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Elucidating the Role of Soluble Galectin-9 in B-cell Acute Lymphoblastic Leukemia Pathogenesis

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Date

# Elucidating the Role of Soluble Galectin-9 in B-cell Acute Lymphoblastic Leukemia Pathogenesis

By

Raira Ank
B.S., Georgia College and State University, 2017

Advisor: Curtis J. Henry, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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2021

**Abstract**

Elucidating the Role of Soluble Galectin-9 in B-cell Acute Lymphoblastic Leukemia Pathogenesis

By Raira Ank

Hematological malignancies, including B-cell acute lymphoblastic leukemia (**B-ALL**), represent the largest class of cancers in pediatric patients. Risk factors, including obesity, have been shown to significantly decrease survival outcomes as much as 30%. This observation is alarming given The Center for Disease Control and Prevention (**CDC**) prediction that 20% of children ages 2-19 in the United States are obese with these numbers predicted to double within the next decade. These observations emphasize the need to further understand the relationship between obesity, leukemia development, and therapeutic responses.

We demonstrate that adipocyte-secreted factors promote B-ALL pathogenesis in a Galectin-9 (**Gal-9**)-dependent manner. Galectin-9 is a tandem repeat type member of the galectin family which has a wide range of biological functions, including inducing cellular proliferation, promoting apoptosis, and enhancing immune escape of malignant cells. In its soluble form, Gal-9 suppresses T-cell activation at low concentrations and is cytotoxic to T-cells at high concentrations. Mass spectrometry profiling of adipocyte-conditioned media (**ACM**) revealed the presence of several proteases, including thrombin, which has a cleavage site in the Gal-9 linker region.

Given the role of Gal-9 in immunosuppression, and that adipocyte-secreted factors cleave Gal-9 from the surface of B-ALL cells, I **hypothesize** that adipocyte-secreted thrombin cleaves Gal-9 from the surface of B-ALL cells which promotes B-ALL immune evasion.

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## Table of Contents

## Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (**ALL**) are hematopoietic neoplasms arising from B-lineage or T-lineage precursor cells [1]. These neoplasms present with involvement of the bone marrow, peripheral blood, and extramedullary sites. The hallmark features of ALL include chromosomal aberrations and genetic alterations in differentiation and proliferation of the lymphoid precursors [2].

The majority of ALL cases (80%) are pediatric. Although mainly a pediatric disease, ALL incidence follows a bimodal distribution, with the second peak occurring in individuals greater than age 50 [2]. Adult patients have a high response rate to treatment, however only 30-40% reach long-term remission [2]. Furthermore, approximately 20% of pediatric patients will relapse, and unfortunately only 30-50% of relapse patients will be cured [3]. Recent studies show that the mortality rates are higher in pediatric patients with risk factors including Down's syndrome and obesity [3, 4]. Of patients that succumb to this cancer, the most common cause of death is infection, often in the lungs [5].

There are several subtypes of ALL, including B-cell acute lymphoblastic leukemia (**B-ALL**), which is the subtype of study for the work presented in this thesis.

## Clinical Presentation of B-ALL

ALL cases predominantly present in pediatric populations, with an incidence of 3 in 100,000 in patients between ages 0 to 14 [1]. ALL represents 34% of cancer diagnoses in this age group, with a peak between 2 to 5 years of age [1]. ALL typically presents as de novo disease, although rare cases of secondary neoplasms have been recorded. Of all cases of pediatric ALL, approximately 80% are precursor B-ALL.

Studies have identified that certain genetic syndromes, including Down syndrome and Fanconi anemia, are predisposing factors for the development of every ALL subtypes [6]. Other predisposing

factors include excessive exposure to ionizing radiation, pesticides and solvents, and certain viruses, including Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) [2, 7, 8].

Classic chromosomal translocations for B-ALL include t(12;21) [ETV6-RUNX1], t(1;19) [TCF3-PBX1], and t(9;22) [BCR-ABL1], commonly known as the Philadelphia Chromosome (Ph+) [2]. ETV6-RUNX1-driven disease is the most common in pediatric patients, representing 25% of pediatric B-ALL cases and this disease has a favorable prognosis. Ph+ status has the greatest impact on prognosis and treatment course. Historically, patients with Ph+ disease had a 10% 1-year survival rate; however, improvement in treatment strategies and targeted therapies have improved survival [2].

Presentation of disease can be non-specific, with constitutional symptoms (pain, fatigue) and signs of bone marrow failure (anemia, thrombocytopenia, leukopenia). Additionally, patients may present with "B symptoms", which include night sweats, fever, and weight loss. Approximately 20% patients will present with lymphadenopathy, splenomegaly, or hepatomegaly [9]. Patients with mature B-ALL can present with extramedullary site involvement or central nervous system (**CNS**) involvement at time of diagnosis, with the most common symptoms being cranial nerve defects or meningismus [10].

Diagnosis is based on the presence of lymphoblasts in the bone marrow or peripheral blood (at least 20%). Analysis of morphology, flow cytometry, immunophenotyping, and cytogenetics are used for confirmation and for risk stratification [2].

## Current Treatments for B-ALL

Treatment structures are similar for pediatric and adult cases. For standard-risk pediatric ALL, long-term survival has approached 90%. However, nearly 20% of pediatric patients will relapse [3].

Chemotherapy is given in three stages, induction, consolidation (intensification), and maintenance with CNS prophylaxis is given at intervals throughout treatment [2]. The induction stage is intended to achieve remission, while restoring healthy hematopoiesis. Induction therapy is given in

two stages of 4 weeks. Chemotherapeutic agents in this stage include vincristine, corticosteroids, and anthracycline [2]. The use of L-asparaginase is standard in pediatric induction therapy but is not advised in adults due to high rates of adverse effects [2].

Following induction, patients are given consolidation therapy (intensification), which consists of methotrexate and L-asparaginase. High-risk patients undergo allogeneic stem-cell transplants at this stage [2].

Maintenance therapy generally consists of daily administration of 6-mercaptopurine weekly methotrexate, vincristine, and prednisone pulse every 3 months [2]. Maintenance therapy can last anywhere from 2 to 3 years after induction.

Patients with Ph+ ALL undergo targeted therapy. Historically, Ph+ ALL had very low 5-year survival rate (~5-20%), and allogeneic stem-cell transplant was the only chance for cure [2]. The discovery of tyrosine kinase inhibitors (**TKIs**), such as imatinib, nilotinib, and dasatinib, marked a turning point for the treatment of this disease. TKIs inhibit the action of the fusion protein Bcr-Abl by blocking its ATP-binding site and increase the 3-year overall survival rates from 15% to 54% [2]. When used as a frontline therapy, third generation TKIs, ponatinib, have significantly better 3-year overall and event-free survival rates [2].

## Childhood Leukemia and Obesity

The World Health Organization (**WHO**) defines obesity as the excessive or abnormal accumulation of fat tissue that poses a risk to health [11]. Adult obesity is defined as body-mass index (**BMI**) of over 30, while childhood obesity is defined as above the 95$^{th}$ percentile, calculated based on height, weight, and adjusted for age [12]. Obese individuals are at higher risks for chronic noncommunicable diseases, including cardiovascular disease, Type-2 Diabetes Mellitus, inflammatory bowel disease, and cancers [11, 12].

While improvements in the treatment of pediatric B-cell malignancies have led to an overall survival of 80-90%, emerging studies have shown that survival rates decline around 30% for patients who are overweight or obese at diagnosis [13, 14]. These reports are highly disconcerting, given that the CDC estimates that in the by 2030, 40% of children in the United States will be overweight or obese [15].

## Obesity and Immunity

Obesity is characterized by low-level systemic inflammation, which is negatively associated with immune system function and lifespan in both human and mice [12]. Obesity leads to dysregulated activation of pro-inflammatory immune cells and extensive changes in levels of both pro and anti-inflammatory cytokines. In obese humans, adipocyte accumulation has been documented to promote an immunosuppressive microenvironment, with the onset of chronic inflammation [16], increased infections (including SARS-CoV2) [17, 18, 19], and suboptimal vaccinations responses [20].

### B-cells

B-cells are lymphocytes which elicit the humoral component of adaptive immunity. The primary role of B-cells is the production of high affinity antibodies. B-cells are capable of producing cytokines, although this process requires specific differentiation and activation conditions [21].

In mice fed high fat diet, bone marrow adipocytes secrete soluble factors that recruit myeloid-derived suppressor cells (**MDSCs**). MDSCs, in turn, secrete IL-1β, which inhibits B-lymphopoiesis [22, 23]. Additionally, cells in the early stages of development have reduced expression of early lymphoid commitment markers, such as the B-cell transcription factor PAX5 [24]. The function of splenic B-cells is also altered by adipocytes. Recent studies demonstrate studies that splenic B-cells from obese mice secrete pro-inflammatory cytokines, such as IL-6 and TNFα, at higher levels compared to B-cells -from lean mice. B-cells from obese mice have also been shown to play a role in

the establishment of insulin resistance by inducing the secretion of IFN-γ and TNF-α from T-cells and macrophages [12]. Depleting B-cells using anti-CD20 antibodies ameliorates obesity-induced abnormalities.

Human studies have shown that adipocyte-secreted factors inhibit B-cell development, most commonly at the lymphoid progenitor to pre/pro B cell phase (BILWANI). Obesity is also associated with compromised B-cell responses to infections and vaccines [25, 26, 27]. B-cells from obese donors displayed defects in class switching and somatic hypermutation, both processes necessary for the generation of high affinity antibodies [28].

T Helper Cells

T helper cells function as regulators of both pro-inflammatory and anti-inflammatory processes. There are several subsets of T helper cells, including Th1, Th2, Th17, and Th22, which are characterized by their function and cytokine secretion; however, all subsets express CD4 on their surface [30].

T helper 1 (**Th1**) cells are pro-inflammatory cells, expressing the transcription factor T-bet, and they produce interferon-γ (**IFN-γ**), interleukin 2 (**IL-2**), and tumor necrosis factor alpha (**TNF-α**). Th1 cells are promote inflammation in adipose tissue. In mice fed high fat diets, Th1 cells were more abundant compared to mice fed control diets in both subcutaneous adipose tissue (**SAT**) and visceral adipose tissue (**VAT**) [31]. T-bet knockout mice, which have attenuated Th1 responses, had blunted inflammatory responses and improved glucose tolerance [32, 33].

T helper 2 (**Th2**) cells confer immunity against parasitic infections and play a role in allergic reactions [34]. Th2 cells primarily produce IL-4, IL-5, IL-13, as well as the anti-inflammatory cytokine IL-10. In the context of obesity, Th2 cells have not been thoroughly studied, although the few reports that exist suggest they play an anti-inflammatory role. It has also been demonstrated that Th2 populations decrease in VAT of mice fed high fat diets compared to mice fed control diet [31].

T helper 17 (**Th17**) cells are pro-inflammatory cells that characteristically express transcription factor retinoic acid receptor-related orphan receptor γt (**RORγt**) and STAT3 [31]. Th17 cells primarily stimulate inflammatory processes and regulate the adaptive response to extracellular bacteria and viruses. Th17 cells secrete the cytokine IL-17, as well IL-22 and IL-23. The IL-17 receptor is expresses on monocytes and macrophages and induces a strong pro-inflammatory response. Additionally, IL-17 secretion has been shown to reduce insulin sensitivity in hepatic, muscle, and adipose tissue [31]. Th17 cells have been shown to increase in adipose tissue and to sustain adipose tissue inflammation through a positive feedback loop [31]. Th17 cells stimulate IL-6 and IL-1β production by adipocytes, macrophages, and monocytes [31].

T helper 22 (**Th22**) cells enhance innate immunity through the secretion of IL-22 and assist in the response against bacterial infections on the body surfaces. In obese patients, both Th22 and IL-22 are enriched in adipose tissue and peripheral blood. The role of Th22 cells in adipose tissue has not been fully elucidated, with some studies suggesting it may play a protective role for the development of obesity, while others suggest low levels of IL-22 are unlikely to contribute to obesity pathogenesis and chronic inflammation [31].

Regulatory T Cells

Regulatory T-cells (Tregs) primarily prevent autoreactivity and stop excessive effector T-cell activation, therefore preventing tissue damage during immune responses. Tregs produce inhibitory cytokines, including IL-10 and TGFβ, and interfere with T-cell survival by depleting circulating IL-2 levels in a form of competitive inhibition [31]. Studies have shown that Tregs decrease in the spleen and VAT of obese mice but increase in SAT [31]. In humans, studies have reported conflicting data: some have demonstrated a decrease in Tregs in VAT, whereas others have shown an increase in Tregs in both VAT and SAT of obese patients [31].

Cytotoxic T Cells

$CD8^+$ cytotoxic T-cells (**Tc**) are the primary effector cells of the immune system. Tc recognize antigens presented on MHC class I molecules by antigen presenting cells. Once activated, they gain cytolytic abilities mediated through perforin and granzyme secretion. The main role of Tc cells is to eliminate malignant or pathogen-infected cells. Murine-based high fat diet models have demonstrated an enrichment of Tc in VAT in obese mice [31]. Furthermore, it has been reported that Tc are crucial for the recruitment of macrophages and for the induction of adipose tissue inflammation through production of chemotactic molecules. This demonstrates a role for Tc —adipocyte interaction. Our studies have shown that adipocyte-secreted factors directly suppress the function of murine T-cells leading to compromised cytokine production and the attenuated upregulation of cytolytic mediators in effector T-cells (**Fig. 1)**.

## Obesity and Cancer Immunotherapies

Due to advancements in the field of immunology, immunotherapies, such as immune checkpoint blockade and engineered chimeric antigen receptor (**CAR**) T cells, have become promising treatment modalities for cancer patients with advanced and relapse disease. Despite clinical successes, more than half of patients treated with immunotherapies fail to achieve durable responses [35]. Given that obesity is a risk factor for many cancers and that obesity negatively impacts immune responses, it is likely that obesity also affects clinical responses to immune-based therapies. Some reports have suggested a positive correlation between obesity and cancer immunotherapy efficacy, while others have reported a negative correlation [35, 36]. This "obesity paradox" may be attributed to several factors, including cancer type, lifestyle, clinical and pathological factors [37].

For renal cell carcinoma, clinical trends show a reduced response rate to anti-PD1 treatment in obese patients, accompanied by worse overall and progression-free survival [38]. Boi *et al*

recapitulated these trends using diet-induced obesity mouse models; anti-PD1 combinatorial immunotherapy had reduced efficacy in obese mice bearing established renal tumors. The authors demonstrate that this may arise in part from early, obesity-associated elevations in IL-1β concentrations and the presence of MDSCs. Neutralization of IL-1β restored efficacy to the levels seen in lean mice [38].

McQuade *et al* reported that melanoma patients who were obese at treatment initiation had better outcomes following immune checkpoint blockade. Interestingly, this association was only true for male patients [39]. Additional studies in melanoma have identified that obesity-associated increases in the surface expression of PD-1 allowed for the improved efficacy of anti-PD-1 therapy in obese mice [40].

Given the rise in obesity incidence globally, it is of utmost importance that obesity and its immunomodulatory effects be studied to identify the best therapeutic regimens for this higher-risk population.

## T-cell Mediated Immunity and Galectin-9

As described above, T-cell mediated immunity is significantly modulated by obesity. In our studies, we have identified an adipocyte-secreted factor, Galectin-9, with several immunomodulatory effects, including mediating T-cell responses.

Galectin-9 belongs to the galectin family of proteins, which are non-classically secreted, β-galactoside binding proteins. Galectins are active in a variety of biological processes, including adhesion and migration, proliferation and apoptosis, and immunosenescence and T-cell exhaustion. Galectins are evolutionarily conserved from nematodes to humans. To date, 12 galectins have been identified in humans [41].

Gal-9 is a 34-39 kDa tandem-repeat protein encoded by the LGALS9 gene in human and lgals9 in mice. This lectin possesses two carbohydrate recognition domains (**CRD**), joined by a polypeptide linker domain of variable lengths. The variable linker domain generates three Gal-9 isotypes (short, medium, and long), conferring the isotypes with differing avidities and affinities. The linker domain of Gal-9L has several cleavage sites, including metalloproteinases, elastases, and thrombin. Protease cleavage of Gal-9 produces free N- and C-CRDs [42]. When cleaved, Gal-9 C-CRD has been shown to have increased anti-proliferative and pro-apoptotic activity greater than the N-CRD [42]. These findings suggest that each CRD can form oligomers to exert their activity.

Gal-9 has been shown to have several roles in immunity. In the thymus, Gal-9 is involved in T-cell selection and induces apoptosis of double-positive or double-negative thymocytes [41], most likely through binding its many receptors on T-cells including CD44, TIM3, 4-1BB. Recent studies suggest that the role of Gal-9 in immunomodulation is two-sided.

When Gal-9 binds T-cell immunoglobulin domain and mucin domain protein 3 (**TIM3**), it induces apoptosis of Th1, Th17 and cytotoxic T-cells; however, it does not induce the apoptosis of TIM3-positive Tregs [41]. In the context of cancer immunotherapy efficacy in models of colon adenocarcinoma, Yang *et al* showed that PD-1 binds Gal-9, attenuating the Gal-9/TIM3-induced apoptosis. The addition of an anti-Gal-9 therapy induced antitumor activity, suggesting that Gal-9 is a viable and promising target for immunotherapy [43].

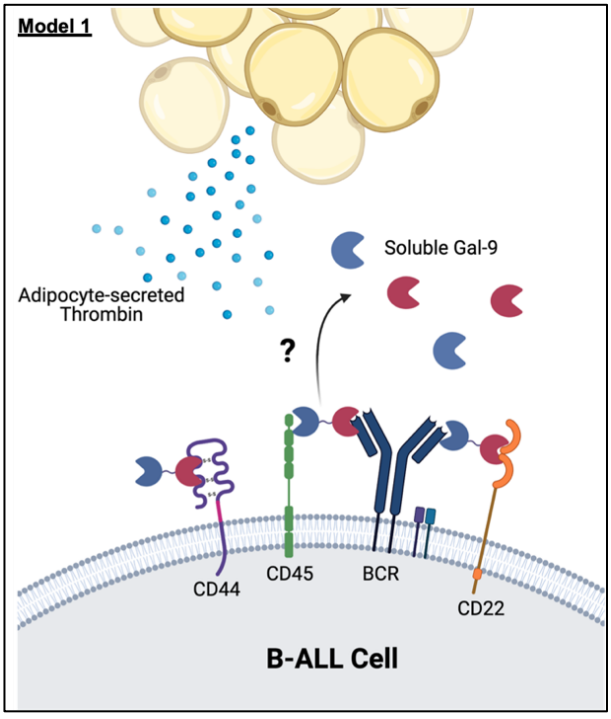
Antibodies to 4-1BB, another Gal-9 binding partner, are currently being investigated to augment immunity against cancer, as well as inhibit autoimmune diseases. Madireddi *et al* showed that the action of these antibodies is dependent on Gal-9 binding 4-1BB at a different binding site. When engaged by the agonist, Gal-9 assists in the clustering of 4-1BB molecules and acts as an immunoregulatory checkpoint [44].

Gal-9 is a required factor for the expansion of adaptive or induced Tregs (iTregs) [45]. Using Gal-9 deficient mice, Wu *et al* showed that while animals have normal T-cell development in the lymphoid organs, they have reduced frequency of iTregs, but not in thymus-derived natural Tregs (nTregs) [45, 46].

In several mouse-based studies of autoimmune diseases, Gal-9 treatment induces T-cell apoptosis and ameliorates autoimmunity in an exogenous manner [47]. However, in models of T-cell mediated colitis, Gal-9 augments proximal TCR signaling and potentiates the disease, highlighting the duality of Gal-9 in the immune system [47] and the context specific studies which need to be conducted on this fascinating lectin.

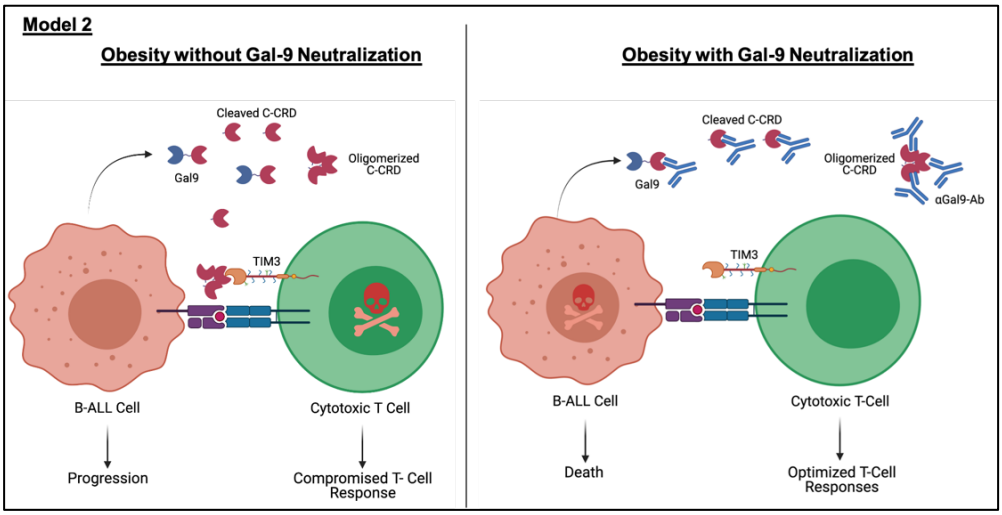
## Summary Models

**To determine how thrombin impacts Gal-9 cleavage from B-ALL cells**. In preliminary studies, we have demonstrated that soluble Gal-9 levels increase when human B-ALL cells are cultured in ACM. Furthermore, mass spectrometry analysis identified that thrombin is secreted by adipocytes which is interesting due to published reports of a thrombin cleavage site int the Gal-9 linker region. Based on these observations, I ***hypothesize*** **that thrombin cleaves Gal-9 from the surface of human B-ALL cells** (**see Model 1**).




*Model created by author using BioRender.*

**To determine how soluble Gal-9 modulates B-ALL immune evasion**. Recent studies have demonstrated that T-cell mediated immunity is required for eradicating leukemia cells. Recently, soluble Gal-9 has emerged as a potent suppressor of T-cell mediated immunity. Given the T-cell suppressive effects of soluble Gal-9 and our data demonstrating that adipocyte-secreted factors significantly increase Gal-9 cleavage from B-ALL cells, I ***hypothesize*** **that Gal-9-mediated immune evasion increases B-ALL pathogenesis** (**see Model 2**).




*Model created by author using BioRender.*

## Scope of Thesis

The aim of this thesis is to elucidate the role of soluble Galectin-9 in the pathogenesis of B-ALL in obese backgrounds. Given that obesity is associated with immunosuppression [48]. We sought to determine how adipocyte-secreted factors impact the immunogenicity of human B-ALL cells. Current literature demonstrate that Gal-9 has extensive immunomodulatory effects, but to our knowledge the relationship between Gal-9, obesity, and B-ALL progression has not been studied.

Based on our results demonstrating that adipocyte-secreted factors cleave soluble Gal-9 from the surface of human B-ALL cells, I ***hypothesize*** that obesity potentiates tumorigenesis by increasing soluble Gal9 levels, which attenuates T-cell mediated immune surveillance of B-ALL cells.

## Materials and Methods

### Murine T-cell Activation Assay:

Murine splenocytes were isolated from lean mice and CD4+ and CD8+ T-cells were purified using magnetic-activated cell sorting (**MACS**). 50,000 cells of each subtype were plated in triplicates in 96-well plates. Cells were cultured in unconditioned media, SCM, and ACM and stimulated (PMA 1-25 ng/mL, Ionomycin 1 μg/mL) for 72 hours. Post-stimulation, cells were stained for the appropriate markers for each experiment and analyzed via flow cytometry.

For recombinant Gal-9 experiments, cells were plated as above and cultured in unconditioned media cells were stimulated with PMA/Ionomycin for 24 hours. Following incubation, cells were treated with varying concentrations of murine recombinant Gal-9 (1ng/ml, 5 ng/ml, 25 ng/ml) for 48 hours. Cells were stained for the appropriate marker (CD44) and analyzed via flow cytometry.

Flow cytometry data was analyzed using FlowJo Software. Statistical analysis was performed using Student's T test in Graphpad Prism. *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$.

### Human T-cell Activation Assay:

Human PBMCs were obtained from lean and obese patients with B-ALL. PBMCs were stimulated with PMA/Ionomycin. After 72 hours, PBMCs were stained for surface markers (CD4, CD8, CD44), for cytokines (IFN-γ and TNF-α), and for cytolytic machinery (Perforin, Granzyme B).

Flow cytometry data was analyzed using FlowJo Software. Statistical analysis was performed using Student's T test in Graphpad Prism. *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$.

### Mass spectrometry Analysis:

Unconditioned, bone marrow stromal cells (**SCM**), and adipocyte conditioned media (**ACM)** was analyzed in the Emory Integrated Proteomics Core (**EIPC**) using state-of-the-art Orbitraps for high resolution mass spectrometry. Protein identiifcation was performed using public bioinformatic

databases and analysis resources (including DAVID and STRING) to annotate proteins and to generate testable signaling networks/pathways.

**Soluble Gal-9 Detection via ELISA**:

Human B-ALL cell lines (REH, KOPN8) were cultured in unconditioned (**RPMI**), SCM, and ACM for 24 hours. Supernatants were collected and soluble Gal-9 levels were detected via ELISA (R&D Systems Quantikine ELISA Human Galectin-9).

For recombinant thrombin experiments, a human B-ALL cell line (Nalm6) was cultured in RPMI with varying concentrations of human recombinant thrombin (0.1ng/mL, 1.0ng/mL, 2.0ng/mL) for 24 hours. Supernatants were collected and soluble Gal-9 release was quantified via ELISA (R&D Systems Quantikine ELISA Human Galectin-9).

Standard curve was interpolated, and statistical analysis was performed using a paired T test in Graphpad Prism. *=$p < 0.05$, ****=$p < 0.0001$

**Diet-Induced Obesity Mouse Model Survival Experiment:**

The diet-induced obesity model best replicates the development of human obesity [55]. C57BL/6 mice were place on control (10% kcal fat) or high fat (60% kcal fat) diets for 2 months. Mice were injected intravenously with murine B-ALL (Bcr-Abl+/Arf-null) cells ($10^5$-$10^6$ cells/mouse) and monitored for disease progression and survival (n=10 per group). Statistical analysis performed using log rank test. ****=$p < 0.0001$

**Blinatumomab-Induced T-cell Cytotoxicity Assay**:

Primary healthy donor CD8+ T-cells and Nalm-6 cells (a human B-ALL cell line) were pre-treated for 24 hours with RPMI, SCM, or ACM. Cells were re-counted and plated at 1:1 effector-target ratio in RPMI, SCM, or ACM + vehicle (PBS) or 7 ng/mL blinatumomab for 72 hours.

Viability was measured by flow cytometry and percent lysis was calculated. T-cell proliferation was assessed using flow cytometry.

Flow cytometry data was analyzed using FlowJo Software. Statistical analysis was performed using a one-way ANOVA with multiple comparisons in Graphpad Prism, followed by Tukey test. *=p <0.05

Percent lysis was calculated using the following equation:

%Lysis = 100 x ((%Treated Sample [Dead Nalm6] - %Vehicle Sample [Dead Nalm6]) / (100 - %Vehicle Sample [Dead Nalm6])

## Results

### Adipocyte-secreted factors attenuate primary murine T-cell function.

Murine T-cells were cultured in unconditioned media, SCM, and ACM. Proliferation was significantly attenuated in CD4 and CD8 T-cell subsets when cells are activated in ACM compared to unconditioned and SCM (**Fig 1A-B**). Additionally, CD4 and CD8 T-cells demonstrate impaired

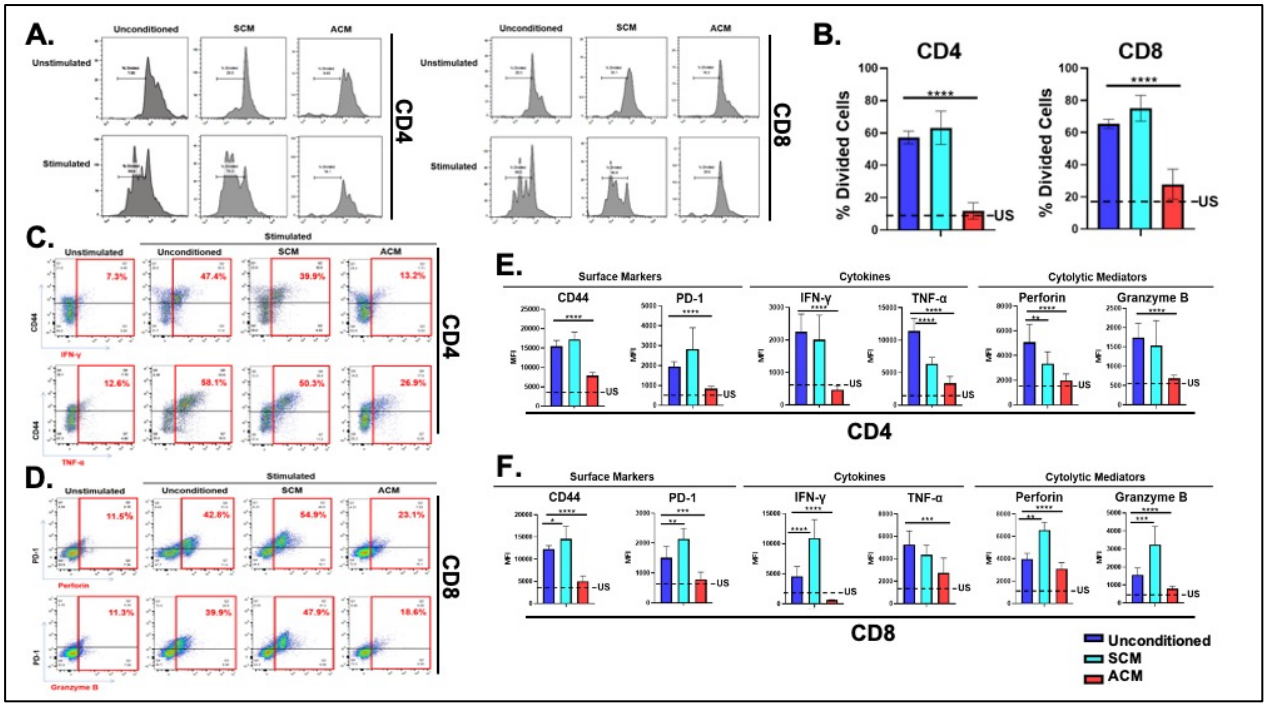



*Figure 1: Significance denoted by: *=p <0.05, **=p <0.01, ***=p <0.001, ****=p <0.0001. Experiment performed by author and Anthony Ross, M.D. Analysis performed by Anthony Ross, M.D.*

function, resulting in decreased TNF-α, IFN-γ, Perforin, and Granzyme B production (**Fig 1E-F**). Furthermore, we saw decreased surface expression of the activation marker, CD44, and of the inhibitory marker, PD-1. Together, these data suggest that adipocyte-secrete factors inhibit proper T-cell function.

**T-cell function is attenuated in obese patients without leukemia compared to healthy weight peers.**

Human PBMCs were isolated from healthy and obese leukemia patients. T-cells from obese patients were dysfunctional highlighted by a decrease in cytokine production (IFNγ/TNFα) and reduced protein levels of cytolytic mediators (Perforin/Granzyme B) (**Fig 2**). However, unlike murine T-cells, human T-cells stimulated from obese patients will B-ALL upregulated the activation marker CD44 to higher levels than observed on T-cells stimulated from lean patients with this disease.

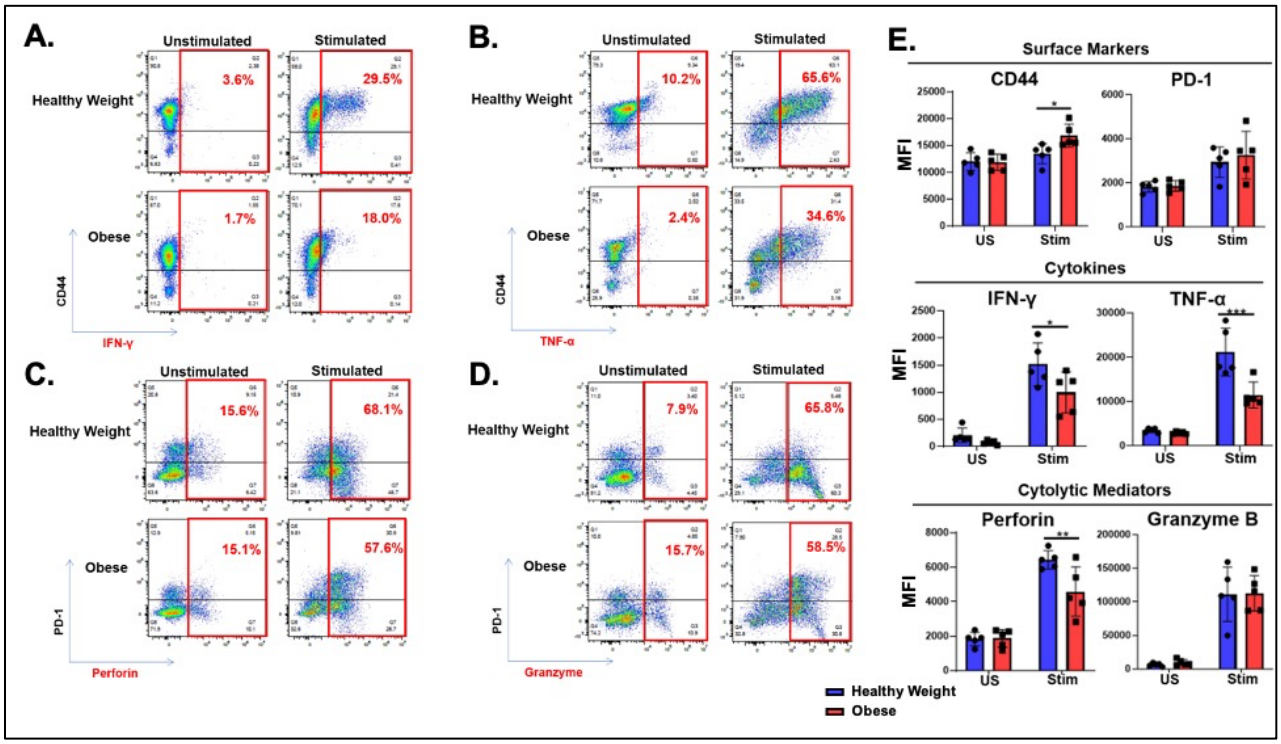



*Figure 2: Significance denoted by: *=p <0.05, **=p <0.01, ***=p <0.001. Experiment performed by author and Anthony Ross, M.D. Analysis performed by Anthony Ross, M.D.*

Together, these data demonstrate that T-cell function is significantly suppressed in obese pediatric patients with B-ALL.

**Proteases identified through mass spectrometry analysis of adipocyte-conditioned media.**

Approximately 200 proteins were identified in the mass spectrometry analysis of unconditioned media, SCM, and ACM. In **Table 1**, five representative proteins are shown in their respective media. Of note, Thrombin was identified only in ACM. Thrombin is serine protease, most known for its role in coagulation and they are currently no reports demonstrating production by adipocytes. Interestingly, the Gal-9 linker domain contains a Thrombin cleavage site which may contribute to the release of Gal-9 from the surface of B-ALL cells in obese microenvironments.

| Proteins Detected in Conditioned Media | | | |
|---|---|---|---|
| **Protein Detected** | **UCM** | **SCM** | **ACM** |
| **Thrombin** | - | - | + |
| **MMP21** | - | - | + |
| **Fibronectin** | - | + | + |
| **IF135** | - | - | + |
| **HSP90** | - | + | - |

*Table 1: Samples prepared by Miyoung Lee, Ph.D. Spectrometry performed by Emory Integrated Proteomics Core. Data table by author and Jamie A.G. Hamilton, B.S.*

**Adipocyte-secreted factors increase soluble Gal-9 levels.**

When exposed to adipocyte-secreted factors, human B-ALL cells (REH and KOPN8) expressed higher levels of Gal-9 at the cell surface (**data not shown**). Similarly, soluble Gal-9 levels were also significantly higher when REH and KOPN8 cells were cultured for 24 hours in ACM relative to the other conditions tested (**Fig 3**).

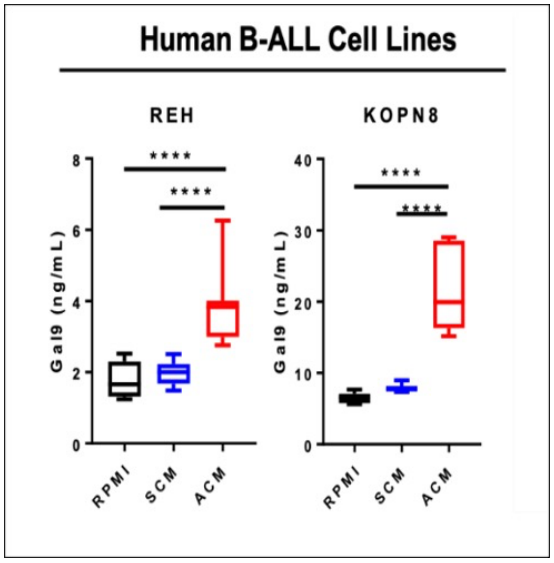



*Figure 3: Significance denoted by: ****=p <0.0001. Experiment and analysis performed by Miyoung Lee, Ph.D.*

**Human recombinant thrombin cleaves Gal-9 from human B-ALL cells.**

Human B-ALL cells (Nalm6) were cultured in unconditioned media for 24 hours and then treated with recombinant Thrombin. Nalm6 cells express low surface Gal-9 levels, and very little Gal-9 is detected in the media when these cells are cultured in media alone. However, when treated with high concentrations of recombinant Thrombin, soluble Gal-9 significantly increased (**Fig 4**).

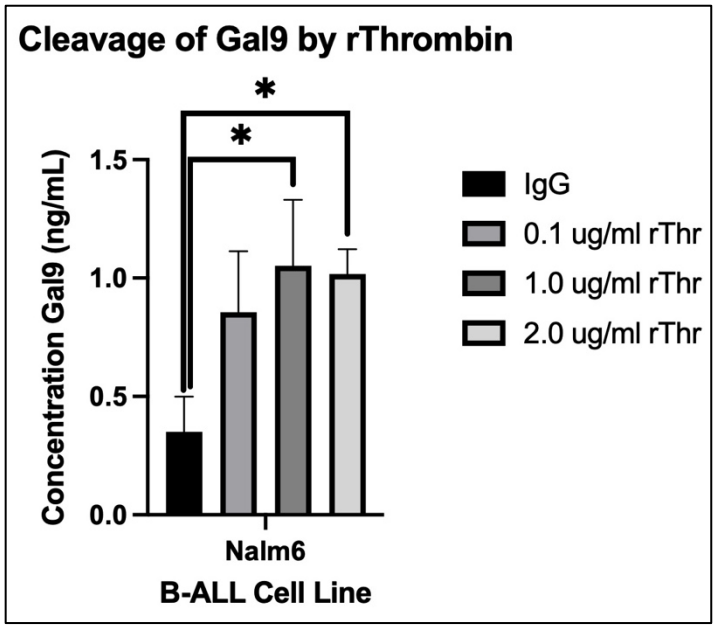



*Figure 4: Significance denoted by: *=p <0.05. Experiment and analysis performed by author.*

Importantly, these experiments are currently being repeated are B-ALL cells are first treated with TNF-α to upregulate Gal-9 surface expression.

**Obesity potentiates B-ALL development.**

Lean and obese C57BL/6 mice were injected with Bcr-Abl+ murine B-ALL cells and monitored for disease progression and survival. Approximately 40% of lean mice survive until 70 days post-transplantation. However, obese

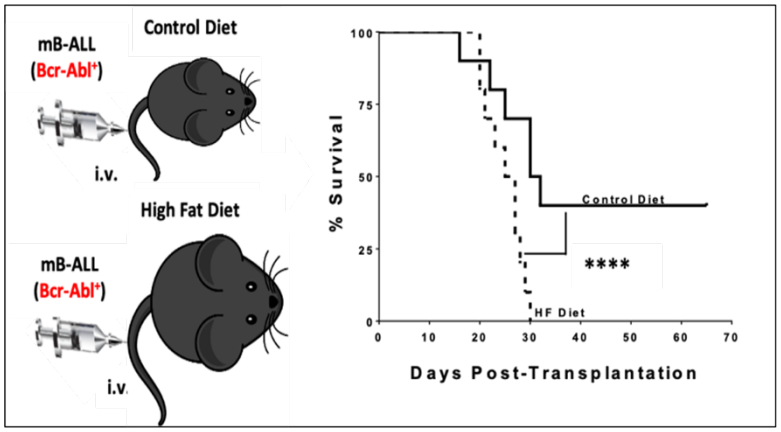



*Figure 5: Significance denoted by: ****=p <0.0001. Experiment and analysis performed by Miyoung Lee, Ph.D.*

mice have significantly reduced survival and do not survive past 30 days post-transplantation (**Fig 5**).

**Adipocyte-conditioned media reduces blinatumomab-induced CD8$^+$ T-cell mediated cytotoxicity.**

Healthy donor CD8+ T-cells were co-cultured with human B-ALL cells (Nalm6) and treated with either vehicle or blinatumomab. Blinatumomab is a bispecific T-cell engager (BiTE) class of FDA-approved immunotherapies used as treatments for refractory and relapsed B-ALL [49]. Blinatumomab targets CD19 on the malignant B-cells and CD3 on T-cells to bring the two in proximity [49]. Furthermore, the T-cell targeting portion of this drugs serves to activate T-cells by inducing CD3-mediated signaling [49]. In this experiment, blinatumomab was used as a tool to determine the potency of T-cell mediated cytotoxicity in the presence of adipocyte-secreted factors. T-cells cultured in ACM killed 30% fewer target cells compared to cytotoxicity observed in unconditioned media and 50% less compared killing observed in SCM (**Fig. 6A-B**). T-cell proliferation was also significantly reduced in ACM relative to the other conditions tested (**Fig 6C**).

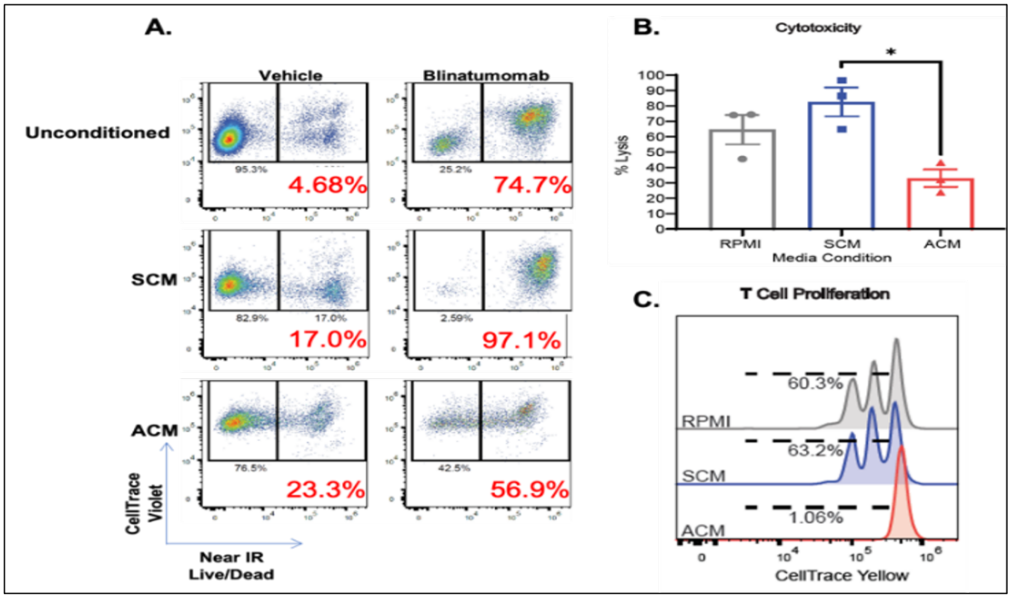



*Figure 6: Significance denoted by: *=p <0.05. Experiment and analysis performed by Priscilla Do, Ph.D.*

**Recombinant Gal-9 treatment attenuates murine T-cell activation.**

Treatment of murine T-cells with recombinant Gal-9 recapitulated the decrease in the activation marker, CD44, seen when murine T-cells were treated with adipocyte-secreted factors (**see Figure 1**). Surprisingly, this suppression in CD4+ and CD8+ T-cell populations was observed at levels significantly lower than quantified in our thrombin cleavage experiments (**see Figure 4**). These data indicate that Gal-9 has direct immunoinhibitory effects on cytotoxic T-cells and T-helper cells.

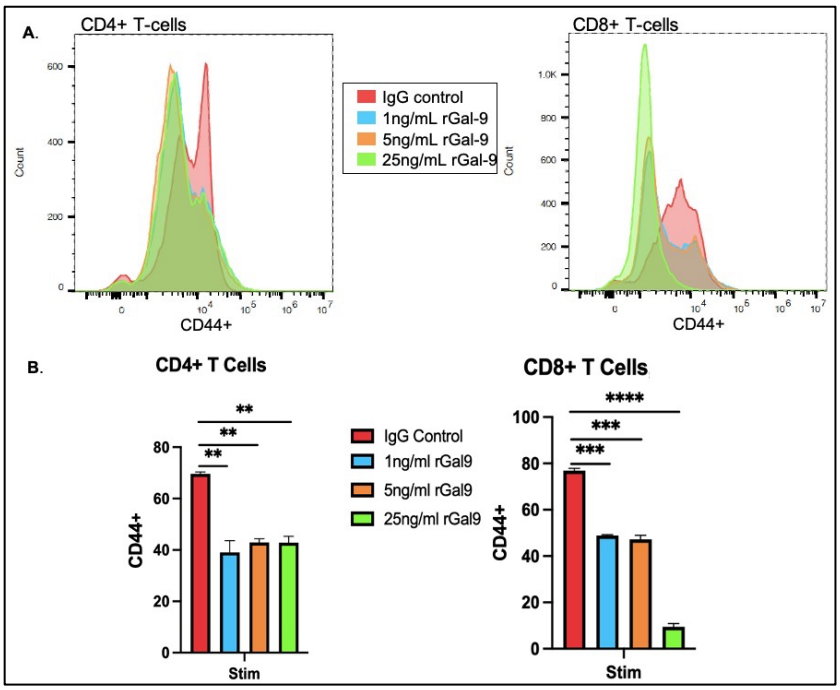



*Figure 7: Significance denoted by: **=p <0.01, ***=p <0.001, ****=p <0.0001. Experiment and analysis performed by author.*

## Discussion

To study the impact of obesity on B-ALL development and immunity we used the diet-induced obesity (DIO) murine model. When mice were fed high fat diet (60% kcal from fat), relative to control diet (10% kcal from fat), survival significantly decreases post-implantation of murine B-ALL cells (**Fig 5**). In addition to potentiating B-ALL development, adipocyte-secreted factors directly suppress the function of murine and human T-cells leading to compromised cytokine production and the attenuated upregulation of cytolytic mediators in effector T-cells (**Figs. 1 and 2**).

Given that obesity is associated with immunosuppression [48], we next sought to determine how adipocyte-secreted factors impact the immunogenicity of human B-ALL cells. In preliminary

studies, we have found that culturing murine and human B-ALL cells in ACM, but not UCM or SCM, increases surface expressed and soluble levels of Gal-9 (**Fig 3**). The adipocyte-induced increase in soluble Gal-9 levels is alarming because soluble Gal-9 suppresses T-cell function at low levels of stimulation and excessive stimulation results in T-cell death [43, 50, 51]. Despite the immunosuppressive effects of soluble Gal-9, there are no reported studies demonstrating immunomodulatory functions of surface expressed Gal-9. Furthermore, to our knowledge, there are no published reports describing the impact of obesity on soluble Gal-9 levels in modulating B-ALL development.

Given the increased levels of soluble Gal-9 observed in B-ALL/ACM cultures, we performed mass spectrometry on ACM to identify potential adipocyte-secreted factors that could cleave Gal-9 from the surface of B-ALL cells. Of the ACM identified proteins, thrombin was nominated as our top candidate due to a thrombin-cleavage site located in the Gal-9 linker region [42]. To our knowledge, our data represents the first observation of thrombin production by adipocytes. Although we are not aware of studies demonstrating thrombin secretion from adipocytes, adipocytes are known to secrete coagulation factor VII upon stimulation with the pro-inflammatory cytokine TNF-α [52]. Notably, protease cleavage of Gal-9 produces free N- and C-CRDs [42]. The C-terminal is capable of oligomerization, and in this state, promotes T-cell death when it binds to T-cell immunoglobulin 3 (Tim-3) which is expressed on T-cells [42, 53, 54], suggesting that thrombin-mediated release of Gal-9 from B-ALL cells may potentiate tumorigenesis and attenuate T-cell mediated immune surveillance. The newly identified relationship between thrombin and B-ALL tumorigenesis begs the question of whether treatment with anti-coagulants or thrombin inhibitors would be protective against leukemia. To our knowledge, there are no published studies on this topic and this relationship remains to be determined.

In future studies, we will determine if antibody-mediated neutralization of Gal-9, combined with conventional therapy, improves T-cell mediated elimination of B-ALL in murine models of obesity.

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