## Specific Aims

**Background**: Acute Myeloid Leukemia (AML) is a clonal malignancy of the bone marrow derived from rare leukemia stem cells (LSC). Conventional chemotherapy results in remission in up to 80% of patients, however, the 5-year survival rate for those with AML has lingered at 25% for over the past decade. This discrepancy is due to the fact that most patients relapse and relapsed disease is deadly. Chemoresistant LSCs are the most probable source of relapse, implicating LSCs as the critical target for curing AML.

Allogeneic stem cell transplant (allo-SCT) remains the only curative therapy for many patients with AML. The graft-versus-host response induced by the introduction of foreign immune cells demonstrates that LSCs are vulnerable to destruction by the immune system. However, the mechanism of allo-SCT success can also be hazardous, resulting in high rates of treatment-related mortality and morbidity. Therefore, there is a need for novel approaches to exploit the immune system while limiting toxicity to eliminate LSCs.

CD200 is a known stem cell marker. We have demonstrated that CD200 is significantly over-expressed in a subset of LSCs when compared to normal hematopoietic stem cells and when compared to their corresponding blasts. CD200 has also been implicated as a poor prognostic marker in AML. Functionally, CD200 has been shown to be immunosuppressive by inhibiting macrophage and NK cell function and also inducing regulatory T cells. Monoclonal antibodies against CD200, like samalizumab, are currently in Phase I/II clinical trials. At present, this therapy has not been translated to specifically target AML LSCs to facilitate clearance by the patient's immune system.

The **long-term goal** of this project is to understand the patient-specific immune evasion mechanisms of leukemia stem cell populations to improve personalized immunotherapies and, ultimately, eradicate AML. The **objective of this proposal** is to determine the functional role of CD200 in the context of AML LSCs and explore the potential therapeutic benefit of targeting CD200 on LSCs. I **hypothesize** that, in a subset of AML, high expression of CD200 is an LSC-specific mechanism for evading T cell mediated cell death and CD200 blockade will result in the clearance of LSCs by the immune system. To address this hypothesis, I will:

**Aim 1: Develop and apply a novel computational method to systematically characterize CD200 distribution on AML LSCs, blasts, and immune cell subsets in primary human AML samples.** Checkpoint inhibitors are now being translated to AML therapy; however, comprehensive characterization of these markers on AML and immune cell subsets has not been performed. CyTOF allows, for the first time, the simultaneous quantification of a number of known and emerging immunosuppressive proteins on AML LSCs, blasts and bone-marrow resident immune cell subsets. While there exist multiple methods for CyTOF data visualization, few allow for quantitative summaries within and comparisons between samples. *The motivation of this aim* is twofold, to (1) build an extension to the SCAFFoLD platform to automatically detect and quantify differences in cellular composition and/or immunophenotypes between samples to, ultimately, (2) characterize and compare of the distribution of CD200 and other immune modifiers, on the surface of LSCs, blasts, immune cells at the single-cell level.

**Aim 2: Determine the role of CD200 on the cytotoxic function of CD8+ T cells.** It was observed that patients with high levels of CD200 on AML blasts had significantly compromised cytotoxic T cell function when compared to those with negligible CD200. However, the functional role of CD200 on cytotoxic T cells has yet to be explored. The *motivation of this aim* is to determine the effect of CD200 on CD8+ cytotoxic T cell mediated cell death and on effector T cell function in AML. I will use a series of mixed lymphocyte reaction experiments using cells with both pharmacological and biological inhibition of CD200 to look at differences in AML cell death ($^{51}$Cr-release assay) and effector T cell functionality (cytokine flow assays).

**Aim 3: Evaluate the utility of CD200 inhibition as a mechanism for eliminating AML LSCs *in vivo*.** In the context of AML, where the disease can destroy the resident immune system, an effective immune mediated approach requires a both strategic therapy and administration. In the Tet2-/-Flt3$^{ITD}$ murine leukemia model, disease evades the immune system and is refractory to standard chemotherapy; much like the human disease. The *motivation of this aim* is to use this murine model to evaluate the potential clinical utility of treating patients with CD200+ LSCs with a CD200 antibody at remission. Efficacy of CD200 antibody therapy will be determined by time to relapse and overal survival. The LSC specific inhibition will be determined using a limiting dilution assay.

**Innovation:** The results of this proposed work will demonstrate the utility of specifically targeting LSCs with biologically relevant immunotherapy as a curative approach to AML. Moreover, the novel CyTOF methodology developed here has the potential to easily be modified to track virtually any cell population(s) for either clinical observation or scientific discovery.

## Background and Significance

**Most patients with Acute Myeloid Leukemia (AML) die from relapsed disease.** AML is a clonal malignancy where immature blast cells accumulate in the bone marrow and outcompete normal hematopoiesis resulting in systemic consequences such as anemia and increased susceptibility to infection. Frontline chemotherapy regiments result in remission in up to 80% of patients, however, most will relapse[2]. Refractory or relapsed disease is the leading cause of death in AML patients[3, 4].

**Leukemia stem cells are the likely source of relapsed disease.** Similar to normal hematopoiesis, populations of AML cells are organized as a hierarchy with the LSC residing at the apex and being the rarest (Fig. 1)[5]. Leukemia stem cells (LSCs) are functionally defined as cells capable of engrafting and recapitulating disease in sub-lethally irradiated NOD/SCID mice[6]. In AML, LSCs comprise a small fraction of the overall disease (likely <0.3%), are largely quiescent, and capable of both long-term self-renewal and production of more differentiated leukemic blasts. Besides their role in disease initiation, LSCs are also hypothesized as the likely source of relapse. Due to the quiescent nature of the LSCs, they are capable of evading the effect of the majority of chemotherapeutic agents that rely on active cell cycling for cytotoxicity[7]. AML patients with a more "stem-like" disease, as defined by gene expression profiling, have significantly worse overall survival when compared to patients with a



**Figure 1. Hierarchical structure of normal hematopoiesis and leukemogenesis[1]**

more differentiated phenotype[8]. Therefore, designing novel combination therapeutics that effectively clear bulk disease while also eradicating the residual LSCs is critical for curing AML.

**"Immunotherapy" is the only curative treatment for most AML.** Immunotherapy has seen unparalleled success using checkpoint inhibition in groups of melanoma[9] and non-small cell lung cancer[10]. However, curing cancer with the immune system was actually first described in AML over 40 years ago[11]. It was demonstrated that immune cells reconstituted from donor marrow were capable of eliminating residual leukemia cells and curing patients. Allogeneic stem cell transplant after myeloablative chemotherapy is still the best option for reducing the likelihood of relapse[12]. This suggests that LSCs can be destroyed by the immune system. Despite this knowledge, immunotherapy has not yet been optimally exploited for the treatment of AML. Popular checkpoint inhibitors are now being explored in AML patients with mixed results[13]. PD-1 inhibitors (nivolumab) have had limited success in patients with hypomethylating agents[14]. However, many of these trials are driven by drug availability rather than the biology of the disease.

**CD200 is a marker of stem cells.** CD200 is a type-1 transmembrane glycoprotein that is a member of the immunoglobulin superfamily[15]. This protein is broadly expressed on multiple cell types including myeloid, lymphoid, and epithelial, while the CD200 receptor (CD200R) expression is strictly confined to myeloid and a subset of T cells [16]. This unusual pattern of expression is highly conserved across species and has been hypothesized as a mechanism for local immune regulation and tolerance[17]. CD200 has also been characterized as a stem cell marker with expression correlating with epithelial stem cell markers in brain, breast, colon, and prostate cancers[18]. In AML patients, high CD200 expression is significantly enriched in CD34+, or stem-like, disease [19].

**CD200 is immunsuppressive.** CD200 has been shown to have an immunosuppressive effect on macrophages[20] and NK cells[21]. In patients with AML, high CD200 expression correlated with a high prevalence of FOXP3+ regulatory T cells, a known predictor of poor outcome[22, 23]. A CD200-Ig fusion protein has been shown to induce the expression the enzyme IDO from in plasmocytic dendritic cells, which further enhanced the immunosuppressive microenvironment[24].

**CD200 is clinically relevant in AML.** Having high CD200 expression on bulk AML disease reduces the odds of a complete remission by 50% and these patients have significantly worse overall survival when compared to patients with negligible CD200 expression[19]. CD200 has now been implicated to play a role in both liquid[25, 26] and solid tumors[27], thus, spurring the development of a targeted antibody. Samalizumab is a humanized monoclonal antibody against CD200 that specifically blocks the binding of the CD200 receptor. Phase I clinical
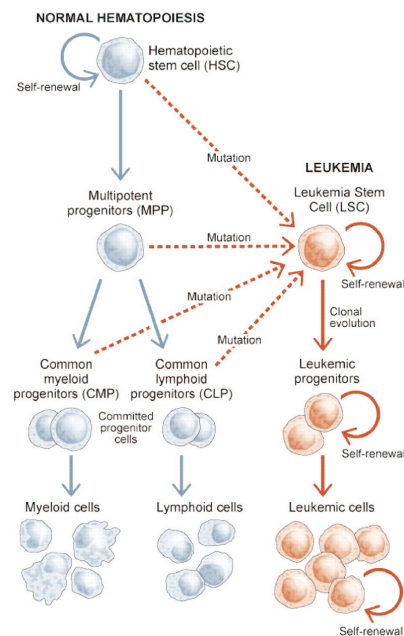
trial data suggests that CD200 antibody therapy has potent biological activity and limited toxicity[28]. However, the utility of samalizumab as a single agent against bulk disease is limited thus far.

**Gap in knowledge** While it is known that CD200 plays a role in immune suppression through a variety of mechanisms and is an established stem cell marker in multiple cell types, it has not been studied as an LSC-specific mechanism of immune evasion, particularly in AML. This proposal will be the first to characterize the immune checkpoint profile of LSCs and strategically target CD200 to aid the immune system in eliminating them.

**Innovation** The *conceptual innovation* of this grant is breaking down the AML disease into biologically relevant subunits that can be studied individually and then targeted in concert. Specifically, LSCs will be studied for their unique immune properties and strategically targeted to prevent relapse. The *technical innovation* includes the novel methodology and visualization tools that will be made publicly available as an extension to the SCAFFoLD analysis. These tools will be the first step towards making the CyTOF platform systematic and reproducible for use in both scientific discovery and clinical observation.

## Approach

### Preliminary Data

    I analyzed 3 publicly available datasets that contained microarray gene expression profiling on sorted LSCs, blasts, and normal hematopoietic subsets[29-31]. I have shown for the first time that CD200 is significantly overexpressed at the mRNA level in a subset of LSCs when compared to normal HSCs and to their more differentiated progenitors and blasts (Fig. 2). This pattern is robust and appears in multiple, independently generated datasets.

### Methods

    *1) CyTOF* Identifying and understanding the behavior of LSCs amongst bulk disease and the immune microenvironment requires a multi-parametric single-cell "microscope". Mass-cytometry, more commonly known as cytometry by time-of-flight (CyTOF), is a novel hybrid of mass spectrometry and flow cytometry that provides a high-dimensional proteomic quantification with single-cell
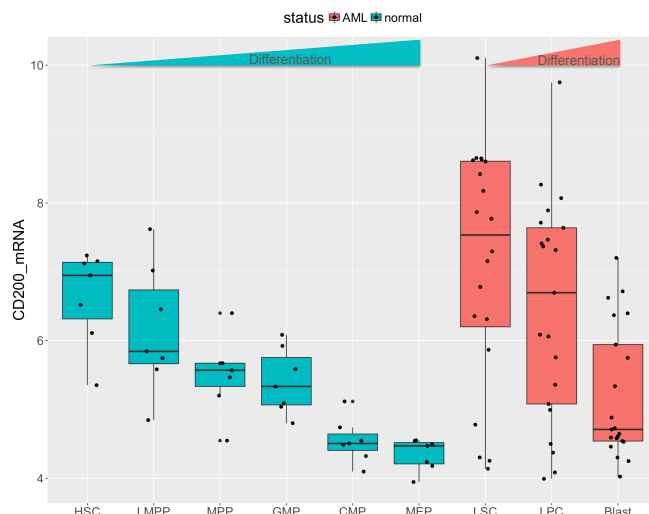
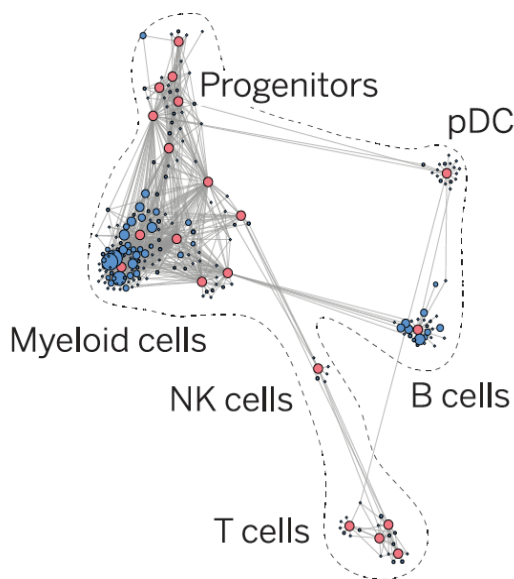**Figure 2. CD200 mRNA expression across normal and leukemic cell subsets[29]**

resolution. With CyTOF, cells are stained with antibodies conjugated to rare, heavy metal ions instead of fluorochromes and passed one by one through a mass spectrometer[32, 33]. This currently allows for the simultaneous quantification of 40 proteins; a number that will continue to grow with additional conjugated metals. CyTOF introduces a high-dimensional data structure that requires sophisticated statistical techniques for visualization and quantification.

    *2) SCAFFoLD:* One such visualization method, uses single-cell analysis by fixed force and landmark-directed maps (SCAFFoLD) and allows for the exploration of previously uncharacterized disease cell types in the context of the well-established cellular composition of normal hematopoiesis (Fig. 3)[34]. This computational method uses the immunophenotype of normal cells as a foundation reference map onto which novel cell clusters are mapped, providing a data-driven representation of the distribution of the heterogeneous AML disease. While SCAFFoLD is a powerful tool for visualizing and understanding AML on a sample-by-sample basis, it does not currently allow for an objective metric of comparison between samples[35].

**Figure 3. SCAFFoLD visualization of normal hematopoiesis with landmark nodes (red) and clustered cell data (blue).**

**Specific Aim 1: Develop and apply a novel computational method to systematically characterize CD200 distribution on AML blasts, LSCs, and immune cell subsets in primary human AML samples.**

Rationale: Checkpoint inhibition is now being translated to AML therapy. There are currently more than 10 Phase I/II clinical trials recruiting AML patients for PD-1, PD-L1, or CTLA-4 blockade as single-agent agents or in combination with traditional therapies [clinicaltrials.gov] [36, 37]. Despite the number of trials, there is limited science on the expression of immunosuppresive proteins on AML and neighboring immune cell subsets. To date, there has been only one study to comprehensively characterize well-known checkpoint ligand/receptors on AML and T cells across patients using multi-color flow cytometry[38]. Novel immune markers, like CD200, remain completely unexplored at the single cell level. The high dimensionality of CyTOF allows, for the first time, the simultaneous investigation of a large number of immune modifiers on AML blasts, LSCs and a variety of immune cell subsets. While CyTOF data analysis with SCAFFoLD is a powerful visualization tool, there are no existing methods to quantify or statistically compare data in this form. *The motivation of this aim* is twofold, to (1) build an extension to the SCAFFoLD platform that will allow for the (2) characterization and comparison of the distribution of CD200 and other immune modifiers, on the surface of LSCs, blasts, and bone marrow resident immune cell subsets at the single-cell level. *I hypothesize* a Euclidean distance scoring metric will be able to systematically identify outliers while simultaneously providing summary data for normal cell populations. I further hypothesize that the cells enriched for known stem cell markers (including CD34, CD123, or TIM-3) will have the highest CD200 expression and that these CD200 high populations will be distinct to AML.

Experimental Design: **1.1) Develop novel method for identifying unique subsets of AML and the corresponding immune microenvironment**

Within a given SCAFFoLD projection map, I will define a deviation score for each cluster of cells as the Euclidean distance, $\sqrt{(y_i - y_j)^2 + (x_i - x_j)^2}$, where $(x_j, y_j)$ is the center of a given cell cluster, $j$, in the 2-dimensional graph and $(x_i, y_i)$ are the coordinates of the nearest landmark node, $i$. The null distribution of deviation scores will be generated using resampling techniques from 5 biological replicates of normal bone marrow from different human donors. This distribution will be used to identify clusters that fall greater than 4 standard deviations ($4 \times [1.35 \times IQR]$) from the nearest landmark. I will evaluate these methods using an existing CyTOF dataset containing both normal and AML bone marrow. Test sets will be engineered by resampling from the normal sets and spiking in varying numbers of immunophenotypically abnormal AML cells.

Anticipated Results: I expect that this metric will be sensitive enough to detect clusters of cells that are distinct from normal populations, including AML blasts and LSCs, as well as T cell populations with increased activation. I also expect that this approach will provide summary values (cell abundance and individual protein intensity) for given normal cell populations that can be compared between CyTOF samples.

Potential Pitfalls and Alternative Approaches: In my CyTOF experience, the abundance of normal cells varies between samples from different individuals but the immunophenotypes of known cell subsets are highly consistent. Therefore, I do not anticipate high variability in deviation scores for normal cells. However, if I do see greater than anticipated variance, I will increase the number of normal biopsies used to establish the null deviation score distributions. A conceptual caveat of this method is that it relies on a well-designed CyTOF antibody panel. Normal cell populations that are present in the sample but not accounted for by the antibody panel will be identified as outliers by this approach.

**1.2) Characterize CD200 expression across AML LSCs, blasts, and immune cell microenvironment**

This aim will be conducted as part of a Phase Ib/II study of Avelumab (anti-PDL1) in combination with 5-azacytidine for the treatment of AML. In this study, bone marrow biopsies and peripheral blood from patients will be collected at baseline. Patient samples will be frozen and banked until 30 baseline samples are collected. Bone marrow biopsies from 5 age-matched normal donors have already been banked. All cells will be thawed and processed simultaneously to eliminate potential batch effects. The samples will be stained according to optimized lab protocols[39]. The antibody panel has been strategically designed to include markers that will distinguish AML blasts, LSCs, and immune cell subsets, along with antibodies to explore the distribution of immune regulatory proteins (Fig. 4). Samples will be

| Immuno-phenotyping | Immune modulators |
|---|---|
| CD3 | CD80 (CTLA-4L) |
| CD4 | CD86 (CTLA-4L) |
| CD8a | CD137 (41BB) |
| CD25 | CD152 (CTLA-4) |
| CD27 | CD200 |
| CD28 | CD200R1 |
| CD44 | CD223 (LAG-3) |
| FOXP3 | CD272 (BTLA) |
| HLA-DR | CD273 (PD-L2) |
| CD33 | CD274 (PD-L1) |
| CD34 | CD278 (ICOS) |
| CD38 | CD279 (PD-1) |
| CD45 | CD357 (GITR) |
| CD90 | OX40 |
| CD117 | TIGIT |
| CD123 | |
| TIM-3 | |
| CD16 | ☐ T cell |
| CD19 | ☐ AML/LSC |
| CD11b | ☐ other |
| CD33 | |
| CD56 | |
| CD41 | |

**Figure 4. CyTOF antibody panel**

processed consecutively on a Helios machine. Immunophenotypic markers will be used to manually distinguish the known myeloid and lymphoid cell subsets in the normal marrow and to construct the SCAFFoLD maps for AML patient samples. Immune modulatory proteins, including CD200 and CD200R, will be projected onto the SCAFFoLD maps. The method described in Aim 1.1 will be used to identify outlier populations and shifts in cellular composition.

Anticipated Results: I anticipate that high CD200 expression will be most prevalent in cell clusters with high known AML stem cell markers and that the intensity and/or number of CD200+ cells will be greater in approximately half of AML patient samples when compared to normal marrow. I also expect CD200R will be expressed on effector T cells and that the expression will be higher or more prevalent in T cells from patients with high levels of CD200 on LSCs.

Potential Pitfalls and Alternative Approaches: Although many published CyTOF experiments are performed on samples after undergoing at least one freeze-thaw cycle, it is still not known whether using fresh versus frozen samples has a significant effect on the distribution of any cell surface markers. While I do not expect sample storage to affect the overall results, I will confirm by running 5 pairs of fresh and freeze-thawed AML samples using the same antibody cocktail to compare between-sample variability. If the variability due to preparation outweighs that of potential batch effects, I will modify this protocol to run samples fresh on the day of collection. A conceptual limitation of this approach is the loss of spatial information in the bone marrow as aspirate cells are in suspension; meaning that some relevant populations of the bone marrow will not be observed and cells from the same aspirate may or may not be neighbors in the bone marrow architecture. An extension of the CyTOF platform is currently being optimized to allow for multi-parameter measurements while maintaining tissue structure. Profiling cells in the context of their environment would be a future direction for this study.

**Specific Aim 2: Determine the role of CD200 on the cytotoxic function of CD8+ T cells.**

Rationale:   In the normal immune response, CD200 has been proposed to play numerous roles in immunosuppression including; inhibiting mast cell degranulation[40], down-regulating macrophage activity[16, 20], and shifting the cytokine environment from Th1 to Th2[41]. In AML, CD200 expression on blasts has been shown to significantly reduce NK cell cytolytic capacity[21]. Other studies have observed that bulk CD200 expression in AML is positively correlated with abundance of regulatory T cells[23] and negatively correlated with memory CD4+ T cell function[42]. While CD8+ T cell abundance was comparable among AML patients, those with high bulk CD200 expression were associated with significantly compromised cytotoxic T cell function[42]. However, whether or not such suppression was a direct result of CD200 has yet to be addressed. The *motivation of this aim* is to determine the effect of CD200 on targeted T cell mediated cell death and on CD8+ effector cell function in AML. I *hypothesize* that CD200 expression on AML LSCs suppresses T cell dependent cytotoxicity by inhibiting the secretion of necessary cytolytic enzymes.

Experimental Design: **2.1) Determine the influence of CD200 surface expression on cytotoxic T cell killing.** Two human AML derived cell lines selected for high cell surface CD200 expression (KG1 and Kasumi1) will be transduced with a CRISPR construct to ensure homozygous knockout of CD200. Chromium release assays will be performed as previously described to determine percent lysis of target cells[43]. Briefly, wild-type (WT) and knockout (KO) cell lines will be labeled with radioactive chromium 51 ($^{51}$Cr). In parallel, 3 effector cell groups will be prepared from a single, normal PBMC sample; CD8+ cells (positively selected by magnetic beads), CD8 depleted PBMCs (negatively selected by magnetic beads), and whole PBMC. Then, target $^{51}$Cr labeled leukemia cells will be co-cultured with the effector cells at varying effector:target (E:T) ratios for 5 hours. Radioactive chromium released into the supernatant will be quantified using a gamma counter. Three biological replicates will be performed.

Anticipated Results: I expect that the cells with CD200-KO will have a higher $^{51}$Cr levels in the supernatant, indicating a higher cell lysis when compared to their WT counterpart and that the differential chromium release will only be significantly different in effector cell populations containing CD8+ T cells.

Potential Pitfalls and Alternative Approaches: One limitation of this approach is the assumption that CD200R is expressed at similar intensity on normal and AML derived CD8+ T cells. To address this potential caveat, I could isolate non-leukemic PBMCs from AML patients with high CD200 expression for the $^{51}$Cr assay. A second limitation is that CD200 expression may not be exclusive to LSCs, so this assay will more generally determine the effect of CD200 on the surrounding T cells.

**2.2) Determine whether CD200 antibody blockade is sufficient for T cell mediated cytotoxicity.** Two human CD200+ AML cell lines (KG1 and Kasumi1) and 2 CD200+ sorted AML patient samples will be treated with the CD200 blocking antibody, samalizumab, at 1 of 4 escalating doses or with an IgG2 control for 5 hours.

As in Aim 2.1, cells will then be incubated with $^{51}$Cr and co-cultured with the effector cell populations at varying E:T ratios. Killing will be determined by the relative radioactive chromium release into the supernatant. Biological replicates will be performed 3 times.

Anticipated Results: I expect to see significantly increased target cell death in both AML cell lines and patient samples treated with the CD200 antibody in a dose dependent manner. Again, I anticipate this differential killing to occur only in effector cell groups containing CD8+ T cells.

Potential Pitfalls and Alternative Approaches: In addition to the limitation addressed in Aim 2.1, a potential caveat of this aim is the assumption that CD200 is an exclusive checkpoint ligand on these LSCs. There is correlative evidence to suggest that patients with high CD200 expression also have high PD-L1 levels[44]. This could be addressed using CyTOF to look at co-expression of checkpoint ligands at the single cell level to identify patient specific combination blockade therapy.

**2.3) Test whether CD200 has a functional effect on the cytokine production of effector CD8+ T cells from AML patient samples.** Bone marrow T cells from AML patients will be isolated using magnetic beads and stimulated using plate-bound CD3 antibody. Activated CD8+ T cells will be treated with a protein transport inhibitor, brefeldin A, to ensure intracellular cytokine accumulation and then co-cultured with an equal number of KG1 cells (as a source of CD200 ligand) with and without a CD200 blocking antibody. Controls will include activated T cells co-cultured with KG1 CD200-KO cells both with and without the CD200 blocking antibody. Cells will be washed, fixed in paraformaldehyde, and stained for the cell surface antibodies, CD3, CD8, and CD200R. Cells will then be permeabilized to allow for intracellular staining of the cytolytic markers, IFN-$\gamma$, granzyme B, and TNF-$\alpha$, and measured by flow cytometry[45].

Anticipated Results: I expect that effector cells unable to bind CD200 will have increased production of the cytolytic enzymes, indicating an enhanced effector response when compared to cells capable of binding CD200.

Potential Pitfalls and Alternative Approaches: Using a cell line with a corresponding knockout as a control, should allow for the investigation of the specific effect of CD200. However, there is a possibility that there are other molecules on KG1 cells that have a stronger inhibitory effect that could mask that of CD200. If changes in cytokine production are minimal, I will perform the same experiment using a soluble CD200-Ig protein to eliminate confounding[24].

**Specific Aim 3: Evaluate the utility of CD200 inhibition as a mechanism for eliminating AML LSCs *in vivo.***

Rationale: Immunotherapies that rely on an intact immune system are particularly challenging in the context of AML, where disease progression is directly related to a decrease in normal immune cell production. In this case, strategic timing of therapy may be as critical as the therapy itself. The optimal time to target LSCs expressing CD200 would likely be after the patient is in remission following myeloablative chemotherapy; when leukemia burden is low, enrichment of LSCs is high, and the immune system is attempting to rebuild. To model this *in vivo*, I will use the Tet2-/-Flt3$^{ITD}$ murine model[46] that provides the opportunity to study AML in the context of an intact immune system and is refractory to chemotherapy, consistent with most human disease. Mouse CD200 gene expression patterns are similar to that in human with the highest expression in mouse LSCs (LSKs) when compared to progenitors and higher in AML primed mouse cells (Tet2-/-Flt3$^{ITD}$) when compared to wild type (Fig. 5). CD200/CD200R expression is highly conserved between mouse and human[17, 47]. The *motivation of this aim* is to use this murine model to evaluate the potential clinical utility of treating patients with CD200+ LSCs with a CD200 antibody. I *hypothesize* that specifically blocking CD200 in remission will strip LSCs of their immune privilege and make them vulnerable to T cell mediated cytotoxicity.



**Figure 5. CD200 mRNA expression in WT and Tet2-/- and Flt3$^{ITD}$ mice**

Experimental Design: **3.1) Test CD200 inhibition as a mechanism for eliminating residual leukemia burden.** A cohort of 30 recipient WT C57Bl/6 CD45.1 mice will be injected IV with $1\times10^6$ Tet2-/- Flt3$^{ITD}$ CD45.2 AML stem cells and followed until the average disease burden is approximately 20% in the peripheral blood. At this point (day 0), low dose cytarabine treatment (100mg/kg/day) will be administered for 7 days by
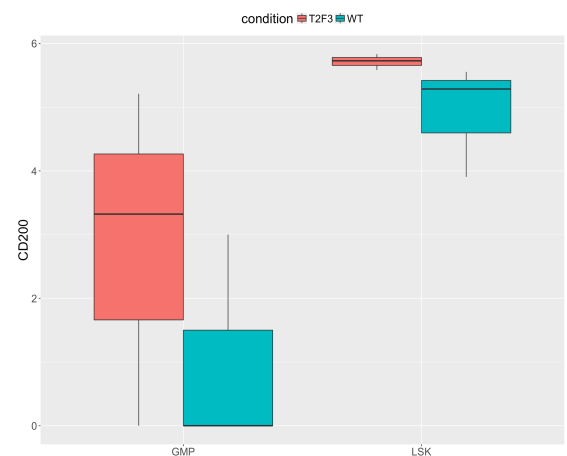
intraperitoneal injection in combination with doxorubicin treatment for 3 days(3/mg/kg/day)[46, 48]. After one week of recovery, mice will randomly assigned to 3 groups and refractory disease burden will be determined by peripheral blood measurements. Each group will receive either high dose anti-mouse CD200 IgG2 blocking antibody, low dose CD200 antibody, or an IgG control that will be administered daily by intravenous injection for 10 days (Fig. 6). The effects of CD200 antibody therapy on LSC clearance will be quantified every 7 days using peripheral blood measurements of CD45.2 positivity, percent WBC, time to relapse, and overall survival.
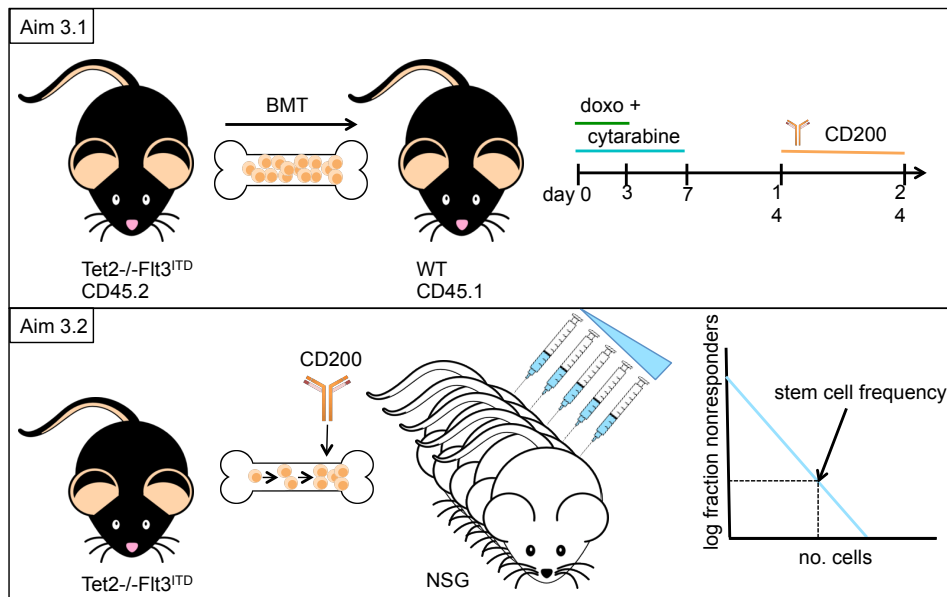


Figure 6. Aim 3.*1 In* vivo experiment strategy. Aim 3.2 Limiting dilution assay schema

Anticipated Results: I expect that mice treated the CD200 antibody will have reduced CD45.2 positivity, reduced WBC count, increased time to relapse, and improved overall survival in a dose dependent manner.

Potential Pitfalls and Alternative Approaches: Previous studies have suggested that doxorubicin and cytarabine treatment fail to significantly inhibit the function of the normal immune cells responsible for adequate antibody or cell-mediated cytotoxicity. In fact, there is even evidence to indicate that these chemotherapies can cooperate to elicit adaptive and innate immune response[49-51]. Therefore, I do not expect the pretreatment with chemotherapy to affect the function of the CD200 antibody. However, I would be able to confirm the presence and activity of major immune cell subsets in the bone marrow microenvironment using the longitudinal CyTOF measurements. If necessary, I will adapt the dose or chemotherapeutic agent to ensure the presence of necessary cells for antibody therapy. Additionally, the IgG2 antibody was selected because it is the least likely member of the immunoglobulin family to activate antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC)[52]. While I expect that the majority of CD200 cell destruction to be attributable to T cell toxicity, there may still be off-target antibody effects on normal CD200 expressing cells that could result in toxicity. To address this caveat, I could procure an antibody with a chimeric IgG2/IgG4 constant region that is incapable of inducing CDC or ADCC, thereby reducing toxic side effects of the therapy[25].

**3.2) Determine reduction in AML LSCs due to CD200 therapy** To evaluate whether CD200 therapy specifically recognizes and eliminates CD200+ LSCs, I will perform serial limiting dilution competitive transplantation assays *in vivo* to quantitatively compare the abundance of LSCs treated with a blocking CD200 antibody versus an IgG control[53]. Nine Tet2-/-Flt3$^{ITD}$ mice with approximately 50% blasts in the peripheral blood will be randomly assigned to 1 of 3 treatment groups; high dose CD200, low dose CD200, or an IgG control. After 9 days of treatment, the mice will be sacrificed and leukemic blasts will be harvested. Four dilutions of pooled cells will each be injected into 5 sub-lethally irradiated NSG secondary recipients (5 mice/dilution x 4 dilutions x 3 treatment groups). After 12 weeks, mice will be sacrificed and LSC frequency will be calculated based on engraftment in the bone marrow (Fig. 6)[54].

Anticipated Results: Because CD200 is enriched in LSC cells, I anticipate that the number of leukemia stem cells will be significantly reduced after CD200 antibody therapy in a dose dependent manner. Ideally, mice treated with a high CD200 dose would have complete ablation of LSCs and no engraftment ability in secondary recipients.

Potential Pitfalls and Alternative Approaches: One potential limitation of this assay is that relies on engraftment for LSC quantification. While CD200 is implicated as a stem cell marker, its effect on LSC function (or engraftment potential) is unknown. Therefore, CD200+ LSC elimination *in vivo* may be confounded by a possible role of CD200 in engraftment potential. Elucidating the role of CD200 in AML stem cell function is a future direction of this study.

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