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The Role of the Glucocorticoid Receptor in $CD8^+$ T cells

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An abstract of a thesis submitted to the Faculty of Emory College of Arts and Sciences of Emory University in partial fulfillment of the requirements of the degree of Bachelor of Sciences with Honors

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Abstract

The Role of the Glucocorticoid Receptor in $CD8^+$ T cells

By Emma Giarracco

Exogenous glucocorticoid (corticosterone) administration is a common therapeutic in cancer patients due to its ability to reduce inflammation and manage pain. Commonly dexamethasone—which reduces overall inflammation by lowering the activity of the immune system and slowing the body's response to disease or injury— is used in combination with a wide variety of cancer treatments to help mitigate the side effects of cancer or its treatment. Immunology-based cancer treatments rely on immune activation and re-functionalization to respond to cancer and other chronic infections. It is predicted that steroid administration has a negative impact on the efficacy of immunology-based cancer treatments. Glucocorticoids can be examined using the murine lymphocytic choriomeningitis virus (LCMV) model. Previous studies (Acharya et al., 2020) indicate that glucocorticoids —when acting through the glucocorticoid receptor (GR) —modulate immune function and the immune response. Their effect ranges from cytokine suppression, alteration of thymocyte development, to immunosuppression. GR causes transcriptional changes via direct DNA binding, and in turn, controls otherwise lethal immune activation. In this research the role of GR in immune activation is studied through the generation of GR T cell knockout (KO) mice, allowing us to study the role of GR in only activated $CD8^+$ T cells. We find that the Cluster of Differentiation 8 ($CD8^+$) T cell glucocorticoid receptor (GR) is required for survival of the chronic (clone 13) LCMV infection. These data also show increased interferon-γ (IFN-γ) levels in P14 KO mice after chronic viral infection in the clone 13 strain of LCMV. Through P14 LCMV T Cell Receptor (TCR) transgenic mice and congenic markers we can characterize the GR signaling pathway in $CD8^+$ T cells throughout the course of chronic infection, giving better insight into the interaction between GR and immunotherapy and improving the efficacy of future treatments dependent on immune activation.

The Role of the Glucocorticoid Receptor in $CD8^+$ T cells

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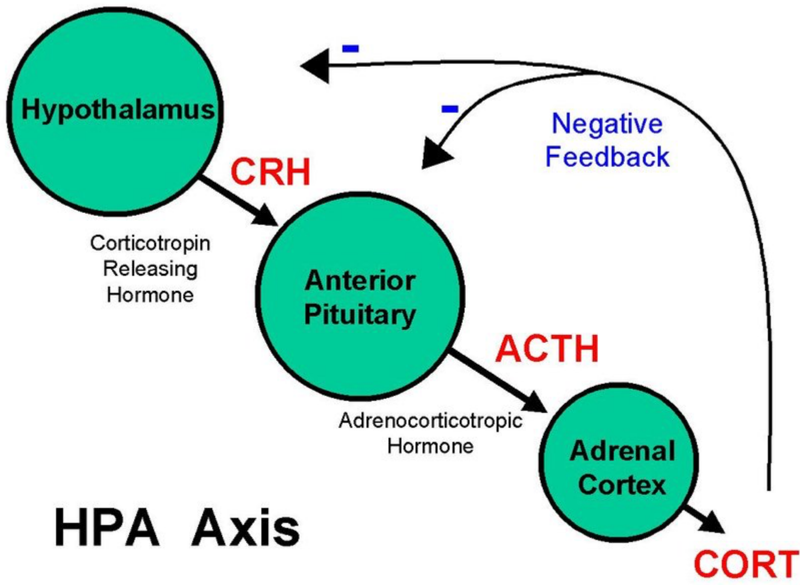
Introduction

Immunology-based cancer treatments have revolutionized the field of oncology, generating hope for patients with no existing treatment options and improving outcomes for a wide variety of cancer types. Immunotherapy is unique in that it targets host immune cells to manipulate their function and re-energize a dysfunctional immune response. This targeting is dependent on immune activation and the generation of inflammation in response to an injury or infection. Steroid hormones are often administered to patients to help cope with fatigue and pain, improve appetite, and reduce systemic inflammation or other side effects of chronic inflammation or treatment for cancer. Steroid hormones are lipophilic, squalene derivatives with a characteristic 4 ring structure that allows them to directly cross cell membranes. They vary by the functional groups attached to the 4-ring core and the oxidation state of the rings. There are five types of steroid hormones: mineralocorticoids, androgens, progestogens, estrogens, and glucocorticoids. Glucocorticoids (GCs) are a specific type of steroid hormone which was discovered with the isolation of steroidal compounds from adrenal extracts by Edward Calvin Kendall in 1946 (Simoni et al., 2002). Their therapeutic potential was unlocked through successful anti-inflammatory treatment of a patient with rheumatoid arthritis (Hench et al., 1949). GCs are part of a class of intracellular nuclear receptors that bind directly to DNA to upregulate their transcription. GR causes both transcriptional inductions of anti-inflammatory protein-coding genes and repression of pro-inflammatory transcription factors.

Glucocorticoids are expressed not only in the immune system but also the endocrine system as the biological response to stress. Glucocorticoids are synthesized in the adrenal cortex, and they control metabolism in muscle, adipose, liver and bone. They affect the circadian rhythm and sleep-wakefulness cycle and are regulated in a diurnal rhythm and peak at the onset of

waking. Glucocorticoids are crucial for mobilizing energy stores. Glucocorticoids include cortisol which is controlled via the hypothalamic-pituitary-adrenal (HPA) axis. Cortisol regulation and its function is vital for life as it controls regulation of blood pressure, the immune system, maintains blood sugar by balancing insulin and helps the body respond to stress. The HPA axis begins with the release of corticotrophin-releasing hormone (CRH), which initiates the release of adrenocorticotrophin (ACTH). ACTH triggers the release of glucocorticoids which travel throughout the body and act on peripheral tissues. Glucocorticoids are regulated via negative feedback inhibition where circulating glucocorticoids mitigate continued CRH and ACTH secretion. The HPA pathway maintains homeostasis in acute stress by connecting the central nervous system and the endocrine system but can become dysregulated under conditions of acute stress caused by environmental factors or infection.

Figure 1: HPA axis (Xiao, 2015)



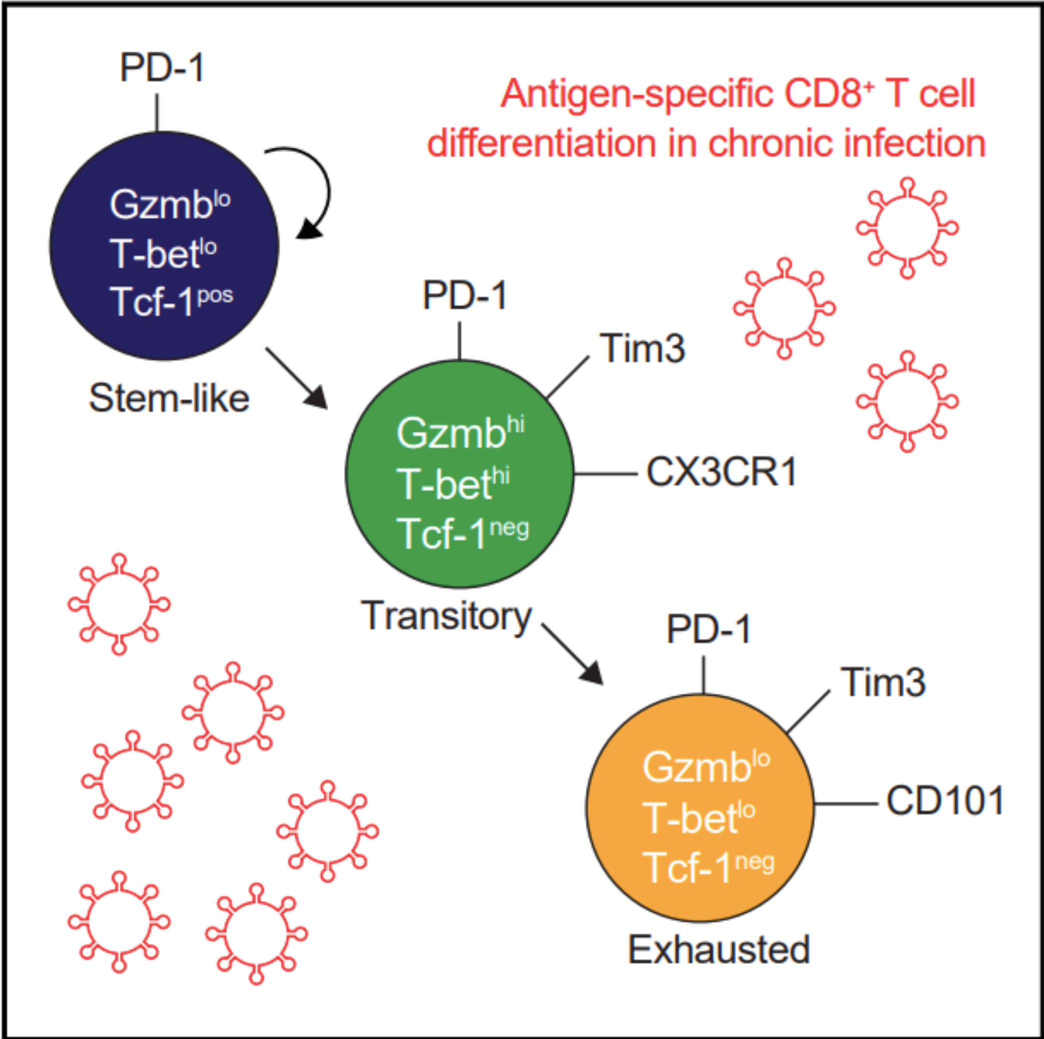
GR is expressed virtually in all human tissues and organs including CD8^{+} T cells, or Cytotoxic T Lymphocytes (CTLs), and CD4^{+} T cells, or helper T cells (Th). CD8^{+} T cells recognize peptides associated with Class I MHC (major histocompatibility complex) and CD4^{+} T cells recognize MHC II-associated peptides. Protein presentation and recognition on the MHC surface are dictated by appropriate hydrophobic/hydrophilic interactions between conserved amino acid side chains in the active site. Both use T cell receptors (TCRs) on their surface to recognize their cognate antigen and generate an appropriate immune response to foreign pathogens. Glucocorticoids affect the immune response of both CD4^{+} (helper) and CD8^{+} (cytotoxic) T cells in different ways. In helper T cells, glucocorticoids block cytokine production affecting B cell activation and reducing the number of antibodies released. In killer T cells, GR causes upregulation of Interleukin-10 (IL-10) which is a regulatory cytokine with a crucial role in the suppression of inflammatory processes (Adam et al., 2018). Coupled together, GR signaling serves to decrease the immune response.

The ingenuity of immunotherapy relies on its ability to target regulatory cell surface proteins on the surface of CD8^{+} T cells such as Programmed Cell Death-1 (PD-1) or its corresponding ligand PDL-1. Successful targeting of these cell proteins restores native immune function by returning CD8^{+} T cells to their effector state after they have become functionally 'exhausted' by persistent antigen stimulation during chronic infection. Successful inhibitors of PD-1/PD-L1 include peptides, small molecule chemical compounds, and by far most commonly antibodies (Guo et al., 2020). Side effects of checkpoint inhibitors are many and may include diarrhea, rashes, kidney infections, and/or pneumonitis (inflammation in the lungs) (American Cancer Society, 2019). Previous studies implicate GR in activating PD-L1 expression leading to decreased activity of cytotoxic T cells. (Deng, 2021). It is strongly predicted that immunotherapy

in the absence of additional exogenous corticosterone administration could improve the efficacy of treatment despite increased inflammation. This could prove a potentially effective treatment strategy for patients without comorbidities requiring continuous immunosuppression (in the case of autoimmune disease or organ transplant).

The pathway of $CD8^+$ T cell differentiation in chronic infection was initially explained by Se Jin Im et al. in *Nature* in 2016. Chronic infections such as cancer are associated with expression of the PD-1 inhibitory receptor as well as $CD8^+$ T cell dysfunction and eventual exhaustion. Im investigated virus specific $CD8^+$ T cells that proliferate after PD-1 blockade in the lymphocytic choriomeningitis virus (LCMV) murine model. These PD-1^+ $CD8^+$ T cells were characterized as "stem-like" due to their ability to self-renew and differentiate into terminally exhausted $CD8^+$ T cells. These cells were characterized by the presence of the T cell factor-1 (Tcf-1) transcription factor. This stem-like $CD8^+$ T cell population (PD-1^+ Tcf-1^+) was further characterized in Hudson et al., 2019 into two additional distinct cell populations: transitory and exhausted. Transitory $CD8^+$ T cells express the chemokine receptor CX3CR1, pro-inflammatory cytokines, as well as granzyme B that all serve to create an effector-like transcriptional signature. These transitory cells—with the effector-like transcriptional signature—can successfully bind to and kill virus infected cells. Successful PD-1 pathway blockade increases the number of transitory $CD8^+$ T cells suggesting they are crucial for successful immunotherapy before their transition to a terminally exhausted $CD8^+$ T cell with limited effector function. The figure below is a graphical abstract from Hudson et al., 2019 showing the intracellular and extracellular markers of $CD8^+$ T cell differentiation in chronic infection. This thesis focuses on stem-like PD-1^+ Tcf-1^+ $CD8^+$ T cells and transitory Tcf-1^- $Tim3^+$ $CD8^+$ T cells. Previous work indicates that GR expression potentially impacts thymocyte development and exhaustion pathways.

Figure 2: Differentiation pathway of CD8$^+$ T cells with Tim3 and CX3CR1 markers. Hudson et al., 2019.



Lymphocytic choriomeningitis virus (LCMV) is a rodent meningitis infection that is largely responsible for our understanding of modern immunology. LCMV was initially isolated by Charles Armstrong in 1933 during an encephalitis outbreak in St. Louis, MO, U.S.A. Though not ultimately responsible for the encephalitis epidemic, LCMV has allowed us to gain insight into MHC restriction, T cell memory, chronic infection, T cell exhaustion and overall immune

pathology in disease. In initial LCMV research, many different viral isolates were identified due to differences in viral persistence. In 1984, Ahmed et al. studied different LCMV isolates by observing virological outcome after infecting mice with virus from the brain, spleen, lung and liver. These experiments lead to the characterization of LCMV Clone 13 which was one of many splenic clones that created a persistent LCMV infection, unable to be cleared by CTLs. Clone 13 was a variant of the parent Armstrong LCMV strain with substitutions at five of 10,600 nucleotides. A substitution of two amino acids resulted in the phenotypic difference between an acute Armstrong infection and a chronic Clone 13 infection. This was crucial in proving small amino acid substitutions could be responsible for observed changes in infection lifetime not only in LCMV but also in HIV and hepatitis E. Our understanding of adaptive immunity has largely been influenced by T cell immune responses using the LCMV model due to the ability of LCMV to generate a large and specific T cell response.

The adaptive immune response is impacted to a great extent by the principle of MHC restriction—Dohtery and Zinkernagel won the Nobel prize for its discovery. The LCMV model created a fundamental understanding of how CTLs kill their MHC restricted targets via simultaneous recognition of foreign peptide on an activated dendritic cell or other antigen presenting cell and MHC antigen. Though MHC structure differs slightly between MHC I and MHC II and foreign peptides range in length from 9-22 amino acids, the binding principles are similar between MHC I and MHC II. MHC restriction refers to the specific T cell-MHC interaction only when the appropriate foreign peptide is presented. After development in the thymus, T cells undergo a rigorous selection process to eliminate the presence of self-reactive T cells. T cells that have a high affinity for self-antigens are eliminated via apoptosis. This is necessary to prevent the development of fatal autoimmune reactions that would destroy healthy

tissues and organs. After selection, fully developed T cells have T cell receptors (TCRs) that bind to MHC with an extraordinary degree of specificity—with $CD8^+$ T cells binding exclusively to MHC I and $CD4^+$ T cells binding exclusively to MHC II. MHC restriction improves TCR specificity so that antigen is bound only in the presence of peptide-MHC complexes. This particular mechanism affects all of immunology research and was discovered through the LCMV murine model.

Another important component of the adaptive immune response is memory. LCMV enabled the study of T cells that remember their cognate antigen following the initial response to infection. After complete antigen depletion, later immune responses are characterized by a faster and more powerful T cell response. These studies allowed for specific proof of memory in the immune response. Though T cell memory is now a widely accepted immunological phenomena it was only possible through studies with LCMV and TCR transgenic mice. Lastly, and most relevant to the development of immunotherapy, was the use of LCMV model to understand immune dysfunction, especially immune exhaustion. In 1993, Moskophidis et al. introduced the concept of exhaustion to describe loss of T cell function in chronic infection marked by an increase in inhibitory signals and diminishing cytotoxic ability. Investigations by Ahmed and others into $CD8^+$ T cell inhibitory pathways generalized immune exhaustion in other infections such as HIV and Hepatitis B and C. Exhausted $CD8^+$ T cells display inhibitory markers such as PD-1 and TIGIT. PD-1 blockade has been a highly successful immunotherapy through the ability to re-functionalize $CD8^+$ T in chronic infection and TIGIT inhibition has shown promising results in some clinical trials when used in combination with PD-1 (Roche Pharmaceuticals Tiragolumab) though more studies need to be done to prevent an extremely elevated and potentially fatal immune response. Immune exhaustion and markers of $CD8^+$ T cell dysfunction

continue to be understood using the LCMV model, and exhausted $CD8^+$ T cell populations including Tcf-1^- $Tim3^+$ cell populations.

This thesis investigates the role of GR using $GR^{fl/fl}$ gzmb cre^+ mice as well as P14 gzmb cre^+ with the murine LCMV model. This approach validates the importance of the glucocorticoid receptor in steroid hormone signaling and activation of immune cells. These results indicate that $CD8^+$ T cell glucocorticoid receptor (GR) is required for survival of LCMV clone 13 infection—$GR^{fl/fl}$ knock out (KO) mice are unable to survive past day 10 of this study. Usage of P14 mice with gp33 transgenic TCR's specific for LCMV was required for further study. P14 mice allow for selective knock out of the glucocorticoid receptor in activated $CD8^+$ T cells with the use of the granzyme b (gzmb) promoter and a highly specific immune response. Analysis of data from P14 mice revealed TIGIT and CD226 are T cell checkpoint inhibitory receptors (IR) and costimulatory receptors respectively that are upregulated in $CD8^+$ T cells during antigen specific expression. WT cells express more inhibitory TIGIT, and KO cells express more costimulatory CD226 highlighting future markers of immunosuppression in the presence of GR. Taken together, this work indicates the importance of GR as a component of immunosuppression that is necessary to prevent hyper immune activation that would damage healthy tissues.

Methods

Types of Mice used throughout experiments

Many types of mice were used throughout the experiments. Granzyme b (gzmb) causes recombination (cre) mice are cre under the control of gzmb promoter. Gzmb is expressed by activated $CD8^+$ T cells and is a marker of cytotoxicity. For a $CD8^+$ T cell to kill a virus infected

cell, it requires the production of perforin and gzmb. Perforin binds to cell membranes and creates small holes through which gzmb is shuttled into a virus infected cell. Gzmb triggers an enzymatic cascade that causes cellular apoptosis. Cre stands for causes recombination. These mice are bred in house such that when gzmb is expressed, it knocks out the floxed gene which means the gene is flanked by lox p. GR flox mice–GR gene (*Nr3c1)*—are floxed at exon 3. When a mouse has the gzmb cre and GR floxed modification together, GR is knocked out in any $CD8^+$ T cell that has ever been activated. The P14 gene in mice is a transgenic TCR for LCMV gp33. These mice are bred in lab to elicit a specific immune response to LCMV. Adoptive transfer experiments utilize CD45.1: a congenic marker/allele of the CD45 gene. This gene has no functional consequences and is a marker used in lab to compare endogenous vs. transferred cell populations in adoptive transfer experiments. C57BL/6J mice are from Jackson Laboratories. All mice were genotyped for CD45, P14, cre and GR flox and breeding cages were set up accordingly using only mice homozygous for floxed GR in all experiments. Mice were separated into granzyme B $cre^{+/-}$ groups where they were differentiated by GR knockouts in activated T cells in the cre^+ group. All mouse work was approved through the appropriate regulations as indicated by the Emory University Institutional Animal Care and Use Committee (IACUC) all required mouse handling trainings were completed before beginning experimentation.

Corticosterone Enzyme-linked immunoassay (ELISA)

ELISA is a plate-based assay where hormones (as well as antibodies and proteins) can be quantified. In ELISA, antibodies bind to corticosterone after antigen is immobilized to a solid surface and complexed with an enzyme-linked antibody. Mice were infected with either LCMV clone 13 or Armstrong strains. Serum samples were taken, and all serum samples were processed

and stored at -80° C until Biotechne Corticosterone Parameter Assay Kit (KGE009) was used. 50 μL of primary antibody solution was added to all samples except non-specific binding wells and incubated for 1 hour. Recommended aspiration and washing instruction protocols were executed followed by pretreatment and diluent application. Conjugate, substrate and stop solutions were added according to manufactures instructions. All measurements were read at 450nm within 30 minutes using a wavelength correction of 540 nm or 570 nm.

Adoptive cell transfer experiments

LCMV cells isolated from spleen, liver, lung and blood from 7 days after clone 13 infection were separated appropriately based on tissue type. $CD8^+$ T cells were labeled with fluorescent dyes according to the manufacturer's instructions. CD45.1/CD45.1 and CD45.1/CD45.2 donor mice were bled, and lymphocytes were isolated by a histopaque gradient. Cells were mixed at a 1:1 ratio and stained for flow cytometry. Mixture was transferred intravenously via tail vein injections to recipient mice with a composition of 1,000 total P14 cells. One week after transfer cells were isolated from host mouse spleen, liver, lung and blood and stained following intracellular cytokine staining experiments.

Intracellular cytokine staining experiments

For intracellular staining, the eBioscience Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00) was used for fixation, permeabilization, and intracellular staining according to the manufacturer's instructions. Cells were fixed to stabilize the cell membrane and then antibodies for intracellular staining were diluted with eBioscience Foxp3 permeabilization

buffer. Intracellular cytokine staining allows for dual analysis of surface molecules and intracellular antigens using single cell flow cytometry.

Mouse infection experiments

All mice studied were infected with LCMV strain Armstrong or clone 13, which cause acute or chronic infection, respectively. LCMV is a natural rodent infection commonly used in immunological studies (Zhou et al., 2012). In the clone 13 infection, C57BL/6JB6 (B6) 6–8-week-old mice received intraperitoneal (i.p.) injections with 300 µg of CD4-depleting antibody GK1.5 (Bio X Cell) or CD8-depleting antibody (clone 2.43 Bio X Cell) one and two days before intravenous (i.v.) injection with 2 x 10^6 pfu LCMV clone 13. After $CD4^+$ T cell depletion, the LCMV infection is unable to be cleared and lasts the lifetime of the mouse. One- and two-days pre-infection, anti-$CD4^+$ antibodies were injected to deplete $CD4^+$ T cells, and PBS with no T cell depletion served as a control.

Flow cytometry analysis

Flow cytometry uses hydrodynamic focusing to focus cells into a streamline with the same velocity. Cells pass through a laser light and the corresponding scattered light is collected by a detector. Flow cytometry measures excitation and emission of fluorescently labeled antibodies, it requires fluorophores attached to antibodies to detect both intracellular and extracellular markers of interest. Fluorophores accept light energy at a given wavelength and rapidly emit light energy at a longer wavelength; the difference between the excitation max and the emission max is called the Stokes shift. Electrons of fluorophores move from a resting to excited state and undergo a conformational change. The energy is released as heat and fluorescence as the electron returns to the resting state. This allows for multi-parametric

measurement and analysis of a single cell by analyzing visible light scatter in two different directions. Forward Scatter (FSC) indicates the size of the cell at 90° and Side Scatter (SSC) indicates the internal complexity/granularity of the cell (McKinnon, et al.). FSC and SSC data are combined to create scatter plots that give information about forward scatter and side scatter.

The Flow cytometer used was a Becton Dickinson five laser Cytek Aurora instrument capable of excitation from 355nm to 633nm and detecting absorbance from 365nm to 810 nm. Single cell suspensions were stained with antibodies and/or tetramer in PBS with 2% fetal bovine serum (FBS) and 2 mM EDTA for extracellular targets. BD Cytofix/Cytoperm was also used for fixation of non-intracellularly stained cells. MHC I tetramers were prepared in lab by Dr. Hong Wu. Multi-parameter flow cytometry can be challenging due to spectral overlap between fluorochromes'. All samples were corrected with compensation to mitigate the effects of overlaps in fluorescent emission spectra of different fluorescent dyes and prevent the observation of double populations via the creation of mutually exclusive markers. This is always required in flow cytometry due to wide ranges of fluorescence between fluorophores that are close in wavelength. A single stain cell or bead sample for every fluorophore and dye used was prepared and a compensation algorithm was applied to achieve correct values. Samples were analyzed using FlowJo. Figures, graphs, and statistical analysis were created using GraphPad Prism v10 software.

Bulk RNA Sequencing

RNA sequencing allows us to understand the impact of gene expression on the development of disease using massively high-throughput and single nucleotide resolution. Bulk RNA sequencing allows for transcriptional analysis of cell populations that is more cost effective than single cell RNA sequencing and insightful. It reduces the number of data points allowing for

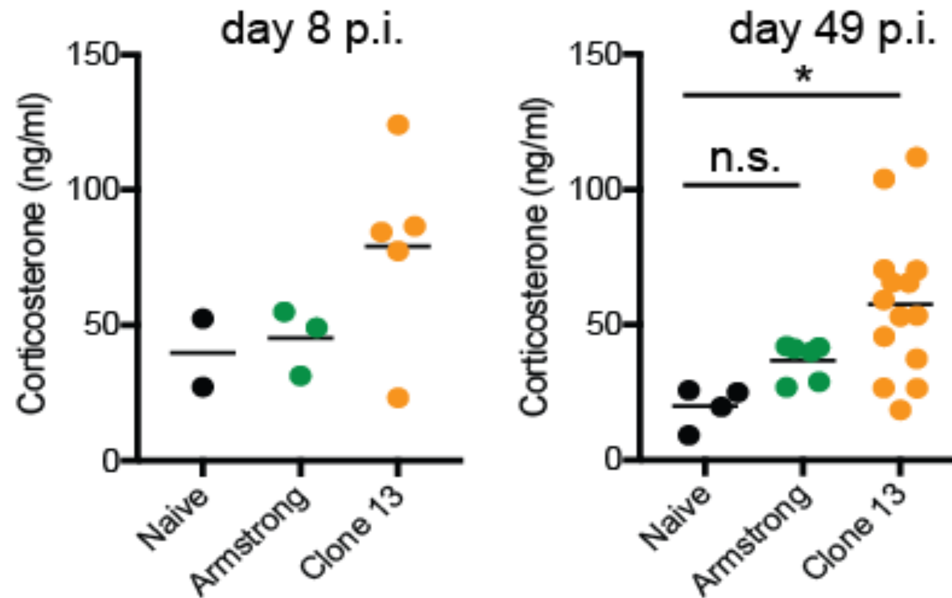
simplified analysis yielding more information about gene regulation and phenotypic variation in each cell population. Isolation of RNA is possible using Qiagen AllPrep Micro kit following manufacturer's instructions. RNA is purified using the RNeasy MinElute spin column. LongncRNA, rRNA, small ncRNA and mRNA are isolated and then undergo Poly-A selection, Ribo-depletion, or size selection before being converted to cDNA. cDNA is construction by reverse transcription and is used to create the sequencing library and PCR amplification and sequencing reveals gene expression. RNA sequencing was performed at the Yerkes Nonhuman Primate Genomics Core.

Results

Exploration into glucocorticoids in LCMV infection

Initial exploration into glucocorticoid levels in LCMV infection began with an ELISA to measure corticosterone concentrations. Mice are nocturnal, and corticosterone concentrations rise and fall with biological circadian rhythms. Blood was sampled from mice at the same time in the afternoon where corticosterone levels reach their minima for each ELISA. Serum from samples was compared between naïve mice and mice with a chronic and acute LCMV infection. Figure 3 depicts findings.

Figure 3: Glucocorticoids are elevated after chronic infection with LCMV clone 13. ELISA was used to measure corticosterone concentrations at day 8 and day 49 post infection. The results revealed a significant difference in corticosterone levels at day 49 in the acute model and suggested elevation at day 8.

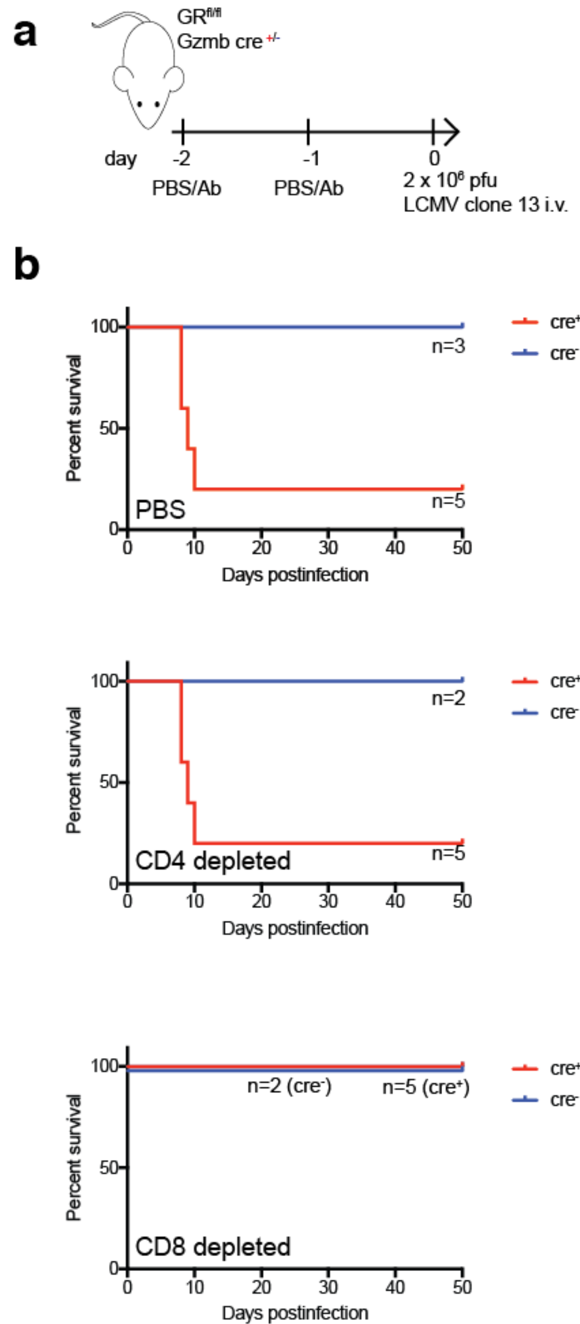


$GR^{fl/fl}$ Gzmb $cre^{+/-}$ Infection Experiments

Significantly elevated corticosterone levels encouraged investigation into the role of glucocorticoids in LCMV infection—specifically their effect on $CD4^+$ and $CD8^+$ T cells using $GR^{fl/fl}$ Gzmb $cre^{+/-}$ mice. To isolate the effect of GR on $CD4^+$ and $CD8^+$ T cells, three separate experiments were conducted. The control experiment injected PBS intraperitoneally into mice and the other experiments used intraperitoneal injections of 300 µg of $CD4^+$ depleting antibody GK1.5 (Bio X Cell) or $CD8^+$ depleting antibody (clone 2.43 Bio X Cell) respectively. The results are depicted in Figure 4b.

Figure 4a: GR$^{fl/fl}$ Gzmb cre$^{+/-}$ LCMV infection scheme

Figure 4b: The CD8$^+$ T cell glucocorticoid receptor (GR) is required for survival of LCMV clone 13 infection. CD4$^+$ and CD8$^+$ T cell populations were depleted separately in cre$^+$ (GR KO) and cre$^-$ mice (control). CD4$^+$ T cell depletion had no effect on murine survival in GR KO mice.

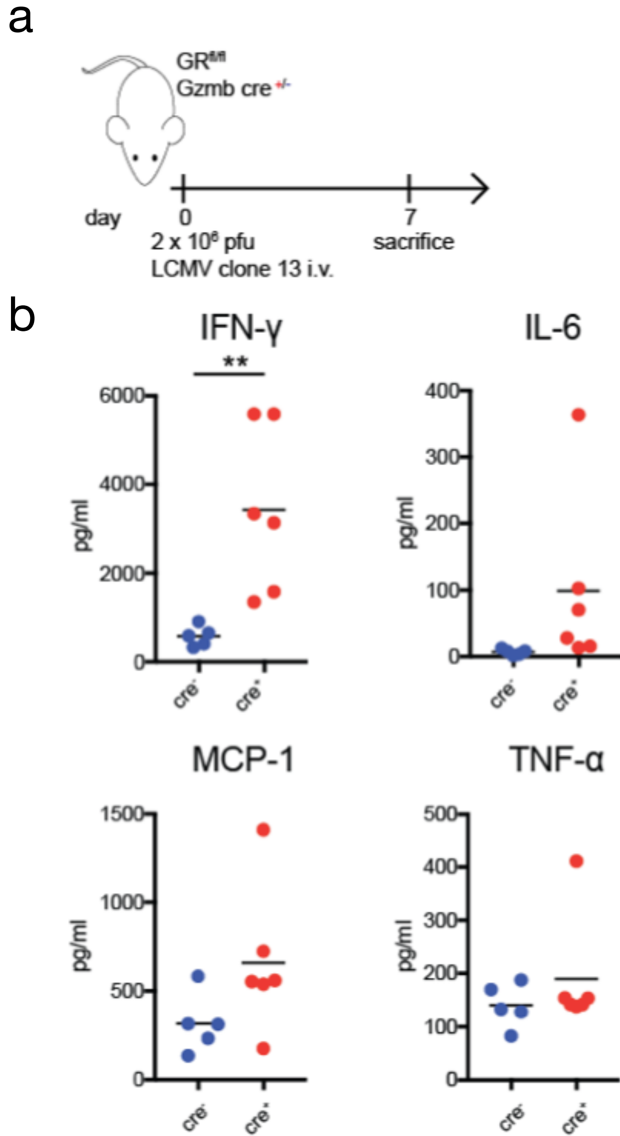


The $CD8^+$ T cell glucocorticoid receptor proved to be crucially important in moderating the murine immune response. When $CD8^+$ T cells were depleted in GR KO mice, all mice survived throughout the duration of the experiment. These findings inspired investigation into cytokines released by cytotoxic T lymphocytes and other CTL intracellular and extracellular markers. Cytokines are hormone-like messengers that facilitate communication between immune cells during infection and create a systemic inflammatory response. If not carefully controlled, a cytokine storm can over-recruit CTLs and create systemic inflammation. Four cytokines were investigated including IFN-γ, IL-6, MCP-1, and TNF-α which are commonly measured in immune research and have biological effects. Interferon- γ (IFN-γ) is a cytokine released by activated $CD8^+$ T cells and it indicates the strength of the immune response. IFN-γ upregulates the expression of class I MHC molecules in the cell environment making it easier for $CD8^+$ T cells to find, recognize and destroy their targets. TNF-α stands for Tumor Necrosis Factor and help T cells kill tumor cells, virus infected cells as well as activating other types of immune cells. Monocyte Chemoattractant Protein-1 (MCP-1) regulates migration and infiltration of monocytes and macrophages, and Interleukin-6 (IL-6) stimulates acute phase protein synthesis as well as production of neutrophils in the bone marrow. Mouse inflammation panels showed a significant difference in levels of IFN-γ. An increase in IFN-γ levels in GR KO cells indicates a stronger immune response as expected.

Figure 5a: GR$^{fl/fl}$ gzmb cre^{+} infection scheme

Figure 5b: GR$^{fl/fl}$ gzmb cre^{+} mice have elevated levels of IFN-γ at day 7

Cytokines were measured from serum using BioLegend LEGENDplex mouse inflammation panel at day 7 post infection. Cytokines investigated include IFN-γ, IL-6, MCP-1, TNF-α with no significant difference in GR$^{fl/fl}$ gzmb cre^{+} mice.



P14 GR KO Experiments

The following experiments investigate GR in P14 mice with modified TCR's specific to LCMV. GR KO in all $CD8^{+}$ T cells create a fatal KO, and the P14 model can be used to study mice past day 8 with a highly controlled, elegant, specific immune response. The analysis was done using a flow cytometer gated on gp33 cells which have a specific MHC peptide on LCMV specific TCRs.

Figure 6: Adoptive transfer experiment using CD45 transgenic markers in P14 mice.

P14 mice with TCR's specific for LCMV are marked with an alternative allele of the CD45 congenic marker. KO mice (cre^{+} $GR^{fl/fl}$) are marked with CD45.1/CD45.1. Control mice (cre^{-} $GR^{fl/fl}$) are marked using CD45.1/CD45.2. This provides information on which cell population expands preferentially for analysis. Cells are simultaneously transferred to CD45.2/CD45.2 C57BL/6J recipient mice and infected with LCMV clone 13.

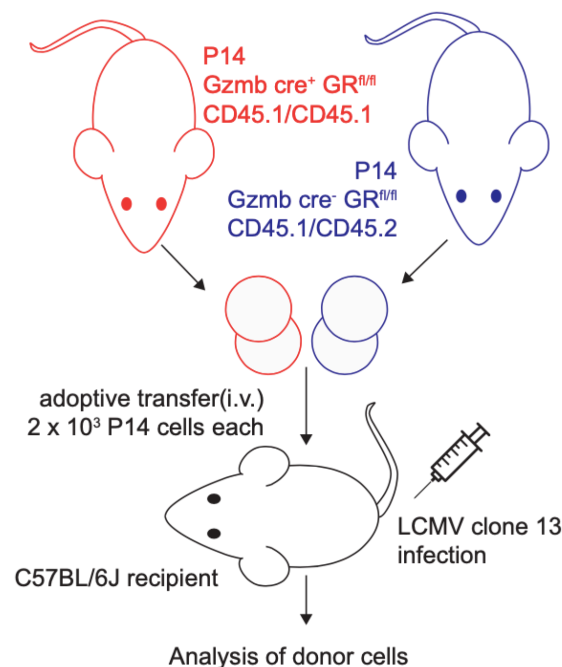
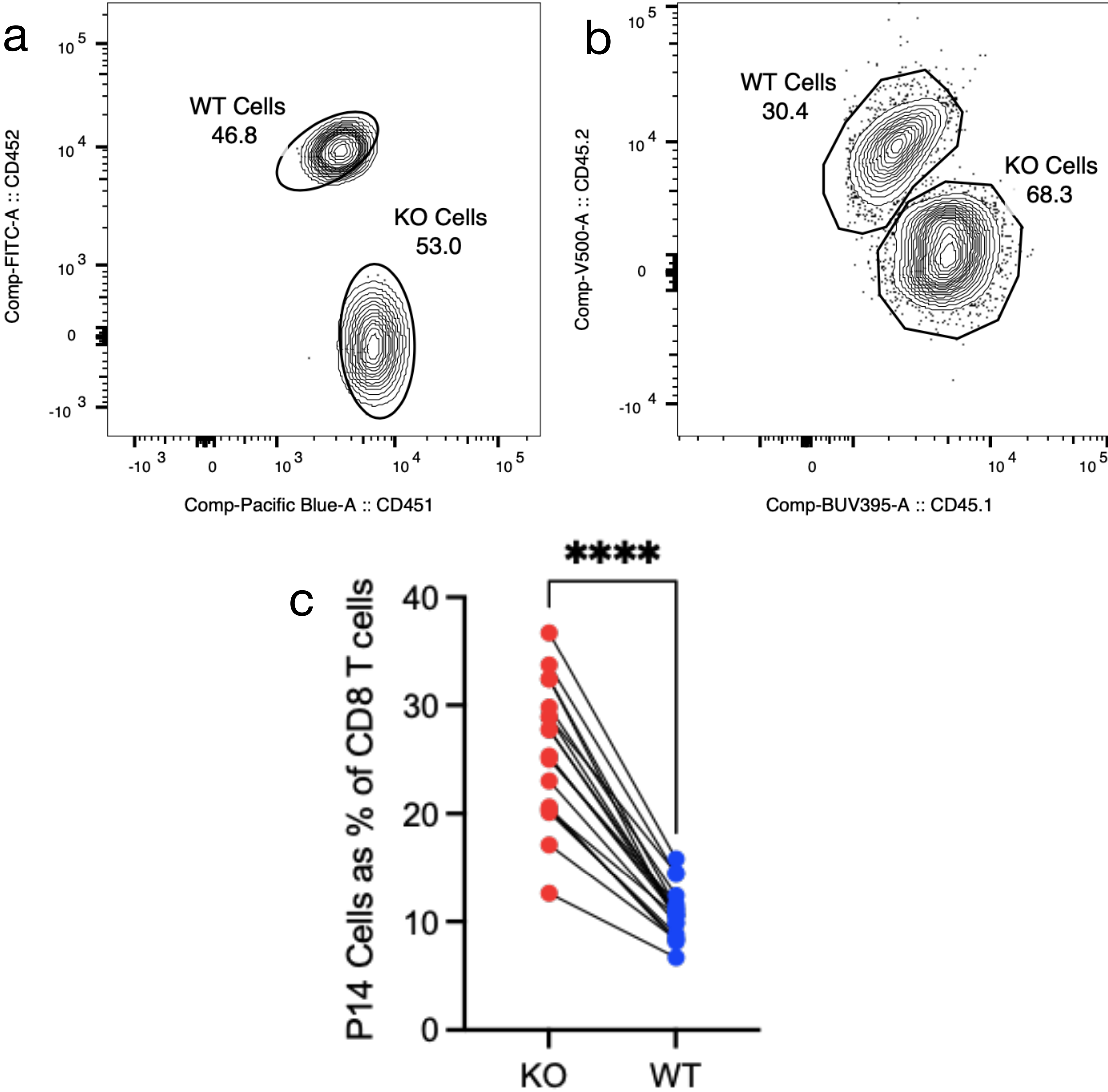


Figure 7: P14 KO CD8$^+$ T cells expand preferentially over WT

Figure 7a: Initial population of WT and KO cells prior to transfer from host to recipient mouse display an approximately 1:1 ratio. Gated on gp33$^+$ CD8$^+$ T cells.

Figure 7b: Cell population of WT and KO cells 7 days post-infection. KO cells preferentially expand over WT cells. Gated on CD45.1 T cells.

Figure 7c: Paired t test run on the difference in cell frequencies between KO and WT cells at day 7 post infection. **** indicates a significant difference with p <0.0001.

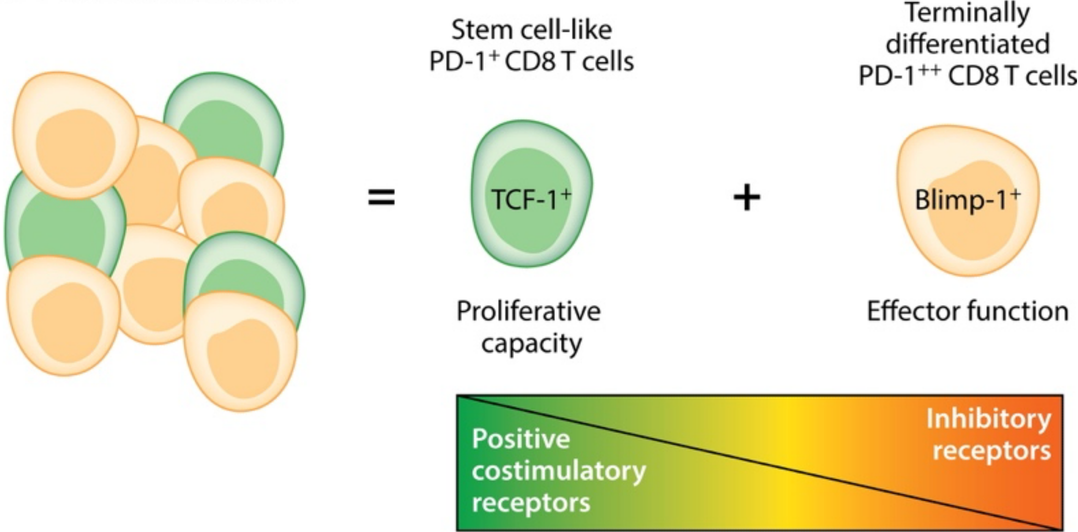


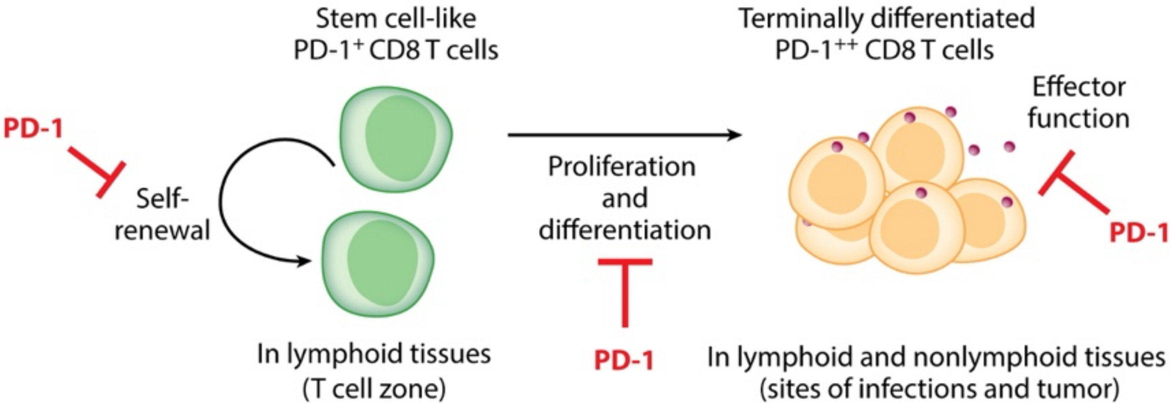
As depicted in **Figure 7c** KO P14 cells preferentially expand and out compete WT at day 7 post infection.

P14 GR KO impact on CD8^{+} T cell differentiation

Hashimoto et al., 2018 identified two specific sets of CD8^{+} T cell populations that develop in chronic infection. CD8^{+} T cells in the pathway to cell exhaustion undergo a transition from stem-cell like PD-1^{+} T cells with a proliferative capacity to terminally differentiated PD-1^{++} CD8^{+} T cells with effector function. PD-1 is a checkpoint inhibitor receptor that blocks division and differentiation from stem-like to effector CD8^{+} T cells. Tcf-1 is an intracellular transcription factor that controls longevity of CD8^{+} T cells and is associated with long-lived stem-like CD8^{+} T cells. Tcf-1^{+} cells respond well to PD-1 blockade or another checkpoint inhibitory receptors but do not exhibit cytotoxic effects. Klrg1 is a cell surface protein that is expressed on short-lived effector CD8^{+} T cells; more Klrg1 is associated with more cytotoxicity. The figure below is taken from Hashimoto et al. 2018 and was later elaborated on by Hudson et al. in 2019 with additional identification markers defining a transitory state as discussed previously.

Figure 8a and 8b: Differentiation pathway in $CD8^{+}$ T cell exhaustion with intracellular Tcf-1 and extracellular Klrg1 markers (Hashimoto et al., 2018)





Examples of Tcf-1 vs Gzmb and Tcf-1 vs Klrg1 staining

Figure 9c: Tcf-1$^+$ cells indicate stem-like CD8$^+$ T cells that turn into terminally differentiated effector cells. Gated on P14 WT cells on the left and P14 KO cells on the right.

Figure 9d: Klrg1 is a marker expressed on effector cells and not Tcf-1$^+$ cells. Gated on P14 WT cells on the left and P14 KO cells on the right.

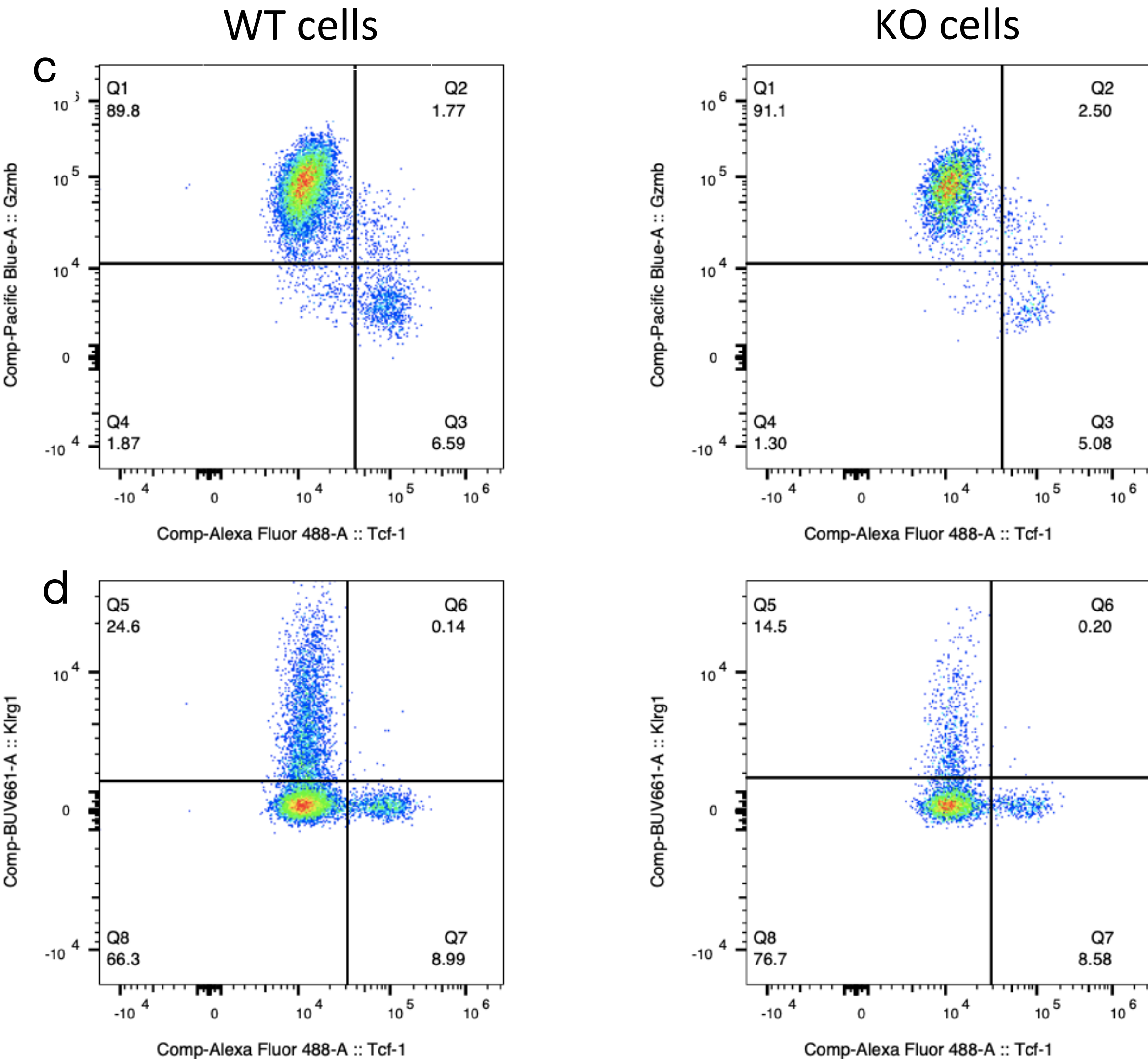
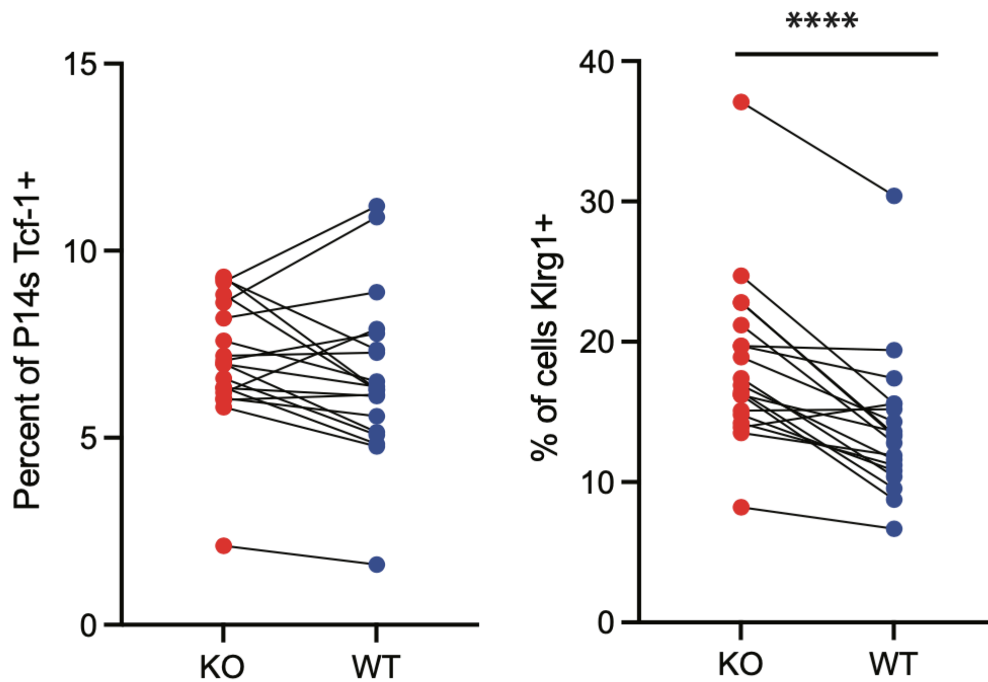


Figure 10a: No significant difference in Tcf-1 expression between P14 KO and WT cells

Figure 10b: Higher Klrg1 expression in P14 GR KO cells within the Tim3$^+$ Tcf-1$^-$ subset

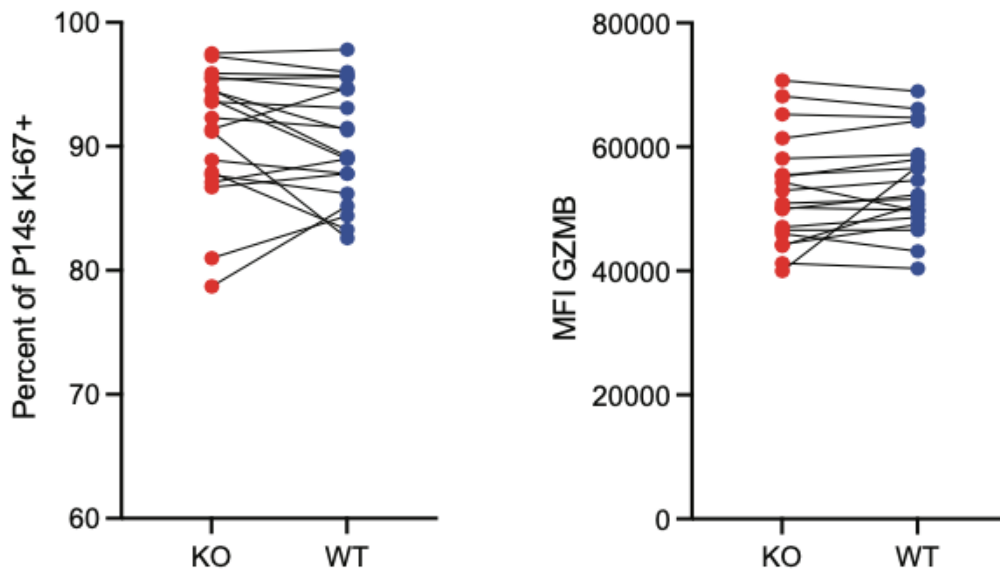


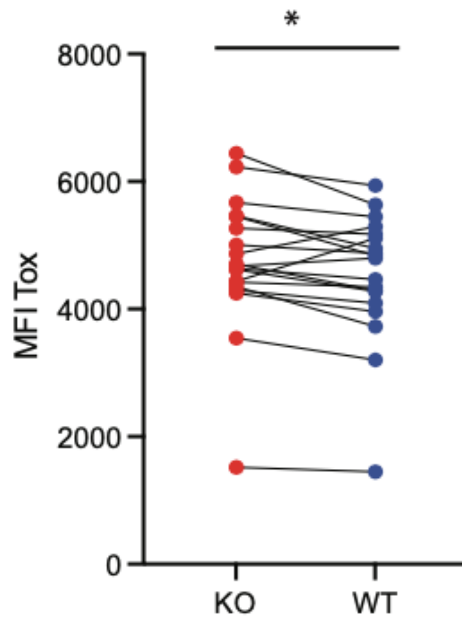
Though the results show no significant difference by day 7 in the percent of Tcf-1$^+$ cells between KO and WT, among the Tcf-1$^-$ cells there is a higher percentage of Klrg1$^+$ KO cells which are markers of better effectors.

Investigation into CD8$^+$ T cell functional markers revealed more specific insights into differences between KO and WT cells. First investigated was Ki-67 which is a marker of division. Ki-67 exhibited no significant difference between KO and WT cells and both populations seemed to divide at an equal rate. Next the Mean Fluorescence Intensity (MFI) of gzmb and Tox was investigated using flow cytometry and analyzed using FlowJo. MFI is a measure of fluorescence using fluorescently labeled antibodies that bind to specific compounds.

Gzmb is a protein CD8+ T cells use to kill target cells and was significantly different among WT and KO cells. Gzmb is highly correlated to effector function as CTLs use perforin and gzmb to kill target cells. Tox is a transcription factor associated with exhaustion is significantly higher KO in comparison with WT cells. Elevated Tox indicates heightened exhaustion in P14 GR KO cells in comparison with WT.

Figure 11: Expression of CD8+ T cell functional markers Ki-67, Gzmb, and Tox on KO and WT cells. MFI measured using FlowJo at day 7. * Indicates a significant difference with $p < 0.01$ and **** indicates a significant difference with $p < 0.0001$.





Additional investigation into CD8$^+$ T cell functional markers examined the role of co-stimulatory and checkpoint inhibitory markers. TIGIT is a T cell checkpoint inhibitory receptor (IR) that is upregulated in CD8$^+$ T cells during antigen specific expression. CD226 is a T cell checkpoint co-stimulatory receptor (IR) that is also upregulated in CD8$^+$ T cells during antigen specific expression. Both CD226 and TIGIT bind onto CD155. CD155 is on T cells and somatic cells whereas TIGIT and CD226 are specific to T cells. TIGIT and CD226 work in opposing ways. When CD155 binds CD226, the T cell is stimulated, and when CD155 binds TIGIT, the T cell is inhibited.

Figure 12: TIGIT is expressed preferentially in WT cells

Figure 12a: Histogram of TIGIT expression, an inhibitory receptor.

Figure 12b: Paired t test run on the MFI of TIGIT between KO and WT cells at day 7 post infection. WT cells have higher TIGIT expression. **** indicates a p value of <0.0001.

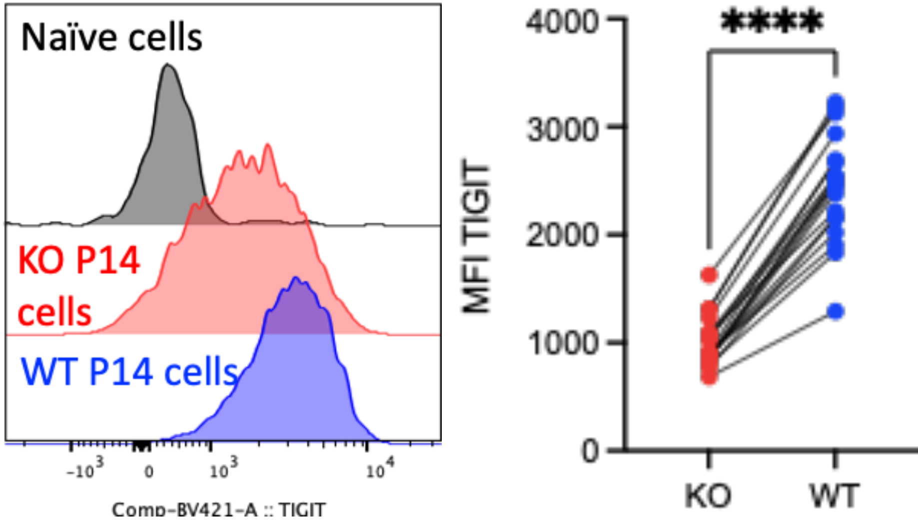
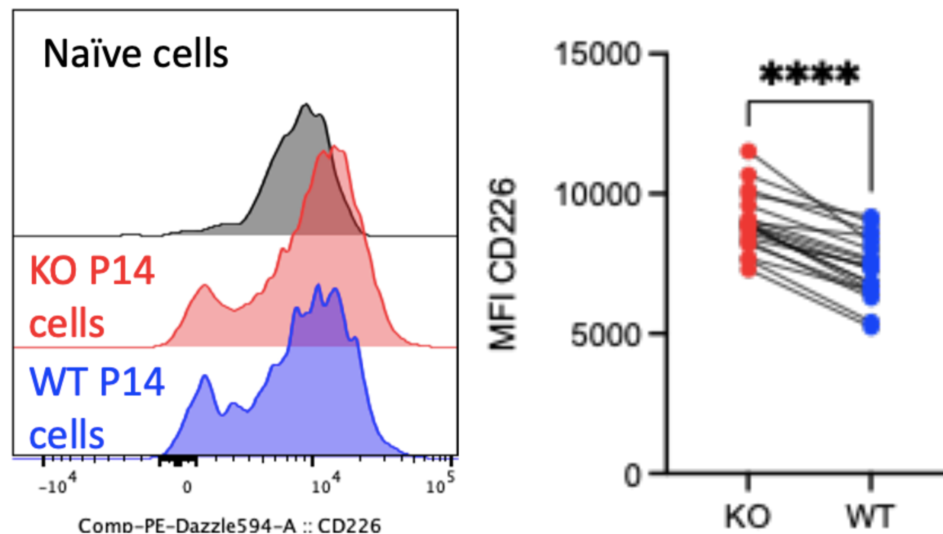


Figure 13: CD226 expressed preferentially in KO cells

Figure 13a: Histogram of CD226 expression, co-stimulatory receptor (IR)

Figure 13b: Paired t test run on the change in cell population between KO and WT cells at day 7 post infection. KO cells express CD226 preferentially. **** indicates a p value of <0.0001.



The TIGIT and CD226 results coupled together show more co-stimulatory activity in the P14 GR KO cells and more inhibitory activity in the WT cells which together indicate P14 KO cells are more activated than the WT cells.

Bulk RNA sequencing was performed on Tim3$^+$ KO and WT P14 cells. Bulk RNA sequencing allows for analysis of CD8$^+$ T cell functional markers that don't have antibodies available for flow cytometry staining. As discussed previously, Tim3$^+$ is a differentiation marker exhibited by transitory and terminally differentiated CD8$^+$ T cells. All Tim3$^+$ cells are Tcf-1$^-$, meaning Tcf-1 and Tim3 are mutually exclusive markers for stem-like cells. The following genes were examined for their difference between KO/WT cells as well as potential functional implications. Shumba et al., 2018 previously showed that *CXCR4* is upregulated by GR. *CXCR4* is a chemokine receptor that is a marker of cell migration, hematopoiesis and cell homing, as

well as retention in the bone marrow. *Nr3c1* is the gene that encodes GR; expression of cre causes a frameshift mutation creating a nonfunctional protein yet the RNA transcript remains intact in KO cells. *IL-10* is an anti-inflammatory cytokine with immunosuppressive activity. *Il7r* is the receptor for IL-7 and is important for T cell survival. *Bcl2* is important for cell survival and inhibits apoptosis. *CXCR4*, *Nr3c1*, *IL-10*, *Il7r* and *Bcl2* were all higher in P14 WT cells in comparison with P14 KO cells. *Xcl1* binds XCR1 and is important for antigen presenting cell (APC)-T cell interactions and remained higher in KO cells in comparison with WT cells.

Figure 14: RNA sequencing on KO and WT P14 cells

Tim3^{+} KO and WT P14 cells were isolated 7 days after infection with LCMV clone 13 infection. RNA was isolated and sent for sequencing. Volcano plot with selected genes (CXCR4, Nr3c1, IL-10, Xcl1, Bcl2, Il7r) is shown.

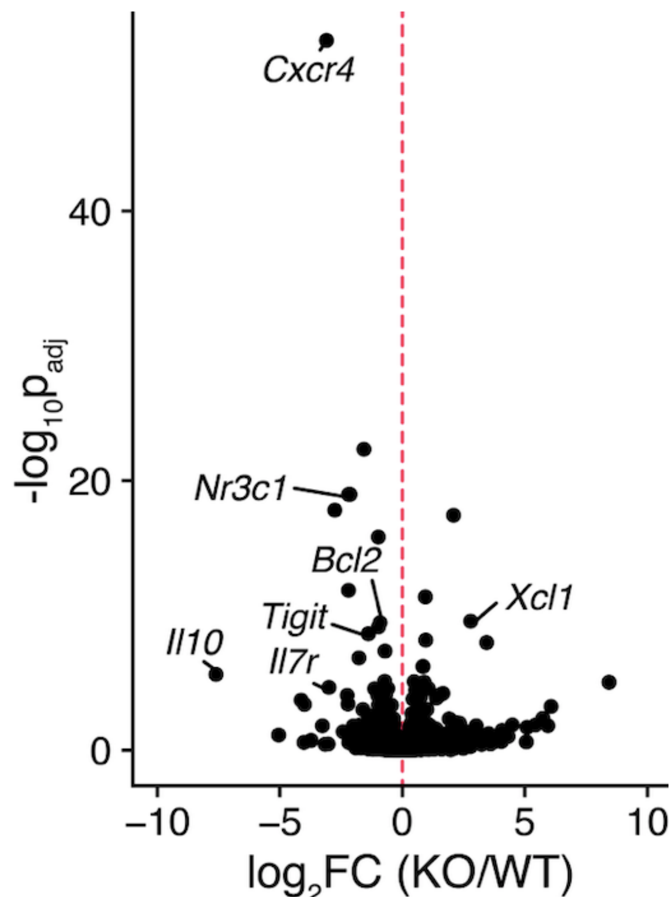
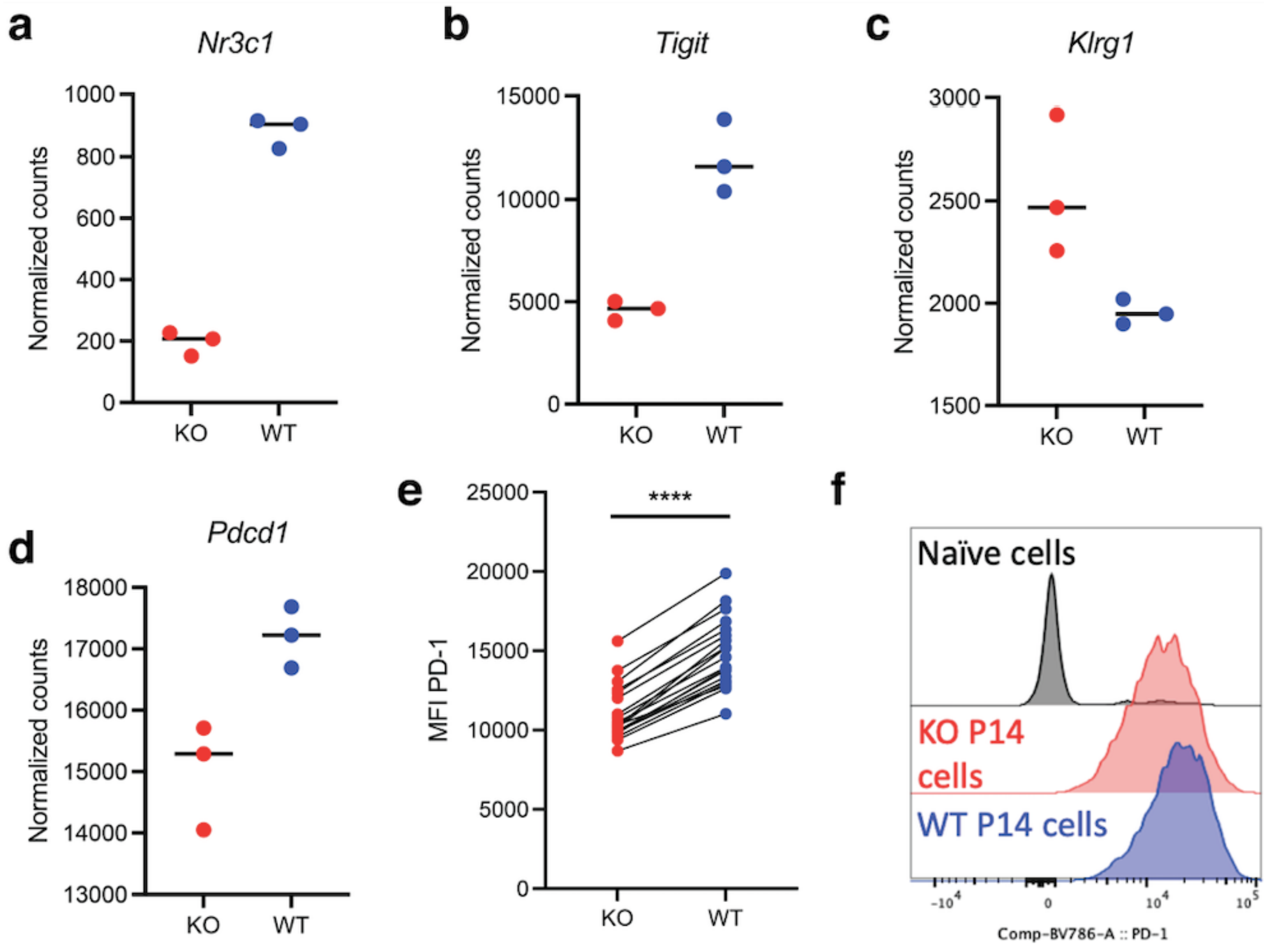


Figure 15: Example RNA sequencing data for four selected genes – *Nr3c1* (encodes GR), *Tigit*, *Klrg1* and *Pdcd1* (encodes PD-1). RNA sequencing results are consistent with **Figures 10 and 12** (Klrg 1 and TIGIT). Figure 13e shows PD-1 MFI flow cytometry data where P14 KO cells are 35% higher than P14 WT in PD-1 expression. **** indicates a p value of <0.0001. Figure 14f shows PD-1 staining for Naïve, P14 KO and P14 WT cells.



Conclusion

Initial experiments showed elevated glucocorticoids in chronic infection and encouraged investigation into the function of the glucocorticoid receptor in the immune system—specifically its function on T cells. Verges et al., 1989 also showed elevated glucocorticoid levels in HIV and Zhang et al., 2018 showed elevated cortisol in hepatitis B cirrhotic patients suggesting elevated glucocorticoids in a variety of chronic infections. In figure 3, we learned that the $CD8^+$ T cell glucocorticoid receptor (GR) is required for survival of LCMV clone 13 infection—proving GR has both an immunosuppressive and lifesaving function. GR suppresses antigen-stimulated inflammation and reduces the cytotoxic immune response by decreasing systemic IFN-γ as shown by elevated levels of IFN-γ in gzmb cre^+ $GR^{fl/fl}$ mice in figure 5. Adoptive transfer experiments proved P14 GR KO $CD8^+$ T cells out-compete P14 GR WT $CD8^+$ T cells in chronic infection. Investigation into subtypes of $CD8^+$ T cells through their well-defined exhaustion pathway in chronic infection showed that the generation of stem-like cells ($Tcf\text{-}1^+$ $CD8^+$ T cells) is not affected by GR at day 7. Within the $Tim3^+$ effector $CD8^+$ T cell subset, a higher percentage of GR KO $CD8^+$ T cells expressed Klrg1 indicating higher cytotoxicity/better effector ability in this population. Analyzing $CD8^+$ T cell functional markers using flow cytometry showed significantly higher Tox suggesting increased exhaustion P14 GR KO cells vs. WT. $CD8^+$ T cell inhibitory and co-stimulatory receptors were also analyzed. Co-stimulatory receptor CD226 was significantly higher in P14 KO cells and checkpoint inhibitor TIGIT was significantly lower in P14 KO cells suggested broader activation in P14 KO cells. PD-1 was also investigated and like TIGIT was lower in P14 KO cells. RNA sequencing information on terminally differentiated $Tim3^+$ $CD8^+$ T cells revealed lower chemokine *CXCR4* expression, anti-inflammatory cytokine *IL-10*, *Bcl2* and *Il7r* (markers of cell survival and apoptosis inhibition)

and *Nr3c1* in P14 GR KO cells. *Xcl1* was higher in P14 GR KO cells. All together these results give insight into the specific impact of GR on $CD8^+$ T cell function to improve targeted immunotherapies in patients with exogenous corticosterone administration.

Discussion and Future Direction

Though much remains to be explored about glucocorticoids and their impact on $CD8^+$ T cell function, this work created opportunities for specific exploration of different co-stimulatory and checkpoint inhibitory receptors which may be effective targets for patients with exogenous corticosterone as part of a chronic infection treatment strategy. Future treatment strategies and potentially effective targets could include antagonizing TIGIT. TIGIT's function as a checkpoint inhibitor is important for immunosuppression—blocking its function could potentially overcome the effect of the GR KO in P14 $CD8^+$ T cells. Current clinical trials testing TIGIT and PD-1 combination therapies indicate this dual approach could also be effective approach in controlling inflammation in a P14 GR KO population. Similarly, CD226 which improves $CD8^+$ T cell function, could also be agonized to improve cytotoxic ability in WT cells. Boosting the effector function of WT cells might be useful for patients who must remain on long-term steroid usage due its life-saving function for transplant tumors or autoimmune disease. Additional studies need to be conducted that continue to examine TIGIT markers later in infection past day 7. P14 GR KO mice allow for an elegant study of the impact of the LCMV's infection impact specifically on T cells but there are other ways to manipulate GR KO cells in the system. Future experiments could employ the use of the Ert2 cre system. Ert2 cre causes recombination and KO of the GR gene when tamoxifen is administered to mice. This would allow us to study a chronic infection and subsequently knock out GR later in the infection rather than at the onset and observe its impact. This study could shed light on the impact of P14 GR KO cells on a $Tim3^+$ exhausted cell

population. Additional follow up experiments could agonize or antagonize the glucocorticoid receptor itself within P14 WT cells in combination with PD-1 blockade.

Additionally, due to unexpected circumstances all P14 mice died between day 11-19 and were unable to be studied until day 49. Accidentally, too many P14 $CD8^+$ T cells were transferred to the recipient, and the mice were unable to survive. Repeating these experiments would help to give data past day 7 for a more longitudinal study of the impact of GR on $CD8^+$ T cells. T cell functional markers including Tox, Ki-67 and gzmb as well as co-stimulatory CD226 and inhibitory TIGIT could be reanalyzed at later time points to provide more insight into how functional marker change in the chronic infection.

Bulk RNA sequencing data was performed on $Tim3^+$ cells and highlighting additional selected genes. Existing bulk RNA sequencing could examine additional genes in the Tcf-1^+ stem-like population. Additionally, bulk RNA sequencing data showed a wide variety of differences between P14 KO and WT cells in selected genes which allows for the identification of averages of the genes of many cells and mask individual differences. Single cell RNA sequencing would provide more insights in heterogeneity of P14 KO cells and allow for understanding of gene expression profiles of individual cells. Existing research indicates that even within phenotypically homogenous cell subsets bulk RNA-seq can fail to capture true cell transcriptome (Kurkurba et al., 2015). This would be useful for identify functional differences within the Tcf-1^+ or $Tim3^+$ cell populations.

This work was largely exploratory but could be highly useful for future experiments exploring the impact of GR on PD-1 blockade. Setting up an experiment comparing antagonizing and agonizing GR cells treated with PD-1 blockade and identifying functional markers using flow cytometry and or RNA sequencing could help to improve PD-1 based immunotherapies or

create successful combination therapies. Future GR experiments can answer questions and create treatment opportunities for those suffering from diseases specifically related to glucocorticoids. One such disease is Cushing's syndrome that is the result of an excess of cortisol. Iatrogenic Cushing's syndrome is the most common form of hypercortisolism though it can also be caused by elevated endogenous cortisol levels as the result of adrenal tumors. The symptoms of Cushing's syndrome can create obesity (with fat distribution concentrated around the core of the body), and result in facial redness and puffiness, diabetes, female facial hair, proximal muscle weakness, fatigue, and excessive thirst. If surgical intervention is not an option (in absence of an adrenal tumor), regulation of GR (either via TIGIT or PD-1 blockade) could be an effective way to control elevated cortisol levels and reduce the severity of symptoms of patients. In contrast, Addison's disease is the result of adrenal insufficiency and results in erroneously low levels of glucocorticoids. Symptoms of Addison's include weight loss and decreased appetite, low blood pressure, hypoglycemia, nausea/diarrhea/vomiting, abdominal pain, muscle and joint pain as well as body hair loss. Addison's disease can be life threatening if untreated. Upregulating CD226 or other costimulatory signals could potentially provide an alternative treatment from prednisone or dexamethasone or traditional exogenous steroid administration.

References

Acharya, N.; Madi, A.; Zhang, H.; Klapholz, M.; Escobar, G.; Dulberg, S.; Christian, E.; Ferreira, M.; Dixon, K. O.; Fell, G.; Tooley, K.; Mangani, D.; Xia, J.; Singer, M.; Bosenberg, M.; Neuberg, D.; Rozenblatt-Rosen, O.; Regev, A.; Kuchroo, V. K.; Anderson, A. C. Endogenous Glucocorticoid Signaling Regulates $CD8^+$ T Cell Differentiation and Development of Dysfunction in the Tumor Microenvironment. *Immunity* **2020**, *53* (3), 658-671.e6. https://doi.org/10.1016/j.immuni.2020.08.005.

Adam, M.; Kooreman, N. G.; Jagger, A.; Wagenhäuser, M. U.; Mehrkens, D.; Wang, Y.; Kayama, Y.; Toyama, K.; Raaz, U.; Schellinger, I. N.; Maegdefessel, L.; Spin, J. M.; Hamming, J. F.; Quax, P. H. A.; Baldus, S.; Wu, J. C.; Tsao, P. S. Systemic Upregulation of IL-10 (Interleukin-10) Using a Nonimmunogenic Vector Reduces Growth and Rate of Dissecting Abdominal Aortic Aneurysm. *Arterioscler. Thromb. Vasc. Biol.* **2018**, *38* (8), 1796–1805. https://doi.org/10.1161/ATVBAHA.117.310672.

Addison’s disease https://www.mayoclinic.org/diseases-conditions/addisons-disease/symptoms-causes/syc-20350293 (accessed 2022 -03 -18).

Ahmed, A.; Salmi, L. D.; Butler, J. M.; Michael B, A. OLDSTONE From the Department of Lmmunology. In *Suppression of Cytotoxic T Lymphocyte Response and Viral Persistence BY*.

Barber, D. L.; Wherry, E. J.; Masopust, D.; Zhu, B.; Allison, J. P.; Sharpe, A. H.; Freeman, G. J.; Ahmed, R. Restoring Function in Exhausted $CD8^+$ T Cells during Chronic Viral Infection. *Nature* **2006**, *439* (7077), 682–687. https://doi.org/10.1038/nature04444.

Bio-Rad. Fluorescent compensation - flow cytometry guide https://www.bio-rad-antibodies.com/flow-cytometry-fluorescence-compensation.html?JSESSIONID_STERLING=09EDB498BCB1AC1E8406347868E4AE37.ecommerce2&evCntryLang=US-en&cntry=US&thirdPartyCookieEnabled=true (accessed 2022 -03 -18).

Brewer, J. A.; Khor, B.; Vogt, S. K.; Muglia, L. M.; Fujiwara, H.; Haegele, K. E.; Sleckman, B. P.; Muglia, L. J. T-Cell Glucocorticoid Receptor Is Required to Suppress COX-2-Mediated Lethal Immune Activation. *Nat. Med.* **2003**, *9* (10), 1318–1322. https://doi.org/10.1038/nm895.

Cole, T. J.; Blendy, J. A.; Monaghan, A. P.; Krieglstein, K.; Schmid, W.; Aguzzi, A.; Fantuzzi, G.; Hummler, E.; Unsicker, K.; Schütz, G. Targeted Disruption of the Glucocorticoid Receptor Gene Blocks Adrenergic Chromaffin Cell Development and Severely Retards Lung Maturation. *Genes Dev.* **1995**, *9* (13), 1608–1621. https://doi.org/10.1101/gad.9.13.1608.

Deng, Y.; Xia, X.; Zhao, Y.; Zhao, Z.; Martinez, C.; Yin, W.; Yao, J.; Hang, Q.; Wu, W.; Zhang, J.; Yu, Y.; Xia, W.; Yao, F.; Zhao, D.; Sun, Y.; Ying, H.; Hung, M.-C.; Ma, L. Glucocorticoid Receptor Regulates PD-L1 and MHC-I in Pancreatic Cancer Cells to Promote Immune Evasion and Immunotherapy Resistance. *Nat. Commun.* **2021**, *12* (1), 7041. https://doi.org/10.1038/s41467-021-27349-7.

Giorgio Caratti, P. P. Glucocorticoids: restoring balance during stress https://www.endocrinology.org/endocrinologist/130-winter18/features/glucocorticoids-restoring-balance-during-stress/ (accessed 2022 -03 -18).

Guo, L.; Wei, R.; Lin, Y.; Kwok, H. F. Clinical and Recent Patents Applications of PD-1/PD-L1 Targeting Immunotherapy in Cancer Treatment-Current Progress, Strategy, and Future Perspective. *Front. Immunol.* **2020**, *11*, 1508. https://doi.org/10.3389/fimmu.2020.01508.

Hashimoto, M.; Kamphorst, A. O.; Im, S. J.; Kissick, H. T.; Pillai, R. N.; Ramalingam, S. S.; Araki, K.; Ahmed, R. $CD8^+$ T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. *Annu. Rev. Med.* **2018**, *69* (1), 301–318. https://doi.org/10.1146/annurev-med-012017-043208.

Hench, P. S.; Kendall, E. C. The Effect of a Hormone of the Adrenal Cortex (17-Hydroxy-11-Dehydrocorticosterone; Compound E) and of Pituitary Adrenocorticotropic Hormone on Rheumatoid Arthritis. *Proc. Staff Meet. Mayo Clin.* **1949**, *24* (8), 181–197.

Hudson, W. H.; Gensheimer, J.; Hashimoto, M.; Wieland, A.; Valanparambil, R. M.; Li, P.; Lin, J.-X.; Konieczny, B. T.; Im, S. J.; Freeman, G. J.; Leonard, W. J.; Kissick, H. T.; Ahmed, R. Proliferating Transitory T Cells with an Effector-like Transcriptional Signature Emerge from PD-1+ Stem-like $CD8^+$ T Cells during Chronic Infection. *Immunity* **2019**, *51* (6), 1043-1058.e4. https://doi.org/10.1016/j.immuni.2019.11.002.

Iyer, S. S.; Cheng, G. Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. *Crit. Rev. Immunol.* **2012**, *32* (1), 23–63. https://doi.org/10.1615/critrevimmunol.v32.i1.30.

Jin, H.-T.; Anderson, A. C.; Tan, W. G.; West, E. E.; Ha, S.-J.; Araki, K.; Freeman, G. J.; Kuchroo, V. K.; Ahmed, R. Cooperation of Tim-3 and PD-1 in $CD8^+$ T-Cell Exhaustion during

Chronic Viral Infection. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (33), 14733–14738. https://doi.org/10.1073/pnas.1009731107.

Johnston, R. J.; Comps-Agrar, L.; Hackney, J.; Yu, X.; Huseni, M.; Yang, Y.; Park, S.; Javinal, V.; Chiu, H.; Irving, B.; Eaton, D. L.; Grogan, J. L. The Immunoreceptor TIGIT Regulates Antitumor and Antiviral CD8(+) T Cell Effector Function. *Cancer Cell* **2014**, *26* (6), 923–937. https://doi.org/10.1016/j.ccell.2014.10.018.

Junker, J. P.; van Oudenaarden, A. Every Cell Is Special: Genome-Wide Studies Add a New Dimension to Single-Cell Biology. *Cell* **2014**, *157* (1), 8–11. https://doi.org/10.1016/j.cell.2014.02.010.

Kukurba, K. R.; Montgomery, S. B. RNA Sequencing and Analysis. *Cold Spring Harb. Protoc.* **2015**, *2015* (11), 951–969. https://doi.org/10.1101/pdb.top084970.

Liu, S.; Trapnell, C. Single-Cell Transcriptome Sequencing: Recent Advances and Remaining Challenges. *F1000Res.* **2016**, *5*, 182. https://doi.org/10.12688/f1000research.7223.1.

McKinnon, K. M. Flow Cytometry: An Overview. *Curr. Protoc. Immunol.* **2018**, *120* (1), 5.1.1-5.1.11. https://doi.org/10.1002/cpim.40.

McLachlan, J. Immune Checkpoint Inhibitors and Their Side Effects. *Pathology* **2019**, *51*, S17. https://doi.org/10.1016/j.pathol.2018.12.036.

Oakley, R. H.; Cidlowski, J. A. The Biology of the Glucocorticoid Re- Ceptor: New Signaling Mechanisms in Health and Disease. *J. Allergy Clin. Immunol* **2013**, *132*, 1033–1044.

Parham, P. Putting a Face to MHC Restriction. *J. Immunol.* **2005**, *174* (1), 3–5. https://doi.org/10.4049/jimmunol.174.1.3.

Shimba, A.; Cui, G.; Tani-ichi, S.; Ogawa, M.; Abe, S.; Okazaki, F.; Kitano, S.; Miyachi, H.; Yamada, H.; Hara, T.; Yoshikai, Y.; Nagasawa, T.; Schütz, G.; Ikuta, K. Glucocorticoids Drive Diurnal Oscillations in T Cell Distribution and Responses by Inducing Interleukin-7 Receptor and CXCR4. *Immunity* **2018**, *48* (2), 286-298.e6. https://doi.org/10.1016/j.immuni.2018.01.004.

Steroids impair immunotherapy for brain cancer https://www.cancer.gov/news-events/cancer-currents-blog/2020/brain-cancer-immunotherapy-steroids-limit-effectiveness (accessed 2022 -03 -15).

Swarbrick, M.; Zhou, H.; Seibel, M. MECHANISMS IN ENDOCRINOLOGY: Local and Systemic Effects of Glucocorticoids on Metabolism: New Lessons from Animal Models. *Eur. J. Endocrinol.* **2021**, *185* (5), R113–R129. https://doi.org/10.1530/EJE-21-0553.

Tarumi, T.; Kravtsov, D. V.; Zhao, M.; Williams, S. M.; Gailani, D. Cloning and Characterization of the Human Factor XI Gene Promoter: Transcription Factor Hepatocyte Nuclear Factor 4α (Hnf-4α) Is Required for Hepatocyte-Specific Expression of Factor Xi. *J. Biol. Chem.* **2002**, *277* (21), 18510–18516. https://doi.org/10.1074/jbc.m201886200.

Tidy, D. C. Cushing's syndrome https://patient.info/hormones/cushings-syndrome-leaflet (accessed 2022 -03 -18).

Tikhonova, A. N.; Van Laethem, F.; Hanada, K.-I.; Lu, J.; Pobezinsky, L. A.; Hong, C.; Guinter, T. I.; Jeurling, S. K.; Bernhardt, G.; Park, J.-H.; Yang, J. C.; Sun, P. D.; Singer, A. Aβ T Cell Receptors That Do Not Undergo Major Histocompatibility Complex-Specific Thymic

Selection Possess Antibody-like Recognition Specificities. *Immunity* **2012**, *36* (1), 79–91. https://doi.org/10.1016/j.immuni.2011.11.013.

Timmermans, S.; Souffriau, J.; Libert, C. A General Introduction to Glucocorticoid Biology. *Front. Immunol.* **2019**, *10*, 1545. https://doi.org/10.3389/fimmu.2019.01545.

Van Laethem, F.; Tikhonova, A. N.; Singer, A. MHC Restriction Is Imposed on a Diverse T Cell Receptor Repertoire by CD4 and CD8 Co-Receptors during Thymic Selection. *Trends Immunol.* **2012**, *33* (9), 437–441. https://doi.org/10.1016/j.it.2012.05.006.

Verges, B.; Chavanet, P.; Desgres, J.; Vaillant, G.; Waldner, A.; Brun, J. M.; Putelat, R. Adrenal Function in HIV Infected Patients. *Eur. J. Endocrinol.* **1989**, *121* (5), 633–637. https://doi.org/10.1530/acta.0.1210633.

Wen, L.; Tang, F. Boosting the Power of Single-Cell Analysis. *Nat. Biotechnol.* **2018**, *36* (5), 408–409. https://doi.org/10.1038/nbt.4131.

Whirledge, S.; Cidlowski, J. A. Glucocorticoids, Stress, and Fertility. *Minerva Endocrinol.* **2010**, *35* (2), 109–125.

Wikipedia contributors. Glucocorticoid receptor https://en.wikipedia.org/w/index.php?title=Glucocorticoid_receptor&oldid=1041500250.

Xiao, P. *A Modeling Study in the Regulation of Stress on Neuronal Plasticity. Doctoral Dissertation*.

Zhang, J.; Ma, J.; Wang, H.; Li, J. Correlation between Cortisol Levels and Concurrent Infection for Hepatitis B Cirrhosis Patients and Countermeasure Analysis. *Exp. Ther. Med.* **2018**. https://doi.org/10.3892/etm.2018.5738.

Zhou, X.; Ramachandran, S.; Mann, M.; Popkin, D. L. Role of Lymphocytic Choriomeningitis Virus (LCMV) in Understanding Viral Immunology: Past, Present and Future. *Viruses* **2012**, *4* (11), 2650–2669. https://doi.org/10.3390/v4112650.

Zinkernagel, R. M.; Doherty, P. C. MHC Restricted Cytotoxic T Cells: Studies on the Biological Role of Polymorphic Major Transplantation Antigens Determining T Cell Restriction Specificity. *Advances in Immunology* **1979**, *27*, 51–177.

Zinkernagel, R. M.; Doherty, P. C. Restriction of in Vitro T Cell-Mediated Cytotoxicity in Lymphocytic Choriomeningitis within a Syngenic and Semiallogeneic System. *Nature* **1974**, *248*, 701–702.