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CHARACTERIZATION OF JARID1B EXPRESSION DURING CD8 T CELL DIFFERENTIATION

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ABSTRACT

The overall goal of this project was to elucidate the role of the Jarid1b histone demethylase in the maturation and differentiation of CD8+ T cells. It is known that Jarid1b downregulates the oncogenic miR-17-92 cluster, which in turn drives terminal effector differentiation and compromises memory potential by promoting excessive proliferation during acute infection. ***Thus, our hypothesis was that Jarid1b is rapidly downregulated during effector differentiation to promote expansion of antigen-specific CD8+ T cells, but it is preferentially expressed in memory-fated CD8+ T cell subsets to promote memory function and longevity.*** The first aim of the experiment was to establish a western blot protocol to detect murine Jarid1b. Bone marrow cells were selected for this initial step as they are enriched in stem cells that are known to express higher levels of Jarid1b. The next aim of this study was to assess expression levels of Jarid1b in mature CD8+ T cells at the naïve, effector, and memory differentiation states. After establishing these levels, the subsequent steps involved creating retroviral constructs that possessed the ability to knock down the JARID1B gene through RNA interference. The most efficient of these constructs would be selected and used to transfect lymphocytes to knock down the JARID1B gene, inject these lymphocytes into P14 mouse models and observe their immunological fates following stimulation with LCMV. Western blot analysis revealed that Jarid1b was downregulated at day 2 of splenocyte maturation and continued through to day 8 of maturation, where the downregulation was most pronounced. Flow cytometry was run to characterize the splenocytes and determine their true state of immunological development. Following the preparation of five Jarid1b-knockdown retroviral constructs, western blot analysis was used to determine that construct #2 most effectively knocked down Jarid1b and was selected for use in the transfection of the 293T cell line. Future studies which will involve *in vivo*

knockdown of Jarid1b in antigen-specific CD8 T cells to determine the functional significance of Jarid1b during effector and memory CD8 T cell differentiation.

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Chapter 1

Introduction

CD8+ T cell differentiation. Following the presentation of a foreign antigen to a CD8+ (cytotoxic) T cell in lymphoid organs, as well as interaction with proper costimulatory molecules (such as CD28) and stimulation of cytokine receptors (i.e. the receptor for IL-12), mass division and expansion occurs. This, in turn, generates a functional population of effector T cells that serve to clear the antigen. But shortly after this expansion process, a vast majority of these cells (upwards of 90%) undergo death by apoptosis, and the small population of T cells that remain form the memory T cell population (Obar & Lefrancois, 2011). Following this transformation, interaction with cytokines IL-7 and IL-15 are required for persistence of the memory T cells, which will then become central memory cells or effector memory cells, depending on their expression of CD62L (also called L-selectin) and chemokine CCR7 (Kalia, Sarkar, & Ahmed, 2010). The transformation from naïve T cell to effector to memory is a complicated process that relies on a number of crucial checkpoints that alter cellular transcription.

Controlling cellular transcription. Transcription of the cellular genome is staggeringly complex and controlled at a number of different levels. Transcriptional control can include the principle of an inducible system (constitutively inactive unless an inducer is present) or repressible system (constitutively active unless a repressor is present). The *lac* operon is the classic example of these phenomena. There are general transcription factors that place polymerases on certain promoter regions of DNA to promote gene expression, and contrarily there are silencer regions of DNA at which other transcription factors prevent expression. But the

chromatin of the nucleus can also be remodeled in order to alter patterns of gene expression, and under this heading falls the modification of the histones around which DNA is wound.

Histone modification. Altering the histone in turn alters the ability of transcription factors to bind and initiate genomic transcription. Histone demethylases are responsible for performing these actions by removing methyl, dimethyl, or even trimethyl groups from particular amino acid residues on designated histones. When a histone demethylase recognized a certain amino acid residue, it recruits cofactors (for example FAD) and catalyzes the demethylation reaction (Pedersen & Helin, 2010). A crucial step in regulation of transcription is the methylation of the fourth lysine residue of Histone H3 (H3K4) (Dey et al., 2008). Methylation of this lysine has been shown to regulate the binding of RNA polymerase to active genes. The loss of the methyl group plays a role in cellular differentiation. It was determined that the JARID1 family of histone demethylases (also called the lysine (K) demethylase 5, or KDM5, family) is responsible for the developmental changes stemming from the loss of the methyl group on H3K4. Two members of this family, Jarid1a and Jarid1b (also known as *KDM5a* and *KDM5b*, respectively), were deemed to be particularly crucial to events in the cell cycle (Dey et al., 2008).

The Jarid1b histone demethylase. Jarid1b was determined to have an N-terminal JmjN domain, an ARID domain (involved in protein–DNA interactions), a PHD domain, a JmjC domain (which contain the catalytic pocket required for demethylation), a zinc finger (involved in protein–protein interactions), and 2 C-terminal PHD domains. The Jmj (jumanji) domains are most critical for the demethylation function of the protein product (Xiang et al., 2007). The expression of this gene in normal adult body tissue is restricted but was found to be more highly expressed in breast cancer and prostate cancer (particularly so if the prostate cancer was metastatic) (Xiang et al., 2007). With respect to the prostate, it was found that Jarid1b regulates transcription of cellular Androgen Receptor that has a critical role in the development of prostate cancer (Xiang et al., 2007). Jarid1b was shown to be highly expressed in some melanoma cells in

of late-stage tumors, and similar results were observed in cultured cell lines from melanoma tumors (Roesch et al., 2011). A link between the expression of Jarid1b and slow-proliferation was suggested by the fact that Jarid1b-positive cells lacked a substantial marker of proliferation, ki-67 (Roesch et al., 2011). Subsequent experimentation was performed to corroborate this potential relationship to observe the retention of dye in cellular membranes and determine division rates of cells that highly expressed Jarid1b. It appeared that even in quickly-dividing, highly proliferative melanomas, the most consistent member of the jumonji family of proteins associated with subgroups of cells that were slow to divide was Jarid1b. Moreover, this sub-population of slowly-dividing cells among the population of melanoma cells could be observed both *in vitro* and *in vivo* (Roesch et al., 2011). Knockdown of Jarid1b (*in vitro*) in three different populations of melanoma cells did not eliminate the initiation of proliferation, but 10 days after the knockdown, the previously-increasing proliferation gradually leveled off (Roesch et al., 2011).

Jarid1b and the miR-17-92 cluster. Of particular interest is Jarid1b's relationship with the miRNA cluster miR-17-92. In order for the body to properly generate terminal blood cells, it depends upon transcription factors and microRNAs as regulators of the process. MicroRNAs can be individually coded, or instead can be sequenced as a cluster of RNAs that are expressed from a single transcriptional unit (a polycistron) (Pospisil et al., 2011). The polycistronic miR-17-92 cluster encodes seven different microRNAs that serve as regulatory elements for proliferation, apoptosis, and cellular development. Upon cellular differentiation, the expression of this cluster is decreased. Through relatively recent experimentation, Jarid1b was shown to cause the repression of the cluster by demethylating H3K4 of the miR-17-92 promoter, rendering its gene as heterochromatin. It is recruited there by the early growth response protein 2 (Egr2) after is induction (Pospisil et al., 2011). The miR-17-92 cluster is oncogenic. Its expression causes proliferation of innate immune cells. Downregulation of the cluster is associated with the differentiation of innate immune cells into macrophages, and overexpression of the cluster

prevents this differentiation and instead induces mass proliferation of the macrophage precursor (Pospisil et al., 2011).

The association between expression of the miR-17-92 cluster and memory T-lymphocytes is less clear than the relationship with macrophage differentiation, however it has been shown that when the cluster's expression is downregulated in naïve T-lymphocytes, the subsequent maturation of the T-lymphocyte favors memory T cells over effector T cells (Khan, 2013).

The expression of Jarid1b and its implications. The primary purpose of the following experiment was to elucidate the relationship between Jarid1b and the differentiation of 5-10% of immune effector cells into memory T cells. ***The presented hypothesis is that Jarid1b is rapidly downregulated during effector differentiation to promote expansion of antigen-specific CD8+ T cells, but it is preferentially expressed in memory-fated CD8+ T cell subsets to promote memory function and longevity.*** To test this hypothesis, previously-constructed siRNA clones that serve to knock down the Jarid1b gene were grown in 293T cells used to produce Lymphocytic Choriomeningitis Virus (LCMV) vectors that bore the constructs. The 293T cell line – derived from the human embryonic kidney – were selected for the transfection because of their relative ease of growth and their high transfectability. Western blots were run to determine which of the constructs was most successful at knocking down the Jarid1b gene, and also to establish the relative levels of Jarid1b expression in mouse splenocytes.

The P14 mouse model. P14 mice are transgenic mice that bear CD8 T cells that are specific for the gp33 epitope of LCMV. These receptors are stimulated selectively and exclusively by this epitope (Pircher, 1989). This transgenic system provides a large number of CD8+ T cells of a single specificity for analysis. Thus the retrovirus was selected as the model system to deliver the siRNAs that would be used to silence the Jarid1b gene in P14 splenocytes and lymphocytes in this study.

Plans were made to select the retroviral constructs that best knocked down the Jarid1b gene, grow it out via 293T culture, infect T-lymphocytes from P14 mice, and then inject those cells back into P14 models and observe their immunologic fate (that is, the final percentages of effector T cells and memory T cells in the mouse model). These steps are subject for future continuation of this research. Over the course of the experiment, Jarid1b was successfully identified via western blot in bone marrow lysate and, subsequently, was identified in naïve and memory splenocytes. It was also noted that in splenocyte maturation, Jarid1b was downregulated at the day 2 and day 8 stage.

Chapter 2

Materials and Methods

Splenocyte Isolation from P14/B6 Mice. After harvesting the spleens of P14 or B6 mice, each spleen was added to a tube of RPMI on ice. The spleen prep was then poured into a petri dish and cut into 2-3 pieces with a frosted glass slide. The frosted ends of the slide were used to extract the cells from the spleen, leaving behind the connective tissue (stroma). The same RPMI was added to the petri dishes. The liquids and their cell contents were returned to their original tubes and were centrifuged for 10 minutes at 1200 rpm at 4°C with brakes. Supernatant was poured off, and tubes were rubbed against a peg rack to suspend and dissociate the pellet. 1 mL of .83% ammonium chloride was added and vortexed to mix. The cells sat for 1 minute and RPMI was used to top off the tubes. The samples were again centrifuged for 10 minutes at 1200 rpm at 4°C with brakes. Supernatant was discarded again. Tubes were rubbed on a peg rack, and then 2 mL of RPMI was added to the tube and mixed.

Bone Marrow Isolation from B6 Mice. Legs were removed from B6 mice and the femurs were isolated. Other connective or muscular tissue was cleaned off. The ends of the femurs were cut off and a syringe with a 26 ½ gauge needle was used to wash the bone marrow from the femur's interior using a total volume of 5 mL of RPMI. The liquids and their cell contents were placed in 10 mL tubes and were centrifuged for 10 minutes at 1200 rpm at 4°C with brakes. Supernatant was poured off, and tubes were rubbed against a peg rack. 1 mL of .83% ammonium chloride was added and vortexed to mix. The cells sat for 1 minute and RPMI was used to top off the tubes. The samples were again centrifuged for 10 minutes at 1200 rpm at 4°C

with brakes. Supernatant was discarded again. Tubes were rubbed on a peg rack to break the pellet, and then 2 mL of RPMI was added to the tube and mixed.

Antibodies and Flow Cytometry. The following antibody markers were used in the flow cytometry analysis: CD8 (to identify T-cell populations), CD44 (which remains lowly expressed on naïve immune cells and increases upon their immunological activation by antigen, thus being an indicator of T-cell activation), CD127 (the expression of which rapidly decreases during infection and again rises during the formation of memory), KLRG-1 (the distinguishing marker for effector T cells used to identify memory precursor effector cells (MPECs) and short-lived effector cells (SLECs)), CD62L (the homing marker for secondary lymphoid tissue which decreases during the transition from naïve to effector and increases on lymphoid-resident memory cells during the effector to memory transition), CD11c (identifies myeloid cells), and B220 (for the identification of B-cells). No intracellular markers were used. Software used to analyze flow cytometric readouts was FlowJo V9.7.2 (Macintosh).

Western Blotting. Western blots were the primary mode of protein detection throughout the course of experimentation. All western blots were run on a Bio-Rad Mini-PROTEAN® Tetra Cell electrophoresis and transfer system. Primary antibody used for the detection of Jarid1b protein product on PVDF membrane was Bethyl JARID1B Antibody (rabbit anti-Jarid1b) (A301-813A) and secondary antibody was ABCAM goat anti-rabbit HRP. Primary antibody used for the detection of β-actin protein product on PVDF membrane was abcam Anti-β-actin antibody (ab8227). The DNA ladder used as a standard marker was the Bio-Rad Precision Plus Protein™ WesternC™ Standards. Sample volumes loaded into wells were based on a BSA standard-curve determined by spectrometry readout. Loaded samples were run on an electrophoresis gel (polyacrylamide, 4-15%) for 1-2 hours as was determined to be necessary. The gels were subsequently transferred to PVDF membrane that was first prepped with a 5-second soak in 100% methanol and a second soak in transfer buffer. Transfer was set to run for 2 hours at 80V. Post-

transfer membranes were blocked in 5% milk solution (1% BSA in the case of the blots with the actin control bands). Primary antibodies were administered at 4°C and allowed to sit overnight. In the case of blots with actin control bands, the membranes were cut to separate the Jarid1b anticipated zone from the actin anticipated zone. They were then incubated in their respective primary antibodies (1:5000). The membranes were then given five 5-minute washes of PBST and were soaked in solution with secondary antibody (1:50,000). Following another five 5-minute washes with PBST, the membranes were placed on a holder film and developed. Exposure time varied as was necessary for amount of protein in the samples and the determination of signals.

Retroviral construction. LCMV retroviral DNA containing the siRNAs that would be used to knock down the Jarid1b gene in P14 splenocytes and T-lymphocytes were previously prepared by colleague Jeongin Son. The protocol followed is outlined in the materials and methods component of Araki et al, 2009.

Transfection of the 293T cell line. To augment the initial population of siRNA-containing LCMV, the 293T cell line was transfected. Parts of this protocol were also performed on my behalf by colleague Jeongin Son. Frozen 293T cells were thawed in a 37°C water bath and were transferred to a tube containing 10 mL of culture medium. The tube was then centrifuged for 10 minutes at 12,000 RPM at 4°C.The supernatant was discarded and the pellet was broken by tapping. 2 mL of culture medium was then added and the contents of the tube were mixed by pipette. The pellet was then transferred to a culture dish with 10 mL of culture medium and incubated for three days. Cells were split 4x to 6x every 2-3 days when the culture reached 70-90% confluence. To split cells, the culture was washed with PBS and 2 mL of .5 Mm EDTA solution with .05% Tryp was added. It was then incubated at 37°C for 4 minutes. The dish was tapped, and 10 mL of culture medium was taken and set aside for a later wash. The dish surface was then washed with 4 mL of Tryp/EDTA solution three times. The cell suspension was mixed by pipette and transferred into the 10 mL of culture medium that had been set aside prior. The

cells were centrifuged for 10 minutes at 1,200 rpm. The supernatant was discarded, and the pellet was tapped to break. The cells were then resuspended in 15 mL of culture medium and counted. 2 x 10^6 cells were seeded in a T25 flask with antibiotic-free FCS and glutamine medium. After approximately 20 hours, transfection began at 75% confluence. Seven tubes were prepared for five clones, the positive control, and the negative control. In this order, each tube received 9 µL of the FuGENE reagent, 3µg of DNA, and 91 µL of SFM. Tubes were incubated for 30 minutes at room temperature. These mixtures were added to the seeded cells in a drop-wise fashion evenly throughout the dish. Cells were cultured for 26 hours and were subjected to fluorescent microscopic analysis.

Experimental Mice. C67BL/6 mice were purchased from the Jackson Laboratory and bred to continue the stock. These mice were fed on readily-available LabDiet 5058 PicoLab® Mouse Diet 20 ground pellets. All animals in this study were used in accordance with University Institutional Animal Care and Use Committee guidelines and were exclusively handled by colleague Yevgeniy Yuzefpolskiy. Mice were euthanized via exposure to CO2 gas and, immediately thereafter, cervical dislocation.

Chapter 3

Results

Characterization of Jarid1b expression in bone marrow cells. The first stage of the experiment – to ensure efficacy of Jarid1b detection – was largely successful. Bone marrow was then isolated from two B6 mice and run through flow cytometry analysis to determine its constituent cells. Bone marrow was selected for this characterization stain as it is the center of hematopoiesis, giving rise to the immune cells of the body and would provide ample amounts of the protein of interest across an array of immune cells. Figure 1A shows the percentage of B220+ cells (B cells) in the marrow sample (approximately 35%, slightly over 10^7 cells) and the percentage of CD11c+ cells (monocytes/macrophages/neutrophils) in the sample (approximately 1.5%). The frequencies of CD3+ CD8+ cells (cytotoxic T cells) and CD3+ CD8- cells (T-helper cells) are presented in Figure 1B at approximately .5% and .9% respectively. 78.5% of cytotoxic T cells were activated, and 83% of T-helper cells were activated (Figures 1C and 1D, respectively) as determined by high expression of CD44.

Jarid1b detection in bone marrow lysate via western blot. Using frozen bone marrow preparations from B6 mice (previously isolated by a lab colleague) the western blot that was run clearly returned bands for the JARID1B protein product (and its JARID1A protein product counterpart, as the antibodies were polyclonal and cross-activation occurred) (Figure 2B). A diagnostic Jarid1b band has a size of 180-190 kD. When carrying out the blot, volumes were calculated such that approximately 500,000 cells were loaded into the well for Jarid1b detection

in the bone marrow sample. Using the generated standard curve, 20 µL were used in the same well to achieve the desired level of testable protein.

Analysis of CD8+ T cell differentiation from naïve to effector to memory cells, via flow cytometry. The second portion of the experiment, concerning Jarid1b expression in splenocytes at varying stages of immunological activity, involved flow-cytometric analysis of the constituents of splenocytes from B6 and P14 mice as well as western blots to detect JARID1B gene product. In the case of each splenocyte type (naïve, day 2, day 8, and mature), the splenocytes of only one mouse were analyzed. CD8+ populations were examined in the isolated splenocytes, and the size of this population was (expectedly) observed to be smallest (13.3% of live cells) in naïve splenocytes, largest in day 8 splenocytes (35% of live cells), and lingered at 9.14% of live cells in memory splenocyte samples (Figure 3A). Gated on these populations of CD8+ cells, the analysis then divided these cells into those that were CD44-low (in the case of the naïve splenocytes) and CD44-high (in the case of day 2, day 8, and memory splenocytes). The size of this population grew as time passed after initial infection and lingered at 52% in memory splenocytes (Figure 3A and 3B). From these CD8+ CD44 populations, the frequency of CD127, KLRG-1, and CD62L were established (Figure 3A). As expected, CD127+ cell populations decreased rapidly following infection and were lowest in day 8 splenocytes. Memory splenocytes showed the re-establishment of this population. The KLRG-1 population, the diagnostic marker for effector T cells, was virtually nonexistent in any splenocyte prep except for those day 8 splenocytes. CD62L populations decreased significantly at day 8 post-infection, but were reestablished in memory splenocytes. These patters observed in naïve, effector, and memory cells are consistent with expression patterns historically reported in the field of immunology. Thus this has been a successful validation of the CD8+ T cell differentiation states that were used in subsequent experiments regarding stage-specific Jarid1b expression patterns.

Analysis of Jarid1b expression at distinct stages of CD8+ T cell differentiation. A number of western blots were performed to determine the degree of Jarid1b expression during the various immunological stages of splenocyte function. The initial blot performed sought to identify Jarid1b in naïve, day 2, and memory splenocytes (Figure 4A). The β-actin "housekeeping" gene was used as a control marker. From the resulting blot, it appeared that Jarid1b was downregulated at around day 2 of splenocyte maturation post-infection with LCMV. A subsequent blot was attempted, including the day 8 stage as well as the previously blotted stages. However, despite a number of attempts, all blots either returned no bands, or returned inconclusive bands (images of these films not included in the manuscript). A subsequent blot (performed by colleague Jeongin Son) returned proper output bands (Figure 4B). From this blot, it appeared that the downregulation of Jarid1b gene product observed on day 2 of splenocyte maturation continued and was more pronounced on day 8 of maturation. However the memory splenocytes demonstrated levels of Jarid1b comparable to the naïve splenocytes that did not encounter infection with LCMV (Figure 4B).

Growing Jarid1b knockdown retrovirus stock. The third component of the experiment was to create a retrovirus that contained a knockdown sequence (via siRNAs) to cause a loss of JARID1B gene function and then use the retroviral construct to infect a 293T cell line and generate lymphocytes without functional Jarid1b. The retroviral construct was transfected into 293T cells using the pCL-Eco plasmid that contained the gag, pol, and env genes required to properly construct the LCMV retrovirus. Five retroviral constructs were produced, used to infect P14 effector cells and a western blot was performed to determine which of the five constructs most successfully knocked down JARID1B gene product in these cells. Construct #2 of 5 was deemed to be the most efficient at knocking down Jarid1b and was selected for use in the experiment.

Figure 5A shows the flow cytometry results (GFP-fluorescing cells) from the negative (no transfection) cell group, the GFP control (transfection with GFP, but not the Jarid1b knockdown), and the true transfection with knockdown construct #2. These three transfection groups were attempted once and had no replicates. It was determined that transfection with construct #2 was approximately 40% efficient. Visual data of GFP fluorescence of these three cell populations was also collected (Figure 5B).

Chapter 4

Discussion

From the cytometric analysis and the blot films returned from this experiment, it would appear that Jarid1b is downregulated as early as day 2 and as late as day 8 in the maturation of splenocytes post-infection. Given that the polycistronic miR-17-92 cluster is repressed by Jarid1b histone demethylase, it would make logical sense that Jarid1b is downregulated at this time to stop the repression of miR-17-92, thereby allowing for cellular differentiation and proliferation that goes hand-in-hand with immunologic maturation. From the bone marrow blotting, it would seem that Jarid1b is constitutively activated to keep miR-17-92 in check until it is repressed by the need to form immunological memory. A previous study - in which overexpression of the miR-17-92 cluster in bone marrow was shown to lead to lymphomas that lack apoptitic properties of other established lymphomas – corroborates the notion that miR-17-92 must be strictly controlled in bone marrow constituent cells to prevent aberrant proliferation (He et al, 2005).

The possibility of red blood cell contamination in both the bone marrow lysate and the splenocyte lysate cannot be ignored when considering the data readout from the flow cytometer, as well as being a confounder in the construction of the protein standard curves required to properly perform the blotting. On several instances, following the bone marrow and splenocyte isolation protocols, the lysate appeared to have a reddish hue that could imply incomplete lysis of splenocytes. This, in turn, could have led to artificially high levels of protein in the lysate and potentially thrown off the standard curves that were calculated. It may also have contributed to the cross-reactivity of the polyclonal antibody, rendering very strong signals (i.e. the black squares that obscured the bands) even at very low exposure times.

Of course, the issue of replicates must also be addressed, as this study only provided one replicate in each stage of experimentation (with the sole exception of the bone marrow analysis, in which the bone marrow of two B6 mice was analyzed).The accuracy of the expression profiles and the accuracy of the transfection efficiency could be more thoroughly refined if sample sizes were larger to allow for substantially more replicates. While the data presented in this manuscript involve only a limited number of mice that does not make for a statistically significant sample size, repeats of these experimental procedures have been conducted by other members of the laboratory. Ongoing experiments are already underway in the laboratory to confirm the successful knockdown of Jarid1b in effector CD8+ T cells, which will be subsequently analyzed for effector and memory differentiation and function.

Chapter 5

Additional/Future Work

Following the tasks outlined in the previous chapters, the next experimental endeavor would have been to select the best retroviral construct (deemed to be #2), grow it out via another transfection of the 293T cell line, and use it to knock down Jarid1b in isolated P14 T cells. These T cells would then have been injected into P14 mice and their fates would have been observed. The resulting phenotypes would have given information as to the progression (or lack thereof) of immunologic memory in the absence of Jarid1b gene product.

In addition to the continuation of this loss-of-function experimentation, to acquire insight to the function of Jarid1b in both macrophage and T-lymphocyte development, a gain-of-function experiment should also be performed. This project would seek to induce the opposite phenomenon of the Jarid1b knockdown by upregulating or overexpressing the gene in P14 T-lymphocytes. Subsequent injection of the cells into P14 mice and observation of the immune fate would then follow. Presumably, the overexpression (or constitutive expression) of Jarid1b gene product would cause unrelenting repression of the miR-17-92 oncogenic cluster and a subsequent cascade of detrimental immunological events.

Beyond observing the fates of the P14 mice with non-functional Jarid1b gene product, the next logical step would have been to search for the stage at which development and maturation was halted (if indeed the processes were halted at all). It is plausible that splenocyte differentiation would be halted at day 8, given that the expression of Jarid1b again increases from its nadir sometime after this stage. Theoretically, without a second spike in Jarid1b to repress the oncogenic miR-17-92 cluster, cellular division would run unchecked and unhindered. At the same

time, it is not unreasonable to hypothesize that naïve splenocytes would function properly in the absence of Jarid1b (indeed, there might not be a solid naïve splenocyte population, as higher levels of Jarid1b are found in the naïve cells).It would also be prudent to examine the effects of Jarid1b-knockdown in other types of immune cells (B cells, granulocytes, etc.), as well as cancerous cell lines that correlate with the conditions and diseases listed in the first chapter to which Jarid1b was found to be related. Unless the mechanism of differentiation is, at its core, fundamentally different in B cells or granulocytes, it would be logical to assume that a Jarid1b knockdown would render mass proliferation of these other cell types and prevent differentiation past an effector stage.

Appendix A

Figures

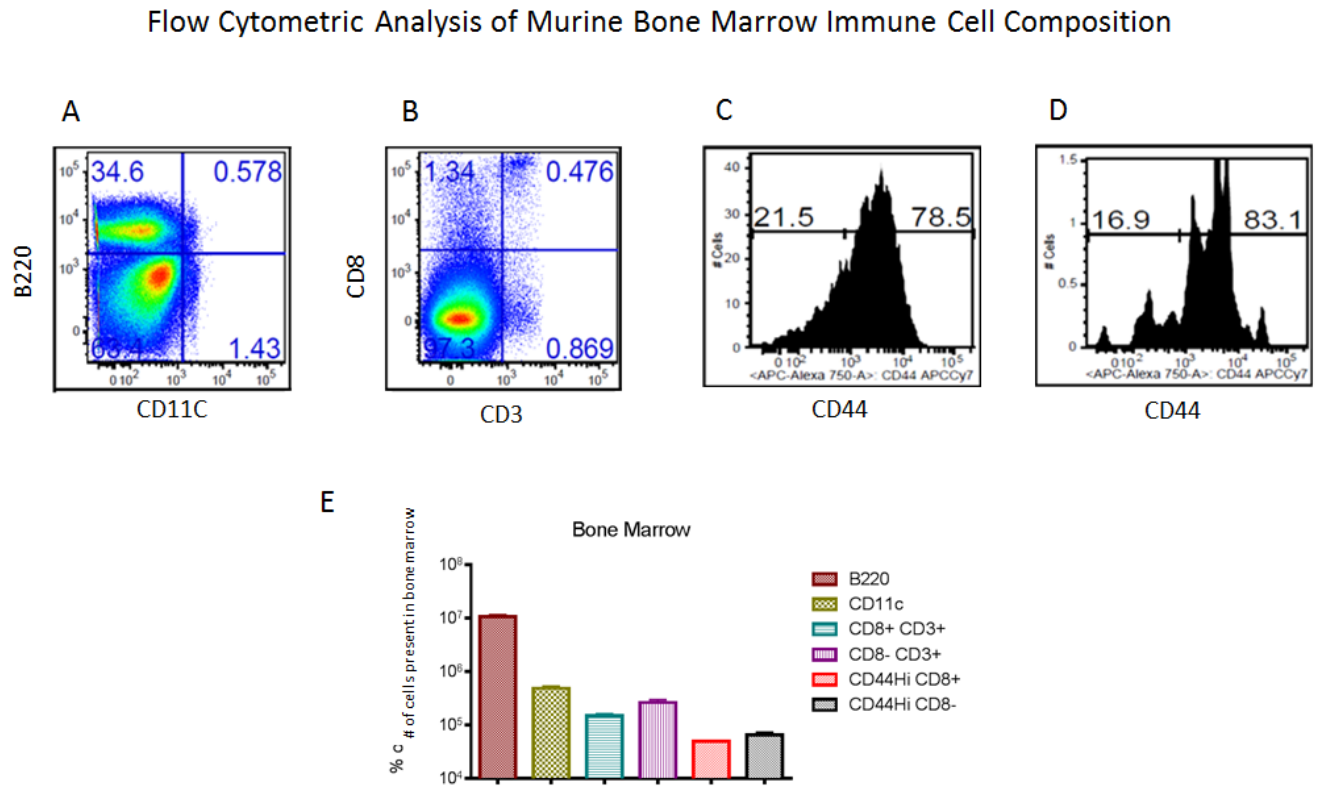
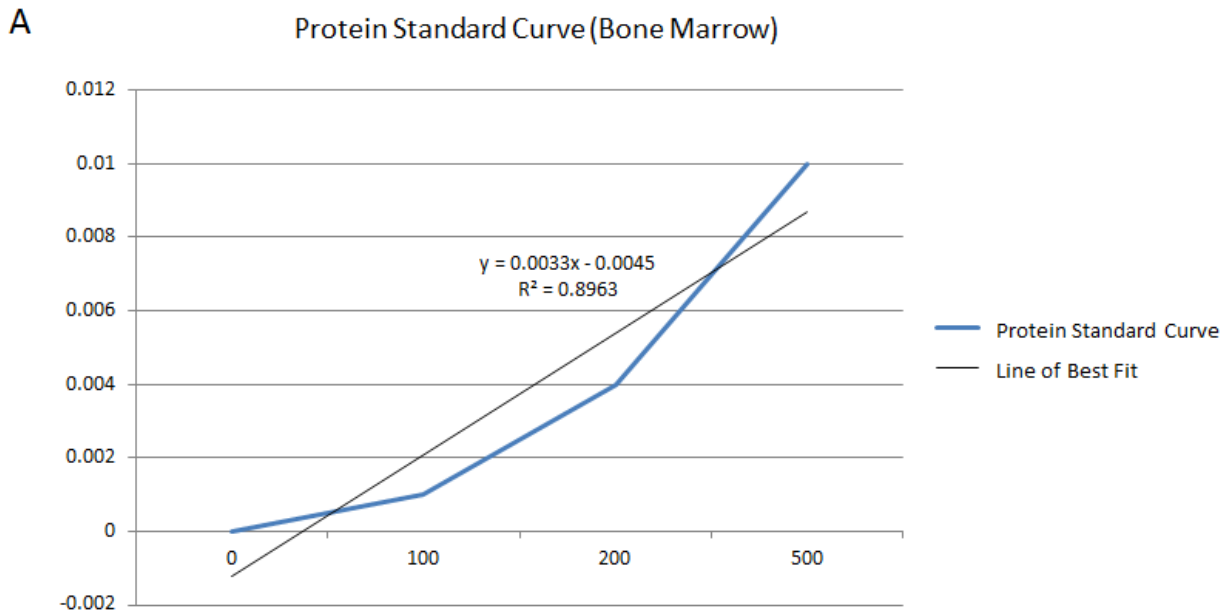


FIGURE 1: **(A)** Flow cytometry output for the bone marrow of one B6 mouse. B220 Markers are plotted against CD11c. **(B)** Flow cytometry output from the same B6 mouse where CD8+ populations are plotted against CD3+. **(C)** Gated on the CD3+ CD8+ population from 3B, a histogram demonstrating the proportion of CD44+ (activated) cells in the population. **(D)** Gated on the CD3+ CD8- population from 3B, a histogram demonstrating the proportion of CD44+ (activated) cells in the population. **(E)** Frequency histogram of cell populations in both B6 bone marrow samples (with error shown).

Detection of Jarid1b Protein Expression in Bone Marrow Cells Using Western Blot

A



B

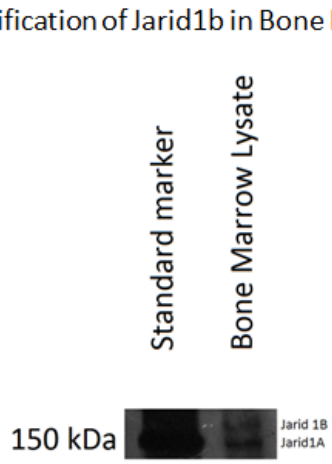


FIGURE 2: (A) The protein standard curve calculated from the subjection of bone marrow isolate samples to spectrometric analysis. The curve was used to determine proper volumes for use in the subsequent western blot **(B)**. Western blot, demonstrating that Jarid1b histone demethylase (as well as its counterpart Jarid1a) were detected in the bone marrow. Exposure time 1 minute.

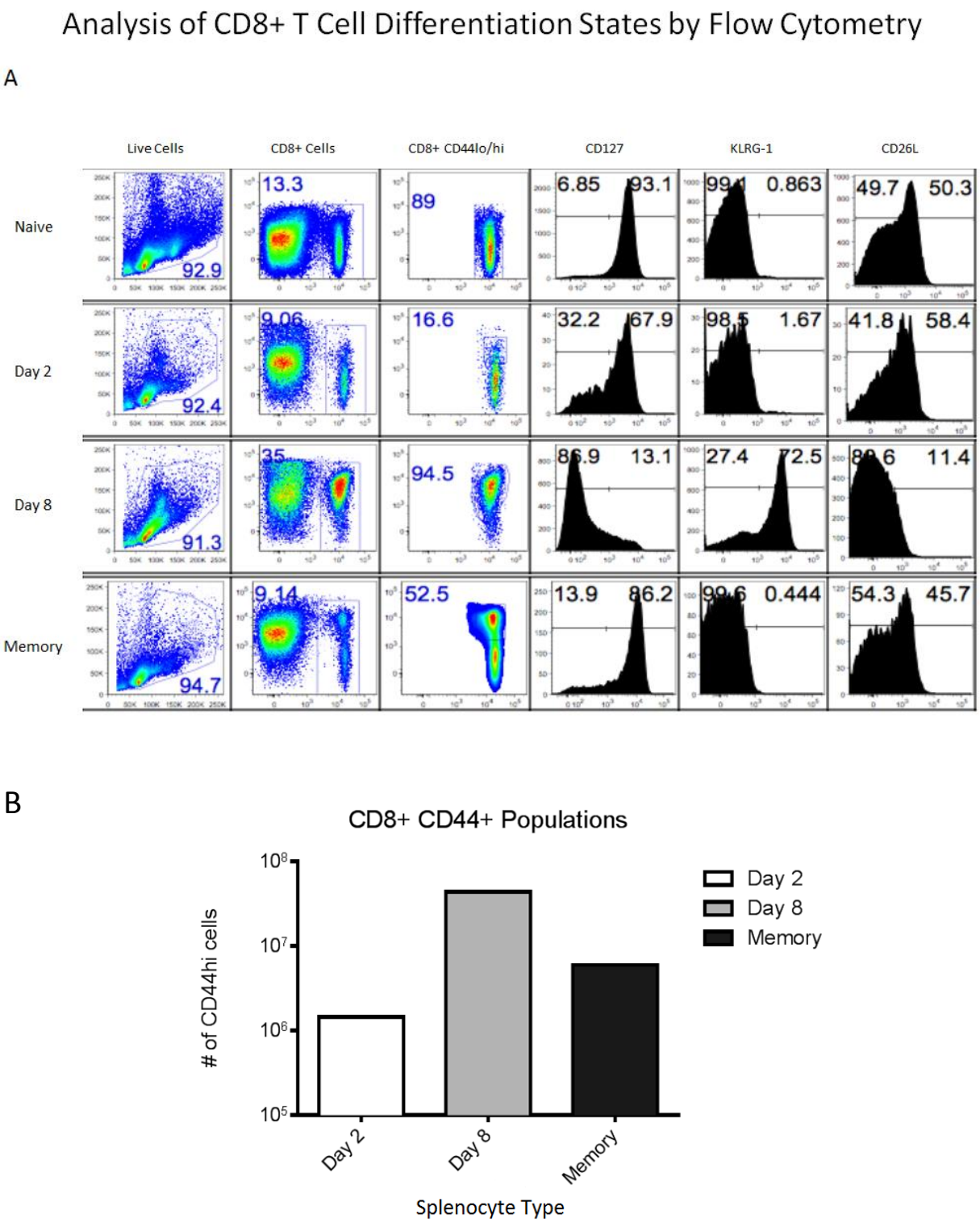


Figure 3: (A) Flow cytometry data to verify the Jarid1b expression profiles. Populations of Naïve, Day 2, Day 8, and memory splenocytes were analyzed first by selecting for CD8+ cells. The gated CD8+ populations were then used to determine the amount of CD44-high (or in the case of naïve splenocytes CD44-low). From this population, frequencies of CD127+, KLRG-1+, and CD62L+ cells were determined and displayed in histograms to verify the formation of memory cells, the presence of effector T-cells, and secondary lymphoid tissue, respectively. **(B)** The number of CD44-high cells from the splenocyte samples. CD44 was expected to rise from Day 2 to Day 8 splenocytes and then persist in memory cells.

Jarid1b Expression Profile in Naïve, Effector, and Memory CD8+ T Cells

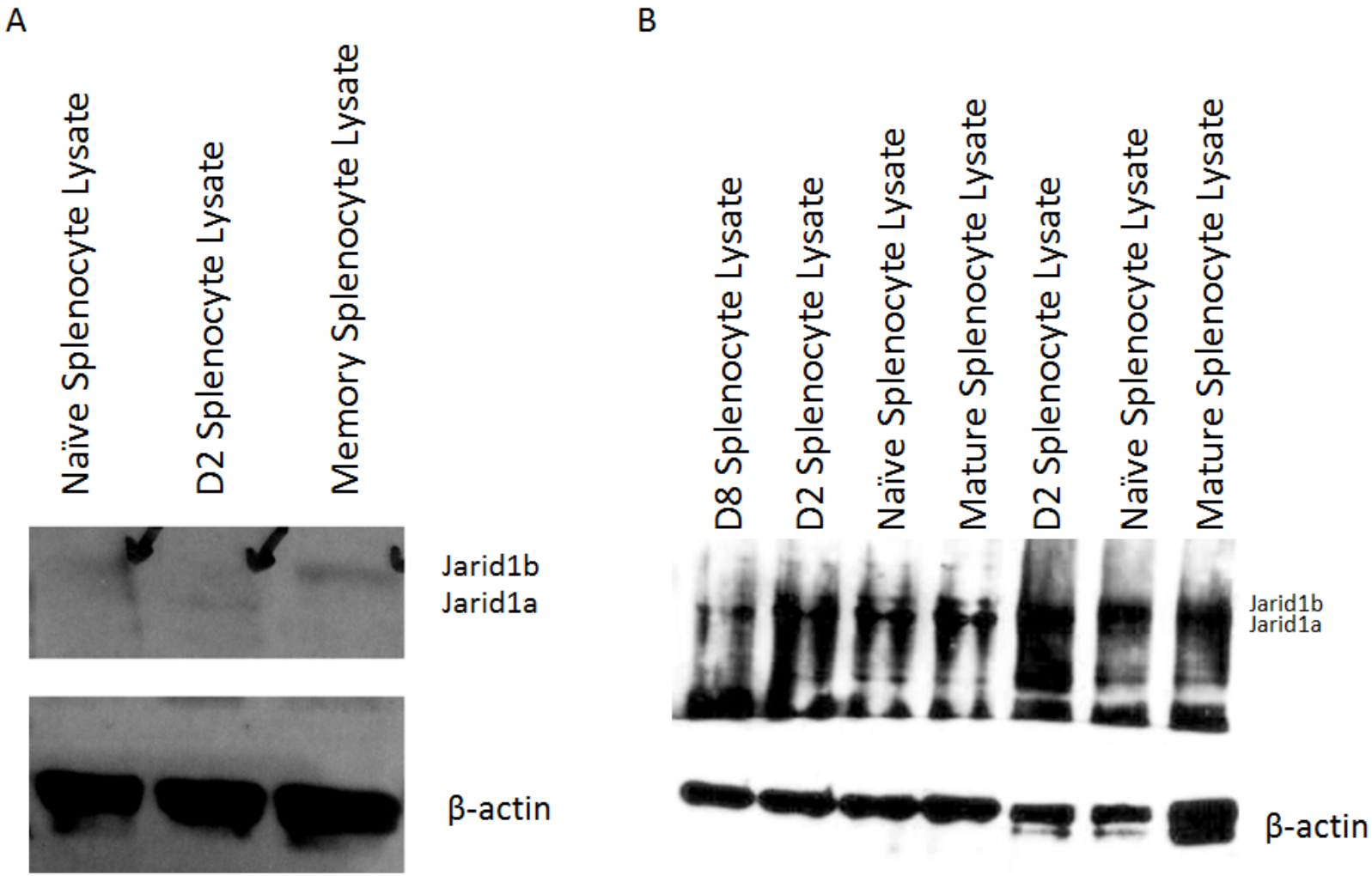
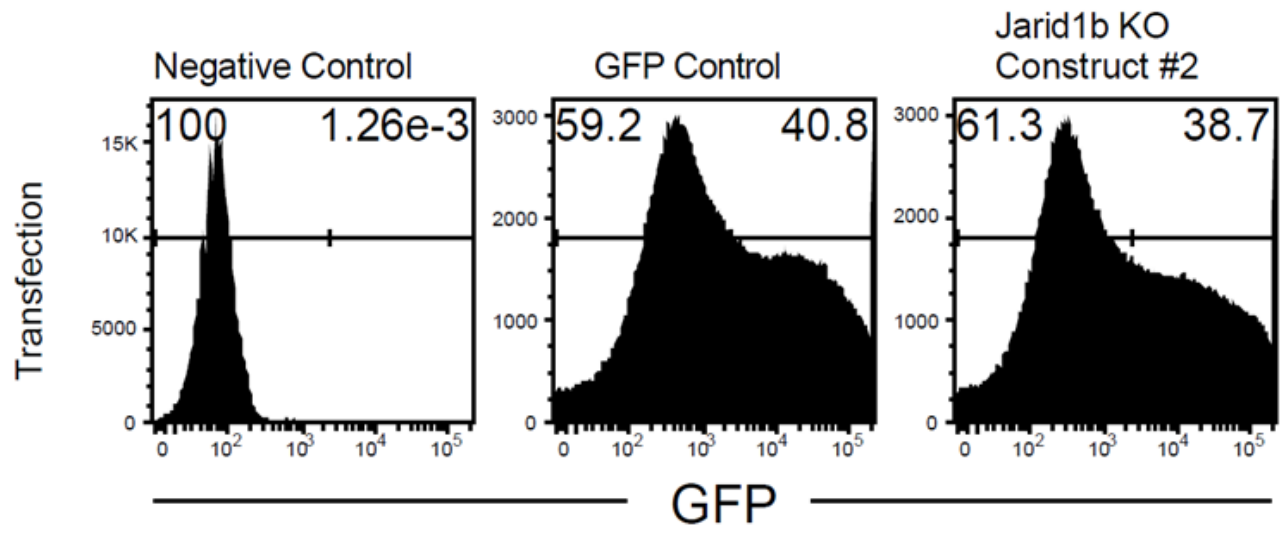


Figure 4: (A) Western blot showing Jarid1b (and its counterpart, Jarid1a) at different stages of splenocyte maturation. β-actin Was used as the constitutively-expressed "housekeeping" gene for comparison. Exposure time 4 minutes. **(B)** Subsequent western blot, run by colleague Jeongin Son. This clearer representation of the initial results also includes the Jarid1b identification for day 8 splenocytes. Exposure time 1 minute.

Analysis of Transfection Efficiencies during Retrovirus Production

A



B

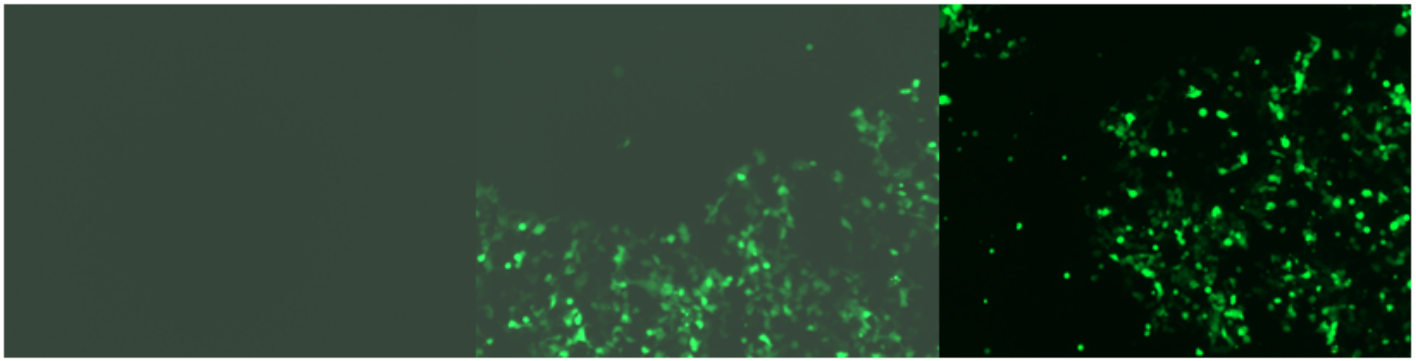


Figure 5: (A) Histogram representation of the negative and GFP controls for the transfection of P293 cells with LCMV containing the Jarid1b KO sequences from the 2nd construct. Transfection with the construct showed an approximate success rate of 40%. **(B)** Visible fluorescence from the GFP control and the Jarid1b KO construct #2 transfection.

Appendix B

List of Acronyms

CD3 – antigen expressed on thymocytes and T cells that denotes the presence of the T-cell receptor.

CD4 – antigen that identifies thymocyte subsets T_H1 and T_H2, as well as monocytes and macrophages.

CD8 – antigen that identifies thymocyte subsets cytotoxic T cells.

CD28 – antigen that serves in the activation of naïve T cells and as a co-stimulator for memory T cells.

CD44 – antigen present on leukocytes and erythrocytes which mediates leukocyte adhesion.

CD62L – antigen present on B-cells, T cells, monocytes, and NK cells that mediates rolling interactions with endothelium.

CD127 – antigen that characterized bone marrow lymphoid precursors, pro-B cells, mature T cells, and monocytes. It is an IL-7 receptor.

KLRG-1 – surface antigen that is upregulated on effector T cells.

LCMV – Lymphocytic Choriomeningitis Virus, member of family Arenaviridae.

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