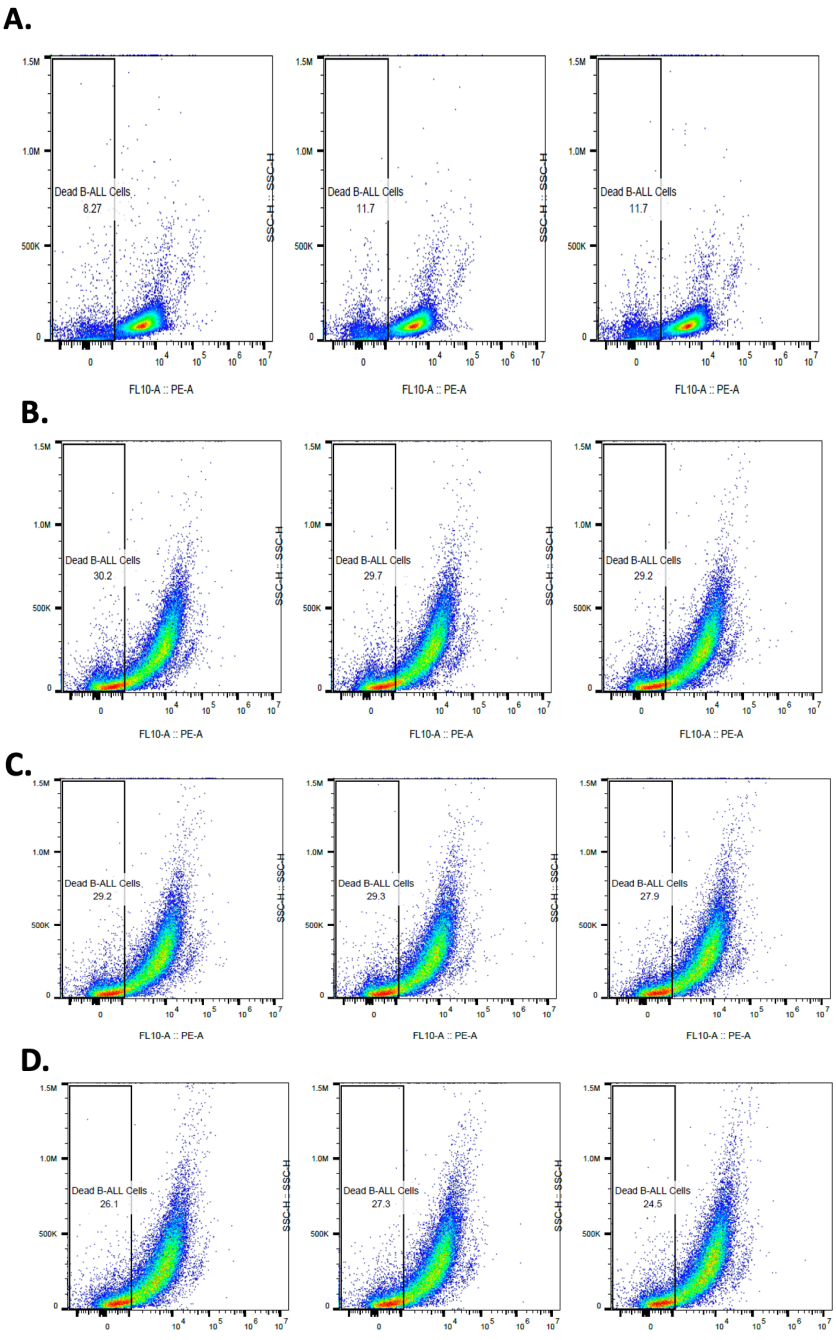

Figure 6. Cell Survival of B-ALL cells when Co-cultured with T-cells for 72 Hours. This depicts the flow cytometer output of the co-culture assay. Both cell types were plated at 10^4. **B. - D.** Jurkat co-cultured with B-ALL at 2:1. **A.**B-ALL alone condition. **B.** B-ALL co-cultured with lowly stimulated T-cells. **D.** B-ALL co-cultured with medially stimulated T-cells. **D.** B-ALL co-cultured with highly stimulated T-cells. Gating was done on dead cells. Percent of B-ALL cells dead is observed.

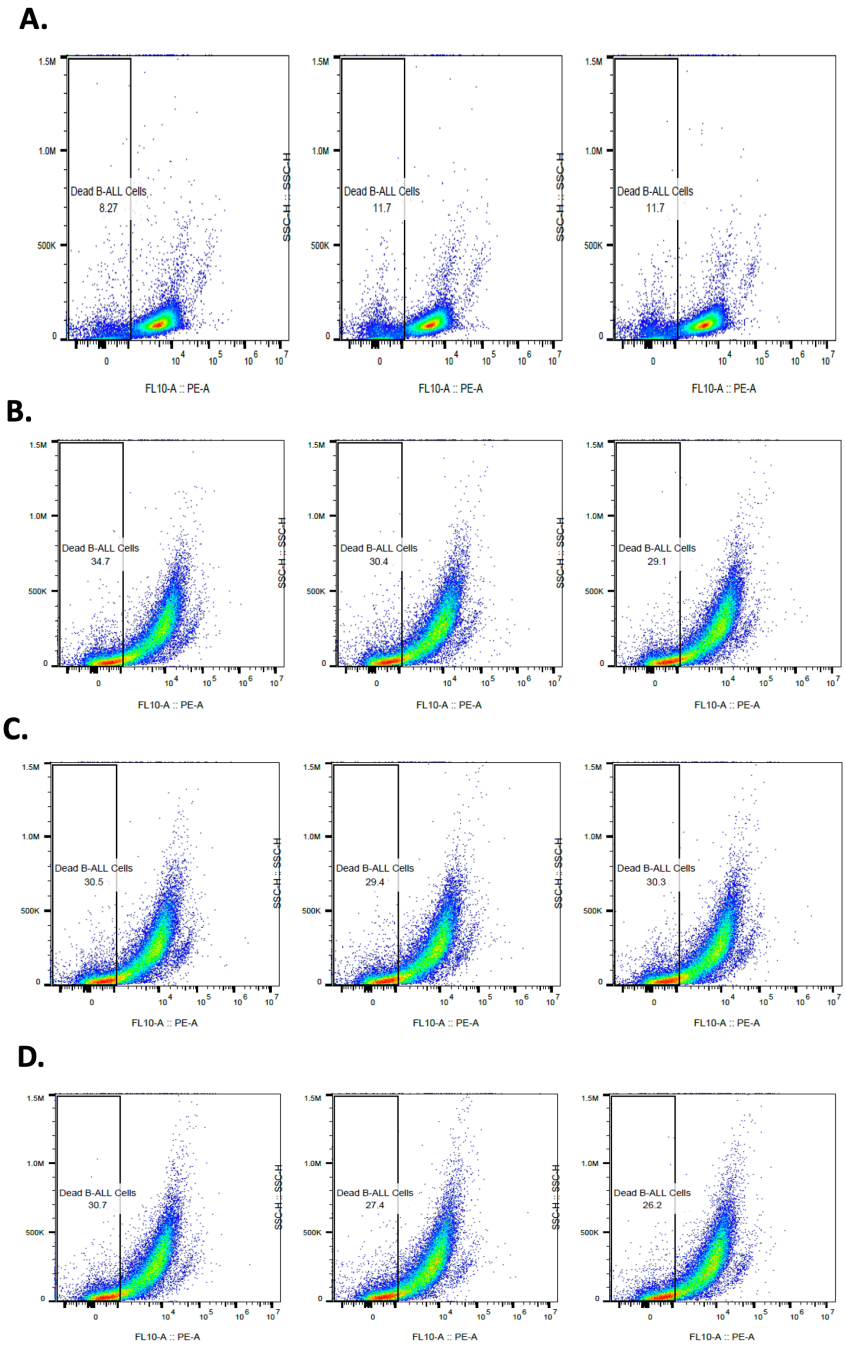

Figure 7. Cell Survival of B-ALL cells when Co-cultured with T-cells for 72 Hours. This depicts the flow cytometer output of the co-culture assay. Both cell types were plated at 10^4. **B. - D.** Jurkat co-cultured with B-ALL at 1:1. **A.**B-ALL alone condition. **B.** B-ALL co-cultured with lowly stimulated T-cells. **D.** B-ALL co-cultured with medially stimulated T-cells. **D.** B-ALL co-cultured with highly stimulated T-cells. Gating was done on dead cells. Percent of B-ALL cells dead is observed.

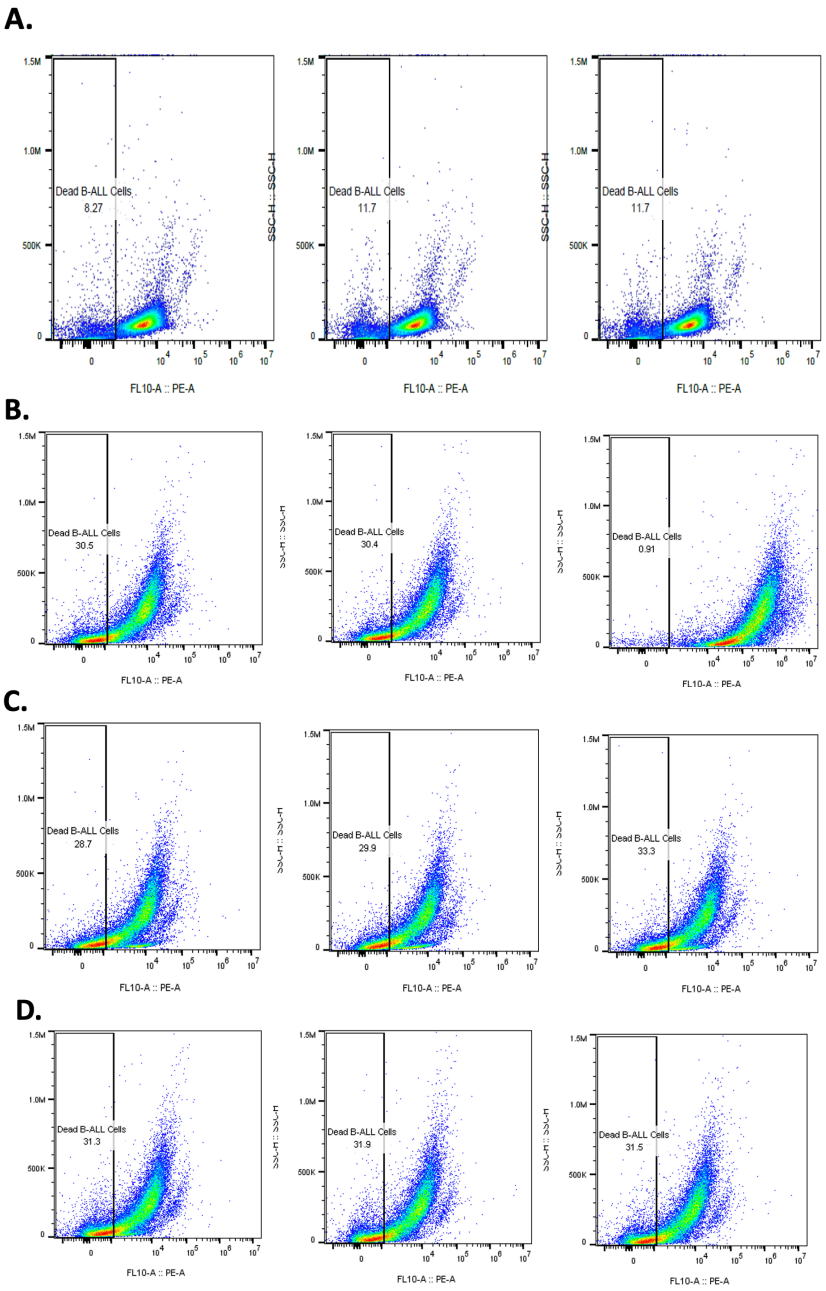

Figure 7. Cell Survival of B-ALL cells when Co-cultured with T-cells for 72 Hours. This depicts the flow cytometer output of the co-culture assay. Both cell types were plated at 10^4. **B. - D.** Jurkat co-cultured with B-ALL at 1:2. **A.**B-ALL alone condition. **B.** B-ALL co-cultured with lowly stimulated T-cells. **D.** B-ALL co-cultured with medially stimulated T-cells. **D.** B-ALL co-cultured with highly stimulated T-cells. Gating was done on dead cells. Percent of B-ALL cells dead is observed.

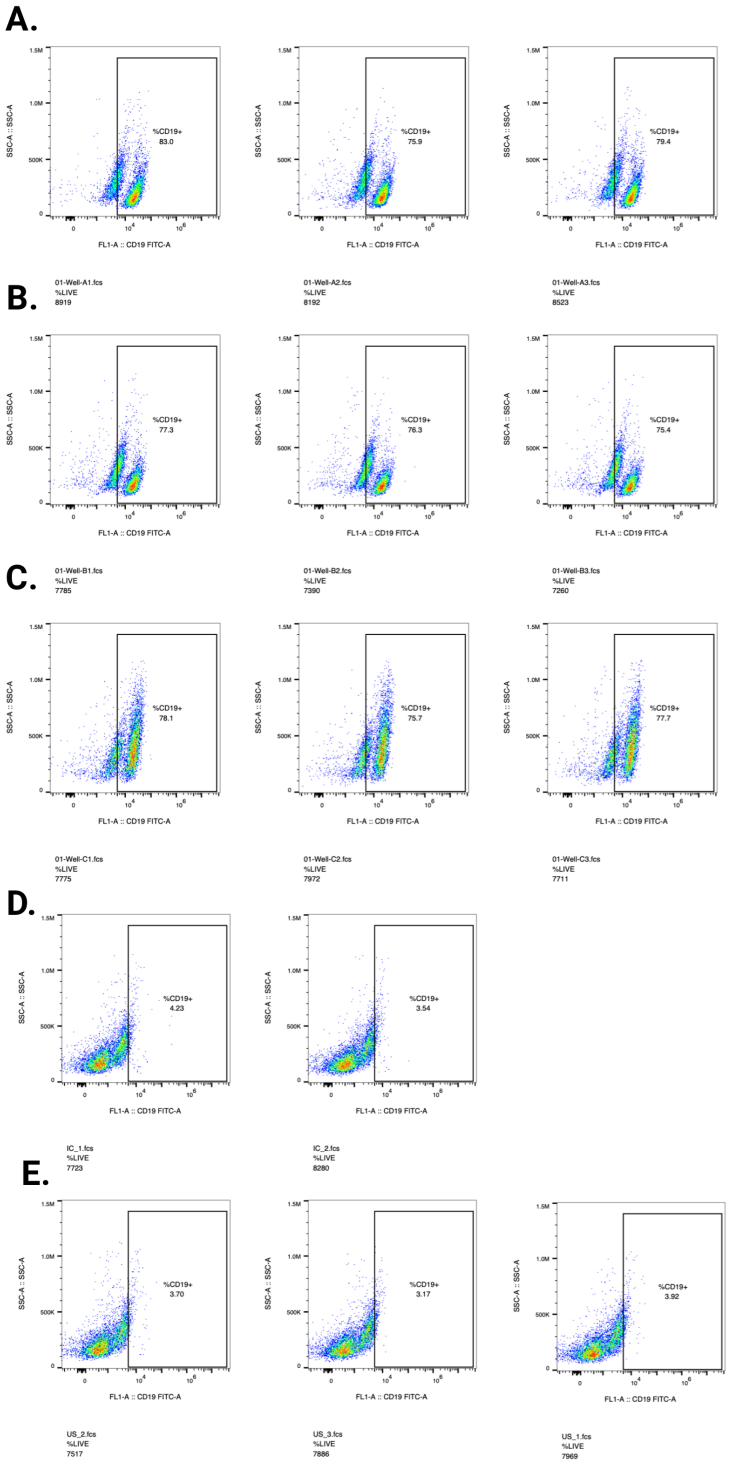

Figure 9. Percent of RCH-ACV B-ALL cells Expressing CD19 (a pan B-cell marker). This depicts the flow cytometer output of the surface expression assay. B-ALL were plated at 10^5. **A.**B-ALL plated in UCM. **B.** B-ALL plated in SCM. **C.** B-ALL plated in ACM. **D.** B-ALL unstained condition. **E.** B-ALL isotype condition. Gating was done on live cells.

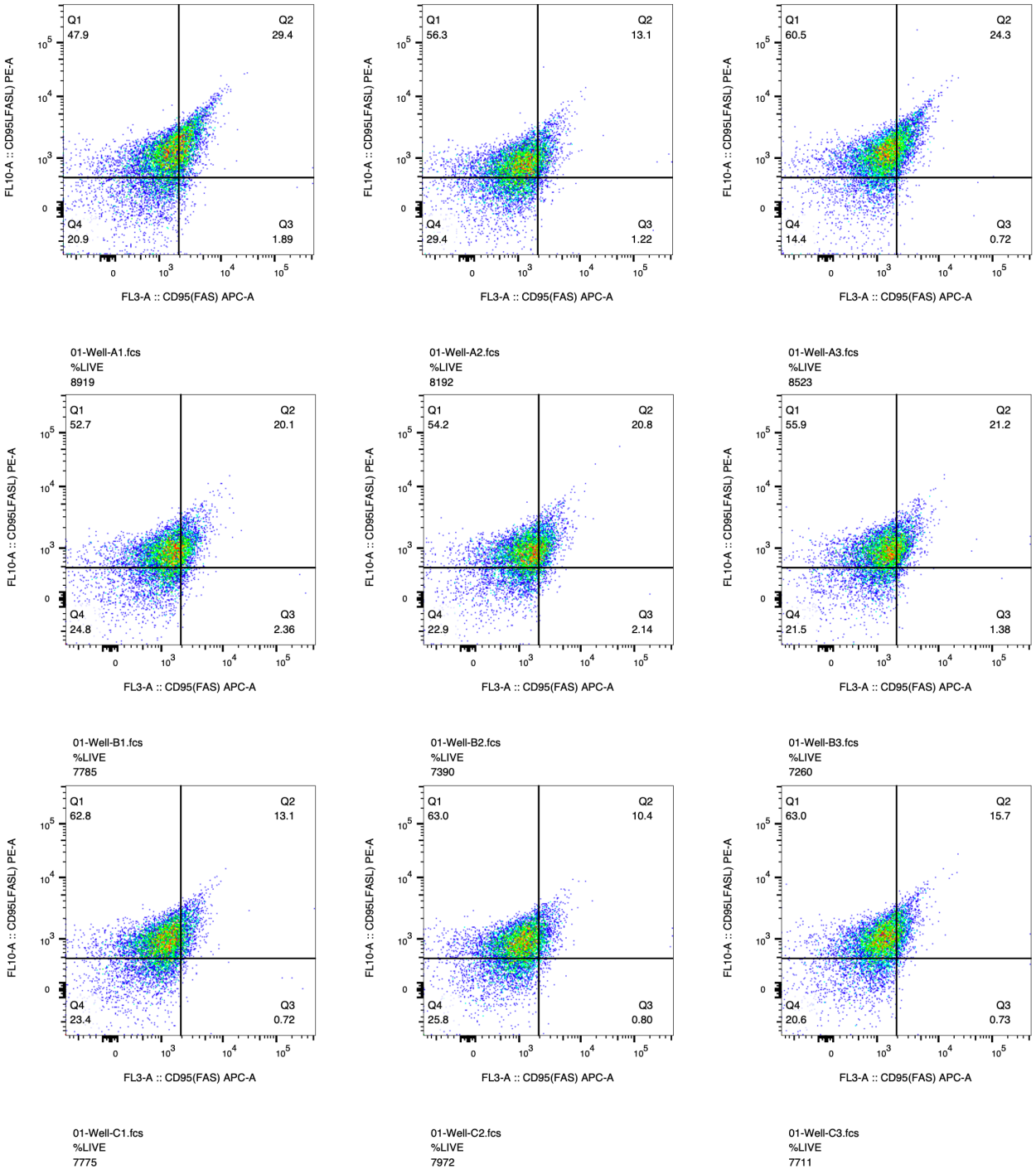

Figure 10. Percent of RCH-ACV B-ALL Cells Expressing CD95 and CD95L in UCM, SCM, ACM. This depicts the flow cytometer output of the surface expression assay. X-axis denotes CD95. Y-axis denotes CD95L. Top row is UCM. Middle row is SCM. Bottom row is ACM. B-ALL were plated at 10^5 in triplicates. Gating was done on live cells.

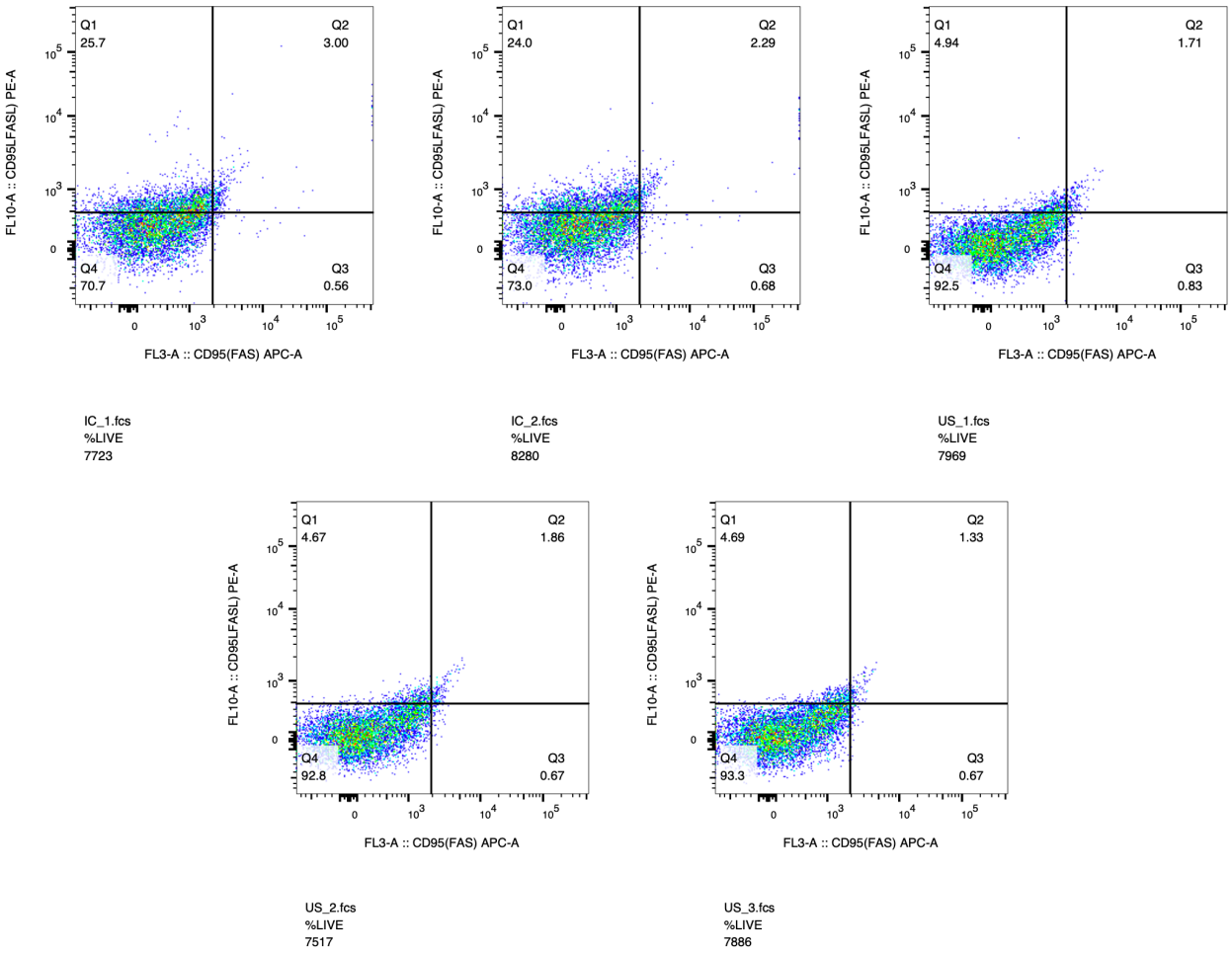

Figure 11. Percent of RCH-ACV B-ALL Cells Expressing CD95 and CD95L in Unstained and Isotype Control This depicts the flow cytometer output of the surface expression assay. X-axis denotes CD95. Y-axis denotes CD95L. First two graphs are isotype control. Last three charts are unstained control. Gating was done on live cells.

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Appendix

Micropropagation of Mesenchyme OP-9 and Generation of ACM/SCM

Grow Phase

1. Split cells every 3-4 days.
    i. Check cells every day for contamination and confluence.
2. When retrieving the T-25 flask from the incubator, always keep the flask tilted upwards to avoid the media from touching cap.
3. Observe cells using the microscope, checking for contamination and confluence
4. Add αMEM bottle and Trypsin bottle to the water bath to warm up for 10-15 minutes
5. Spray down the hood work surface with 70% EtOH.
6. Use glass pipette to aspirate out the old media from the flask.
7. Add ~7-10 mL of sterile 1X PBS to the T-25 flask and swirl gently to wash
    i. Spray with 70% EtOH on all 1XPBS before placing in the hood.
    ii. Be sure to cover the surface of all the cells
    iii. Swirl gently for at least 1 minute
    iv. Aspirate out the 1X PBS using a glass pipette
8. Add ~1-2 mL of Trypsin to T-25 flask, be sure to cover the surface of the cells a. Spray 70% EtOH on Trypsin bottle before placing in the hood

9. Incubate for 3 minutes in 37˚ incubator

10. During Incubation

    i. Remove 2, T-75 flask and label with the following: OP-9 | Date | Initials

    ii. Make sure Trypsin and 1X PBS are back in their respective places.

    iii. Place a 15 mL conical tube into the hood, be sure the tube-holder is sprayed with 70% EtOH

11. After Incubation

    i. Hold T-25 flask up to the light or swirl and you should see the floating cells in the media

    ii. Add 2x or 3x the amount of MEM media to the T-25 flask

        i. This neutralizes the Trypsin

12. Mix well with a pipette

13. Pipette all of the liquid in the T-25 flask into the 15 mL tube

14. Add your 15 mL tube to the lab centrifuge for 5 minutes at 1200 rpm.

15. During Centrifuge

    i. Pipette 18 mL of MEM media to both T-75 flask using

    ii. Clean hood of any unnecessary clutter or trash

16. After Centrifuge

i. Aspirate out the supernatant media

ii. Don't touch the pellet at the bottom

iii. Resuspend your cells in ~4 mL of MEM media and mix well using pipette

iv. Add ~2 mL of cell/media solution from the 15 mL to each T-75 flask

17. Close the cap firmly and place your two T-75 flask into the 37˚ incubator.

18. Check cells every day for the next four days

i. Check cells every day for contamination and confluence

19. Remove all your supplies from hood and wipe down with 70% EtOH.

Initial Plate Phase (10% DMEM) 4 days have passed since you placed cells into T-75 flask.

20. When retrieving the T-75 flask keep the flask tilted upwards, avoid media touching cap

21. Observe cells using the microscope, checking for contamination and confluence

22. Add DMEM with 10%FBS bottle and Trypsin bottle to the water bath to warm up for 10- 15 minutes.

23. Spray down the hood work surface with 70% EtOH.

24. Use glass pipette to aspirate out the old media from the flask.

25. Add ~7-10 mL of sterile 1X PBS to the T-75 flask and swirl gently to wash

26. Spray with 70% EtOH on all 1XPBS before placing in the hood.

27. Be sure to cover the surface of all the cells

i. Swirl gently for > 1 minute

28.Aspirate the 1X PBS using glass pipette

29.Add ~3-4 mL of Trypsin to T-75, be sure to cover the surface of the cells

30.Spray the Trypsin bottle with 70% EtOH before placing in the hood

31.Incubate for 3 minutes in 37˚ incubator

32.During Incubation

i. Spray the Trypan Blue with 70% EtOH and place in the hood along with a strip of parafilm

33.After Incubation

i. Hold T-75 flask up to the light or swirl and you should see the floating cells in the media

ii. Add 2x or 3x the amount of DMEM media to the T-75 flask

i. This neutralizes the Trypsin

ii. Mix well with a pipette

34.Pipette all of the liquid in the T-75 flasks into two 50 mL tubes

35.Add your 50 mL tubes to the lab centrifuge for 5 minutes at 1200 rpm.

36.Aspirate out the supernatant media

i. Don’t touch the pellet at the bottom

37. Re-suspend the cells in 10ml of DMEM media for each flask

38. Remove 10 ul of Trypan Blue and place droplet on parafilm, mix with 10 ul of cells

39. Retrieve a cell counting slide, and add 10ul of the Trypan/Cell solution to A or B well

40. Insert into cell counter

41. Use values to calculate how many 6-well plates you can make and how much more media you need to add for resuspension

    i. 1 x 105cells/well is the current standard for a 6 well plate

42. Add 1 mL of DMEM media to each well, and you will add 1 mL of the cell/media solution a. Final volume: 2 mL with 1x 105 cells in one well

43. Once all plates are made, place in 37˚ incubator for 24 hours.

IOM Plate Phase (DMEM) 24 hrs have passed since you placed cells into plates

42. Add DMEM bottle and IOM to the water bath to warm up for 10-15 minutes

    i. When retrieving the 6 plates, keep the plates level as possible

    ii. Observe cells using the microscope, checking for contamination and stability

    iii. Place the plates in the hood and split them into two equal groups. b. One group of three labeled as below

        i. SCM D1, SCM D2, SCM D3

    iv. Second group of three labeled as below

i. ACM D1, ACM D2, ACM D3

43.Aspirate out all media from all wells using glass pipette

44.Add 2 mL of fresh DMEM media to the SCM plates

45.Add 2 mL of IOM media to the ACM plates

46.Place in 37˚ incubator.

ACM and SCM Collection Phase

47.hours from adding in IOM Media

48.Extract all ACM Day 1 media into a 15 mL tube

49.Extract all SCM Day 1 media into a separate 15 mL tube

50.Centrifuge both for 5 minutes at 1200 rpm

51.Add FBS to the ACM media, to create a 10% solution.

52.Pipette 1 mL of supernatant into 1.5 mL microcentrifuge tubes

53.Label the tops with "ACM D1 date", so media and date

54.Place completed tubes in a labeled box and then into the -80˚ freezer 38. Celebrate your Day 1 Media!

48 hours from adding in IOM Media

55.Extract all ACM Day 2 media into a 15 mL tube

56.Extract all SCM Day 2 media into a separate 15 mL tube

57.Centrifuge both for 5 minutes at 1200 rpm

58.Add FBS to the ACM media, to create a 10% solution.

59.Pipette 1 mL of supernatant into 1.5 mL microcentrifuge tubes

60.Label the tops with "ACM D2 date", so media and date

61.Place completed tubes in a box and then into the -80˚ freezer 46.

Insuline Oleate Medium (IOM) Preparation

1. Prepare 100mM Oleate Stock Solution
    I. Add 1g of sodium oleate to a 50ml conical
    II. Dissolve it in 32.8mL of methanol filtered through .22µM
2. Prepare 1.8mM IOM to make 50mL
    I. Combine .76mL 100mM Oleate and 3mL 30% BSA
    II. Incubate at 37 degrees Celsius water bath for 1 - 2.5 hours
    III. Add 20mL of alpha-MEM media, then add .2% FBS (100µL).
    IV. Add 175nM insulin (1µg/mL) (25µL of 2mg/mL) and 1x P/S (500µL).
    V. Add up to 50mL with media

Flow Cytometry Assay Set-Up Procedure

1. Acquire a 12 well flat bottom plate

2. Place 1 x 10^5 cells/well of at least 80% viability

3. Use laser cell counter to measure viability and number of cells within flask.

4. Find appropriate volumes needed from each cell line flask and place within 15 mL conical.

5. Spin down the 15 mL conicals at 1200 RPM for 5 minutes in centrifuge.

6. Acquire 15 mL conicals that have been spun down. Discard their supernatant.

7. Re-suspend the pellet in 300 micrometers RPMI1640 supplanted with 10% FBS.

8. Place 100 micro-liters of the resuspension in each well per condition.

9. Fill each well up to 2 mL with their appropriate condition. (i.e ACM, SCM, 10% RPIMI, etc.)

10. Place the 12 well plate within the CO_2 incubator for designated time for assay (I.e 24 hours)

11. After the designated time has passed, transfer well content in triplicates into a 96 round bottom well at 300 microliter/well,

12. Once transferred, spin down at 1200 RPM for 5 minutes in centrifuge and discard the supernatant between 300 microliter additions. Repeat steps 11 - 12 until transfer from the 12 well plate Is complete.

13. Add stain prepared at 1:100 to each well except unstained and isotope controls.

14. Add isotype control to designated well.

15. Wrap in tinfoil, place in 4 degree cold unit, and wait 1 hour - 24 hours before you run flow cytometry analysis.

T-cell Activation Protocol

1. Acquire 2e+6 Jurkat T-cells
2. Place within 15 mL conical and spin down at 1200 RPM for 5 minutes within centrifuge.
3. Re-suspend the cells in 1 mL of RPIMI1640 supplanted 10% FBS.
4. Place the entire volume into a 6 flat bottom well plate (2e+6 cells/well)
5. Add 25 micro-liters of PMA/Ionomycin stimulation cocktail per well.
6. Wait 24 - 72 hours before experiment

Co-coculture Experiment Protocol

1. Label B-ALL cells with Cell Trace Yellow (fluoresces in the PE channel) 20min before co-culture with TC
2. Start labeling process by re-suspending 2.2e+6 B-ALL cells in 1 mL of 1X PBS.
3. To make the working CTY solution, dilute the stock (10uL dye : 100ul PBS) in 1X PBS.
4. Add 1 ul of the working solution to the 1 mL of cells in PBS.
5. CTY-labeled human B-ALL cells are plated at 5.0 x 10^4 cells/well in a 96-well U bottom plate
6. Plate B-ALL cells with 24 hour stimulated TC.
7. Follow 96 well diagram below.
    i. The co-culture ratios treatment TC:B-ALL
8. Run flow cytometry

X Stim (10:1)	X Stim (10:1)	X Stim (10:1)		X Stim (1:1)	X Stim (1:1)	X Stim (1:1)		X Stim (1:10)	X Stim (1:10)	X Stim (1:10)
B-ALL Alone	B-ALL Alone	B-ALL Alone		B-ALL Alone	B-ALL Alone	B-ALL Alone		B-ALL Alone	B-ALL Alone	B-ALL Alone
Unlabeled TC	Unlabeled TC	Unlabeled TC		Unlabeled TC	Unlabeled TC	Unlabeled TC		Unlabeled TC	Unlabeled TC	Unlabeled TC

Table 4. Example 96-Well Plate Co-culture Set-up. Horizontal rows represents rows A - C of the 96 well plate. Vertical columns represent columns 1 - 11 of the 96 well plate. This plate is set up in triplicates, as denoted by colors orange, yellow, and green. Each color is further repeated 3 times as denoted by same color columns (i.e columns 1-3, 5-7, and 9-11). Row A contains cell trace orange stained co-culture samples at varying levels of T-cell stimulation with PMA ionomycin (i.e high, medium, low). Row A also contains varying level of TC:B-ALL samples (i.e. 10:1, 1:1, 1:10). Row B contains only stained B-ALL cells. Row C contains only unstained TC. This plate will be placed in a flow cytometer. Entire 96-well plate is not depicted due to not being used.