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Optimizing Dendritic Cell-Tumor Fusion Vaccine for Acute Myeloid Leukemia

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Abstract

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Conventional treatment for acute myeloid leukemia (AML) is with non-cancer selective chemotherapy or allogenic bone marrow transplantations. Developing a treatment option to specifically target AML without damaging healthy cells is a promising strategy for improving patient prognosis. A common approach within immunotherapy is cancer vaccine synthesis which utilizes the individual's own antigen-presenting cells to enhance anti-tumor T cell activity.

Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system. The DC-AML fusion heterokaryon entity exposes DCs to tumor-associated antigens that are expressed on the tumor cells, allowing DCs to endogenously process and present tumor-associated antigens via major histocompatibility complex (MHC) classes, which can then elicit T cell responses.

The organization of this study included four parts: harvesting bone marrow from FLT3L injected mice, harvesting bone-marrow derived dendritic cells, fusing dendritic cells with murine AML cell line, C1498, and testing the efficacy of the fused vaccine in a mixed lymphocyte reaction (MLR) assay. Previous studies in our lab has shown that FMS-like tyrosine kinase 3 ligand (FLT3L) injections in mice bone marrow donors increased the content of pDCs in their graft. We showed that mouse receiving FLT3L injections had increased number of nucleated cells in the bone marrow. We also showed that FLT3L had a proliferative effect on dendritic cell expansion in vitro when added to adherent and nonadherent bone marrow cultures, but this increase in dendritic cells was not observed when only adherent cells were cultured with FLT3L. In addition, dendritic cells in FLT3L-supplemented cultures had increased CD80 and B220 expression levels, but decreased CD11c expression, which is consistent with pDC phenotype.

Results from the fusion percentages fusion of vaccine reveal that an extra two-time serum-free RPMI wash prior to fusion with polyethylene glycol improves fusion percentage. Upon testing for the efficacy of the vaccine in MLR assays, we observed that T cells primed with irradiated C1498 had enhanced cytotoxic activities compared to non-specific T cells.

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Introduction

Overview of Acute Myeloid Leukemia

Leukemias are a group of blood cancers in which mutations bypass regulatory signals during the differentiation of blood hematopoietic cells, leading to increased proliferation and accumulation of a clonal population of leukocytes. Leukemias are often classified by their rate of growth, and the cell of origin that was mutated during hematopoietic differentiation of either myeloid progenitor cells or lymphoid progenitor cells (1). Acute myeloid leukemia (AML), is the most common type of acute leukemia in adults, primarily amongst the elderly (2). AML is distinguished by its fast-growing cancerous development originating from mutations occurring from myeloid progenitor cells within the bone marrow. Upon abnormal differentiation, the mutated blasts accumulate and primarily infiltrate hematopoietic tissues including bone marrow and blood (3).

Since AML develops in the bone marrow with broad dissemination within the blood circulatory system, treatment is different from those applicable to solid malignancies that may be amenable to localized treatment with surgery or localized radiation (4). Treatment for AML in young patients is usually based upon intensive non-cancer-selective chemotherapy and allogeneic hematopoietic stem cell transplantation. However, not all AML patients are eligible for an allogeneic transplant given their co-morbidities and age. In addition, allogeneic transplantation may result in donor-graft-derived auto-immune disease in patient, termed graft-versus-host-disease (GvHD) (4). The pharmacological therapies to prevent and treat GvHD in addition to therapies directed towards treating leukemia leads to increased treatment-related mortality among AML patients who undergo allogeneic transplantation. The 5-year overall

survival (OS) for AML rate is 27% (5). For the elderly population (age >65), the prognosis is shorter with a median OS of 9 months (6).

Personalized Vaccine

Developing a new targeted AML-specific treatment option for elderly AML patients could improve these patient's cancer prognosis and outcome. A recent therapeutic approach involves the manufacture of a personalized cancer vaccine, whereby a patient's AML cells are fused with their autologous dendritic cells to trigger cytotoxic T cell activity directed against the patient's own AML(7). Results of the clinical trial that enrolled 17 transplant-ineligible patients who received vaccination of an autologous AML-DC vaccine during post-induction remission reported an increase of AML-specific CD4+ and CD8+ T cells. The 4-year progression free survival rate of vaccinated patients was 71% while the median PFS and overall survival were not reached (Figure 1, top) (8). Outcomes for these patients were compared to historical comparator groups of 309 acute leukemia patients randomized to receive either high dose cytarabine (HiDAC) chemotherapy or multiagent chemotherapy without vaccination (Figure 1, bottom) (9). The median overall survival among patients treated with chemotherapy without post-remission vaccination was 2.8 years (9). While these data do not represent the "gold-standard" of a randomized clinical trial, they support the hypothesis that post-remission vaccination with AML-DC fusion vaccines confers a survival benefit to AML patients compared to traditional consolidation chemotherapy.

The fusion of DCs and tumor cells produces a heterokaryon entity that expresses marker characteristics of DCs as well as those antigens associated with the tumor (10). The DC-AML fusion exposes DCs to tumor-associated antigens that are expressed on the tumor cells, allowing DCs to endogenously process and present tumor-associated antigens via major histocompatibility

complex (MHC) classes (10). Through the presentation of MHC, antitumor immune responses of CD4+ and CD8+ T cells are activated. The optimal ratio of dendritic cell to tumor cells varies amongst different DC-tumor fusion vaccines. The addition of polyethylene glycol (PEG) to a mixture of DC and autologous cancer cells is critical to the successful fusion of dendritic and tumor cells by integrating the cellular membranes of the two cell types (10). PEG dehydrates the lipid plasma membranes to force them close together into a rigid state (11). Additionally, subsequent short-term culture of fused cells can improve fusion percentage, but longer-term cultures lead to over-growth of unfused tumor cells (10).

Dendritic Cell Maturation Overview

Dendritic cells are the most potent antigen-presenting cells for triggering humoral immune response (8). Fusing dendritic cells with tumor cells exposes tumor antigens to dendritic cells, enhancing tumor-specific activity (10). To induce the development of dendritic cells, a cocktail of GM-CSF, IL-4 and TNF-α is added to freshly harvested bone marrow cultures (12). Given their short lifespan, dendritic cells develop continuously from bone marrow progenitor cells under the regulation of cytokines, especially granulocyte-macrophage colony-stimulating factor (GM-CSF) (13). GM-CSF promotes maturation of bone marrow progenitor cells into three main subsets of dendritic cells, migratory DCs, lymphoid-resident DCs, and plasmacytoid DCs. Co-culturing GM-CSF with interleukin-4 increases the expression of mature dendritic cell markers including CD80 and CD86 (12) (Figure 3). Adding IL4, an additional cytokine, to the bone marrow cultures helps prevent differentiation of monocyte progenitors into macrophages while increasing the production of dendritic cells since granulocytes, macrophages, and DC have common progenitors (14).Adding IL-4 to developing dendritic cells also triggered autocrine production of IL-4 in DC during dendritic cell maturation. The optimum concentration

of IL-4 for culture is 10 ng/mL to 15 ng/mL (Figure 3) (12). Additionally, the synergistic effects of tumor necrosis factor-alpha (TNF-α) and Interleukin-4 (IL-4) cytokines co-stimulates the M-O7e cytokine dependent human myeloid cell line to by secret granulocyte-macrophage colony-stimulating factor (GM-CSF), which induces anti-tumor activity (13).

Effect of FLT3L on Dendritic Cells

A primary way to improve efficiency in dendritic cell-tumor fusion vaccine manufacturing is to increase the number of dendritic cells available for fusion, especially important given the high ratio (3:1) of dendritic cells to tumor cells during fusion. FMS-like tyrosine kinase 3 ligand (FLT3L) promotes plasmacytoid dendritic cells (pDCs) differentiation and proliferation from myeloid and lymphoid lineage progenitor cells *in vitro* (15). Usually, more conventional DCs (cDCs) are produced in bone marrow than pDCs (15). Plasmacytoid dendritic cells may be particularly attractive as the basis for creating cancer vaccines. In response to foreign nucleic acids, pDCs augment a rapid immune response by secreting large amounts of type I interferon (IFN-α/β) (16). While pDC are critical for the initiation of immune responses they have lower antigen-presenting capacity than cDC due to lower expression of MHC-II (16).

The limited antigen presentation capacity of pDC are mitigated by their capacity to differentiate into cDCs (15). Due to their plasticity and potent type 1 IFN production, pDC development and function is an area of high interest. Our lab has published that FLT3L treatment of murine bone marrow donors increased the survival and decreased GvHD severity in allogeneic BMT recipients while maintaining GvL effect (17). Murine bone marrow donors models that received FLT3L injections had increased numbers of pDC (17). Recipients of an allo-BMT transplant in which donors were FLT3L-treated had increased survival and decreased

GvHD when compared to outcomes of mice transplanted with cells from PBS-treated donors (17). Since FLT3L treatments produced a protective effect against GvHD by increasing pDC content in donor graft, culturing dendritic cells from FLT3L *in vivo* treated murine bone marrow may result in a graft with the optimal balance between GvL and GvHD, due in part to increased numbers of donor pDCs in the allograft. Therefore, we wanted to test dendritic-tumor cell fusion vaccine from dendritic cells generated from the bone marrow of mice pre-conditioned with FLT3L injections.

Effect of VIP Antagonist on Dendritic Cells

Vasoactive intestinal peptide (VIP) is a neuropeptide secreted primarily by the gastrointestinal tract and neurons but is also shown to be secreted by cells in the myeloid and lymphoid lineages during inflammation (18). Bone-marrow-derived DC cultured with VIP have been shown to be effective in treating auto-immune diseases and sepsis, since the VIP-treated BM progenitors differentiate into tolerogenic DCs that trigger T cell anergy and regulatory T cells (19). While VIP induces immunosuppressive dendritic cells, the VIP antagonists VIPhyb and ANT08 may elicit stronger immune responses to AML.

VIP binds to VPAC1, VPAC2, and PAC1 receptors where VPAC1 and VPAC2 receptors are found on T-cells and dendritic cells (20). The secretion of VIP by lymphocytes and non-lymphoid cells leads to enhanced immune suppression by T regulatory cells, decreased T cell proliferation and decreased IL-2 production. The combined effect may be sufficient to eliminate GvHD (21). Dendritic cells cultured with VIP express low levels of CD11c, CD40, CD80, and CD86. Furthermore, treatment of allogenic bone marrow recipients with VIP cultured DCs dampened the effects of GVHD, via downregulation of T cells (22).

Our lab has previously shown that blockade of VIP via an antagonist (VIPhyb) enhances immune responses in cytomegalovirus (mCMV) and induced CD8 T cell-dependent anti-cancer immune responses to acute leukemia in mice (18), (23). Following mCMV infection, the blockade of VIP receptor-signaling, evaluated using VIP-KO mice and VIPhyb injections, increased expression of costimulatory molecules (CD80/CD86) and IFN expression while inhibiting PD-L1 expression, a co-inhibitory molecule of DC (24). However, the role of VIP and VIPhyb in DC development remains unclear. Therefore, we wanted to explore the use of VIP antagonists (VIPhyb or ANT08) added to DC cultures as a means to optimize the biological activity of dendritic-tumor fusion cells.

Hypotheses tested

The potency of dendritic cells can be enhanced by *in vitro* and *in vivo* cytokine and peptide treatments. We hypothesized that FLT3L injection into mice will increase the content of dendritic cells and dendritic cell precursors in the bone marrow. Subsequent culture of these bone marrow cells would result in increased numbers of dendritic cells with pDC characteristics. We also hypothesized that dendritic cells cultured with the combination of FLT3L and VIPhyb would be more potent when used to make DC-leukemia fusion vaccines than conventional dendritic cells such that T cells stimulated by these vaccines will have enhanced leukemia-specific killing compared to T cells stimulated with conventional DC-AML fusion vaccines in which the dendritic cells were cultured without VIP antagonists. Since the efficacy of the dendritic-tumor fusion cell vaccine depends upon co-expression of the MHC and co-stimulatory molecules of antigen-presenting cells along with the respective tumor-associated antigens on leukemia cells, we also hypothesized that DC-C1498 fusion vaccine would stimulate

proliferation of tumor antigen specific T cells with greater C1498-specific cytotoxicity than T cells primed only with irradiated C1498.

Experimental Approach

1. Mice

C57BL/6 Ptprc^{a} Pepc^{b}/BoyJ (CD45.1 transgenic) 4-5-week-old female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6j CD45.2 and B6 Luciferase (CD45.1) mice were bred at Emory. Ethical guidelines according to Emory University's Institutional Animal Care and Use Committee were followed. Animal care guidelines from the National Institutes of Health were followed.

2. FLT3L Induced Murine Bone Marrow Harvest

7.5 ug/25g(mice weight) of human recombinant FLT-3L (CDX301), generously donated by CellDex Therapeutics (Hampton, NJ), was diluted with 1X PBS were injected into the subcutaneous dorsal region of the mouse on Day -4 (Table 1). A second injection was administered on Day -1. On Day 0, mice were euthanized in CO_2 chamber, and tibias and femur bones were collected. and flushed with 25G needle with PBS to extract bone marrow cells. Cell solution was homogenized with 18G needle and filtered through 70 µm cell strainer. Cell suspensions were lysed with ACK Lysis Buffer for 10 minutes, centrifuged at 1500 rpm for 10 minutes at 37 C, and counted using cell counter (Beckman Coulter, Indianapolis, Indiana). Cells were resuspended and plated with complete RPMI 1640 media supplemented with 10% FBS, 100 U/mL of penicillin, 100µg/mL of streptomycin, 2-mercaptoethanol, nonessential amino acids, sodium pyruvate, and glutamine.

Table 1. Timeline of manufacturing cell-fusion vaccine from adherent bone marrow cells	
Event	**Day**
FLT-3L second injection	Day -4
FLT-3L second injection	Day -1
Bone marrow harvest from mice plated with cytokine-free media	Day 0
Non-adherent cells removed and media replaced with RPMI media containing GM-CSF and IL-4 cytokines	Day 1
Transfer cultured cells into G-rex plates	Day 5
Add TNFα to mature dendritic cell cultures	Day 7
Harvest dendritic cells, fuse dendritic cells with C1498 AML cell line, and plate fused cells to allow membranes to recover from PEG	Day 10
Harvest and freeze AML-DC fusion vaccine	Day 13

3. Cytokine-induced Dendritic Cell Culture Following Bone Marrow Harvest

Preliminary method: Lysed adherent and nonadherent bone marrow cells were cultured with complete RPMI media containing 40ng/mL GM-CSF, 5ng/mL IL-4, and 25 ng/mL TNFα cytokines in 6-well polystyrene plates or silicone plated G-rex plates. Cells plated in 6-well polystyrene plates were at concentration of 2.5 million cells/mL while cells plated in G-rex plated were plated at concentration of 1 million or 2 million cells/ml. Media was changed every other day. Adherent cells were scraped off the plate on Day 10 of culture for fusion. In some groups, 200ng/mL human recombinant FLT3L was added beginning on Day 0. FLT3L injected murine bone marrow were plated at either 1 million or 2 million cells/mL in

complete RPMI media. The total volume in each of the wells in the 24 well polystyrene plates was 2.5mL and the total volume of 24 Grex plates was 6 mL. Peptide/cytokine treatments included adding 3 uM of Viphyb or Ant8 and FLT3L at 200ng/mL. Each treatment group had two samples or two repeats. To prepare for flow cytometry characterization, antibodies used for immunostaining were CD86 FITC, I^{AB} Pe, CD80 Pe-CF594, CD45.1 PerCP-Cy5.5, B220 PeCy7, CD45.2 APC, CD11C APC R700, CD3 APC-cy7 , and AquaFluor Live Dead staining. Antibodies were purchased from BD Biosciences (San Jose, CA).

Current method: Lysed bone marrow were cultured in cytokine-free complete RPMI media for 18 hours. Non-adherent cells were removed by gently washing the plates with PBS and adherent bone marrow cells were cultured with 5ng/mL murine recombinant interleukin-4 (IL-4) and 40ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) purchased from Prospec. Regular cell culture maintenance included replacement of all cytokine augmented RPMI media every day. Cell suspension was collected and centrifuged. Old media was discarded, and cell pellet resuspended with cytokine augmented RPMI media. Adherent and nonadherent cells were transferred to G-rex plates purchased from Wilson Wolf (Saint Paul, Minnesota) on Day 5. Adherent cells were scraped with cell scraped after the addition of 10% FBS PBS. On Day 7, 25 ng/mL of cytokine tumor necrosis factor alpha (TNFα) were administered daily until DC harvest. Dendritic cells were harvested from G-rex plates on Day 10.

4. Dendritic Cell-Tumor Vaccine Fusion with C1498

Preliminary method: Warmed PEG added directly to cell pellet before serum-free RPMI wash.

Current method: Appropriate amounts of dendritic cells and tumor cells following fusion ratio of 3:1 (dendritic cells: tumor cells) collected and washed two times with warm serum-free RPMI media. Warmed polyethylene glycol (PEG) was added in a dropwise manner for every 50,000,000 cells in solution. To wash out PEG, 50 mL warmed serum-free RPMI media was added in a dropwise manner and the cell suspension diluted with serum-free RPMI media centrifuged at 1000rpm for 10 minutes. The RPMI after centrifugation was collected and the cell pellet was washed again with 50 mL warm RPMI media. The RPMI supernatant and fresh RPMI were centrifuged again. Fused vaccines were plated at 1 x 10^6 cells/mL in polystyrene plates with 20ng/mL GMCSF for 3 days for cell membrane integrity to recover then fused vaccine were harvested on Day 13 (Table 1). Adherent fused portions of the fused vaccine were harvested via scraping with cell scraper.

5. Immunostaining and Flow Cytometry Characterization of Fused Vaccine

C1498 tumor control, bone marrow control, DC control, mixed C1498 tumor and DC control, fused vaccine, and recovered fused vaccine were characterized by flow cytometry. LIVE/DEAD Fixable Aqua Dead Cell Stain were added to each sample at concentration of 1 µL/1000 µL PBS per sample and incubated for 10 minutes and washed 2 times. Each sample were then incubated with 1 µL of anti-mouse Fc CD16/CD32 blocking antibody per 100 µL staining media for 15 minutes for the binding of Fc receptor to eliminate false positive binding and ensure antigen specific binding . 1 µL of each respective antibody was added to each sample. Antibodies used: CD45.1 PerCP-Cy5.5, CD45.2 APC, CD11c APC R700, B220 PeCy7, I^{AB} Pe-CF594, CD11B Pe, CD80 FITC, and CD3 APC-cy7. Antibodies were

purchased from BD Biosciences (San Jose, CA). Flow cytometry characterization was done by the FACSAria cell sorter (BD Biosciences, San Jose, CA) with a 70 μM nozzle. At least 10,000 events were captured. Flow data were later analyzed and gated by using FlowJo software (Tree Star, Inc.)

6. T Cell Isolation

Splenocytes were collected from C57BL/6j CD45.2 and B6 Luciferase (CD45.1) male or female 4-5 weeks old mice. Spleens were collected at necropsy and flushed with PBS. Cell suspensions were lysed with ACK Lysis Buffer for 10 minutes. Each sample was then incubated with 0.5 μL of anti-mouse Fc blocking antibody per 10 million cells and incubated for 10 minutes. To enrich T cells by negative selection, biotinylated anti-mouse B220, CD49, TER119, and CD11b antibodies (BD Biosciences, San Jose, CA) were added to each sample and incubated for 15 minutes. Antibodies were washed 2 times with PBS and resuspended with MACs Buffer (MAC Miltenyi Biotec, Gladbach, Germany) at a concentration of 70 μL/10 million cells. Magnetic anti-biotin beads were added to the resuspension at concentration of 20μL/10 million cells, incubated for 15 minutes, and washed 1 time with MACs buffer. T cells were then isolated via negative selection through the MACS LS columns (MAC Miltenyi Biotec, Gladbach, Germany),

7. MLR T cell Killing and Proliferation Assays

Proliferation Assay: 100,000 Luciferase-T cells (Luc T cells) were plated in complete RPMI media in clear-bottomed black-plastic 96 well plates in 200 μL of media with 30 units/mL of

IL-2. Plates were coated with anti-CD3e by adding 50 μL of mouse anti-CD3e at a concentration of 1μg/mL per well wrapping plates in parafilm and incubating at 37C for 2 hours. After two hours, 200 μL of PBS were added to each well and plates were centrifuged at 1500 rpm for 5 minutes. Plate was inverted, dried, and washed with PBS again. C1498 cells were irradiated to 30 Gy with 225 Kv X-rays and 17.7 mA for 18 minutes and 41 seconds by Rad Source machine (RS-2000-PRO-225 Biological System). 200,000 irradiated C1498 cells were plated with Luc T cells for antigen priming for 5 days in complete RPMI media (Table 2). Bioluminescent imaging of plates was performed using the IVIS Spectrum imaging system on Day 5 following addition of 0.15 mg/mL D-luciferin to each well. After 1 minute, bioluminescence was measured for 3 minutes (PerkinElmer, Walham, MA). Plating setup is depicted in Figure (5A).

Killing Assay: Similar plating methods as proliferation assay were used, with 100,000 T cells plated in complete RPMI media in clear-bottomed 96 well plates in 200 uL of media. 30 units/mL of IL-2 was added for T cells receiving IL-2 treatment. The bottom of the well was coated with anti-CD3e antibodies by adding 50 μL of mouse anti-CD3e at a concentration of 1μg/mL per well and washed as above. T cells were plated for 5 days. 100,000 of LucC1498 cells as cytotoxicity targets were added to wells containing T cells after 5 days of culture. Plates were imaged with IVIS Spectrum 24 hours later on Day 6 (Table 2). Plating setup is depicted in Figure (5B).

Table 2. Timeline of mixed lymphocyte reaction T cell proliferation and killing assay

Event	**Day**
Isolate T cells from spleens and plate T cells to test proliferation and killing	Day 0
Change media	Day 3
Bioluminescence imaging of T cell proliferation plate. Challenge T cell killing assay plate with LucC1498	Day 5
Bioluminescence imaging of T cell killing assay plate	Day 6

Results

FLT3L Injection to Mice Increased the Numbers of Nucleated Cells in Bone Marrow

To determine the effectiveness of pre-treating mice with FLT3L *in vivo* prior to harvesting bone marrow, the amount of bone marrow cells collected from a mouse that received FLT3L injection was compared to an untreated mouse. (total n =2) We chose the FLT3L injection schedule based on previous work done in our laboratory, which showed that injections performed four days and one day before harvest significantly increased the number of plasmacytoid dendritic cells (pDCs) while other cells, T cells, B cells, NK cells, and hematopoietic stem cells (HSC) in the marrow did not undergo significant changes in quantity (17). Injections were given on Day -4 and Day -1 (Table 1).

7.5 ug of FLT3L were injected into C57BL/6 $Ptprc^{a}$ $Pepc^{b}$/BoyJ mice subcutaneously near shoulder region in 100 µL PBS-diluted doses. Whole bone marrow cells were collected from mice on Day 0 and lysed. Marrow was collected from the tibias and femurs of the mice. The amount of bone marrow cells per mouse long bone was obtained by dividing the total

number of bone marrow collected by the number of long bones in one mouse, four. From the mouse that received FLT3L injection, 20 million bone marrow cells per long bone were collected while 12.5 million bone marrow cells per long bone were collected from mouse without injection (Figure 6).

Compared to the control mouse, FLT3L-treated mouse had increased numbers of nucleated cells in its bone marrow. Therefore, we used a schedule of two FLT3L injections prior to harvesting mouse bone marrow for dendritic cell cultures. After determining that FLT3L increased number of bone marrow cells *in vivo,* we then tested whether FLT3L had a similar effect in increasing dendritic cell proliferation *in vitro*.

FLT3L Increased Number of Dendritic Cells Cultured from Non-Adherent and Adherent Bone Marrow Expansion *In Vitro*

To determine whether FLT3L increased the proliferation of dendritic cells in culture, we compared cell numbers of DC from cultures of bone marrow with and without the addition of 200ng/mL of FLT3L. Lysed bone marrow cells were cultured in 24 well polystyrene plates and 24 well G-rex plates.

We hypothesized that FLT3L added to DC cultures would increase dendritic cell expansion turnover *in vitro,* similar to the results from *in vivo* injections. We found that the addition of FLT3L to bone marrow cultures increased overall dendritic cell numbers (Figure 7A, 7B). Cultures receiving FLT3L treatment had higher average number of dendritic cells per mL compared to treatment groups that did not (Figure 7A). Media in the 24 well plates was also observed to be more yellow than cultures in 24 G-rex cultures. Therefore, we used silicone-

coated G-rex culturing plates to enhance gas exchange. Media was changed by only removing media and without disturbing cells near the bottom of the plate.

While G-rex plating did not show an increase in dendritic cell numbers when compared to 24 well polystyrene plating (Figure 7B), G-rex cultures facilitated better gas exchange, stabilizing the pH of the media, leading to better culture conditions. Therefore, we incorporated G-rex plating into the dendritic cell expansion process. However, inducing dendritic cell development directly from bone marrow cells in G-rex plates does not provide a method of selecting only dendritic cells due to the absence of adherence of dendritic cells onto the silicone membrane at the bottom of each well. We next wanted to develop a way to culture only adherent bone marrow cells to enrich the generation of cultured dendritic cells.

FLT3L Increased B220 and CD80 and Decreased CD11c Markers on Mouse DC in Culture Consistent with pDC Differentiation

To determine whether the addition of FLT3L, VIPhyb, and Ant08 altered dendritic cell phenotype, dendritic cells cultured from G-rex plates were characterized by immunostaining and flow cytometry. Dendritic cells with FLT3L and/or peptides added to cultures were compared against the negative control, C1498 tumor cells as these are CD45.2 positive and CD45.1 negative allowing them to be easily distinguished using flow cytometry. All cultured dendritic cells were CD45.1, CD86, and CD80 positive (Figure 8C). CD45.1 expression was noted, as expected, in bone marrow isolated from transgenic CD45.1 pep-boy mice.

The expression of CD86 marker did not change under the various culture conditions (Figure 8C). Dendritic cells cultured with FLT3L decreased CD11c expression and increased CD80 and B220 expression compared to dendritic cells cultured without FLT3L, consistent with pDC differentiation (Figure 8D). In contrast, the effects of adding VIPhyb to dendritic cell

cultures included decreased CD80, CD11c, and B220 marker expression, consistent with decreased numbers of pDC. The effects of adding the combination of VIPhyb and FLT3L led to increased levels of CD80, CD11c, and B220 expression, mitigating the inhibitory effects of VIPhyb (Figure 8D). Another peptide similar to VIPhyb, Ant8, which contains only one amino-acid difference from VIPhyb, increased CD80 and CD11c expression to a greater extent than VIPhyb (Figure 8D).

Culturing Adherent Bone Marrow Cells Yield Higher Numbers of Dendritic Cells than Culturing Nonadherent and Adherent Cells

To continue optimizing dendritic cell expansion culture conditions, we tried a new culture method. Since previous methods of dendritic cell cultures yielded low number dendritic cells, we removed non-adherent cells eighteen hours after initial plating of bone marrow cells. After consulting the literature, many non-adherent dendritic cells were mature, with some expressing high levels of CD11c (25). Dendritic cells cultured from polystyrene plates followed preliminary protocol where nonadherent and adherent bone marrow cells were cultured with GMCSF and IL-4 cytokines beginning on Day 0. Dendritic cells were scraped on Day 5 and transferred to G-rex plates to recover from scraping. The number of dendritic cells collected on Day 10, the end of culture, was 6% of the initially plated bone marrow cells (Table 3). By contrast, the standard method yielded 27% of mature dendritic cells from the bone marrow when adherent bone marrow cells were cultured with GM-CSF and IL-4 cytokines on Day 1 (Figure 9.) Therefore, to increase dendritic cell proliferation, we initiated cytokine-induced cultures with only adherent bone marrow cells.

Since the previous experiment showed FLT3L increasing dendritic cell number *in vitro* from adherent and nonadherent bone marrow well, we hypothesized that FLT3L would exert a similar effect when only cultured with adherent bone marrow cells. However, FLT3L did not increase dendritic cell number in culture when only adherent cells were cultured (Table 3). The decreasing number of dendritic cells from Day 5 to Day 10 may be due to procedural error. Despite the decrease, the percentage of bone marrow cells recovered, 15% and 14%, is still greater than the 6% recovered from culturing nonadherent and adherent bone marrow cells (Table 3). We chose this dendritic cell culture method for the reminder of the project.

Nonadherent Bone Marrow Cells Collected after 8 Hours and 16 Hours Exhibit Small Phenotypic Difference

To determine an optimal time to remove nonadherent bone marrow cells, we characterized nonadherent bone marrow cells 8 hours and 16 hours after initial plating. Flow cytometry characterization of nonadherent cells collected eight and sixteen hours after initial plating on Day 0 with cytokine free media showed small changes in expression of CD80, MHC-II, B220, CD11c, and CD45.1 cellular markers. The blue peaks represent cells removed after sixteen hours and the red peaks represent cells removed after eight hours of culture. The only slight shift observed between the two peaks was for CD80 marker (Figure 10B). The red peak is shifted right, indicating that more cells expressing CD80 were removed at eight hours compared to sixteen (Figure 10B). Compared to C1498 negative control, the nonadherent cells removed also included some B220, CD11b, and CD11c positive cells, perhaps suggesting that B cells, myeloid cells, and dendritic cells are removed. Additionally, more cells were observed to remain adherent after sixteen hours. To produce highest yield possible of matured dendritic cells, we

continued remaining experiments by removing nonadherent cells after sixteen hours and only culturing adherent cells in media supplemented with cytokines for an additional five days culture in T25 flasks. After 5 days, all cells were transferred into G-rex plates for five more days of culture with either FLT3L added to the cytokine cocktail or continuation of cultures with cytokines only.

The Expression of Dendritic Cell Markers on Adherent and Nonadherent Cells Increased Between Day 5 and Day 10 of Culture

To determine the phenotype of adherent and nonadherent cells collected at Day 5 and adherent dendritic cells at Day 10, we analyzed these cells were analyzed by flow cytometry. Adherent and non-adherent dendritic cells were collected for flow cytometry characterization on Day 5 of culture. All dendritic cells were bone-marrow derived following FLT3L *in vivo* injections and cultured in complete RPMI cytokine media. The remaining cells, including adherent and nonadherent DCs, were transferred into G-rex plates. Compared to negative C1498 tumor control, both adherent and nonadherent Day 5 dendritic cells expressed CD80, MHC-II, and B220 markers (Figure 11C).

Nonadherent cells expressed more B220, a B cell and pDC marker, while adherent cells expressed more MHC-II (Figure 11C). The level of expression for CD80 were similar between the two cell types (Figure 11C). On Day 10 of dendritic cell harvest, the expression levels of CD80, MHC-II, and B220 were increased compared to the levels expressed on Day 5, suggesting that cells had matured (Figure 11C). Therefore, cells cultured from FLT3L-induced bone marrow cells expressed dendritic cell markers on Day 5 and expression of these markers continued to

increase when cultures were continued to Day 10, indicating that dendritic cells matured even after transfer of cells into silicone-coated G-rex plates.

Washing with Serum-Free RPMI Media Improves the Fused-Cell Percentage

To improve fusion percentage of tumor and dendritic cell hybrid, a pilot experiment utilizing fusion of splenocytes and C1498 cells was performed to optimize the conditions for cell fusion. Mixtures of splenocytes and C1498 were washed twice with serum-free RPMI prior to the addition of PEG and compared to previous dendritic cell and C1498 direct fusions in which PEG was added without washing. From the DC-C1498 fusion experiment, dendritic cells cultured from adherent bone marrow in T25 flasks after ten days with respective cytokines were fused with polyethylene glycol (PEG). No live/dead gating during flow cytometric analysis were applied to fused vaccine and vaccine after three days recovery (Figure 12C). The percentage of double positive for the mix control was 8.86% (Figure 12C), and the fused percentage was 11.3%. This results in about 3% of successful fusion percentage. By contrast, the fusion percentage of double positives from two-time wash of serum free RPMI media prior to PEG fusion was 25.8% while the mixed control percentage was 10.5% (Figure 13D). Therefore, a two-time serum free wash with RPMI improved fusion percentage.

We hoped to improve fusion percentage and cell membrane recovery, since the cell population of recovered vaccine were positive from Qdot605: live/dead gate, indicating the dye passed through cellular membrane. To improve fusion percentage, lysed splenocytes from CD45.1 Pepboy mice were fused with CD45.2 C1498. We hypothesized that additional two-time wash of the cell mixture with warm serum-free RPMI could improve direct cell-to-cell contact

and improve fusion efficiency. Results from the splenocyte-C1498 fusion indicated that fusion CD45.1 and CD45.2 positive percentage improved to 32.3% while the mixed control percentage was 11.6% (Figure 13C, Figure 13D). The pilot-study provided a greater fusion percentage. Therefore, future fusions were performed after washing the cell mixtures twice with serum free RPMI.

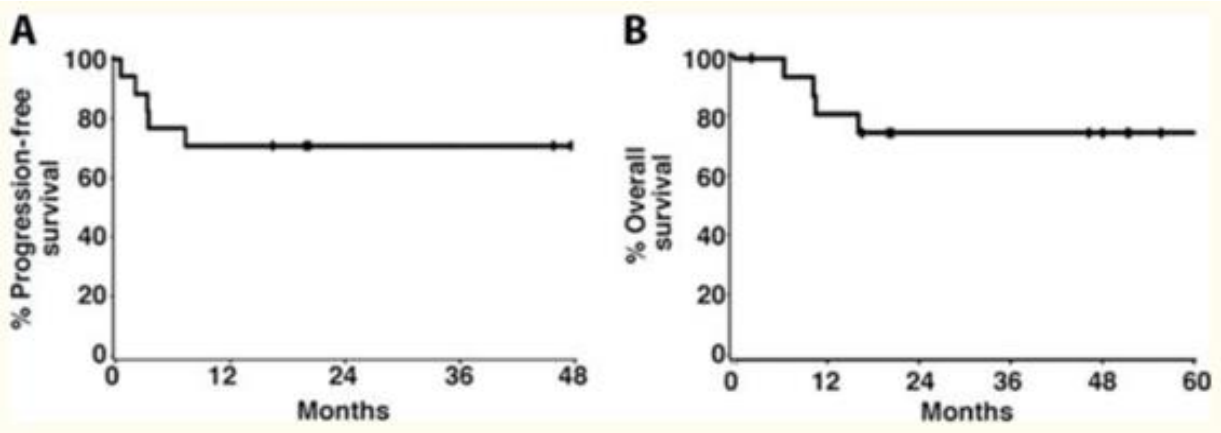
Antigen Specific Killing of C1498 is More Potent than Non-Specific Killing

In preparation for testing the efficacy of fused dendritic cell tumor vaccine in priming T cells *in vivo*, mixed lymphocyte reaction (MLR) assays that included every treatment group except for the fusion vaccine were performed. Two different cultures were used: 1) MLRs to test T cell cytotoxicity that included wild-type T cells primed by co-culture with C1498 as the source for leukemia antigens, with cytotoxicity subsequently assayed using luciferase+ C1498 targets, and 2) MLR that measured proliferation of B6 T cells following co-culture with C1498 in which luciferase+ T cells were co-cultured with wild -type C1498. For the proliferation assay, plates were imaged with bioluminescence for activation of luciferase + T cells following 5 days of culture. For the cytotoxicity assay, T cells primed by 5 days of coculture with irradiated C1498 were added to luciferase+ C1498 with the plates imaged on day 6. In both assays, bioluminescence imaging was performed following addition of luciferin to the media and measuring light emitted in the IVIS.

Although co-cultures of T cells with irradiated C1498 did not increase the proliferation of T cell (Figure 14A), the addition of *in vitro* C1498-primed and activated T cells to luciferase+ C1498 targets resulted in lower levels of leukemia-specific bioluminescence than adding non-primed T cells to luciferase+ C1498 targets, indicating the activated T cells killed more

LucC1498 (Figure 14B). Photographic images of the primed T cells in the bioluminescent killing assay showed large rounded T cells, consistent with activation of T cells during the killing assay (Figure 15). Therefore, T cells primed with C1498 demonstrated a more potent cytotoxic response than non-primed T cells, despite the increase in non-specific T cell proliferation induced by anti-CD3.

Figures



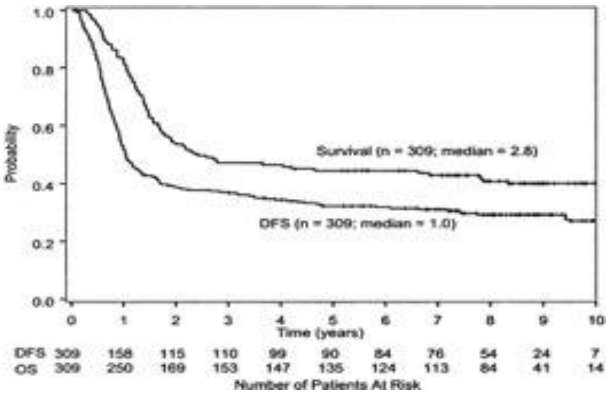


Figure 1. Comparison of median survival time between AML patients on nonrandomized clinical trial for AML-DC fused vaccination (top) and patients receiving high dose chemotherapy on randomized trial (bottom).

Adapted from Rosenblatt et al, 2016 and Moore et al, 2005

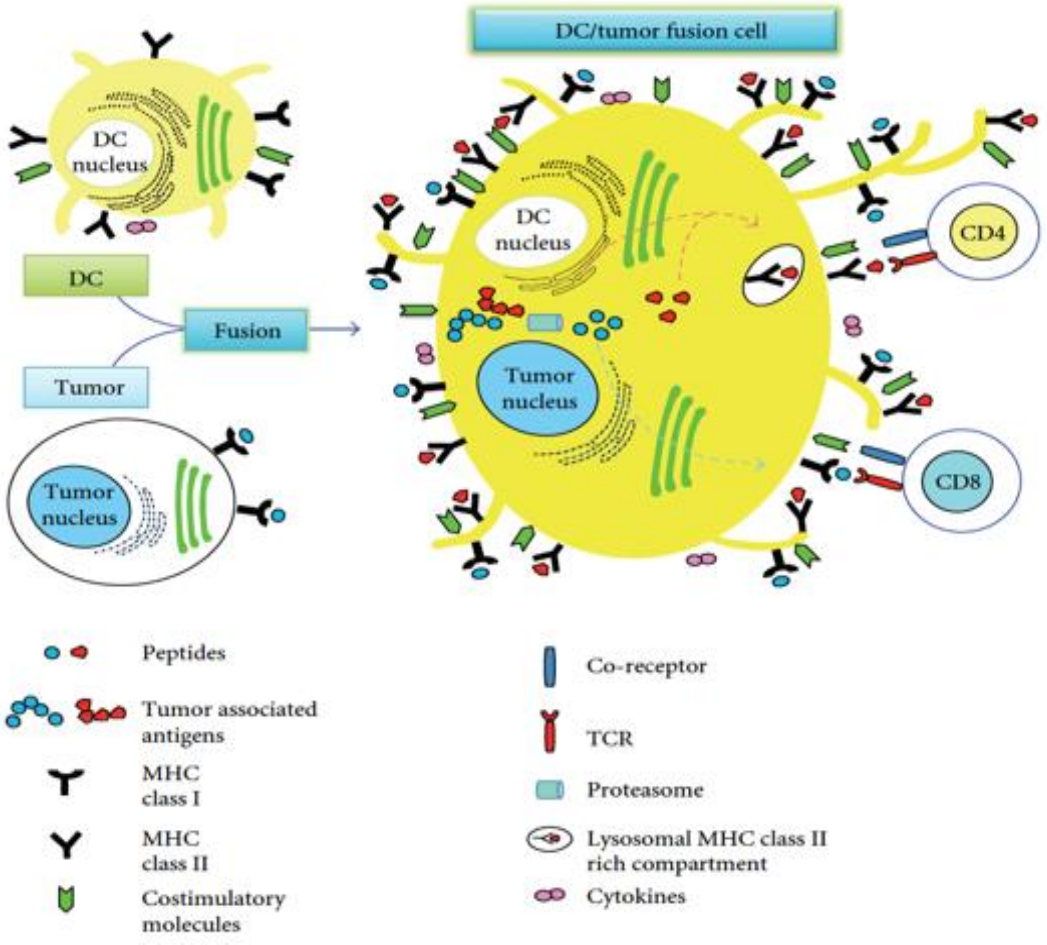


Figure 2. Anatomy of DC/Tumor fusion cell. Adapted from *Koido et al, 2011*.

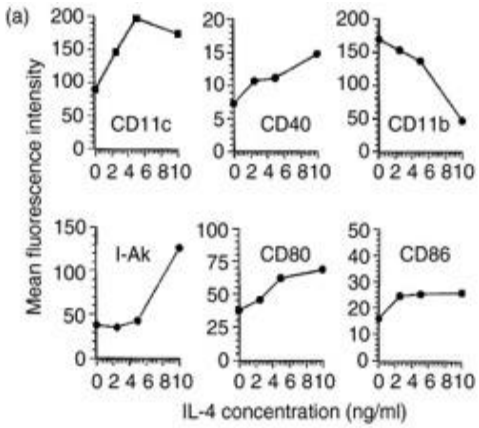
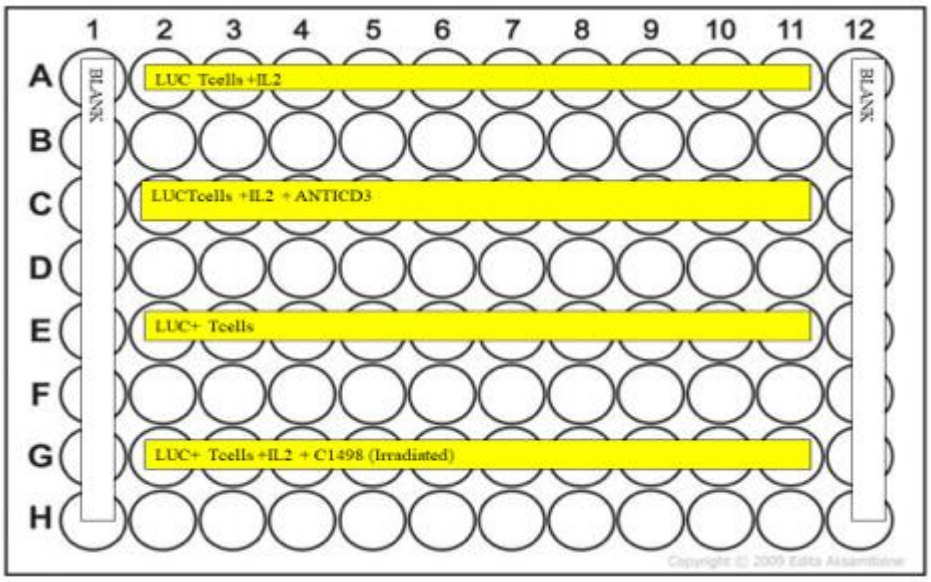


Figure 3. DC cultured with IL-4 increased CD11c and MHC-II expression with small increases in CD40, CD80, and CD86 expression.

Adapted from *Maroof et al, 2011*

A.



B.

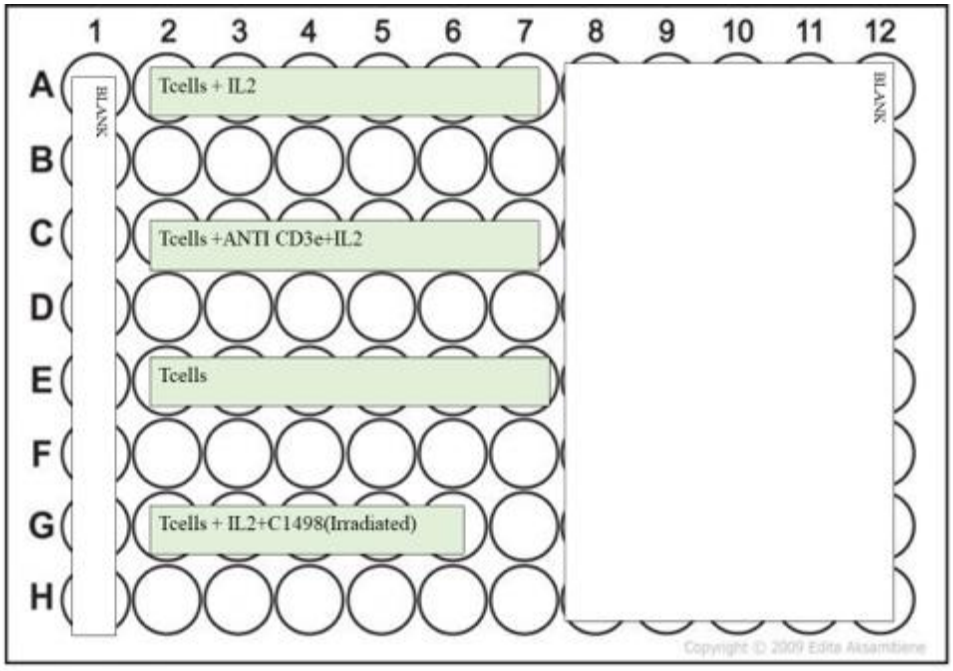


Figure 5. Mixed Lymphocyte Reaction plating schema. Proliferation Assay (A), Killing Assay (B)

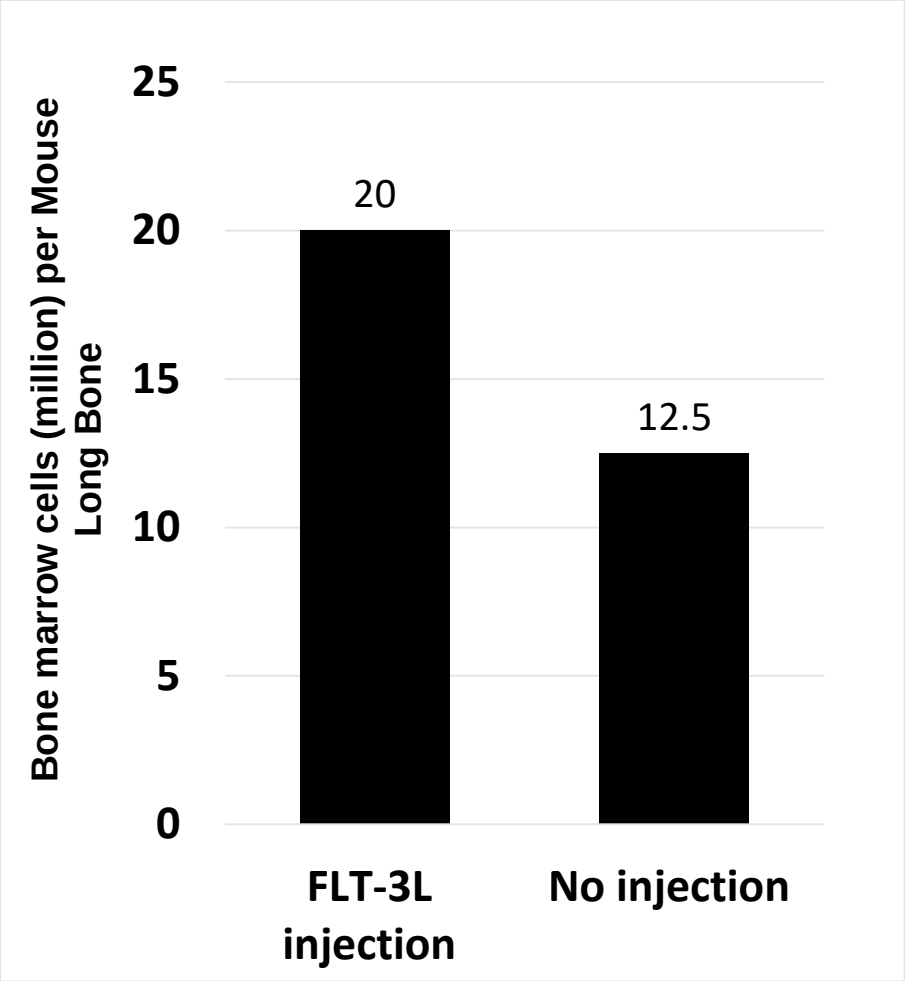
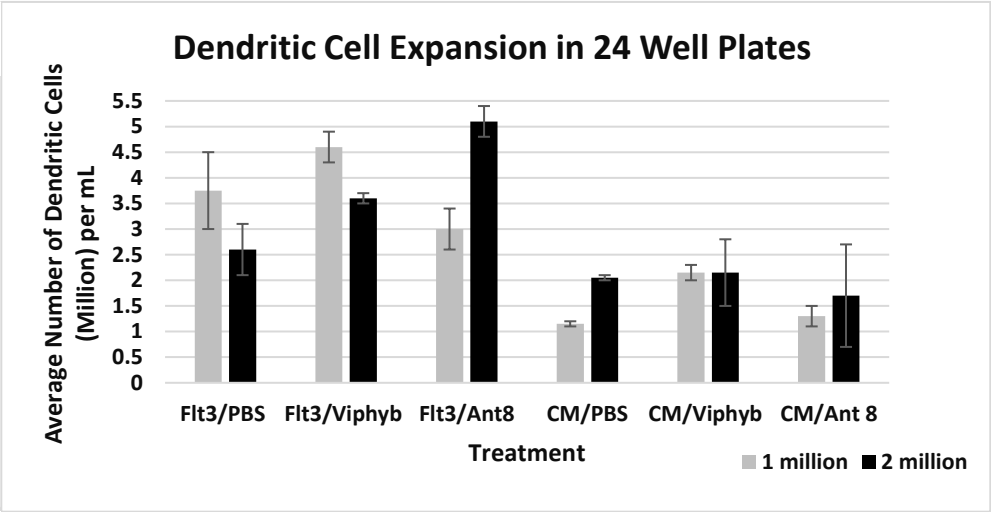


Figure 6. FLT3-L injection *in vivo* increased nucleated cells in bone marrow.

Mice were either injected with 7.5 ug of FLT3L in 100 µL PBS-diluted doses in accordance to the schedule in Table 1. The control mouse received no injections. 1 mouse was used per treatment group. Marrow was collected from tibia and femur of the mice. Total number nucleated cells collected was divided by 4 to obtain the amount of bone marrow cells per mouse long bone.

A.



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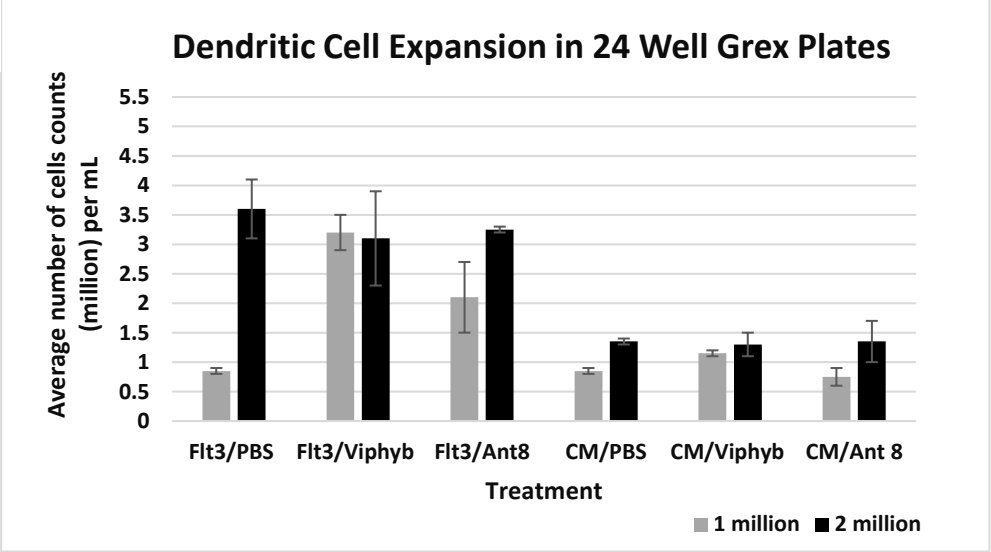


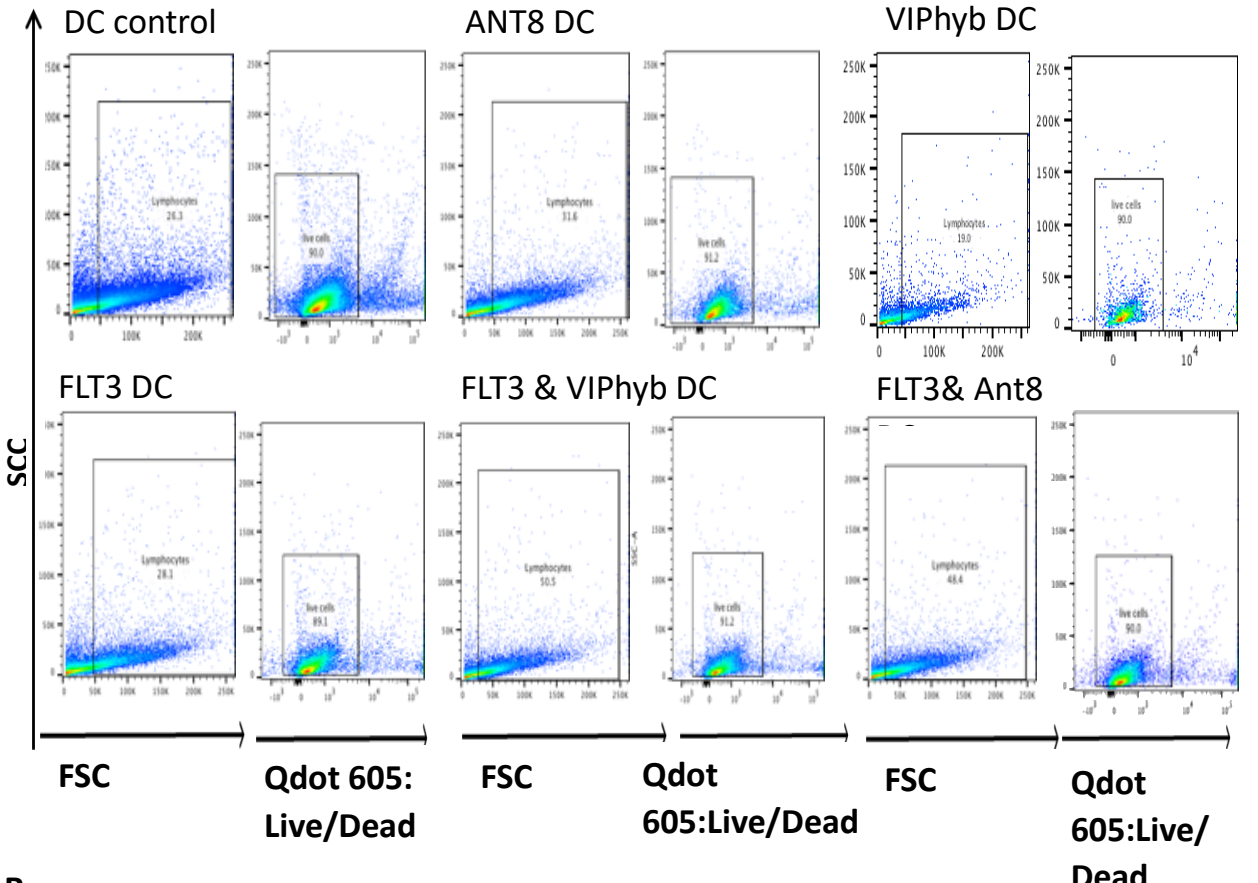
Figure 7. FLT3L increased number of dendritic cells cultured from non-adherent and adherent bone marrow expansion in vitro. G-rex plating showed no increase in dendritic cell numbers.

Gray bars show results from plating at 1 million cells/mL concentration and black bars show results from plating at 2 million cells/mL concentration. Average million number of dendritic cells per mL were taken from two samples. Error bars represents the ranges of these two samples.

(A) Dendritic cells cultured in polystyrene 24 well plates. Total volume of well is 2.5 mL.

(B) Dendritic cells cultured in 24 G-rex silicone plates. Total volume of each well is 6 mL.

A. Gating strategy of peptide-induced dendritic cells



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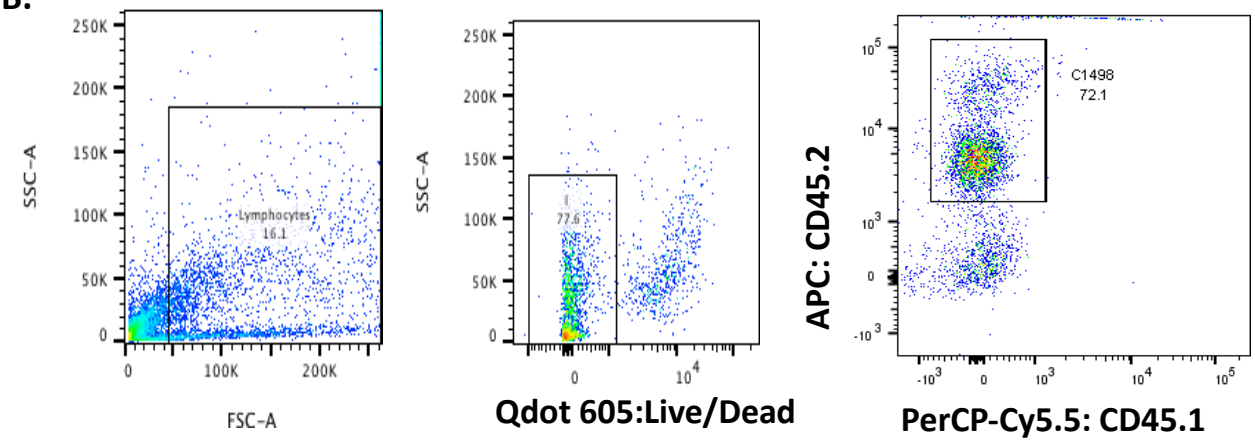
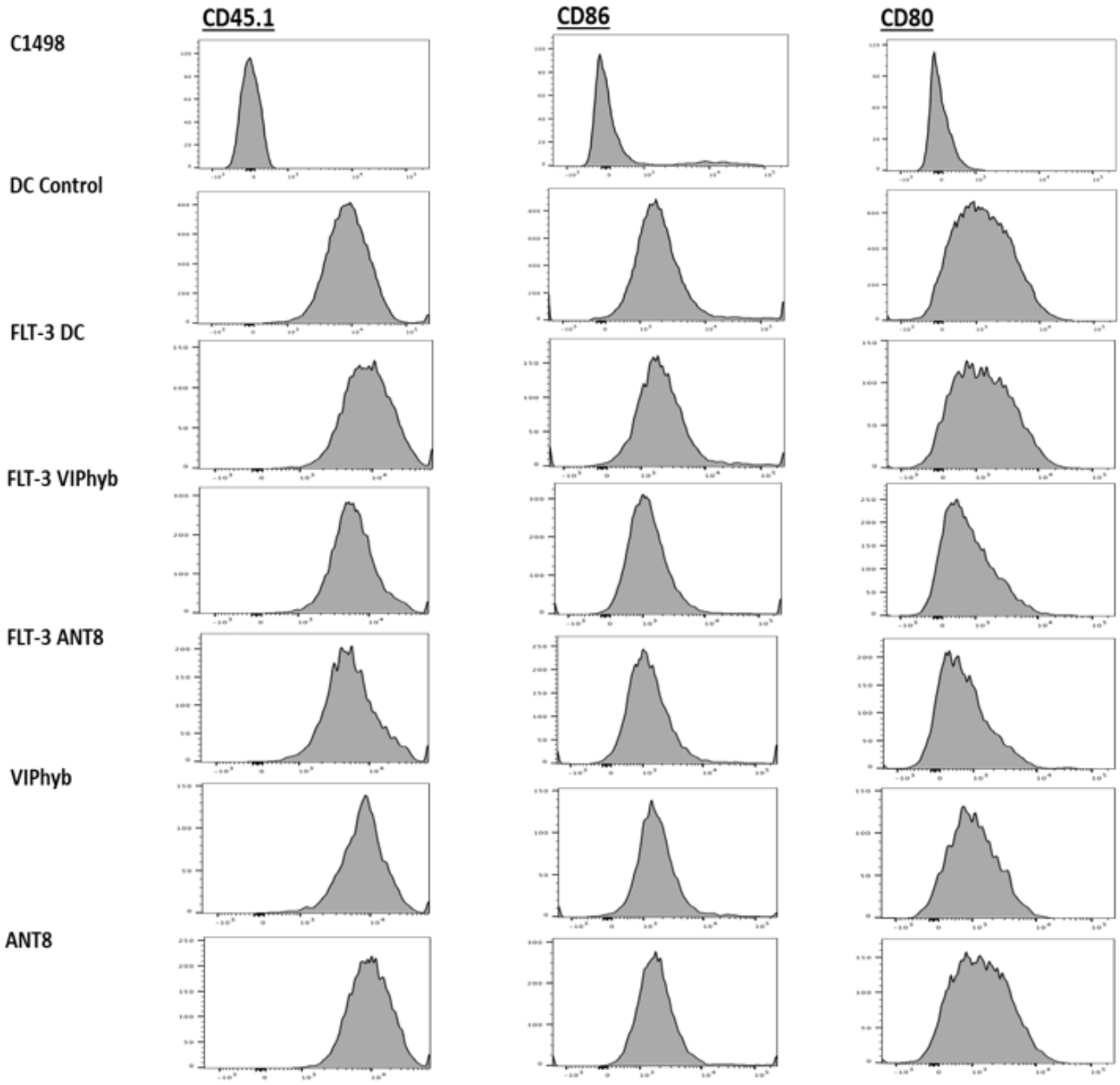
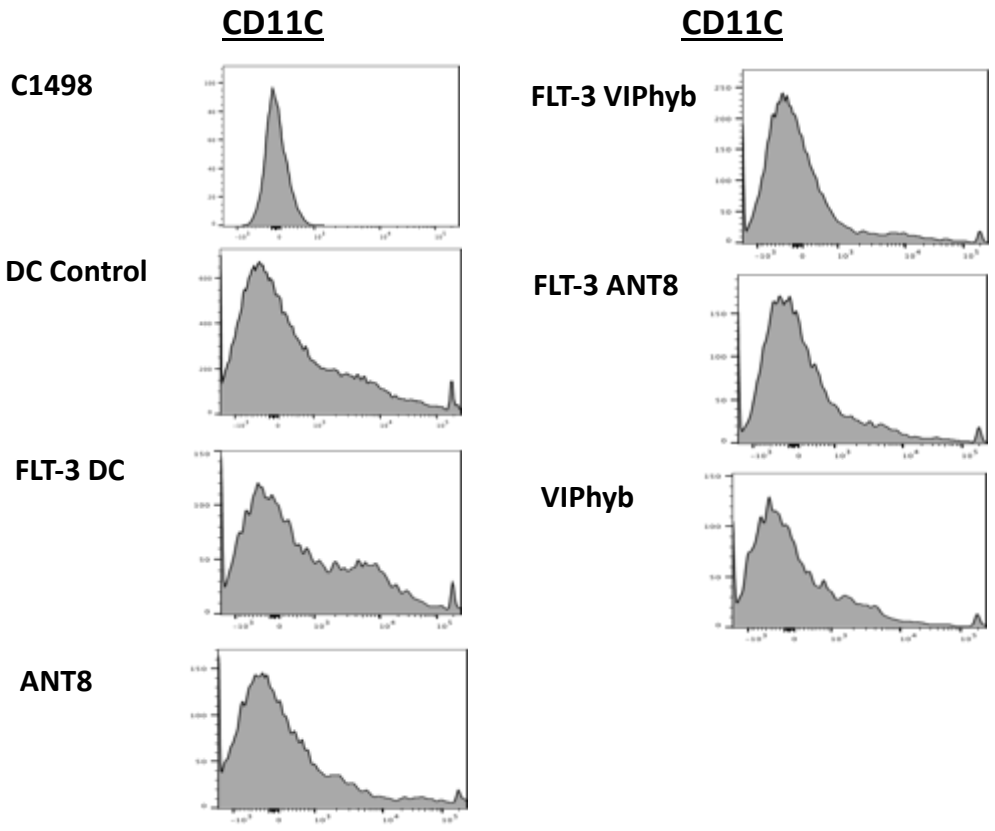
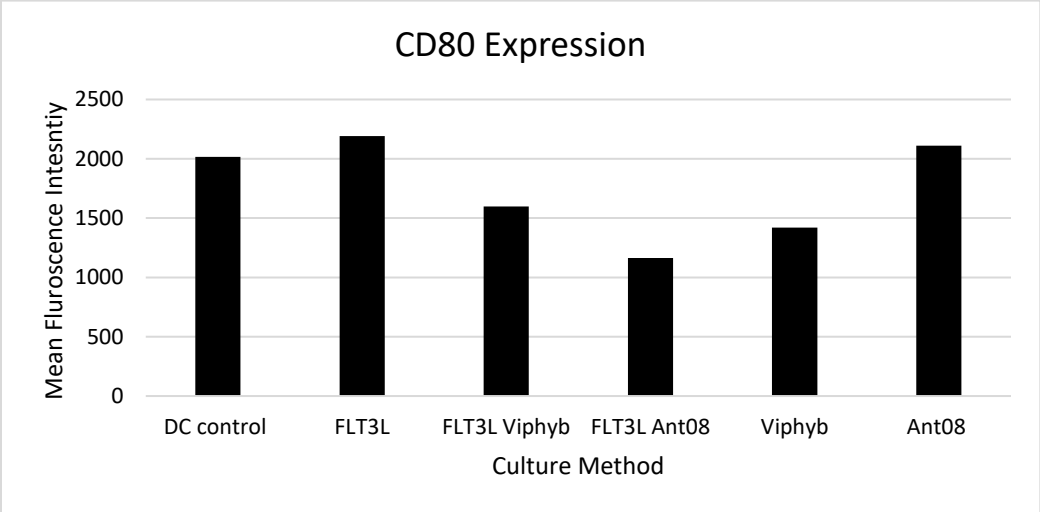


C. Expression markers of peptide-induced dendritic cells





D. Mean Fluorescence Intensity of CD80, CD11C, and B220 expression markers



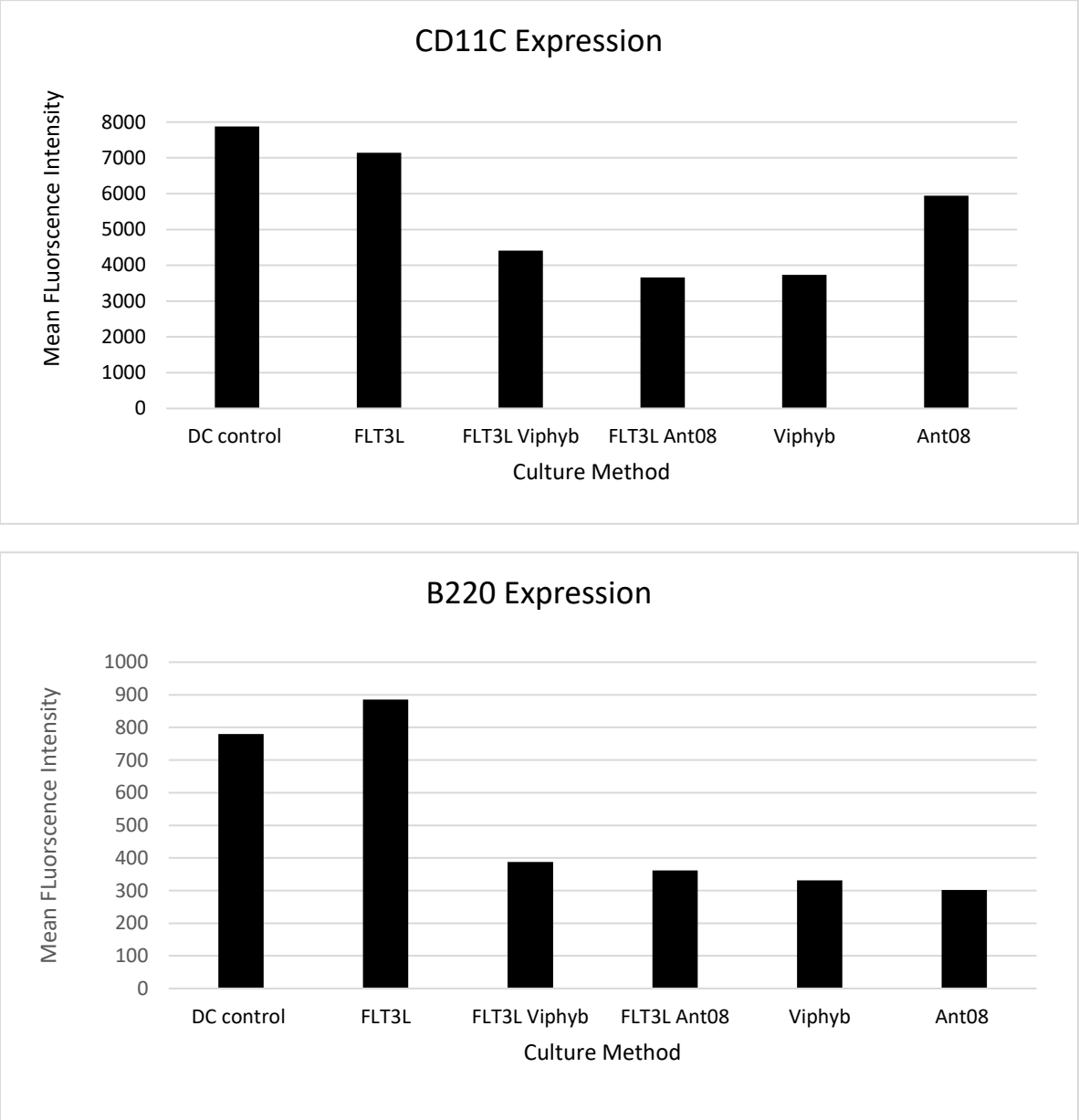


Figure 8. Flow cytometry characterization of dendritic cells cultured from 24 well G-rex plating showed that Viphyb and Ant08 has inhibitory effect on dendritic cell maturation markers.

A. Gating strategy of dendritic cells cultured from cytokine and peptide cultures in G-rex plating.
B. Gating strategy of C1498 negative control
C. Histogram of expression markers
D. Mean fluorescence intensity for CD80, CD11c, and B220 expression

Table 3. Monitoring number of dendritic cells harvested based on culture method				
Culturing method	Number of cells plated on Day 0	Number of cells collected on Day 5	Number of cells collected on Day 10	Percentage of bone marrow matured into dendritic cells
6-well polystyrene plates	10 million cells	N/A	600,000 cells	6%
5 T25 Flasks	100 million	26 million	27 million	27%
FLT3L in 6 T25 flasks	120 million	31 million	18 million	15%
6 T25 flasks	120 million	33 million	17 million	14%

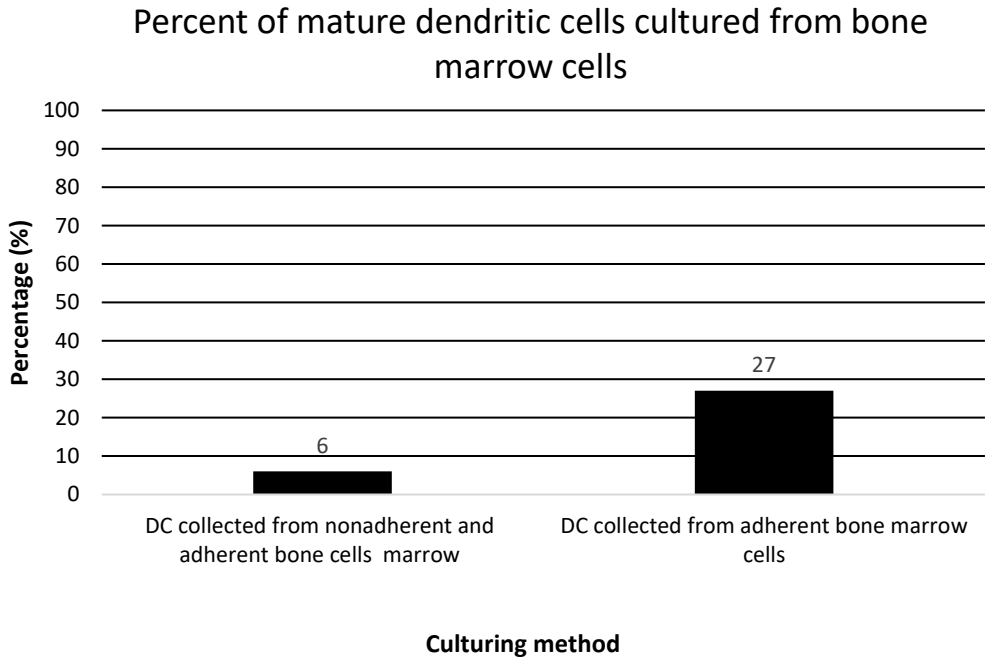


Figure 9. Percent of mature dendritic cells cultured from bone marrow cells between two culture conditions

A.

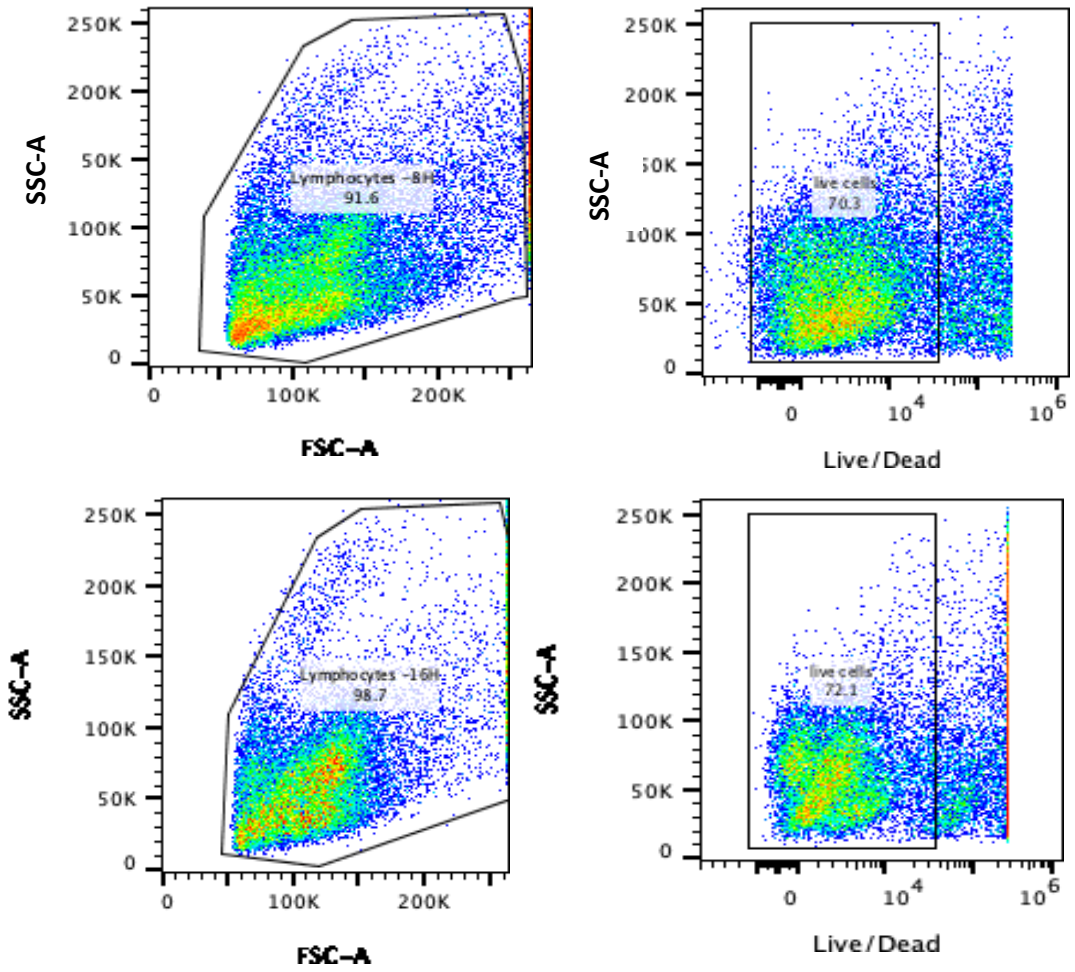


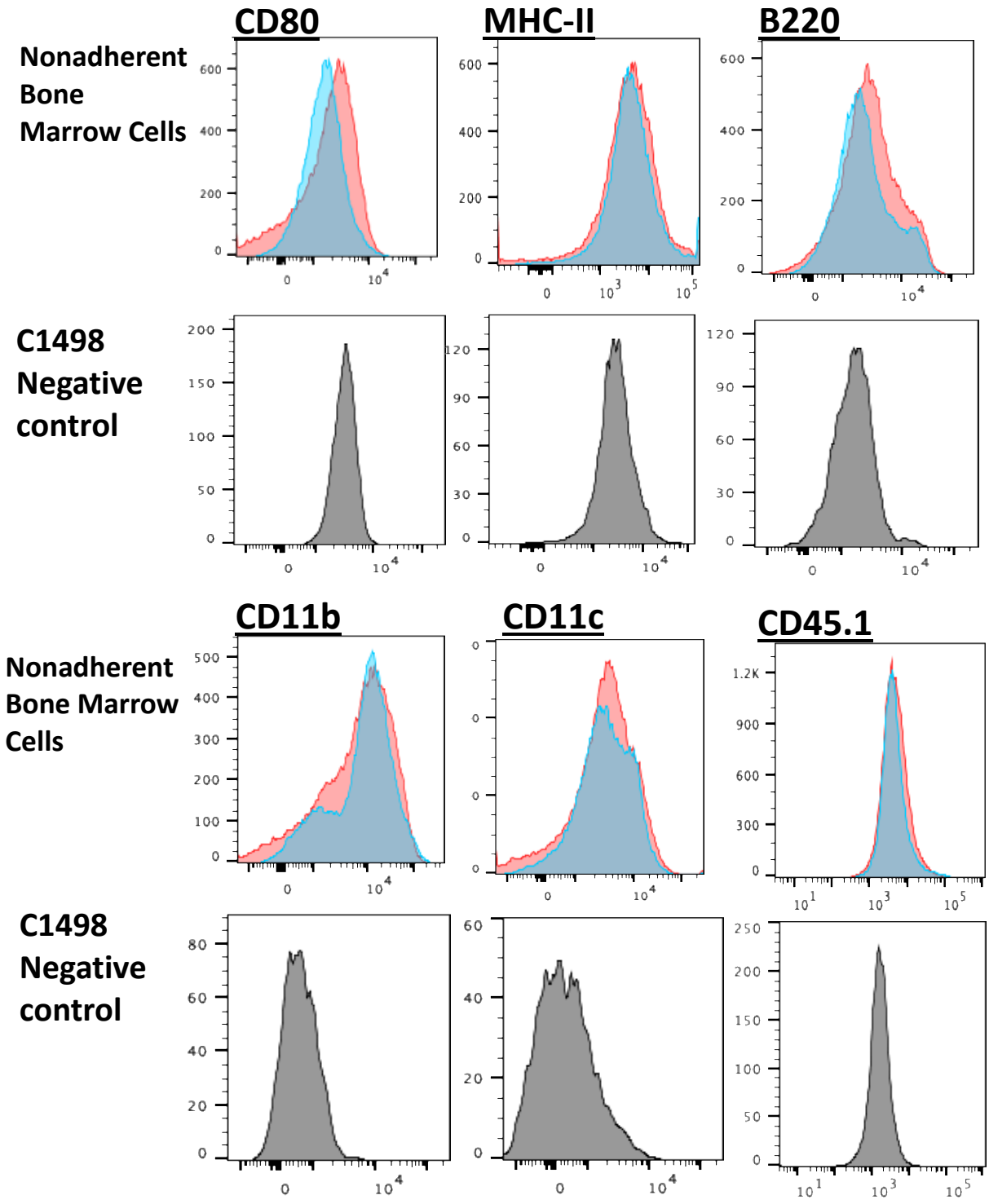
Figure 10. Nonadherent cells display little to no levels of antigen presenting markers.

Bone marrow from FLT3L injected mice were plated in cytokine-free media for 8 and 16 hours.

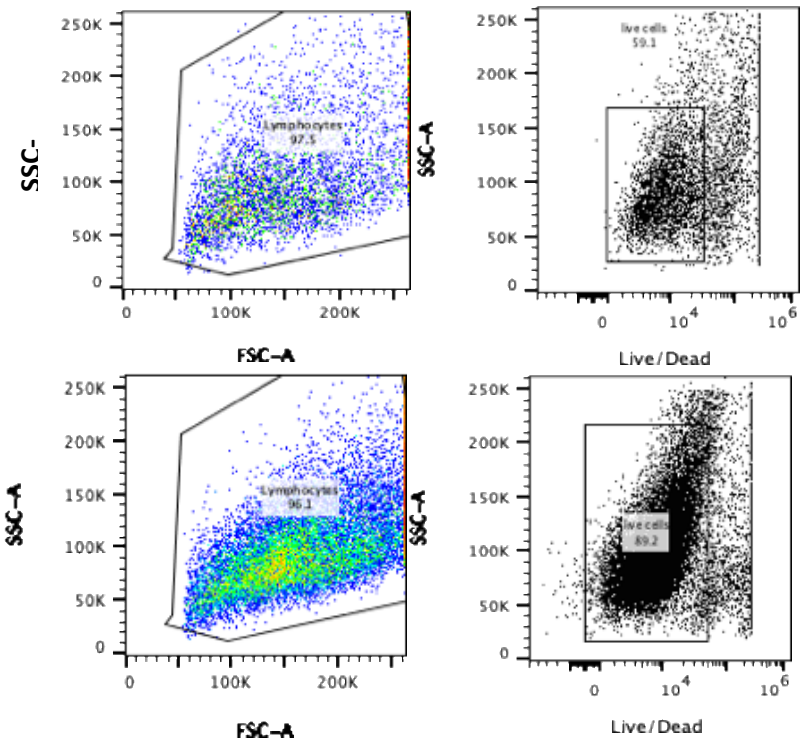
A. Gating strategy of media from bone marrow culture after 8 hours (top)and 16 hours (bottom)

B. Blue is media after 16 hours, red is media after 8 hours. Gray panels are C1498 negative controls

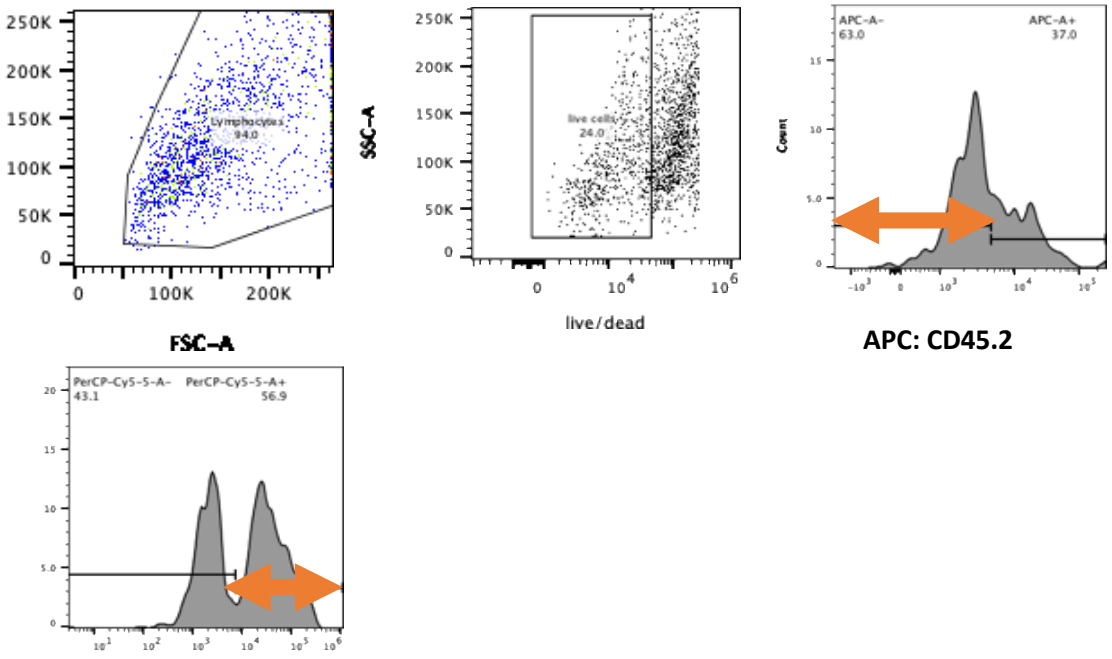
B.


CD80
MHC-II
B220
Nonadherent Bone Marrow Cells
C1498 Negative control
CD11b
CD11c
CD45.1
Nonadherent Bone Marrow Cells
C1498 Negative control

A. Gating strategy of adherent Day 5 DCs (top) and non-adherent Day 5 DCs



B. Gating strategy of DCs from Day 10



C. Level of expression markers

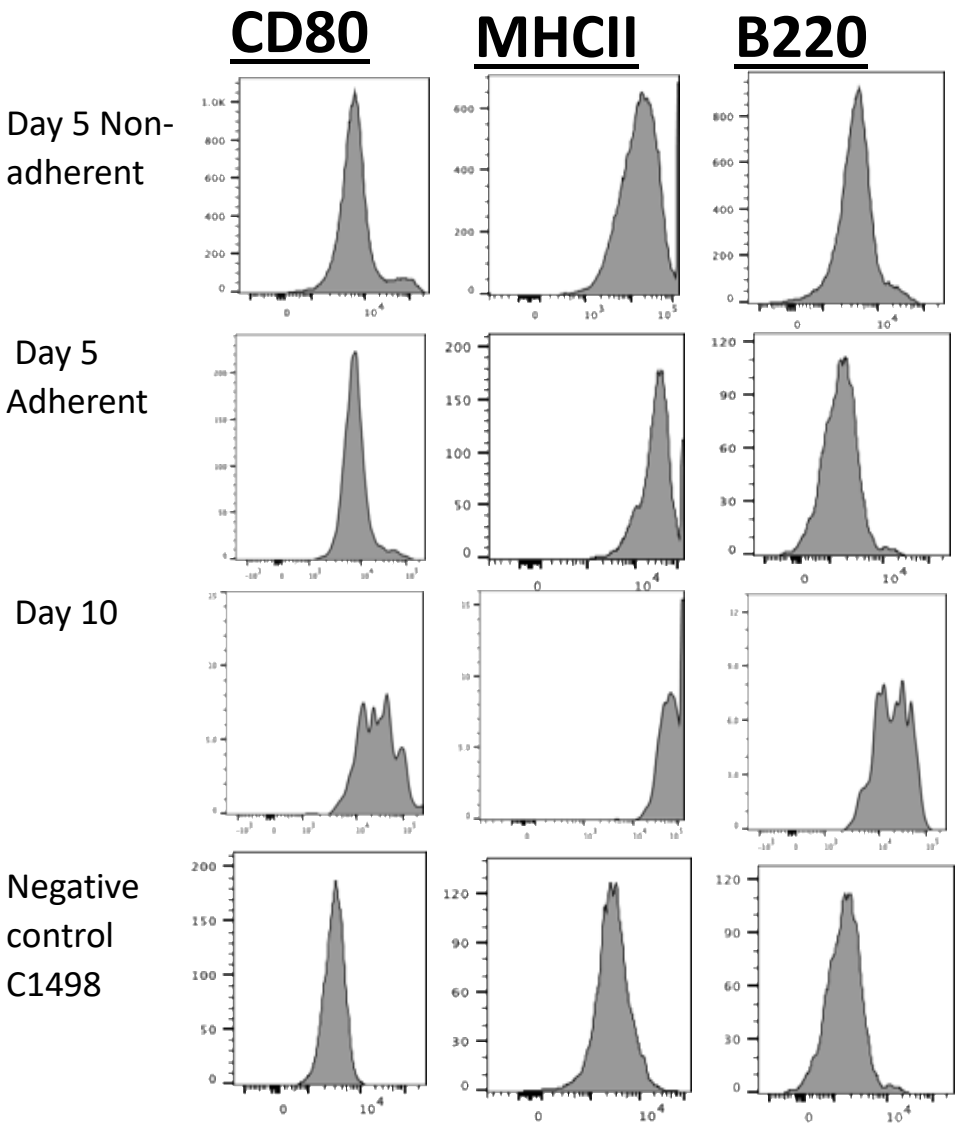


Figure 11. Cell markers CD80, MHC-II, and B220 increased in dendritic cells Day 5 to Day 10.

Cells were cultured scraped from polystyrene flasks on Day 5 and transferred to G-rex plating. Gating order begins from plots from the left to plots on the right.
A. Gating strategy for Day 5 adherent (top) and nonadherent (bottom) DC.
B. Gating strategy for Day 10 DC (top) and C1498 (bottom). Orange arrow indicates the population gated for the next gate.

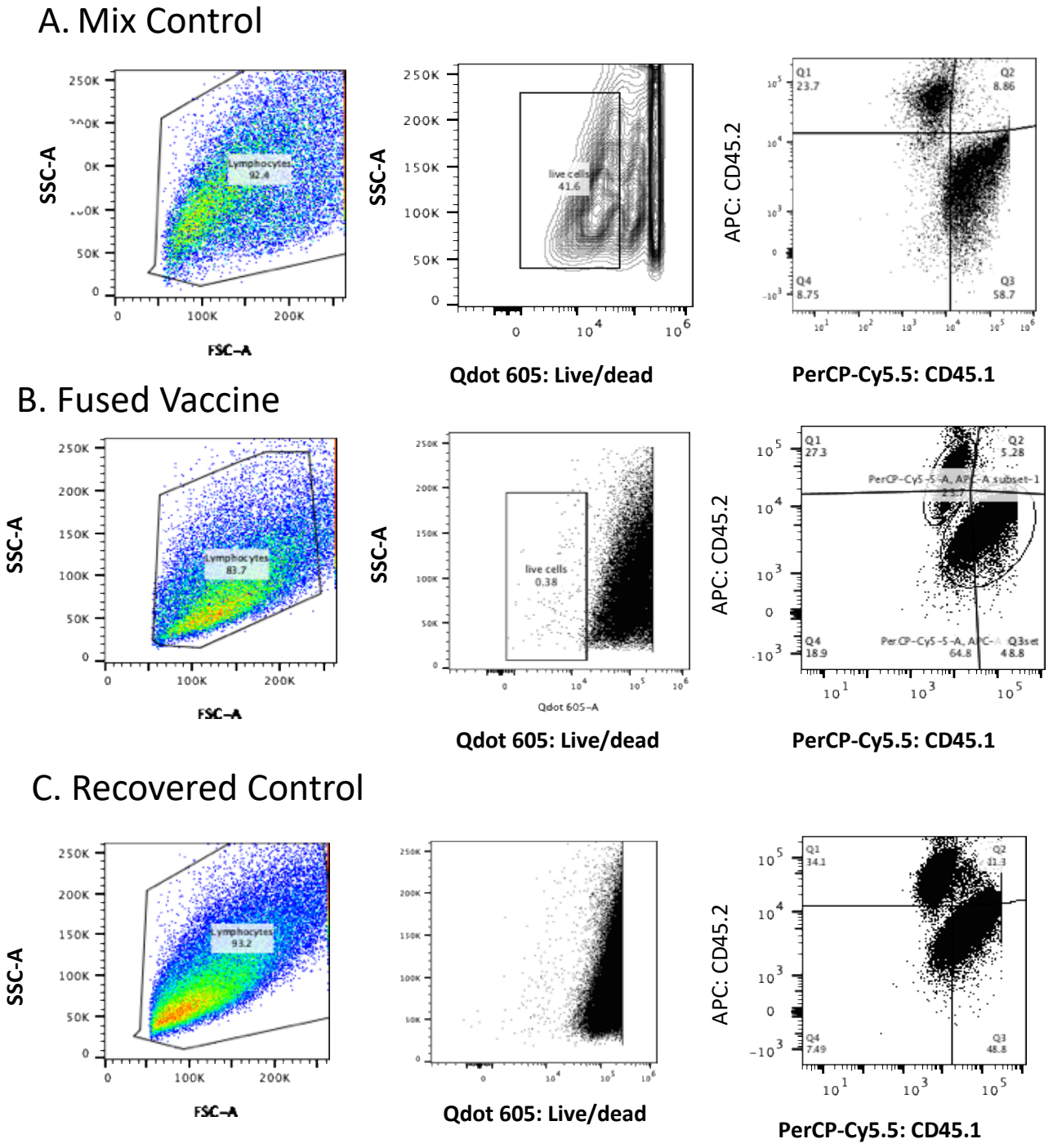
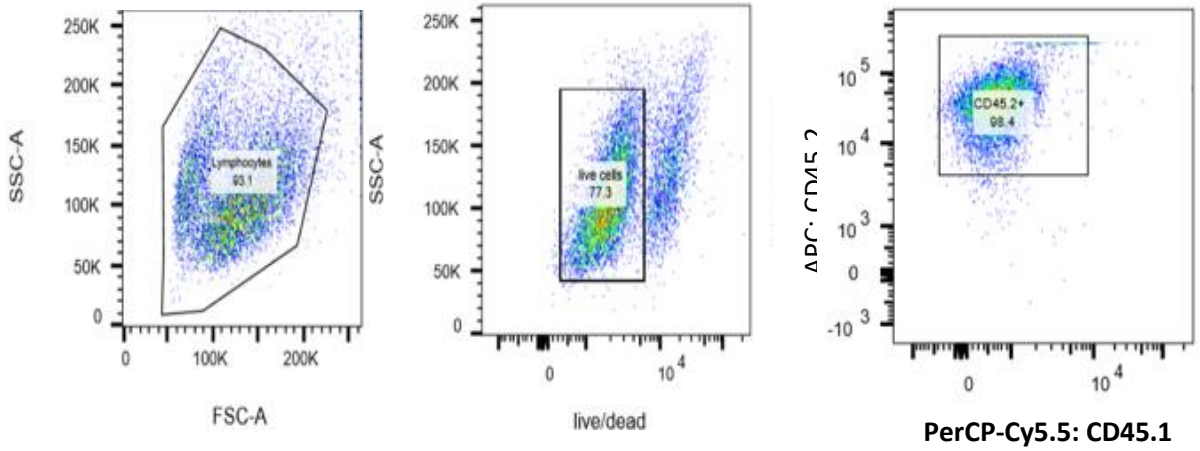


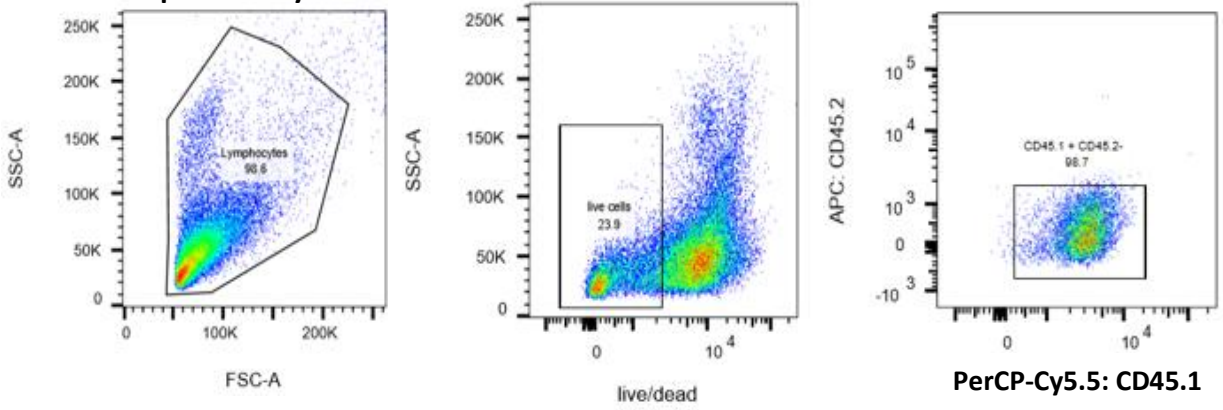
Figure 12. Flow cytometry characterization of dendritic-tumor fused vaccine

A. Gating strategy of C1498 and dendritic cell mixed before fusion
B. Gating strategy of vaccine after PEG fusion
C. Gating strategy of vaccine 3 days after membrane recovery

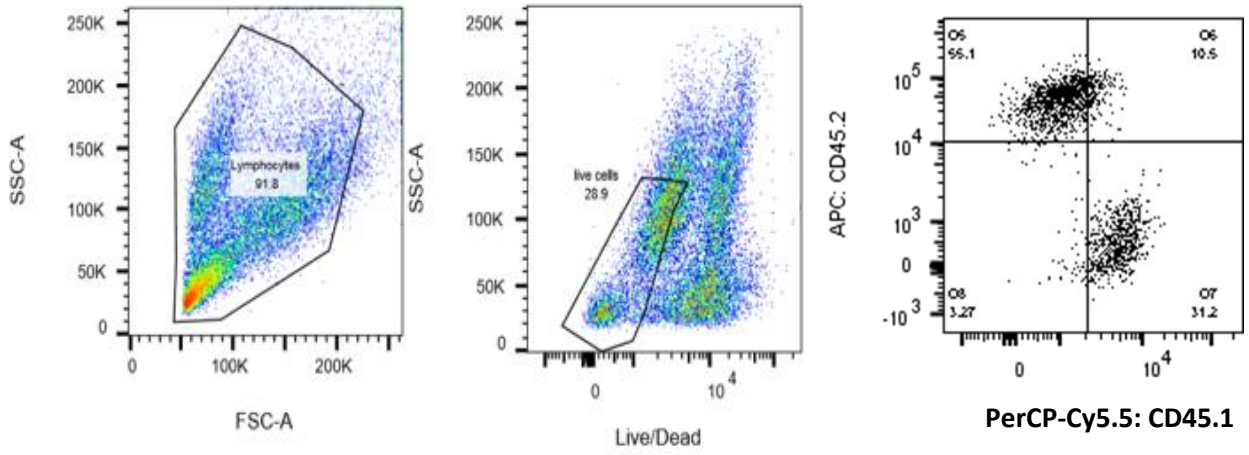
A. C1498 Control



B. Splenocyte control



C. Mixed Control



D. Fused Splenocyte-C1498 after recovery

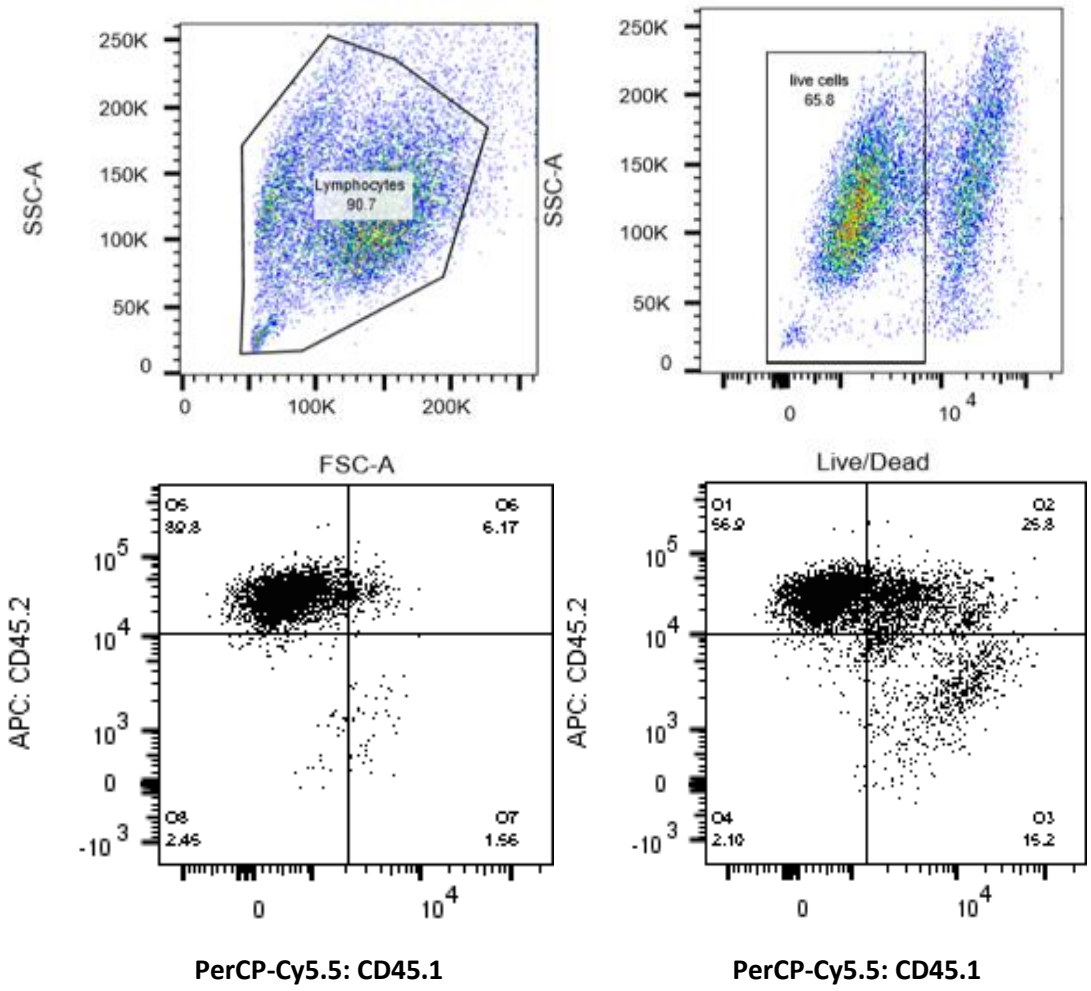
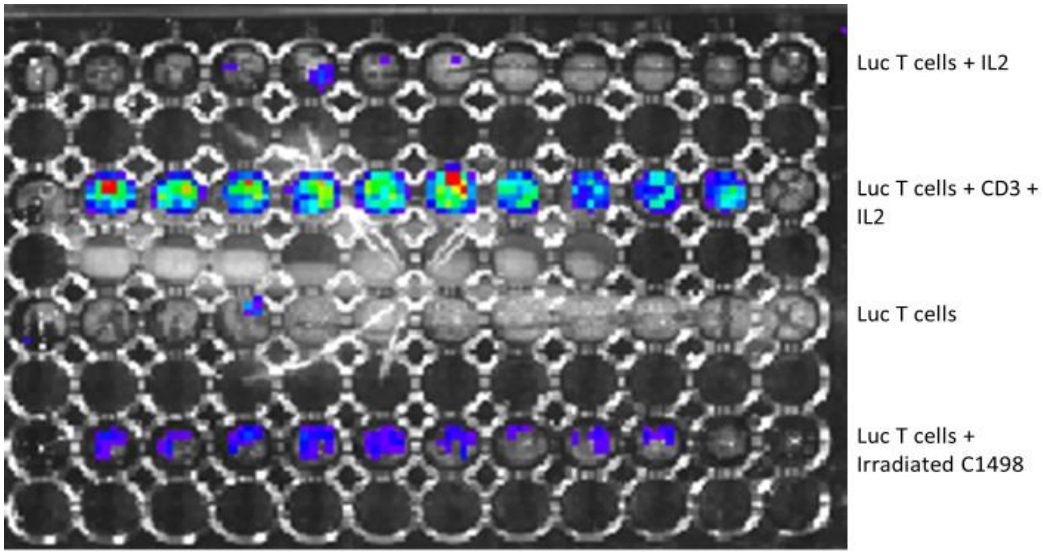


Figure 13. Splenocyte fusion after washing cells with serum free RPMI media increased percentage of fusion cells.

A. Gating strategy for C1498 control
B. Gating strategy for splenocyte control
C. Gating strategy of mixed C1498 and splenocyte control before addition of PEG
D. Gating strategy of vaccine three days after three day recovery. Bottom right panel did not include Live/Dead gate

Fresh splenocytes harvested from CD45.1 mice were directly used for PEG fusion in 3:1 similar to DC fusion methodology. 40ng/mL of GMSCF were added into fusion recovery complete RPMI media cultures.

A.



B.

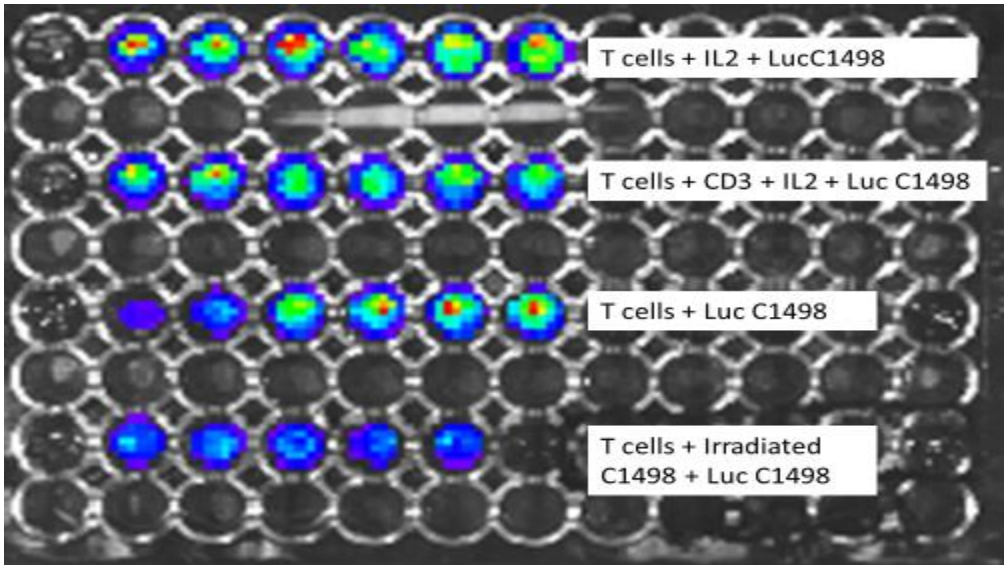


Figure 14. Luciferin bioluminescence plate imaging of mixed lymphocyte reactions

A. Plate imaged on Day 5 measuring T cell proliferation
B. Plate image measuring T cell killing after challenged by LucC1498

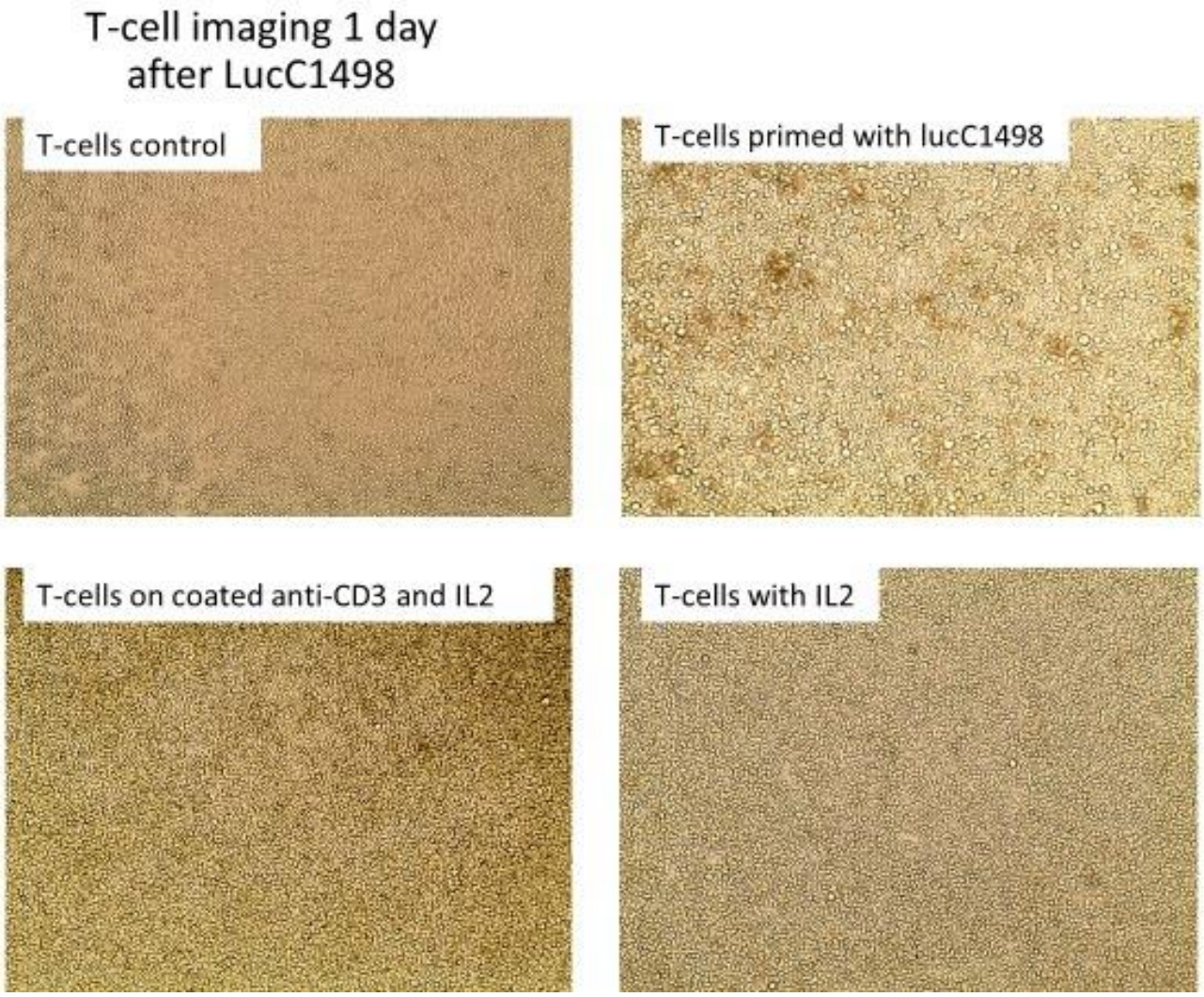


Figure 15. Photographic imaging of T-cells one day after challenging with LucC1498

Discussion and Future Directions

The goal of this study was to produce a murine leukemia-DC fusion vaccine that would enhance the activation and cytotoxicity of T cells directed to leukemia cells. The ultimate application of such a vaccine would be to treat mice with low levels of leukemia (therapeutic vaccine) in order to induce a potent T cell mediated anti-leukemia response, and to treat mice prior to challenge with leukemia (prophylactic vaccine) in order to generate a memory T cell response that would prevent growth of the leukemia cells (26).

To achieve these goals, we initially tested various strategies to produce a fusion vaccine of C1498 and murine dendritic cells. I began this work with four hypotheses. First, I hypothesized that FLT3L injection would increase the numbers of nucleated bone marrow cells, facilitating the generation of bone marrow-derived DC. Our hypothesis was supported by our results, since FLT3L injection *in vivo* resulted in an increase in the numbers of nucleated cells in bone marrow (Figure 6). In a study with transgenic mice designed to express high levels of FLT3L, increased numbers of myeloid and lymphoid progenitor cells were observed in the bone marrow, consistent with our results (27). When bound to its receptor, FLT3L induces development of myeloid and lymphoid lineages while suppressing megakaryocyte and erythrocyte activities within contents of the bone marrow (27). FLT3L administration *in vivo* results in proliferation of both cDC and pDC progenitors. However, proliferation decreases during and after a cell's commitment to differentiating into pDCs; therefore, the FLT3L injections generally increases the population of cDCs more than pDCs (16).

A previous transplant study conducted in our lab showed that murine bone marrow from mice treated with FLT3L injections had increased numbers of pDC (17). An interesting question to further explore is whether more pDC or cDC content are collected following FLT3L injection

and which type of dendritic cell would create a more potent fused vaccine. Since we observed that FLT3L had a positive effect on increasing numbers of bone marrow cells, our second hypothesis predicted that culturing bone marrow with FLT3L *in vitro* would increase dendritic cell proliferation. Our hypothesis was partially supported insofar as the addition of FLT3L to cultures of bone marrow cells increased dendritic cell numbers when both nonadherent and adherent bone marrow cells were cultured. However, this proliferative effect was not observed in adherent bone marrow cells, suggesting that FLT3L only enhances proliferation of the nonadherent bone marrow cells.

When cultured with fresh non-adherent and adherent bone marrow, FLT3L increased the number of dendritic cells *in vitro*. In addition to increasing bone marrow cell count, FLT3L is known to also promote differentiation of BM progenitor cells to pDCs *in vitro* (15). In the same study, pDCs were characterized as smaller and nonadherent cells while cDCs were larger and adherent. Our results showed a lower percentage of dendritic cells harvested following the preliminary method of culture (Figure 9). A possible explanation is that the collection of dendritic cells on day 10 only included adherent dendritic cells. This dendritic cell population may only include adherent cDCs since pDCs are nonadherent.

FLT3L increased the number of dendritic cells collected from non-adherent and adherent bone marrow but had no effect on dendritic cell expansion from adherent bone marrow (Figures 7, Table 3). Dendritic cells cultured from non-adherent and adherent bone marrow included all nucleated bone marrow cells. Although the harvest of these dendritic cells only collected adherent dendritic cells, pDCs precursors have the ability to differentiate back into adherent cDCs (28). The addition of TNF-α also induces an isoform expression marker CD44, which plays a role in cell migration and promotes adhesion (14), (29). Since pDC also exhibit

functional plasticity functions, a possible explanation is that the large amount of pDCs induced by FLT3L *in vitro* reverted into cDCs upon addition of TNF-α, resulting in more dendritic cells to be harvested.

The addition of FLT3L to all nucleated bone marrow cells could have increased the number of dendritic cells, especially since these nonadherent cells could be pDCs. By contrast, the addition of FLT3L to adherent bone marrow may not have stimulated pDC growth since pDCs are nonadherent, resulting no increase in pDCs from adherent bone marrow cells (Table 3). A way to confirm this explanation is to determine whether nonadherent bone marrow cells developed into pDCs. A follow-up experiment could be to collect nonadherent cells on Day 5 while transferring the adherent cells to G-rex plating and characterize the cell population via flow cytometry. If a significant amount of nonadherent cells on Day 5 are pDCs, these cells can be re-plated in polystyrene flasks and stimulated by TNF-α to trigger dendrite adhesion.

We incorporated dendritic cell transfer into G-rex plating to facilitate better gas-exchange of the cultures and avoid the acidification seen in the 24 well polystyrene plate cultures. We also wanted to maintain adding cytokines only to adherent bone marrow cells, since more dendritic cells were harvested from culturing adherent bone marrow cells than non-adherent and adherent bone marrow cells (Table 3). In addition to facilitating gas-exchange, transferring dendritic cells on Day 5 to G-rex plates also allowed the adherent dendritic cells to be efficiently recovered.

We next characterized the phenotype of dendritic cells from G-rex plate cultures. All dendritic cells cultured from G-rex plates expressed high levels of CD80 and CD86, two co-stimulatory molecules necessary to efficiently activate T cells, suggesting that the culture methods we employed can produce mature dendritic cells (Figure 8).The addition of FLT3L to cultures increased CD80 expression, which is critical in presenting peptides on cellular surface

MHC molecules (30). The mean fluorescence intensity of CD80, CD11c, and B220 expression markers of dendritic cells cultured with FLT3L cultured from all nucleated bone marrow may be a mixture of cells with phenotypes of pDC, NK-like cells, and cDC progenitors (16). Since FLT3L injections are known to induce pDC development and that FLT3 surface receptors have been found to be expressed only on CD11c+, MHCII+ dendritic cells, not on B cells, T cells, or NK cells, which highly suggests that the dendritic cells cultured from nonadherent progenitors with FLT3L are pDCs (31). (The last experiment cultured dendritic cells with FLT3L from adherent bone marrow, but these dendritic cells and resulting fused vaccine were not tested or characterized due to the coronavirus pandemic).

pDCs are identified by low expression of CD11c, high expression of B220, low expression MHCII, and absence of CD11b expression (16), (30), (15). cDCs express the opposite pattern of marker expression: higher expression of CD11c, absent expression of B220, and higher expression of MHCII. Results of dendritic cells cultured with FLT3L showed lower CD11c expression and higher B220 expression compared to dendritic cells cultured without FLT3L (Figure 8D). Therefore, FLT3L induced dendritic cells were consistent with a pDC phenotype.

However, FLT3L cultures can induce development of cDCs and pDCs, but co-treatment with GM-CSF and FLT3L inhibits pDC development while allowing cDC differentiation (15). GM-CSF induces STAT5 to suppressively bind to pDC transcription factors, inhibiting pDC development (15). Therefore, the production of dendritic cells with pDC phenotype may have been facilitated by *in vivo* injection of FLT3L rather than *in vitro*.

From the same experiment of dendritic cells derived from nonadherent and adherent bone marrow, the effects of adding a VIP antagonist VIPhyb to the bone marrow cultures were

different from adding FLT3L. Adding Viphyb decreased CD80, CD11c, and B220 marker expression. The addition of FLT3L mitigated this effect and increased relative expression levels of these markers. Dendritic cells cultured with VIPhyb have lower expression of CD11C, absent B220 expression, and lacked increased levels of CD80 and CD86 expression. While the effects of VIPhyb antagonist remains unclear, dendritic cells cultured with VIP (agonist) had a protective effect against lethal GvHD while preserving the GvL effect in mice harboring A20 leukemia (22). In addition, these authors noted that VIP simulated the proliferation and differentiation of regulatory T cells (22).

However, in a study done previously in our lab, VIP-KO mice had a more potent immune response to murine cytomegalovirus (mCMV) than wild-type mice (23). Following mCMV infection, increased expression of MHC-II and CD80 were observed amongst dendritic cells from VIP-KO mice (20). Since the effects of VIP-induced dendritic cells and its antagonist depends on what kind of cells the dendritic cells are testing against (A20 leukemia or mCMV), a better comparison for future experiments in our study would be to compare marker expression in dendritic cells cultured with both VIP (agonist) and VIPhyb (antagonist).

Results from experiments with ANT-08, the other VIP antagonist used in these studies, differed from that of VIPhyb, especially in co-cultured with FLT3L. ANT-08 is a peptide that substitutes the 25^{th} amino acid serine in VIPhyb with leucine, suggesting that leucine may play a pivotal role in the expression of CD80 and CD11c. Culturing dendritic cells cultured with ANT-08 alone also improved CD80 and CD11c expression to a greater extent than VIPhyb.

We hypothesized that fusing C1498 AML cell line with dendritic cells would yield a fusion vaccine that would enhance T cell specific anti-leukemia cytotoxic activity enough to result in elimination of C1498 cells. The preliminary MLR assay showed that the anti-CD3

coating was the most successful treatment for enhancing T cell proliferation, but T cells primed with C1498 had a more potent cytotoxic anti-leukemia effect than non-specific T cells (Figure 14). Therefore, increased non-specific T cell proliferation does not necessarily trigger leukemia specific cytotoxic T cell activities.

Another MLR to include T cells primed with irradiated fusion dendritic and tumor cell fusion vaccine was planned but could not be due to the coronavirus pandemic. A key future goal for this project will be to determine whether a fusion vaccine has a more potent effect in priming T cells to anti-leukemia cytotoxicity than T cells primed with irradiated C1498. The overall goal of this project, also prevented by the coronavirus pandemic, is to test the anti-leukemia activity of the fusion vaccine *in vivo* in both prophylactic and therapeutic settings.

In conclusion, this study demonstrated that FLT3L injections into mice increased the number of nucleated bone marrow cells and the content of dendritic cells progenitors. However, whether FLT3L injections increased the amount of pDC or cDC remains to be defined. FLT3L had a proliferative effect on dendritic cell expansion *in vitro* when added to adherent and nonadherent bone marrow cultures, but this increase in dendritic cells was not observed when only adherent cells were cultured with FLT3L. Dendritic cells in FLT3L-supplemented cultures had increased CD80 and B220 expression levels, but decreased CD11c expression, which is consistent with pDC phenotype. Our studies also pointed towards a method of improving dendritic-cell leukemia cell fusions, as we found improved recovery of DC-AML heterokaryon fusions when cells were washed twice with serum free media prior to PEG fusion. Finally, results from MLR assays demonstrated enhanced cytotoxic capabilities of T cells primed with irradiated C1498 compared to non-primed T cells. This reveals a critical role of T cells exposures to respective tumor antigens in eliciting C1498 specific cytotoxic responses. Hopefully,

presenting these tumor-associated antigens in the context of antigen-presenting cells such as dendritic cells, would induce greater cytotoxic T cell activity.

Works Cited

1. Domen, Jos; Wagers, Amy; Weissman, Irving L. Bone Marrow (Hematopoietic) Stem Cells | stemcells.nih.gov [Internet]. [cited 2018 Dec 9]. Available from: https://stemcells.nih.gov/info/Regenerative_Medicine/2006Chapter2.htm

2. Showel MM, Levis M. Advances in treating acute myeloid leukemia. F1000Prime Rep [Internet]. 2014 Oct 1 [cited 2018 Oct 18];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4191225/

3. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. New England Journal of Medicine. 2015 Sep 17;373(12):1136–52.

4. Curran E, Corrales L, Kline J. Targeting the Innate Immune System as Immunotherapy for Acute Myeloid Leukemia. Front Oncol [Internet]. 2015 Apr 9 [cited 2018 Oct 18];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4391043/

5. Acute Myeloid Leukemia - Cancer Stat Facts [Internet]. [cited 2018 Dec 9]. Available from: https://seer.cancer.gov/statfacts/html/amyl.html

6. Oran B, Weisdorf DJ. Survival for older patients with acute myeloid leukemia: a population-based study. Haematologica. 2012 Dec;97(12):1916–24.

7. Vaccine therapy in hematologic malignancies | Blood Journal [Internet]. [cited 2018 Dec 9]. Available from: http://www.bloodjournal.org/content/131/24/2640.long?sso-checked=true

8. Rosenblatt J, Stone RM, Uhl L, Neuberg D, Joyce R, Levine JD, et al. Individualized vaccination of AML patients in remission is associated with induction of antileukemia immunity and prolonged remissions. Science Translational Medicine. 2016 Dec 7;8(368):368ra171-368ra171.

9. Moore JO, George SL, Dodge RK, Amrein PC, Powell BL, Kolitz JE, et al. Sequential multiagent chemotherapy is not superior to high-dose cytarabine alone as postremission intensification therapy for acute myeloid leukemia in adults under 60 years of age: Cancer and Leukemia Group B Study 9222. Blood. 2005 May 1;105(9):3420–7.

10. Koido S. Dendritic-Tumor Fusion Cell-Based Cancer Vaccines. Int J Mol Sci [Internet]. 2016 May 26 [cited 2018 Dec 9];17(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4926362/

11. Studies on the mechanism of polyethylene glycol-mediated cell fusion using fluorescent membrane and cytoplasmic probes. J Cell Biol. 1983 Jan 1;96(1):151–9.

12. Maroof A, Penny M, Kingston R, Murray C, Islam S, Bedford PA, et al. Interleukin-4 can induce interleukin-4 production in dendritic cells. Immunology. 2006 Feb;117(2):271–9.

13. Danis VA, Franic GM, Rathjen DA, Brooks PM. Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, interferon-gamma (IFN-gamma), tumour

necrosis factor-alpha (TNF-alpha) and IL-6 on the production of immunoreactive IL-1 and TNF-alpha by human monocytes. Clin Exp Immunol. 1991 Jul;85(1):143–50.

14. Sallusto F, Antonio L. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 1994 Apr 1;179(4):1109–18.

15. Chen Y-L, Chang S, Chen T-T, Lee C-K. Efficient Generation of Plasmacytoid Dendritic Cell from Common Lymphoid Progenitors by Flt3 Ligand. PLoS One [Internet]. 2015 Aug 11 [cited 2020 Mar 27];10(8). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4532451/

16. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid Dendritic Cells: Recent Progress and Open Questions. Annu Rev Immunol. 2011;29:163–83.

17. Hassan M, Ulezko Antonova A, Li JM, Hosoba S, Rupji M, Kowalski J, et al. Flt3L Treatment of Bone Marrow Donors Increases Graft Plasmacytoid Dendritic Cell Content and Improves Allogeneic Transplantation Outcomes. Biol Blood Marrow Transplant. 2019;25(6):1075–84.

18. Petersen CT, Li J-M, Waller EK. Administration of a vasoactive intestinal peptide antagonist enhances the autologous anti-leukemia T cell response in murine models of acute leukemia. OncoImmunology. 2017 May 4;6(5):e1304336.

19. Delgado M, Ganea D. Vasoactive intestinal peptide: a neuropeptide with pleiotropic immune functions. Amino Acids [Internet]. 2013 Jul [cited 2020 Apr 4];45(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3883350/

20. Li J-M, Southerland L, Hossain MS, Giver CR, Wang Y, Darlak K, et al. Absence of Vasoactive intestinal peptide expression in hematopoietic cells enhances Th1 polarization and anti-viral immunity in mice. J Immunol. 2011 Jul 15;187(2):1057–65.

21. Li J-M, Petersen CT, Li J-X, Panjwani R, Chandra DJ, Giver CR, et al. Modulation of Immune Checkpoints and Graft-versus-Leukemia in Allogeneic Transplants by Antagonizing Vasoactive Intestinal Peptide Signaling. Cancer Research. 2016 Dec 1;76(23):6802–15.

22. Chorny A, Gonzalez-Rey E, Fernandez-Martin A, Ganea D, Delgado M. Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response. Blood. 2006 May 1;107(9):3787–94.

23. Li J-M, Hossain MS, Southerland L, Waller EK. Pharmacological inhibition of VIP signaling enhances antiviral immunity and improves survival in murine cytomegalovirus-infected allogeneic bone marrow transplant recipients. Blood. 2013 Mar 21;121(12):2347–51.

24. Li J-M, Darlak KA, Southerland L, Hossain MS, Jaye DL, Josephson CD, et al. VIPhyb, an Antagonist of Vasoactive Intestinal Peptide Receptor, Enhances Cellular Antiviral Immunity in Murine Cytomegalovirus Infected Mice. PLoS One [Internet]. 2013 May 27 [cited 2020 Apr 4];8(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3664580/

25. Gong J. Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. Blood. 2002 Apr 1;99(7):2512–7.

26. Feinstone SM, Hu DJ, Major ME. Prospects for Prophylactic and Therapeutic Vaccines Against Hepatitis C Virus. Clin Infect Dis. 2012 Jul 15;55(suppl_1):S25–32.

27. Tsapogas P, Swee LK, Nusser A, Nuber N, Kreuzaler M, Capoferri G, et al. In vivo evidence for an instructive role of fms-like tyrosine kinase-3 (FLT3) ligand in hematopoietic development. Haematologica. 2014 Apr;99(4):638–46.

28. Frontiers | What Makes a pDC: Recent Advances in Understanding Plasmacytoid DC Development and Heterogeneity | Immunology [Internet]. [cited 2020 Mar 29]. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01222/full

29. Haegel-Kronenberger H, Salle H de la, Bohbot A, Oberling F, Cazenave J-P, Hanau D. Adhesive and/or Signaling Functions of CD44 Isoforms in Human Dendritic Cells. The Journal of Immunology. 1998 Oct 15;161(8):3902–11.

30. Schmidt SV, Nino-Castro AC, Schultze JL. Regulatory dendritic cells: there is more than just immune activation. Front Immunol [Internet]. 2012 Sep 4 [cited 2020 Mar 29];3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3432880/

31. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 Ligand Regulates Dendritic Cell Development from Flt3+ Lymphoid and Myeloid-committed Progenitors to Flt3+ Dendritic Cells In Vivo. J Exp Med. 2003 Jul 21;198(2):305–13.