UNIVERSITY OF CALIFORNIA, SAN DIEGO

Therapeutic Potential Of Activating and Inhibitory Immune Pathways On T cells In Chronic and Acute Viral Infection

A thesis submitted in partial satisfaction of the requirements for

the degree

Master of Science

in

Biology

by

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2012

The Thesis of Aleksandr Dolgoter is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2012

DEDICATION

I would like to dedicate this thesis to my parents Pavel and Nina Dolgoter, and my sister Nataliya Dolgoter. I would also like to dedicate this thesis to my girlfriend Christina Ly. They always supported me and encouraged me to succeed. Without their constant support I would not have made it this far.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Elina Zuniga for giving me the opportunity to join her lab and work under the supervision of James Harker to research the immune system's response to infection. I would also like to thank all of the present and past members of the lab, Gavin Lewis, Monica Macal, Eugenia Loureiro, Aaron Chang, Phuc Bao, Charles Hesser, Jessica Daggett, Natalie Warsinger-Pepe, Allison Dudik, Miguel Tam, Roberto Tinoco, Lauren Mack, Ellen Lee. Your help, guidance, answers to my questions, and knowledge have been invaluable throughout the last 2 years and will undoubtedly mitigate my future career choices.

ABSTRACT OF THE THESIS

Therapeutic Potential Of Activating and Inhibitory Immune Pathways On T cells In Chronic and Acute Viral Infection

by

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Master of Science in Biology

University of California, San Diego, 2012

Professor Elina I. Zuniga, Chair

Chronic viral infections present a major health burden worldwide. Using lymphocytic choriomeningitis virus clone 13 (LCMV Cl13), a mouse model of chronic viral infection, I neutralized transforming growth factor beta (TGF-β) and examined the role of gp130 receptor signaling in immune responses. In chronic infection, TGF-β

mediates deletion of virus specific $CD8^+$ T cells. However, treatment with anti-TGF-β neutralizing antibody, early in LCMV Cl13, did not lead to increased virus specific $CD8^+$ T cell numbers and there was no difference in viremia or clearance compared to the PBS treated group. The transmembrane receptor, gp130, is a common signaling protein utilized by the interleukin-6 (IL-6) family of cytokines. Infection of mice with a genetic ablation of gp130 or IL-27 (a IL-6 family cytokine) signaling on T cells led to a complete failure in viral control, with both antibody mediated and cellular immunity being compromised compared to wild type mice. In contrast, gp130 or IL-27 signaling was not found to be important in the control of an acute viral infection. Then I tested the therapeutic potential of this pathway by treating wild type LCMV Cl13 infected mice with IL-6 and HYPER-IL6, a designer cytokine-receptor complex capable of universally activate the gp130 pathway. Mice receiving HYPER-IL6 showed slightly enhanced viral control compared to PBS treated control mice. This study identifies gp130 signaling as a vital component of the immune response during chronic infection, and opens the possibility that therapeutically targeting this pathway directly on $CD4^+$ T cells may enhance viral control.

INTRODUCTION

Hundreds of millions of people worldwide are directly affected by chronic viral infections human immunodeficiency virus (HIV), hepatitis B, and hepatitis C (HCV)[1]. Currently there are no successful vaccines for HIV and HCV, and there are limitations in studying these viral infections *in vivo*, predominantly due to the lack of an effective small animal model. Lymphocytic choriomeningitis virus (LCMV), a natural mouse pathogen, has emerged as a practical way to study viral infections in a controlled laboratory environment. LCMV is a single stranded negative RNA virus of the *arenaveridae* family, which includes other viruses such as Lassa fever, Machupo and Junin[2]. Different strains allow the investigation of acute (Armstrong 53b strain (Arm)) and chronic (clone 13 strain (Cl13)) viral infections. LCMV Arm infects mice for a relatively short period and is cleared 7 to 10 days after infection, primarily by virus specific CD8 T cells[3]. In contrast, LCMV Cl13, which deviates from LCMV Arm by just two amino acid[4], persists in the blood for approximately 2 months post infection (p.i.), and in some tissues for much longer; about 6 months in the brain, and for the lifetime of the mouse in the kidney[5].

Virus specific T cells play an integral role in an effective antiviral response[6] but in chronic viral infections such as HIV, HCV, and LCMV Cl13 are often functionally exhausted and therefore unable to control viral replication. As a chronic viral infection progresses, cytotoxic T cells (CTLs) lose their ability to proliferate, kill infected cells, and produce key cytokines; first initially IL-2, followed by TNF-α, and finally IFN-γ, as they become more exhausted[7,8]. Exhausted T cells upregulate surface markers such as

PD1[9-11] and Lag-3, among others[12]. $CD4^+$ T helper cells play a vital role in supporting both the CTL response[13,14] and cell immunity. As with CTL responses, in chronic viral infection $CD4^+$ T cells also have impaired function, characterized by decreased cytokine production, proliferation and exhaustion[15,16].

Many of these features, and especially the mechanisms that underlie them, have been elucidated using LCMV Cl13 and rapidly translated into human chronic infections such as HIV and HCV[11,17-22], demonstrating the benefits of using LCMV to study common features of chronic viral infection.

Regulators of the Immune System

Identification, control, and eventual clearance of a viral infection by the immune system requires a precisely tailored and regulated response. To achieve this balance a number of activating and inhibitory immune signaling pathways exist within the immune system. These both enhance immune responses upon infection, but also inhibit the immune response once an infection has been resolved or when the magnitude of the immune response has the potential to be detrimental to the health of the host. This is thought to be the case in many chronic viral infections, where T cells become exhausted and deleted prior to viral eradication in order to prevent excessive immunopathology. A number of specific inhibitory pathways have been identified that contribute to virus specific T cell exhaustion in this context. An inhibitory member of the CD28 superfamily, programmed death-1 (PD-1), has been shown to partially regulate the exhaustion of virus specific $CD8^+$ T cells[23]. Exhausted $CD8^+$ T cells have also been found to have elevated levels of negative regulatory receptors, lymphocyte activation gene 3 (LAG-3)[24], and T

cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3)[25]. LAG-3 is a soluble molecule that activates antigen-presenting cells through MHC class II signaling, leading to increased antigen-specific T-cell responses in vivo[26]. LAG-3 has also been recently identified as present on a subset of regulatory T cells (Tregs)[27,28], which play an important role in the maintenance of self-tolerance and immune homeostasis[29]. Tim-3 triggers cell death of Th1 cells upon interaction with its ligand, galectin-9[30] and has been correlated with disease progression in HIV[31], HBV[32], HCV[33,34], rheumatoid arthritis[35], tuberculosis[36], lymphoma[37], and autoimmune diseases, such as multiple sclerosis[38] and lupus[39], amongst others. The cytokine IL-10, produced primarily by infected CD8α- dendritic cells during infection[40], also has an immunosuppressive role of modulating the function of antigen-presenting cells and T cells.

Therefore one potential approach to promoting viral clearance is to selectively block one or more of the inhibitory pathways involved in this immune-suppression, boosting anti-viral immunity to allow viral clearance. One caveat to this approach has been the need to prevent the induction of detrimental immunopathology, and LCMV has provided an ideal platform to test such approaches. Using LCMV Cl13 blockade of a number of inhibitory pathways, including PDL-1[9], IL10R[41], and LAG3 (in combination with PDL-1)[12], and IL10R in combination with PDL-1[42], have successfully been shown to result in eradication of chronic virus.

Alternatively, promoting signaling of positive regulators, via injections of cytokines such as IL-2[43], IL-7[44], and IL-21[45]. These cytokines signal through specific

receptor complexes which share the common γ receptor chain (γC) and belong to the γC family of cytokines. The members of this family of cytokines also have some overlapping immune functions, particularly in regards to T cell expansion, differentiation, and survival. IL-2, the first interleukin molecule to be identified, has been shown to be necessary for the differentiation and proliferation of T cells[46,47]. Treatment with IL-2 in later stages of mice infected with LCMV Cl13, lead to increased survival of virus specific T cells, increased proliferation of memory T cells and decreased viral burden[43]. The cytokine IL-7 is involved with the survival of naïve and memory T cells[48]. Mice infected with LCMV Cl13 receiving IL-7 treatment had an increase in virus specific CD8+ T cell and downregulation of exhaustion markers, such as PD-1[44]. IL-21 is critical for cellular and humoral immunity. It plays an important role in B cell proliferation and regulation of antibody production[49], and the maintenance of virus specific $CD8^+$ T cells[50-52]. Treatment with IL-21 in CD4 knockout mice infected with LCMV Cl13 lead to enhanced virus specific $CD8^+$ T cell numbers and lower viremia[45].

Transforming Growth Factor β (TGF-β)

The cytokine TGF-β exists as three isoforms TGF-β1, TGF-β2, and TGF-β3 and is released in an inactive form by a variety of cells. Once activated, particularly by protease[53] and integrin activity[54], TGF-β can act in both an autocrine or paracrine fashion, causing a broad spectrum of biological effects including cell development, differentiation, proliferation and survival[55,56]. Active TGF-β binding to its receptor induces the phosphorylation of SMAD2/3. SMAD2/3 then dimerizes with SMAD4, and

the complex is translocated to the nucleus to regulate the transcription of a multitude of genes[57]. In the immune system, the predominant isoform is thought to be TGF-β1 and mice with a genetic ablation of TGFβ1 die within a month after birth due to massive inflammation and multi-organ autoimmune disease elicited by activated $CD4^+$ T cells[58-61]. TGFB1's role as negative regulator of T cell immunity is further supported by research from our lab that found TGF-β is involved in apoptosis of virus specific $CD8^+$ T cells during chronic viral infection. Mice with a dominant negative form of TGF-β receptor II expressed on their T cells, display greatly enhanced virus specific $CD8^+$ T cells numbers after LCMV Cl13 infection and clear the infection rapidly. These virus specific T cells were also found to have increase in production of anti-viral cytokines, cytotoxicity, and down-regulation of the inhibitory molecules PD1 and IL-10[22].

IL-6 and the gp130 Family

Our lab has also recently identified a novel role for the proimmflammatory cytokine interleukin-6 (IL-6) as a positive regulator of the immune system during LCMV Cl13 infection. IL-6 binds with its receptor on the cell surface (IL6R), which then dimerizes with gp130, a transmembrane protein that is ubiquitously expressed on the surface of all cells. The dimerization of IL6R and gp130 leads to JAK kinase phosphorylation of the Signal transducer and activator of transcriptions (STAT) family, predominantly STAT-3. Phosphorylated STATs translocate to the nucleus and control the transcription of a wide range of genes[62].

During LCMV Cl 13 infection we found that IL-6 signaling on $CD4^+$ T cells mediates the natural viral control that is eventually seen 60 to 100 days after infection of wildtype mice by enhancing the differentiation of T follicular helper cells (T_{FH})[63]. T_{FH} have been identified as a subset of $CD4^+$ T cells that are primarily responsible for B cell proliferation, survival, and production of virus specific antibodies within the germinal center[64-66]. T_{FH} can be distinguished from other $CD4^+$ T cell subsets by differential cytokine expression, and the expression of a unique repertoire of surface markers[67-70]. T_{FH} produce high levels of IL-21[71], an important cytokine in the proliferation of B cells, and often produce IL-4, but unlike Th2 cells they fail to produce other Th2 cytokines such as IL-13. T_{FH} also express low levels of the master transcriptional regulators T-bet, GATA3, and RORγt, which control the differentiation of Th1, Th2, and Th17 CD4 helper subsets respectively,[66,68,70,72] but they have high levels Bcl6, a negative transcriptional regulator that has recently been shown to control the differentiation of $CD4^+$ T cells into T_{FH}[73]. In order to migrate into the B cell zone of lymphoid tissue T_{FH} express high levels CXCR5, and low levels of CCR7. They also upregulate a number of co-stimulatory molecules such as PD-1, ICOS and CD40L,[66,67,69] which allow them to positively interact with B cells.

During chronic infection IL-6 enhance T_{FH} responses by upregulating Bcl6 expression, this leads to an overall increase in the number of germinal center B cells and the quality of antibodies produced, especially IgG_1[74], essential step in order for the host to control the infection.

Aims and Objectives

In this thesis project I aimed to:

1. Test the therapeutic potential of suppressing a negative regulator of the immune system in chronic viral infection via TGF-β signal blockade.
2. Investigate the role of gp130 signaling on T cells during acute and systemic infections.
3. Explore the therapeutic potential of gp130 signaling pathway

MATERIAL AND METHODS

Mice and viral stocks

WT C57BL/6 were purchased from The Jackson laboratory. IL27Rko, IL6Rko and $gp130^{fl/fl}$ mice were generously provided by Amgen Inc, Dr. Angela Drew (University of Cincinnati), and Dr. Werner Mueller (University of Manchester, UK) respectively. $Gp130^{fl/fl}$ Mice with loxP region on the 16th exon of the gp130 receptor gene were crossed with mice expressing Cre under the CD4 promoter. During development in the thymus, CD4 and CD8 are coexpressed by T cells, before downregulating either one, as a result all T cells have a dysfunctional gp130 receptor on their surface in these *Cd4*-cre $gp130^{fl/fl}$ mice. Mouse handling followed the requirements of the Institutional Animal Care and Use Committee of UCSD and the National Institutes of Health. Mice were bred and managed in a closed facility and unless otherwise stated 6-8 week old mice were infected intravenously (i.v.) with $2x10^6$ PFU of LCMV ARM or Cl 13. Viruses were grown, identified and quantified as described[4]. For the immunofocus assay, Vero cells were placed on a flat well 96 well plate the night before at 30,000 cells per well in 1% Pen/Strep and L-glutamine, and 7% FBS in MEM media. Ten fold dilutions were prepared on another plate in 2% FBS DMEM and transferred onto the Vero cells, after the previous media was flicked and the Vero cells were washed with PBS. After 20 hour incubation at 37° C/5% CO_2, cells were fixed 4% Formaldehyde (half an hour) then blocked (3% BSA, 10% FBS, and 0.3% Triton-X in PBS) for an hour at room temperature. After block was removed, neat hybridoma supernatant with LCMV specific antibodies was added to each well for an hour at room temperature. After

washing with PBS, each well received secondary anti mouse IgG Cy-3 (Jackson Laboratories) 1:1000 in dilution buffer (3% BSA, 0.3% Triton-X in PBS) for 40 minutes at room temperature away from light. After washing with PBS the cells were viewed under an inverted fluorescent microscope.

Weighing Mice

To assess disease progression, mice were weighed throughout infection. Weight of the mice prior to infection was used as a reference point and mice were weighed at approximately the same time of day at each time point to take into consideration daily fluctuations in weight.

In-vivo immunotherapeutic treatment

For human IL6 or Hyper IL-6 treatments, mice received intraperitoneal (i.p.) injections daily with 2000 U from day 5-21 post LCMV Cl13 infection. Hyper IL-6 was generously provided by Dr. Stefan Rose-John (University of Kiel, Germany). For αTGFβ treatments, mice were injected i.p. with 750 ng of 1D11 or an Eli Lilly antibody (mouse IgG1) or Isotype control provided by Eli Lilly, beginning one day before infection, and then every 7 days until day 21 of infection.

Flow cytometry

Fluorochrome labeled antibodies for flow cytometry were purchased from ebioscience or BD biosciences and used to stain blood or spleen cells: anti-CD8-pacific blue, anti-CD4-pacific blue, anti-CD19-PE, anti-CD38-Alexafluor700, anti-GL7-FITC, anti-PD1-PE-Cy7, anti-IFN-γ-APC, anti-TNF-α-FITC, anti-IL-2-PE, and B220-PE. For staining with Db NP396–404 (provided by NIH Tetramer Core Facility; Atlanta, GA) or

Db-GP33–41 (Beckman Coulter; Fullerton, CA) tetramers, cells were incubated for1 hr and 15 min at room temperature. BD phosflow pSTAT-3(pY705)-PE antibody and staining reagents were purchased from BD and carried out according to instructions provided. Cells were acquired using the Digital LSR II flow cytometer (Becton Dickinson, San Jose, CA) and the data was analyzed with FlowJo software.

***Ex-vivo* T cell stimulation**

Blood cells or splenocytes were stimulated with recombinant murine IL-2 (50 U/ml R&D Systems) and Brefeldin A (1 μg/ml; Sigma) and MHC class-I restricted LCMV GP33–41 (2 μg/ml, all >99% pure; Synpep) and cultured for 5 hours at 37^0 C. Cells were then stained for surface expression of CD8, fixed, permeabilized and stained with antibodies for IL-2, IFN-γ, and TNF-α.

ELISA

To determine relative levels of LCMV specific antibody on a sandwich ELISA, LCMV antigen was purified on a renograffin gradient via ultracentrifugation at 15000 rpm, and a Bradford assay was done to determine antigen concentration. A 96 well ELISA plate was coated with 1 μg/ml of antigen. Serial dilutions of serum were carried out and LCMV specific antibody was detected by using purified HRP conjugated anti-mouse Ig, IgG1 or IgG2a (Southern Biotech) antibodies followed by TMB substrate (eBioscience) and absorbance read by spectrophotometer at a wavelength of 450 nm. VSV antigen control was purified in the same way and run alongside samples on the ELISA plate, as a positive control. Antibody avidity was determined by treating samples

with 3 washes of 3 M Urea for 5 minutes each at room temperature following incubation of serum samples and compared to PBS treated samples[75].

Statistical analysis

Non parametric unpaired student's t-tests and ANOVA tests were done using the InStat 3.0 software (GraphPad, CA.).

Chapter 1: Therapeutic Potential of TGF-β Signaling Blockade In Chronic Viral Infection

TGF-β is a potent cytokine with a variety of biological functions. During chronic viral infection, TGF-β can act as a negative regulator of the immune system. Previous work in the lab has shown that signaling by TGF-β leads to apoptosis of virus specific $CD8^+$ T cells[22]. I wanted to test the therapeutic potential of TGF-β signaling blockade in chronic infection. I hypothesized that administration of antibodies that neutralize TGF-β would improve the number of virus specific $CD8^+$ T cells, and result in enhanced viral control.

Results

Between days 1 and 21 post LCMV Cl13 infection there is significant and progressive virus specific $CD8^+$ T cell exhaustion and deletion, and establishment of high viremia[9]. Therefore to test the therapeutic potential of neutralizing TGF-β in preventing T cell apoptosis and dysfunction, we treated LCMV Cl13 infected mice from the day before infection until day 21 p.i.. Mice were injected with 750 ng of two distinct monoclonal anti-TGF-β antibodies, 1D11 (a commonly used, commercially available antibody) and an Eli Lily developed anti-TGF-β (designed for therapeutic use in a number of human diseases)(Figure 1A). Previous testing by Eli Lily revealed that treatment with 0.0375 mg/kg per mouse every 7 days provided optimal neutralization capacity in tumor models. We therefore selected this dosing regime for initial trials in LCMV Cl13 infection.

At day 9 p.i. peak viremia was neither enhanced, nor reduced compared by treatment compared to isotype treated mice (Figure 1B). By day 60 p.i., there was no detectable virus in the blood of all mice. From day 15 until 45 post infection it appeared that the viral titer in the Eli Lilly treated group was lower as measured by immunofocus assay, however by plaque assay these differences were not significant. There was also no difference in viral load between αTGF-β treated and isotype groups in multiple tissues at day 60 p.i. (Figure 1C).

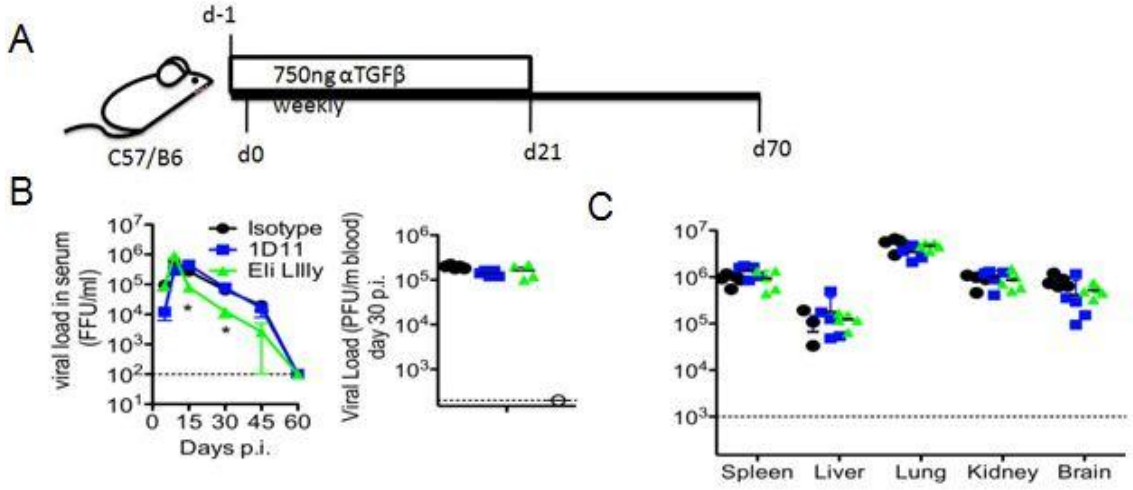

Figure 1: Low Dose TGF-β Neutralization In Chronic Viral Infection Has No Effect on Viral Load. Each mouse was infected with 2x106 PFU of LCMV Cl13 and given 750 ng of αTGF-β (either 1D11, or Eli Lilly(EL), or Isotype Control) once every 7 days from -1 to 21 days p.i..
A) Diagram of experimental design. B) Kinetic of viremia throughout LCMV Cl13 infection determined via immunofocus assay (Eli Lilly found to be significantly lower) and day 30 p.i. via plaque assay, could not confirm result of immunofocus assay.C) Tissue viral load at day 70 p.i. determined via immunofocus assay. No significant difference is observed in viral load of treated and untreated mice. Data representative of 1 experiment of 5 mice per group.

As has previously been reported, in untreated and isotype treated mice normally immunodominant H2-$D^b NP_{396-404}{}^+$ $CD8^+$ T cells were rapidly deletion (data not shown), leaving H2-$D^b GP_{33-41}{}^+$ $CD8^+$ T cells as the dominant epitope specific $CD8^+$ T cell

population. Neutralization of TGF-β with either 1D11 or Eli Lily Ab did not enhance virus specific $CD8^+$

T cell numbers (Figure 2A). $D^bGP_{33\text{-}44}{}^+$ $CD8^+$ T cells from treated and untreated

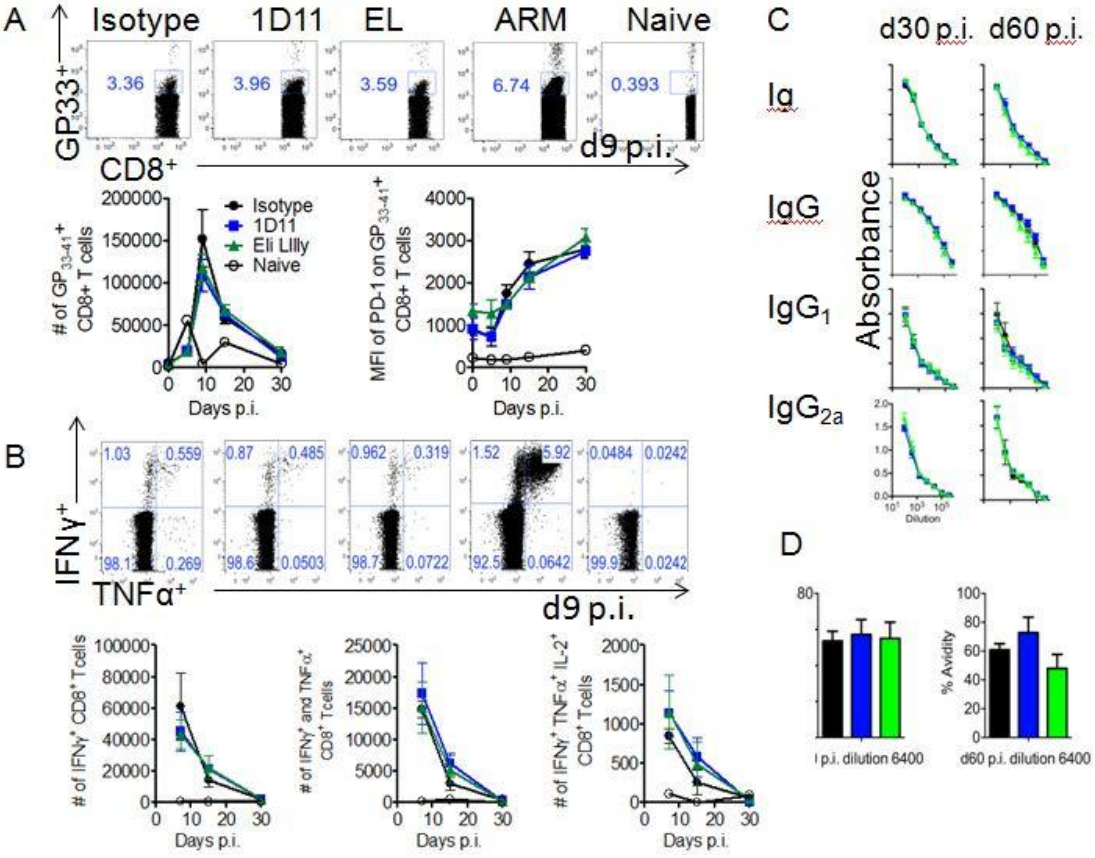

Figure 2: Low Dose TGF-β Neutralization In Chronic Viral Infection Has No Effect on CD8+ T cells. Experimental was carried out as in Figure 1.

A) Representative FACS plot of d9 p.i. with tetramer staining for virus specific H2 DbGP33-41+ CD8+ T cells in the blood and kinetic of total number of GP33-41+ CD8+ T cells and PD1 Mean fluorescence intensity (MFI) on these cells throughout infection. B) Representative FACS plots gated on CD8+ of IFNγ+ and TNFα+ after 5 hour GP33 peptide stimulation d9 p.i. and kinetic of these cells throughout infection. C) Graphs of LCMV specific antibody isotypes in diluted serum d30 and d70 p.i., done via ELISA. D) Avidity assay done on antibodies at d30 and d70 p.i. by treating samples with 3 washes of 3 M urea for 5 minutes. Data representative of 1 experiment of 5 mice per group.

mice also had similar expression of PD-1, a surface marker known to correlate with viral load and T cell exhaustion (Figure 2A) Likewise, loss of function, as measured by comparison to LCMV Arm induced $CD8^+$ T cells, was similar in treated and untreated mice, with the majority of GP_{33-41} stimulated $CD8^+$ T cells producing only IFN-γ, and a small fraction capable of simultaneous production of IFN-γ, TNF-α and IL-2 (Figure 2B) Overall these results indicated that neutralization of TGF-β using this regime was insufficient to prevent virus specific CD8 T cell apoptosis or dysfunction.

Humoral immunity is also known to be required for control of LCMV Cl13, however, at day 30 and day 60 p.i. there were no differences found in serum levels of LCMV specific Ig, IgG, IgG_1 or IgG_{2a} produced between the αTGF-β treated mice and isotype treated mice (Figure 2C). The avidity of LCMV specific antibodies was also unaltered by neutralizing TGF-β (Figure 2D).

Discussion

Here we showed that treatment with two different anti-TGF-β antibodies, at low doses, was insufficient to enhance cellular or humoral immunity against LCMV Cl13 infection. Interestingly at the same time that I was conducting this research, two other studies on TGF-β blockade during LCMV Cl13 infection were published. One approach focused on blocking TGF-β early in LCMV clone 13 infection with high doses of 1D11[76], while another approach published this year attempted to block the TGF-βR after day 21 of infection[77]. Both studies used higher concentrations (400 μg or 1mg/mouse every other day) of 1D11 antibody and found that a significant increase in virus $CD8^+$ could be promoted by this treatment but concluded that this was temporary and did not significant

alter the course of the viral infection. Of note, none of the studies evaluated whether the treatments resulted in attenuation of TGF-β signaling (smad phosphorylation) in virus specific $CD8^+$ T cells after infection, leaving open the possibility that the lack of effect in viral control could be related to insufficient blockade of bioactive TGF-β and/or TGF-β signaling on these cells.

A number of reasons exist for the failure of anti-TGF-β therapy during chronic infection. 1D11 and EL antibodies bind to all three isoforms of TGF-β, this may result in reduced targeting of TGF-β1, the isoform thought to primarily be responsible for $CD8^+$ T cell apoptosis during chronic infection. Location and timing are also a factor; The precise source of TGF-β during LCMV Cl13 is unknown but it can be produced by a wide variety of cells. Indeed we have shown that there is increased TGF-β production by virus specific $CD8^+$ T cells themselves, and if this TGF-β is acting in an autocrine fashion upon the cells, the window of opportunity for blockade of soluble TGF-β may prove very limited. The dose of the αTGF-β used in our study might have been too low to cause a significant biological result in $CD8^+$ T cells. Indeed it would be of interest to look at SMAD2/3 phosphorylation in virus specific $CD8^+$ T cells in the treated and untreated groups to determine if the low dose was effective at blocking sufficient TGF-β signaling, and determine if high doses may be more appropriate.

To further explore whether therapeutic TGF-β signaling blockade in chronic infection can lead to increased survival of virus specific $CD8^+$ T cells and enhanced viral clearance, other therapeutic regimes should be tried. 1D11 and EL antibody bind to TGF-

β1-3 indiscriminately and may trigger compensatory mechanisms; using more specific antibodies that bind specific isoform of TGF-β may increase the likelihood of reducing the apoptosis of virus specific cells. Alternatively combination therapies, where TGF-β signaling could be blocked along with PDL-1 or LAG-3 which are known to alter the function of $CD8^+$ T cells, may improve the efficacy of treatment. Alternatively, blocking TGF-β signaling by targeting TGFβR or molecules downstream of the receptor in $CD8^+$ T cells may be required to overcome the small window of opportunity available, however further understanding of the biology that underlies TGF-β induced apoptosis is required before such approaches can be tested. Other approaches for neutralizing TGF-β signaling would be to target latent TGF-β from becoming active, by blocking protease activity and/or integrin activity. Exploration of these strategies should eventually lead to the optimal manner in which to block the TGF-β pathway during chronic viral infection with beneficial outcome.

Chapter 2: Role Of gp130 In Chronic and Acute Viral Infection

As described in the introduction, we have previously shown signaling of the cytokine IL-6 is essential for control of chronic viral infection[63]. IL-6 was crucial in the development of virus specific T_{FH} cells in this context, and in its absence T_{FH} and anti-viral antibody responses were dramatically reduced. IL-6 is the prototypic member of a family of cytokines that all use the transmembrane protein gp130 for signal transduction. By using *CD4*-cre *gp130*$^{fl/fl}$ mice, which have a conditional ablation of gp130 on their T cells, I wished to confirm the importance of IL-6 signaling on T cells during LCMV Cl13 infection. Given their shared use of gp130, the IL-6 family of cytokines, can play redundant roles in the immune response, however the overall role of this family during chronic viral infection has not been assessed. One potential indicator of gp130 signaling redundancy during chronic infection is the observation that IL-6 is a potent inducer of *Il21* in virus specific CD4$^+$ T cells *ex vivo* (and indeed TFH differentiation, normally a major source of IL-21) but is not required for the production of IL-21 from CD4$^+$ T cells *in vivo* during LCMV Cl13 infection[63]. Indeed IL-27, another member of the IL-6 family of cytokines, has also been shown to potently induce IL-21. IL-21 is critical not only for its ability to promote humoral immunity, but in chronic infection for the maintenance of virus specific CD8$^+$ T cells[50-52,78]. Therefore potential pathway that can lead to its production and overall CD4$^+$ T cell responses in general, are of great interest and I wished to investigate whether other members of the IL-6 family aside from IL-6 might play viral roles in chronic infection.

Results

CD4-cre *gp130*$^{fl/fl}$, IL6Rko and wildtype (WT) mice were infected with LCMV Cl13 and viremia monitored throughout infection (Figure 3A). No difference was found in peak viremia up until 15 p.i. but after day 30 p.i., mice lacking gp130 signaling on T cells failed to control virus and had persistently high viral titer until the conclusion of the

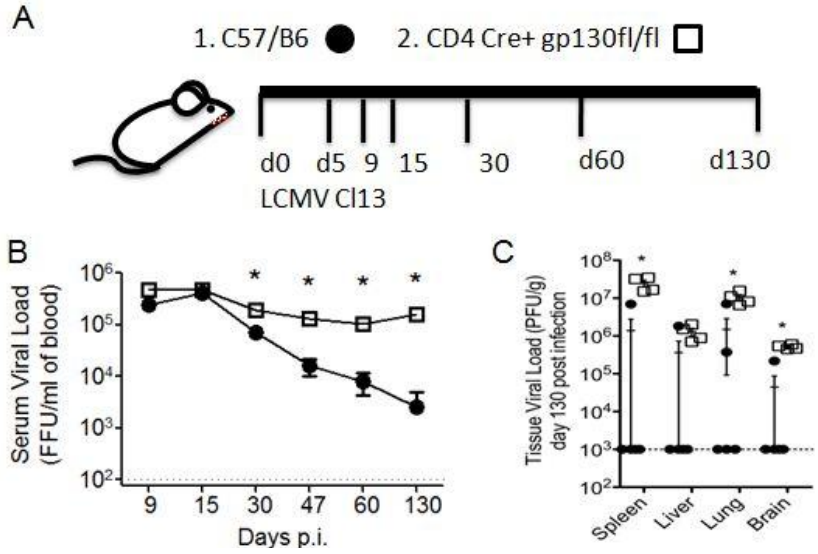

Figure 3: Gp130 Is Essential for Viral Control In Chronic Infection.
WT and CD4-cre gp130fl/fl mice were infected with 2x106 PFU of LCMV Cl13. A) Diagram of experimental design. B) & C) Kinetic of viremia throughout LCMV Cl13 infection (B) and tissue specific viral loads at d70 p.i. (C) were determined by immunofocus assay. Data representative of 2 experiments with 4 mice per group.

experiment, at day 130 p.i., while viral loads in WT mice declined from day 30 p.i. onwards and were undetectable by day 130 p.i. (Figure 3B). Viral load in the tissues was also examined at day 130 post infection in the liver, spleen, kidney, lung and brain. Mice lacking gp130 signaling on T cells had high viral titers present in all tissues compared to wild type mice, where 4 out of 5 mice had undetectable levels of virus (Figure 3C). This

data supported an essential role for gp130 signaling on T cells in controlling chronic viral infection, and also highlighted a potential role for other IL-6 family cytokines, other than IL-6, in this process.

To determine the immunological function of T cell gp130 signaling I analyzed the host immune response to LCMV Cl13. Until day 15 p.i. the number of $PD1^+$ $CD4^+$ T cells (which in chronic infection represent the antigen specific $CD4^+$ T cell compartment) was similar in WT and *CD4*-cre $gp130^{fl/fl}$ mice. By day 30 p.i., however, there was a significant reduction of $PD1^+$ $CD4^+$ T cells in mice lacking gp130, while in WT mice virus specific $CD4^+$ T cells remained at fairly constant levels until day 60 p.i. (Figure 4A). Similar results were obtained for virus specific $CD8^+$ T cell responses, at day 9

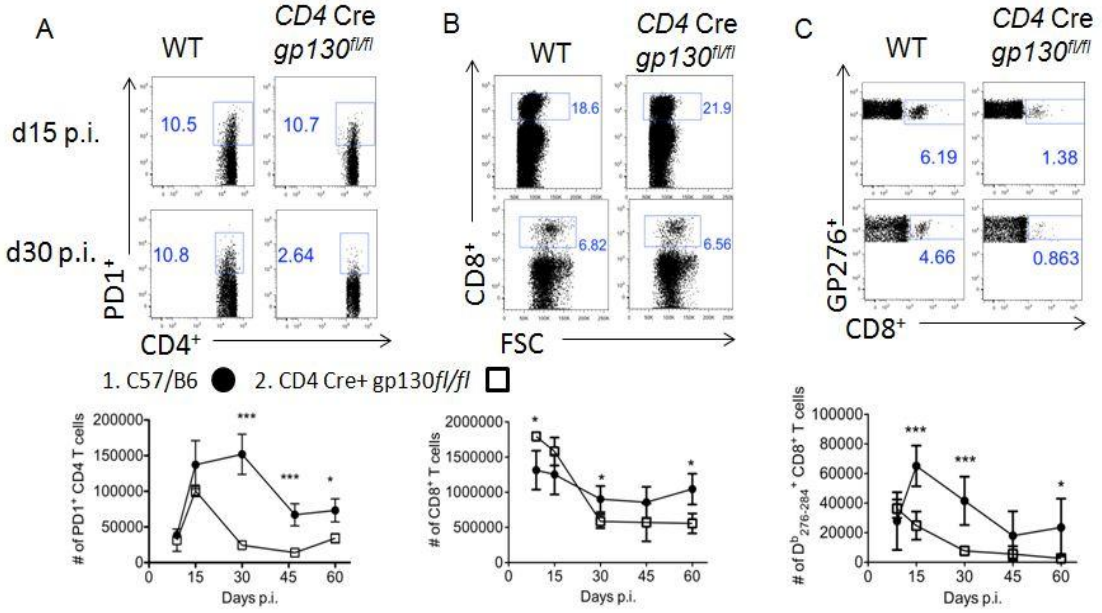

Figure 4: Gp130 Is Essential for Cellular Immunity In Chronic Infection. Experiment was carried out as in Figure 3. A) B) & C)Representative FACS plots at d15 and d30 p.i. and kinetic analysis of total number of antigen specific $CD4^+$ T cells (A), $CD8^+$ T cells (B), and virus specific D^b $GP_{276-284}{}^+$ $CD8^+$ T cells (C) in the blood determined by flow cytometry. Data representative of 2 experiments with 4 mice per group.

infection p.i. the proportion and number of D^b $GP_{276-284}^+$ $CD8^+$ T cells had expanded equally in the WT and *CD4*-cre *gp130*$^{fl/fl}$ mice. By day 30 p.i. total $CD8^+$ T cells were significantly lower in *CD4*-cre *gp130*$^{fl/fl}$ compared to WT (Figure 4B). However, from day 9 until day 60 p.i., the number of virus specific $CD8^+$ T cells continued to decline in mice lacking gp130 signaling on their T cells while WT mice had both a significantly higher number and proportion of virus specific CD8+ T cells in their PBMCs (Figure 4C). In conclusion this data showed that gp130 signaling on T cells was critical for the maintenance, but not formation, of both virus specific $CD4^+$ and $CD8^+$ T cell numbers.

IL-6 deficiency caused a dramatic decline in humoral immune responses to LCMV Cl13 infection, especially titers of LCMV specific IgG_1, which we believe to be the result of reduced T_{FH} induction. Mice lacking gp130 on their T cells, on days 30 and 60 p.i., LCMV specific levels of total Ig, IgG, IgG_1 and IgG_{2a} were all significantly lower compared to wild type mice (Figure 5A). The avidity of this antibody was also found to be greatly reduced in the absence of gp130 signaling on T cells (Figure 5B).In summary during chronic infection *CD4 cre gp130*$^{fl/fl}$ mice produced lower quantity and quality of LCMV specific antibody which was more extreme than observed in total IL6ko mice, where only LCMV specific IgG_1 and antibody avidity were affected[63,74].

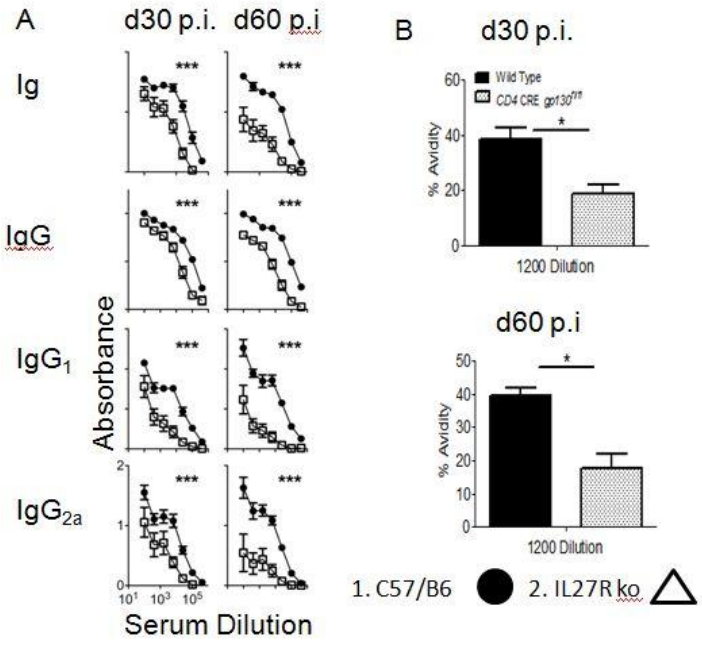

Figure 5: Gp130 is Indirectly Essential for Humoral Immunity In Chronic Infection.
Experiment was carried out as in Figure 3. A) & B) Virus specific antibody(A) and their avidity(B) in diluted serum d30 and d60 p.i., were measured by ELISA. Data representative of 2 experiments with 4 mice per group.

Acute Viral Infection and the importance of Gp130 receptor signaling in T cells

Given the dramatic effect on T cell numbers seen after LCMV Cl13 infection of *CD4*-cre *gp130*$^{fl/fl}$ mice, I wished to confirm whether this signaling was equally important in other acute viral infection. After infection with acute LCMV Arm (Figure 6A), *CD4*-cre *gp130*$^{fl/fl}$ mice were able to clear LCMV Arm infection at a similar rate to WT mice (Figure 6B). The number of antigen specific $CD4^+$ T cells, in this case defined as $CD11a^+$ $CD49d^+$,[79] was similar in *CD4*-cre *gp130*$^{fl/fl}$ and WT mice (Figure 7A). The same was true for the number of H2-$D^b$$GP_{33-41}$$^+$ and H2-$D^b$$NP_{396-404}$$^+$ $CD8^+$ T cells, fitting with the

successful viral control which is predominantly $CD8^+$ T cell mediated in this model(Figure 7B&C). This data supports the conclusion that gp130 signaling is uniquely

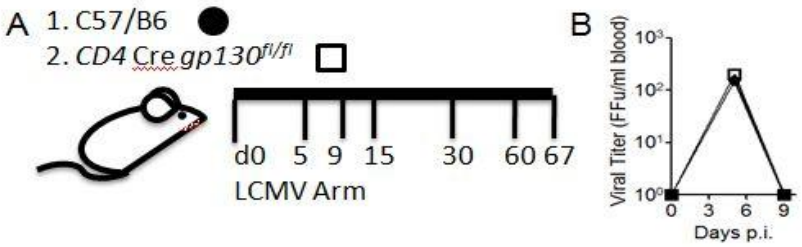

Figure 6: Gp130 Is Not Essential For Viral Control In Acute Infection. WT and CD4-cre gp130fl/fl mice were infected with $2x10^6$ PFU of LCMV ARM. A)Diagram of experimental design. B)Kinetic of viremia throughout LCMV ARM infection determined via plaque assay. Data representative of 2 experiments of 4 mice per group.

required for the survival of antigen specific T cells during chronic viral infection.

Intriguingly, despite limited effects on the number of antigen specific T cells and viral load, there did seem to be a role for gp130 signaling on T cells in humoral immune responses to acute infection. At day 30 p.i. there were equal or lower amount of LCMV specific Ig or IgG_{2a} in *CD4*-cre $gp130^{fl/fl}$ mice compared to WT mice, but they had a significantly higher amount of LCMV specific IgG_1. At day 60 p.i. with LCMV Arm, LCMV specific levels of Ig were similar, but the IgG_1 continued to be much higher in the *CD4*-cre$gp130^{fl/fl}$ mice while IgG_{2a} was lower compared to wild type mice(Figure 7D). This data indicates a role for T cell gp130 signaling in determining the quality of antibody responses during infection, even in the absence of significant reductions in T cell numbers.

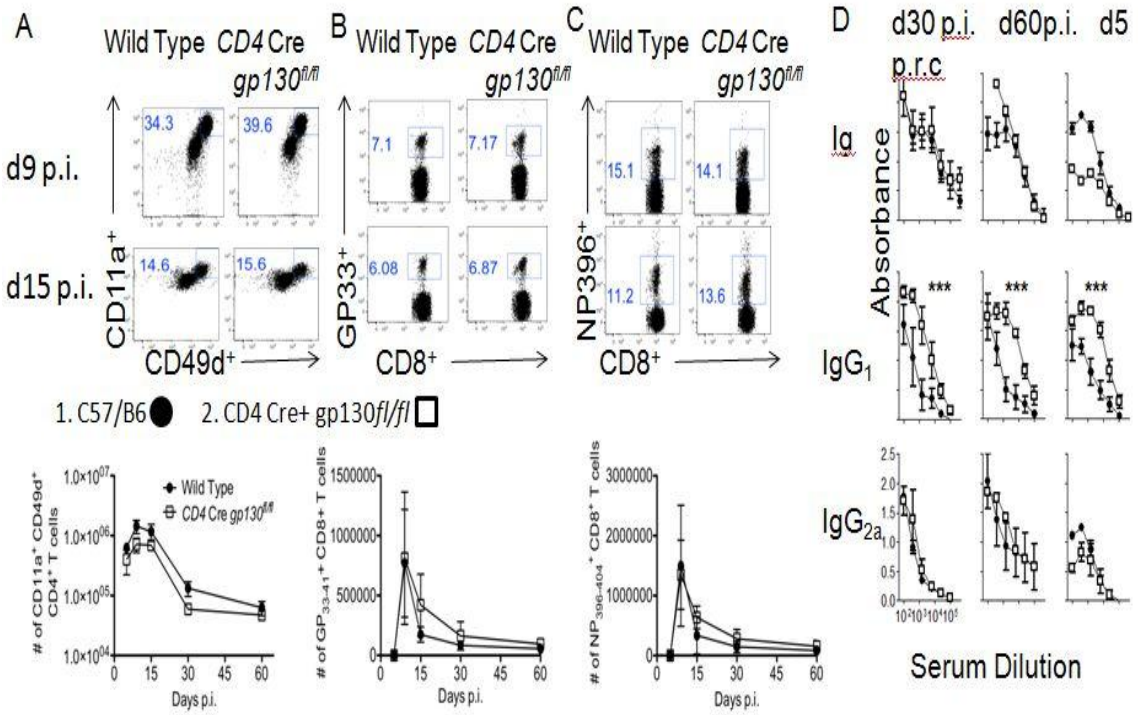

Figure 7: In Acute Infection Gp130 Has a Role In Humoral But Not Cellular Immunity. Experiment was carried out as in Figure 6. A) B) & C) Representative FACS plots at d9 and d15 p.i. and kinetic analysis of total number of antigen specific $CD4^+$ T cells(A), virus specific H2-D^b $GP_{33\text{-}41}{}^+$ $CD8^+$ T cells(B), and immunodominant H2-$D^b$$NP_{396\text{-}404}{}^+$ $CD8^+$ T cells(C) in the blood determined by flow cytometry. D) Virus specific antibody in diluted serum d30, 60 p.i., and d5 post rechallenge (p.r.c.), measured by ELISA. Data representative of 2 experiments with 4 mice per group.

Discussion

Here we found that in chronic infection, in the absence of gp130 signaling on T cells, there was much lower number of virus specific T cells at later stages of infection. *CD4*-cre *gp130*$^{fl/fl}$ mice also failed to mount an effective antibody response, showing the vital role of $CD4^+$ T cells and the IL-6 family of cytokines in this process. Further investigation has revealed that *CD4*-cre *gp130*$^{fl/fl}$ mice have lower numbers of germinal center B cells in their spleen than WT counterparts[63]. With both cellular and humoral

immunity impaired, control of LCMV Cl13 infection is ineffective. The viral titer in the blood in *CD4*-cre *gp130*$^{fl/fl}$ mice was higher than IL6R knockout mice, indicating that other cytokines of the IL-6 family cytokines play a role in viral control (data not shown). It was also confirmed in the lab that gp130 signaling is essential for IL-21 production from splenic virus specific $CD4^+$ T cells. Additionally it was found that direct gp130 signaling on $CD4^+$ T cells, and not $CD8^+$ T cells, is the critical step in the immune response to chronic infection, as it is required for both survival (gp130 deficient $CD4^+$ T cells show higher levels of apoptosis) and the production of IL-21. These defects in turn appear to curb $CD8^+$ and B cell responses.

When mice were infected with LCMV Armstrong it was found that gp130 signaling on T cells had no effect on cellular immunity or viral load in acute infection, but there is an effect on humoral immunity. When T cells lacked signaling through the gp130 receptor there was an increased IgG_1 response. A feature maintained in secondary challenge. Skewed production of IgG_1 is characteristic of Th2 biased immunity or altered T_{FH} function[80,81]. Given the restriction of gp130 deficiency to T cells and the critical role that several of the gp130 cytokines are known to play in $CD4^+$ differentiation, these responses are worthy of investigation in future studie

Chapter 3: Role of IL-27 In Chronic and Acute Viral Infection

As described in the previous chapter I found that gp130 receptor on T cells was essential for viral control, and controlled both cytotoxic and humoral immunity through $CD4^+$ T cell helper responses. I wished to explore which other gp130 cytokines, other than IL-6, were essential for immune response. Several members of the IL-6 family of cytokines are known to influence the immune system. IL-11 has been previously shown to be important in cell survival, particularly dendritic cells[82] and vascular endothelium[83], regulate autoimmune demyelination in the CNS[84], and antigen induced sensitization[85]. LIF has been found to be critical in T cell fate and maturation in the thymus[86] as well as being a key cutaneous anti-inflammatory[87]. Of all the gp130 cytokines however IL-27 is the most studied, particularly in parasitic infections, with regards to innate and adaptive immune responses. Previous studies have shown that innate immunity cells such as macrophages, mast, and NK cells express the IL-27 receptor. When IL27R is genetically ablated in these cells, an increase in activation and production of inflammatory cytokines was observed, which indicates that IL-27 has suppressive role on a variety of immune cells[88-90].

IL-27 has also been shown to play an important role in $CD4^+$ T cell responses. IL-27 has been shown to promote proliferation of naïve $CD4^+$ T cells[91] and is necessary for early development of Th1 response during listeria[92] and the secretion of IL-10 and IL-21 in *in vitro* cultures of T cells[93]. IL-27 not only enhances Th1 response, but also inhibits GATA3 expression[94], a transcription factor that promotes Th2 differentiation[95]. It was also found that in naïve $CD4^+$ T cells, IL-27 is capable of activating JAK2, tyrosine

kinase 2, STAT2, STAT3, and STAT5[96] suggesting that IL-27 may have some overlapping immune functions with IL-6 in chronic infections. In soon to be published work, examination of the ability of these cytokines to induce downstream signaling *ex vivo* in virus specific $CD4^+$ T cells taken from a chronic infection revealed that IL-27 was the only cytokine except for IL-6 that could induce phosphorylation of STAT-3. Given these data we wished to examine if IL27 signaling (via the ablation of the IL-27R, WSX-1) was also required for immunity against chronic viral infections similar to gp130 and IL-6.

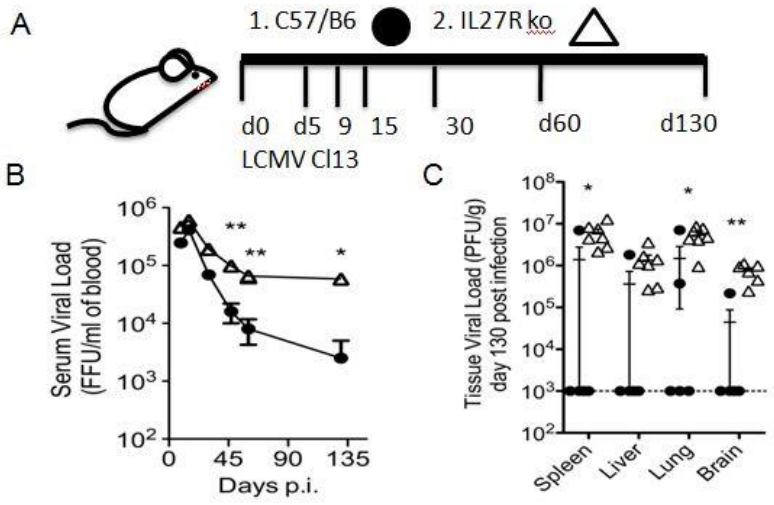

Figure 8: IL-27 Is Essential for Viral Control In Chronic Infection. WT and IL27Rko mice were infected with $2x10^6$ PFU of LCMV Cl13. A) Diagram of experimental design. B) & C) Kinetic of viremia throughout LCMV Cl13 infection(B) and tissue specific viral loads at d130 p.i. (C) were determined by immunofocus assay. Data representative of 2 experiments with 4 mice per group.

Results

IL-27 signaling in Chronic Viral Infection

Infection of IL27R deficient and B6 control mice with LCMV Cl13 showed similar viral loads up until day 30 post infection (Figure 8A), however as we had seen previously with IL-6R ko and *CD4*-cre *gp130*$^{fl/fl}$ mice, after day 45 p.i. IL27Rko mice failed to display viral control and viremia remained high until the conclusion of the experiment, day 130 p.i., while wild type mice successfully cleared infection (Figure 8B). Fitting with this tissue specific viral loads were high in IL-27R ko mice at day 130 p.i. and largely absent in WT mice (Figure 8C).

Examination of IL27R ko mice revealed that the number of total antigen specific CD4$^+$ T cells was similar in IL27R ko mice compared to wild type, though a lower percentage was observed later in infection (Figure 9A). The proportion and number of

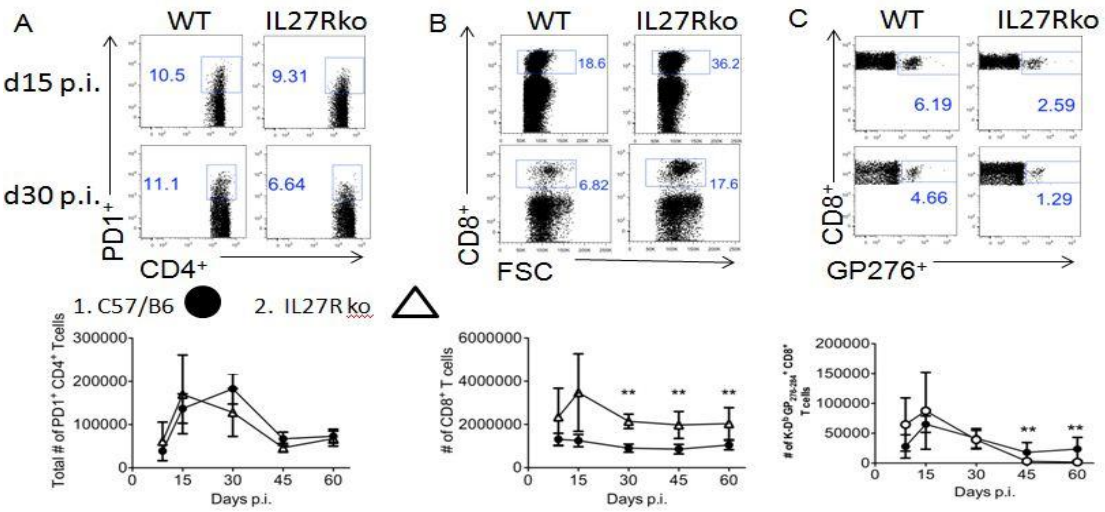

Figure 9: IL-27 Has Vital Role In Cellular Immunity During Chronic Infection. Experiment was carried out as in Figure 8. A) B) & C) Representative FACS plots at d15 and d30 p.i. and kinetic analysis of total number of antigen specific CD4$^+$ T cells (A), CD8$^+$ T cells (B), and virus specific D^b GP$_{276\text{-}284}$$^+$ CD8$^+$ T cells (C) in blood determined by flow cytometry. Data representative of 2 experiments with 4 mice per group.

CD8 T^+ cells was higher in IL27R ko mice compared to wild type mice throughout infection, particularly at day 9 and 15 p.i. (Figure 9B). In contrast the proportion of virus specific H2-$D^bGP_{276-286}{}^+$ CD8 T^+ cells was lower in IL27R ko mice versus wild type mice, meaning the total number of virus specific $CD8^+$ T cells was similar up to day 30 p.i.. As infection progressed, the proportion decreased, but the total number was significantly lower at d45 and 60 p.i. compared to wild type mice (Figure 9C).

IL-27R was essential for development of LCMV specific total Ig, IgG and IgG_{2a} compared to wild type mice at day 30 and 60 p.i.. LCMV specific IgG_{2a} levels in particular were very low at day 30 and 60 p.i. in the IL27R knockout mice (Figure 10A).

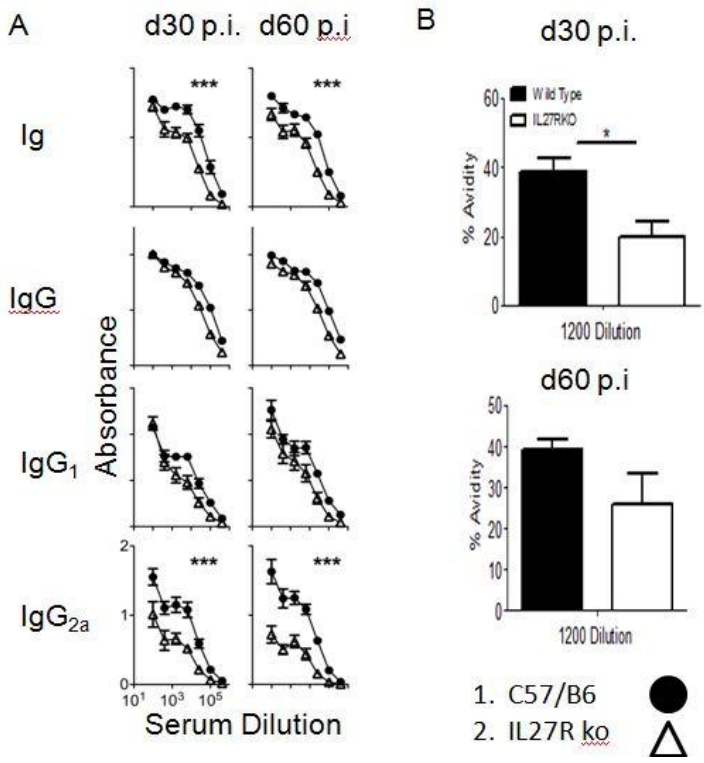

Figure 10: IL-27 is Essential for Humoral Immunity In Chronic Infection. Experiment was carried out as in Figure 8. A) & B) Virus specific antibody(A) and their avidity(B) in diluted serum d30 and d60 p.i., were measured by ELISA. Data representative of 2 experiments with 4 mice per group.

The avidity of LCMV specific antibodies were also found to be diminished at d30 p.i. in IL27R ko mice (Figure 10B). This data suggested that IL-27 is involved in the generation of specific isotypes of antibodies.

IL-27 signaling in Acute Viral Infection

As was seen for LCMV Arm infection of *CD4*-cre *gp130*$^{fl/fl}$ mice, IL-27 signaling was not essential for control of acute infection (Figure 11A), with IL27Rko mice showing

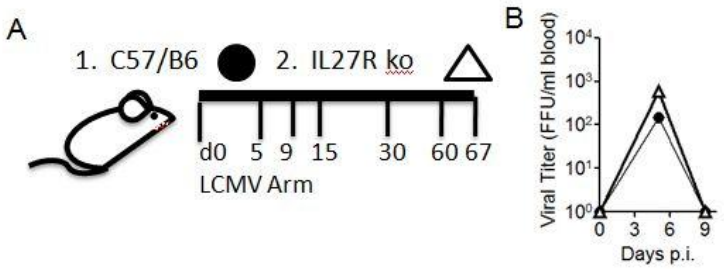

Figure 11: IL-27 Is Not Essential For Viral Control In Acute Infection. WT and IL27Rko mice were infected with 2x10^6 PFU of LCMV ARM. A)Diagram of experimental design. B)Kinetic of viremia throughout LCMV ARM infection determined via plaque assay. Data representative of 2 experiment of 4 mice per group.

similar peak viremia and clearance to that of WT mice (Figure 11B).

In acute viral infection, IL27R ko mice had elevated proportion of antigen specific CD4$^+$ T cells, but similar total number compared to wild type mice throughout acute infection (Figure 12A). IL-27 was found to have an important role in cellular immunity, particularly in the inhibition of CD8$^+$ T cell expansion. As seen chronic viral infection, there was a higher proportion and total number of CD8$^+$ T cells throughout infection (Figure 12B). Virus specific CD8$^+$ T cells were also affected. IL27R ko mice had equal

proportion but higher total number of H2-Db GP_{33-41}^{+} $CD8^{+}$ T cells (Figure 12C). It was also found that IL27R ko mice had a lower proportion but same number of immunodominant $D^{b}NP_{396-404}^{+}$ CD8 T^{+} throughout acute infection, compared to wild type mice (Figure 12D).

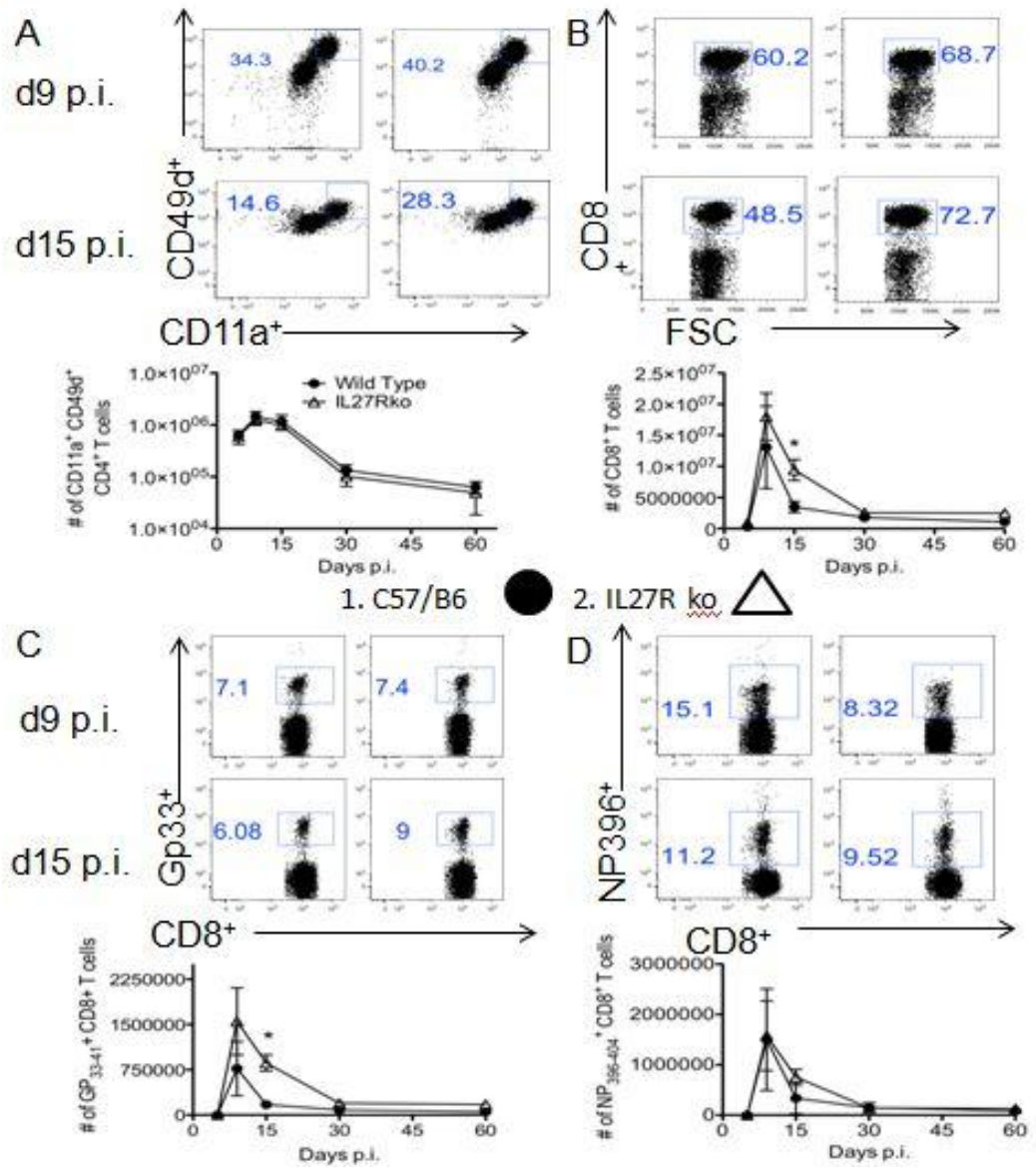

Figure 12: IL-27 Has Vital Role In Cellular Immunity During Acute Infection. Experiment was carried out as in Figure 11. A) B) C) & D) Representative FACS plots at d9 and d15 p.i. and kinetic analysis of total number of antigen specific $CD4^+$ T cells (A), $CD8^+$ T cells (B), virus specific H2-D^b $GP_{33\text{-}41}{}^+$ $CD8^+$ T cells(C), and immunodominant H2-$D^b$$NP_{396\text{-}404}{}^+$ $CD8^+$ T cells(D) in blood determined by flow cytometry. Data representative of 2 experiment with 4 mice per group.

Unlike in chronic infection, IL-27 signaling does not have an effect on humoral immunity in acute infection. At d30, 60 p.i. and d5 p.r.c. it was found that there was no

difference in LCMV specific antibody production of Ig, IgG_1, or IgG_{2a} in IL27R ko mice, compared to wild type mice (Figure 13A).

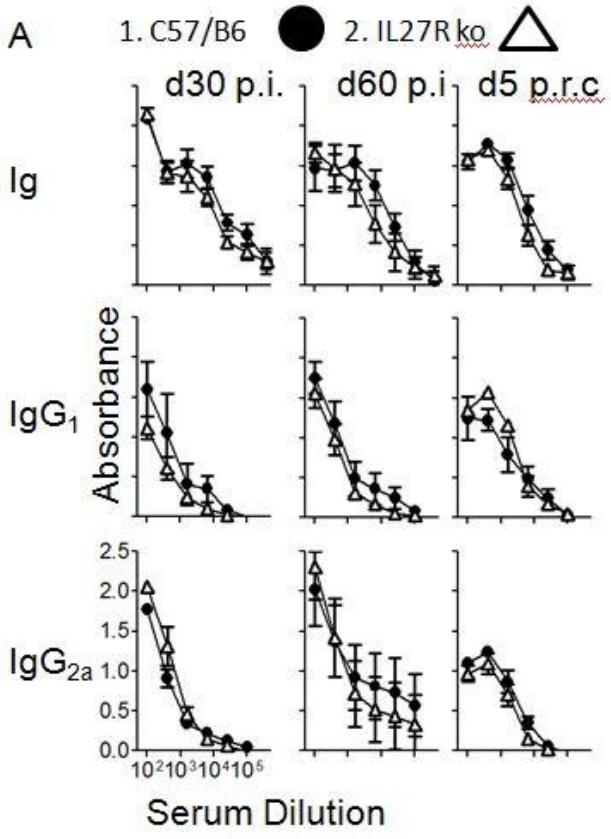

Figure 13: IL-27 Does Not Play a Vital Role In Humoral Immunity In Acute Infection. Experiment was carried out as in Figure 11. A) Virus specific antibody in diluted serum d30, 60 p.i., and d5 post rechallenge (p.r.c.), measured by ELISA. Data representative of` 2 experiment with 4 mice per group.

Discussion:

Here I showed that IL-27 signaling is essential in control of chronic, but not acute, viral infection. Intriguingly the expansion of $CD8^+$ T cells was more substantial in absence of IL-27Rko, although this was more significant in total, rather than virus specific, cells. LCMV Arm clearance is predominantly driven by cytotoxic activity[97], and as with gp130, presence of increased $CD8^+$ T cells in these conditions likely would be beneficial, rather than detrimental, to viral control. Previous studies with done with WSX-1 ko mice in other infectious models indicates that IL-27 may play a role in CD8+ T cell activation and number. It was found that WSX-1ko mice to have increased activation of $CD8^+$ T cells during *Toxoplasma gondii*[98], and in infection of tuberculosis they have higher number of $CD8^+$ T cells in their lungs[99]. Interestingly studies have also shown that IL-27 signaling can also promote the effector function in $CD8^+$ T cells[100], presenting dichotomous function for IL-27 in this part of the immune system.

In contrast to acute infection, under normal WT conditions cytotoxic $CD8^+$ T cells alone are usually insufficient to clear chronic LCMV infection (although they are required for it), and enhanced $CD8^+$ T cell numbers in the absence of IL-27R were less apparent in these conditions as well. This contrast in CD8 responses in acute versus chronic infection in IL27R ko mice is probably due to the dominant role that T cell apoptosis and loss of proliferation play in chronic infection[22,101-103]. In addition I showed that IL-27 signaling is necessary for humoral immunity in chronic, but not acute, infection and lack of IL-27 results in loss of LCMV specific $IgG_{2a.}$ This reinforces the

importance that the humoral immune response has in controlling LCMV Cl13 infection. The maintenance of cytotoxic responses in IL27Rko mice probably allowed control of viremia early in infection, but as the T cells became deleted or exhausted, reliance on humoral immunity appears to increase. Whether IL-27 signaling influences IgG_{2a} responses directly via B cells, or through an accessory cells (e.g. T follicular helper T cell responses) is currently unclear. It is interesting to note however that previous work by our lab has shown that IL-6 defect leads to significant reduction in virus specific $IgG_{1,}$ but not IgG_{2a}, while mice lacking gp130 signaling on their T cells have reduced production of both virus specific IgG_1 and IgG_{2a}. This fits with the hypothesis that IL-27 signaling on $CD4^+$ T cells is vital for the development of IgG_{2a} promoting T cell help, however the decrease of virus specific $CD4^+$ T cell numbers in the absence of gp130 makes it difficult to elucidate its importance in modulation of their antibody producing capacity. In support of IL-27 role in T cell dependent antibody response in a peptide vaccination model IL-27 was found to be important for T_{FH} differentiation. Unfortunately further work in our lab found that total IL-27R ko mice appeared to have no reduction in the number of virus specific T_{FH} in the spleen at day 9 or 30 p.i., or their production of IL-21 and expression of co-stimulatory molecules (Harker, J, personal communication). This observation may support a more direct role for IL-27 in antibody production.

It has also been shown that IL-27 can potently induce IgG_{2a} class switching in human and mouse B cells, and in other models IL-27 signaling is strongly linked to the upregulation of T-bet[96], a transcription factor heavily associated with increased Th1 and IgG_{2a} responses, supporting a direct role of IL-27 signaling in IgG_{2a} responses in mice. It

remains unknown however why such a mechanism would not also be apparent in acute infection, and it may be worthwhile investigating the importance in alternate acute viral infections, such as VSV, where antibody responses are vitally important[104]. Taken together this data places the IL-6 family of cytokines as central for the production of humoral and cytotoxic immunity during chronic viral infection.

Chapter 4: Therapeutic Potential of IL-6 and Hyper-IL6 In Chronic Viral Infection

As described in the previous chapters, I found that gp130 receptor on T cells was essential for viral control, and controlled both cytotoxic and humoral immunity through $CD4^+$ T cell helper responses. I wanted to explore the therapeutic potential of targeting this pathway using human IL-6 and Hyper IL-6, a designer cytokine composed of IL-6 fused to its receptor IL6R. Compared to IL-6, Hyper IL-6 has a longer half life due to its ability to potently activate STAT signaling. In addition, Hyper IL-6 can cause STAT signaling on cells that don't express IL6R because it only needs to bind to gp130, which is ubiquitously expressed[105].

A novel role for IL-6 has been identified by our lab as a positive regulator of the immune system during chronic viral infection. During LCMV Cl13 infection of mice, IL-6 is actually produced in a biphasic manner with an initial peak at day 1-4 p.i. (acute phase), low to undetectable amounts from day 5 to 21 of infection (plateau phase), and the second peak appearing at day 21-24 of infection (chronic phase)[63]. This second period of IL-6 production is essential for viral clearance in chronic viral infection. Previously our lab has administered either a low (20,000 U) and high dose (200,000 U) of IL-6 to mice days 5-11 p.i. twice daily. At the lower dose of IL-6 appeared to have some ability to reduce viral dose, while the high dose was found to be lethal. We hypothesized that therapeutic administration of recombinant IL-6 for the duration of the plateau phase of

IL-6 would enhance viral control by improving cellular and humoral immunity, resulting in accelerated clearance.

Results

Previously we found that treatment with high doses of IL-6 during LCMV Cl13 infection were found to result in mortality around day 10 p.i.. Therefore we chose to treat with the lower dose (20,000 U/day) the duration of the plateau phase, day 5 to day 21 p.i.. Another group of mice was treated with the same low dose of Hyper IL-6. Weight monitoring revealed that while LCMV Cl13 infection (Figure 14A) resulted in significant weight loss (approximately 30% by day 10 p.i.), daily treatment with low dose of IL-6 or Hyper IL-6 neither enhanced, nor reduced this effect, compared to PBS treated mice (Figure 14B). Treatment with IL-6 or Hyper IL-6 was found to be more lethal compared to PBS treated mice, 60% survival for both IL-6 and Hyper IL-6, and 80% PBS. The deaths all occurred at day 9 or 10 p.i., which coincided with peak weight loss of 30% day 10 p.i.(Figure 14C).

IL-6 is thought to signal through STATs, predominantly through the phosphorylation of STAT-3[62]. At day 21 p.i. 30 minutes after IL-6 treatment, levels of pSTAT3 in blood $CD4^+$ and $CD8^+$ T cells, but not CD19+ B cells or non B or T cells, were significantly higher compared to mice that received only PBS (Figure 14E). Mice that received Hyper IL-6 did not have any change in pSTAT3 signal in $CD4^+$ and $CD8^+$ T cells (Figure 14E). This data confirmed that IL-6 could potently induce signaling in $CD4^+$

T cells during infection; however Hyper-IL6 appeared to actually have a reduced capacity to achieve this in the cell types analyzed.

Despite this treatment, IL-6 had no effect on viremia until the natural clearance point of LCMV Cl13 in the blood, as seen in untreated mice (Figure 14D). In addition the

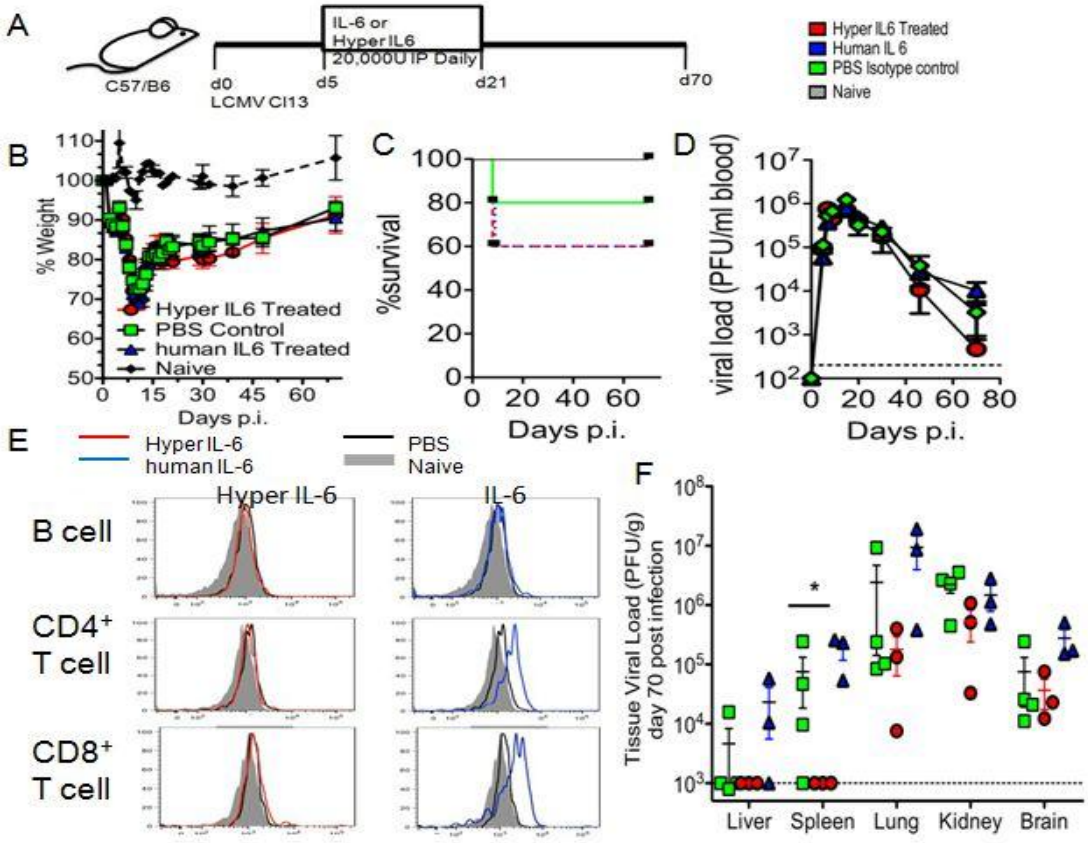

Figure 14: Effects Treatment with Low Dose IL-6 and Hyper IL-6 In Chronic Viral Infection. Each mouse was infected with $2x10^6$ PFU of LCMV Cl13 and treated with 20,000U of human IL-6 or Hyper IL-6 daily from d5 to d21 days p.i.. A) Diagram of experimental design. B) Graph of percent weight of mice throughout infection with LCMV Cl13. C)Survival curve throughout infection with LCMV Cl13. 1 mouse died in PBS control group, while 2 mice died in IL-6 and Hyper IL-6 treated groups around day 9 p.i.. D) Kinetic of viremia throughout LCMV ARM infection determined via plaque assay. E) Representative plots of phosphorylated STAT3 at day 21 p.i. in B cells, $CD4^+$ and $CD8^+$ T cells in mice injected half an hour prior with IL-6 or Hyper IL-6. F) Tissue specific viral load at d70 p.i. determined via plaque assay. Data representative of 1 experiment of 5 mice per group.

viral load in various tissues was also unaffected by low dose IL-6 treatment. On the other hand, mice treated with Hyper IL-6 had slightly lower viral titers in the blood at later stages of infection. It was observed that at day 45 and day 70 of infection, mice receiving Hyper IL-6 had lower blood viral titers, with 2/3 having cleared at day 70 compared to only ¼ in the PBS treated group. Viral load in the tissues was also examined at day 70 post infection; mice receiving Hyper IL-6 had no detectable virus in the spleen, while the majority of the mice in the PBS group still had high levels of LCMV (Figure 14F). No statistical difference was found in the viral load in the kidney, liver, lung and brain. This suggests that Hyper IL-6 has some antiviral effects.

IL-6 or Hyper IL-6 treatment during LCMV Cl13 infection does not enhance $CD8^+$ T cell immunity. Virus specific $CD8^+$ T cell responses were analyzed at various time points post infection. As has previously been reported, in untreated and PBS treated mice the normally immunodominant H2-$D^b NP_{396-404}{}^+$ $CD8^+$ T cells were deleted rapidly, while significant number of H2-$D^b GP_{33-41}{}^+$ $CD8^+$ T cells remained. The virus specific cells expressed high levels of PD-1, (Figure 15A). IL-6 or Hyper IL-6 treatment of mice neither enhanced the number or function of virus specific T cells.

At day 30 and 70 p.i. there were no differences found in serum levels of LCMV specific Ig, IgG, IgG_1 or IgG_{2a} produced between the IL-6 or Hyper IL-6 treated and PBS groups (Figure 15C). To further address the effects of IL-6 treatment on humoral immunity, the splenic B cells analyzed at day 70 p.i. Mice treated with IL-6 elevated numbers of $CD19^+$ B cells, but was not statistically significant (Figure 15B). The total

number of germinal center $GL7^{+}$, IgD^{-} B cells compared to the PBS treated group was also found to be not statistically significant. Treatment with human IL-6 or Hyper IL-6 in chronic viral infection had no considerable effect on humoral immunity compared to to PBS treated mice (Figure 12).

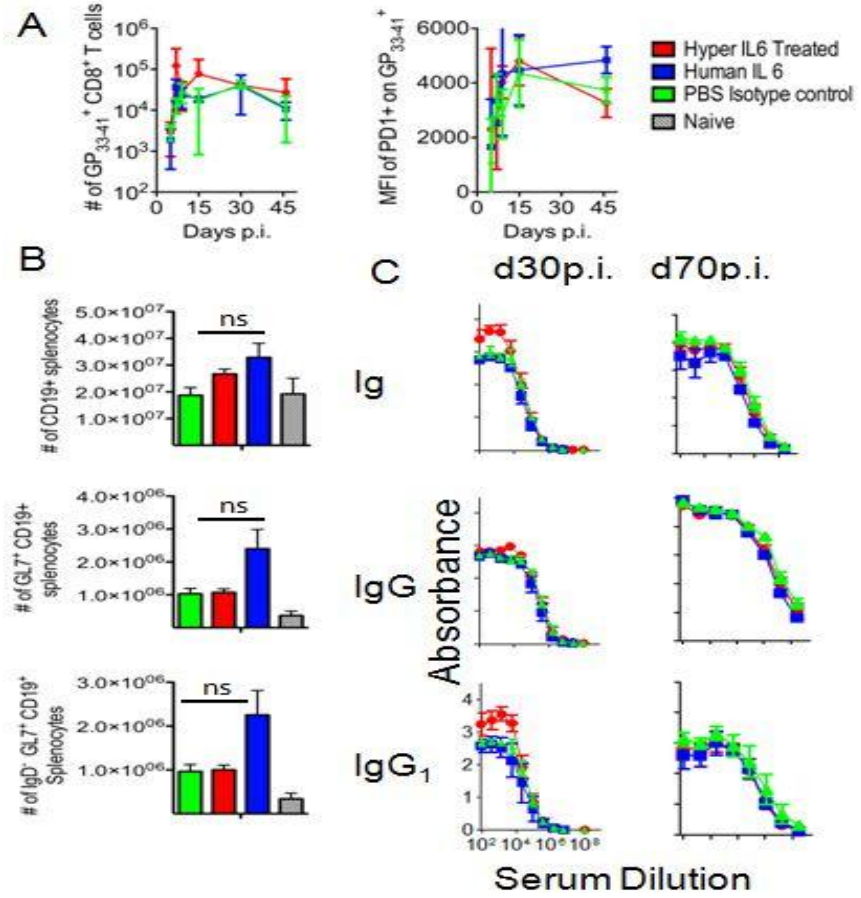

Figure 15: IL-6 Treatment May Play Role in Germinal Center B cells in Spleen. Experiment was carried out as in figure 14. A)Kinetic of number of virus specific H2-D^{b} GP$_{33\text{-}41}$$^{+}$ CD8^{+} T cells in the blood determined by flow cytometry throughout infection with LCMV Cl13 and exhaustion of those cells measured via MFI of PD1. B) Bar graphs of total number of CD19^{+} B cells, and germinal center GL7^{+} CD19^{+}, and IgD- GL7+ CD19+ B cells at d70 p.i. in spleen. C) Virus specific antibody in diluted serum d30 and d60 p.i., done via ELISA. Data representative of 1 experiment of 5 mice per group.

Discussion

To test the therapeutic potential of the gp130 signaling pathway, mice were treated daily with IL-6 or Hyper-IL6 days 5 through 21 p.i.. It was found that at later stages of LCMV Cl13 infection, IL-6 but not Hyper-IL6 treatment, signals on circulating T cells.

There was an increase in pSTAT3 levels in $CD4^+$ and $CD8^+$ T cells, but not B cells, which supports and fits with the mechanism of clearance needing $CD4^+$ T cell help for cellular and humoral immunity[63]. However, IL-6 signaling on the T cells did not lead to a change in their total number compared to untreated groups. There was also no difference in viral load or clearance throughout infection between the treated and untreated groups. Even though mice treated with Hyper- IL6 did not have increased signaling of pSTAT3 in B or T cells, there were some antiviral effects. The viremia of mice treated with Hyper-IL6 was lower at later stages of infection and had undetectable virus in the spleen compared to the untreated group. It is possible that these observations were a result of Hyper-IL6 binding to cells other than circulating B and T cells, and inducing signaling.

The main caveats of cytokine therapy are the unintended side effects due to the broad biological role cytokines have on different cell types. For example, IFN therapy is used in patients with HCV, and is correlated with decreased viral titer[106] but also with a variety of side effects, depending on the individual[107]. The side effect of IL-6 and Hyper-IL6 treatment was the survival rate was 60%. IL-6 has a variety of biological roles that are likely to have contributed to the increased mortality rate. IL-6 is a known pro inflammatory cytokine and also has a variety of effects on the vascular system. This might contribute to why the initial acute phase of IL-6 only lasts for about 5 days in LCMV Cl13 infection, after which there are no detectable levels in the blood until after day 21. And why mice lacking functional suppressor of cytokine signaling 3 (SOCS3) leads to embryonic lethality[108] or mice with SOCS3 deletion in hematopoietic cells, have elevated IL-6 levels and develop lethal inflammatory disease[109,110]. Collectively this data

suggests that cytokine and immunotherapy has antiviral potential, but further studies are necessary to understand the mechanisms and side effects involved. In an attempt to decrease side effects of cytokine therapy and have the desired antiviral effects, the next step would be to try nanoparticle treatments, which would allow for the targeting of a cell type, in this case $CD4^+$ T cells.

GENERAL DISCUSSION

The immune system has evolved over millions of years, its role emerging primarily to determine self from infectious or harmful non-self, has been critical for the survival of mankind. Today chronic viral infections, such as HIV and HCV, present one of the biggest health issues worldwide. Through studying the host immune system and its interaction with viral infectious agents we hope to develop novel techniques and therapeutic strategies.

The study of chronic LCMV Clone 13, versus its acute counterpart LCMV Armstrong, allows for the comparison of viral infections in a small animal model, allowing us to determine the critical steps involved in controlling such infections. During LCMV Cl13 infection mice lacking $CD4^+$, $CD8^+$ T cells, or B cells cannot control the virus[13] indicating that these cell types contributes to clearance. Based on data presented in this thesis project, published[41] and unpublished work ongoing in the lab, we have a working model of the mechanisms that lead to clearance of LCMV Cl13, specifically highlighting the role the IL-6 family of cytokines and CD4 T helper responses in this process:

During the later stages of infection, when the viral persistence has been established, virus specific $CD4^+$ T cells receive signaling through gp130 receptor, promoting survival and proliferation. IL-6 signaling leads to phosphorylation of STAT3 and a distinct pattern of transcription that is not seen at early times post infection. This leads to the upregulation of Bcl6, the master regulator of T_{FH} differentiation. The

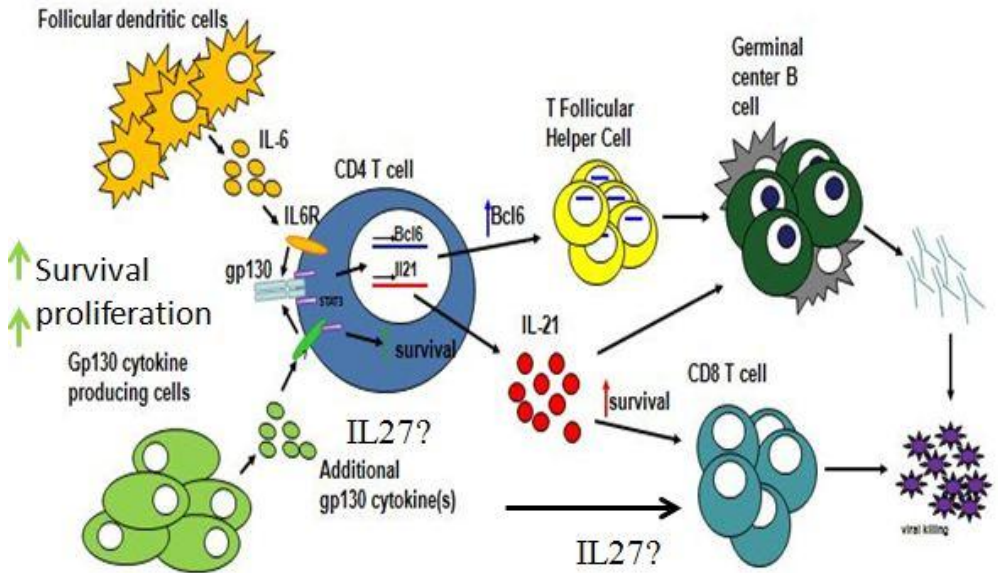

Figure 16: Model of Clearance in LCMV Cl13 and vital role of gp130 Cytokines. Cartoon based on data presented here, published and unpublished work ongoing in the lab. CD4 T cells receive IL-6 signaling and differentiate into T follicular helper cells (**T_{FH}**) through the expression of the master gene regulator Bcl6. **T_{FH}** signaling plays important role in B cell proliferation, survival, and production of virus specific antibodies. Gp130 cytokines signaling on CD4 T cells vital for proliferation and survival, promoting virus specific CD8 T cell survival and cytotoxic activity. Both humoral and cell mediated immunity utilized to clear LCMV Cl13 infection.

resultant accumulation of T_{FH} in chronically infected mice drives enhanced humoral immunity, controlling the virus. We also found evidence for the role of additional gp130 signaling, most likely through IL-27, in both the survival of virus specific $CD4^+$ T cells and their production of IL-21. This is not only critical for humoral immunity, but was also vital for the maintenance of virus specific $CD8^+$ T cell responses. In acute LCMV infection, while $CD4^+$ helper T cells have been shown to be vital for optimal CD8 memory responses[111,112], and long lasting humoral immunity. They are not, however, required for elimination of the primary infection, which is carried out by virus specific

$CD8^+$ T cells. It would be interesting to examine if other acute infections, such as Influenza A virus[113] and VSV[113,114] , which rely on both $CD8^+$ and humoral immunity for viral control, utilize immune mechanisms with similarity to chronic LCMV infection.

Important parallels can be drawn between our observations in LCMV and findings in patients infected with actively replication chronic viral infections such as HIV and HCV. Both HIV and HCV patients have been found to have elevated IL-6 levels, although this has traditionally been observed as a pathogenic biomarker of secondary diseases such as cirrhosis[115-119]. Importantly however, following on from our research a number of labs have gone on to show that HIV, SIV, HBV and HCV infected patients or primates display an accumulation of T_{FH} cells within their virus specific $CD4^+$ compartment[120-123]. In HIV this was found to associate with increased B cell responses and IgG production,[124] while in SIV the accumulation of T_{FH} correlated with the sensitivity of the $CD4^+$ T cells to IL-6 signaling[121].

Unlike LCMV Cl13, there is evidence that HIV in particular has evolved to evade, and even exploit, this anti-viral immune mechanism. Firstly HIV preferentially infects activated $CD4^+$ T cells, directly causing their dysfunction and death[125]. Secondly, select HIV patients develop high affinity, neutralizing antibody responses (known as elite neutralizers), the high mutagenic rate of HIV allows it to escape this pressure rapidly[126-128]. In fact, HIV patients with the lowest viral titers (known as elite controllers) actually have low to undetectable amounts of neutralizing antibody in their blood and control the virus via the presence of virus specific $CD8^+$ T cells[129]. Indeed, HIV is known to present

at high levels in lymphoid follicles and recently published data indicates that HIV and SIV actually replicate at very high levels in T_{FH} cells, without the high levels of $CD4^+$ T cell death seen in other $CD4^+$ effector cells [120,121,124,130]. Leading to the hypothesis that T_{FH} may represent a natural reservoir for HIV, as a source of effector cells localized to the B cell zone and therefore to a large extent protected from anti-viral $CD8^+$ responses.

On the other hand, in both HIV and HCV infected patients the number, and function, of virus specific $CD4^+$ T cells strongly correlates with reduced viral load and disease progression[131]. Indeed in HCV, high $CD4^+$ T cell responses are a hallmark of patients that naturally clear the infection. It has also been found that IL-21 production by $CD4^+$ T cells and serum levels of IL-21 strongly correlate with reduced HIV levels[132,133], although whether this is a result a cause or consequence is difficult to evaluate. Therefore approaches that target gp130 signaling to promote the maintenance and function of virus specific $CD4^+$ T cells could be of great therapeutic potential. The observations of T_{FH} and antibody responses during HIV infection do however highlight a critical point; that the use of any single immune pressure capable of significantly affecting viral load, may inevitably result in viral escape from this pressure and the maintenance of persistence.

One potential approach to solving this issue is the use of combination therapy. Enhancing T cell helper responses, and simultaneously stimulation virus specific $CD8^+$ T cells would represent one logical concept. In HIV elite controllers (who have known protective HLA types), or individuals who are able to spontaneously clear HCV, strong CTL activity correlate with increased viral control[18,129,134,135]. In this thesis I investigated

one pathways linked to this process, by neutralizing TGF-β, since TGF-β signaling leads to apoptosis of virus specific $CD8^+$ T cells in chronic LCMV infection[22], and enhanced TGF-β signaling and virus specific $CD8^+$ T cell apoptosis are observed in patients infected with HIV[136] and HCV[137]. Indeed in individuals who are co-infected with HIV and HCV, lack of TGF-β production by hepatitis C virus-specific T cells has been attributed to HCV clearance during acute phase[138]. While the regime I used was unsuccessful, other regimes have been shown to mediate stronger effects, and coupling these with targeting $CD4^+$ T cell help may prove an effective strategy. Alternatively there are a number of other candidate targets such as PD-L1, PD-1, Lag-3 and Tim-3, that may also be blocked in order to boost $CD8^+$ T cell mediated immunity. Finally, these immuno-therapeutic approaches could be coupled to existing anti-viral therapies (HAART for HIV[139,140] or ribavirin for HCV[140]) in order to prevent escape mutants, and hopefully full viral eradication.

Finally, the observations made in this study are not only relevant to the field of chronic infections. Promoting enhanced humoral immunity is a fundamental objective of vaccine development, and mechanisms that play a central role in the generation of T_{FH} are therefore of great interest. In addition, while enhanced antibody responses are of great benefit during infections, the production of auto-reactive antibodies is thought to play a pathogenic role in auto-immune disease such as Lupus Erythematosus[141], arthritis[142,143] and Sjorgren's syndrome[144]. Indeed enhanced production of IL-21, and recently the presence of T_{FH}, have been observed in several of these diseases[145,146]. The data obtained

through this thesis may therefore contribute to our enhanced understanding of $CD4^+$ T cell immunity in a broad spectrum of conditions.

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