UNIVERSITY OF CALIFORNIA SAN DIEGO

The role of gut microbiome composition and IL-27 in regulating B cell responses to chronic viral infection

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by

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The Thesis of Warren W. Y. Ko is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2020

DEDICATION

This thesis is dedicated to my family.

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LIST OF ABBREVIATIONS

Dendritic cells (DC)

Natural Killer cells (NK)

Pattern Recognition Receptors (PRRs)

Toll-like Receptors (TLR)

Retinoic acid Inducible gene-I-like Receptors (RLR)

Microbe-associate molecular patterns (MAMP)

Type I Interferons (IFN-I)

Human Immunodeficiency Virus (HIV)

Hepatitis C virus (HCV)

Hepatitis B virus (HBV)

Hepatitis B core antigen (HBcAg)

Hepatitis B e-antigen (HBeAg)

Cytotoxic T Lymphocyte (CTL)

Lymphocytic Choriomeningitis Virus Clone 13 (LCMV Cl13)

Programmed cell death protein 1 (PD-1)

Plasma Cell (PC)

Memory B cell (MBC)

Germinal center (GC)

Antibody secreting cell (ASC)

Hypergammaglobulinemia (HGG)

Neutralizing antibody (nAb)

Segmented filamentous bacteria (SFB)

Small intestinal lamina propria (SILP)

Short chain fatty acid (SCFA)

Taconic mice (TAC mice)

Jackson mice (JAX mice)

Co-housed Taconic mice (TAC coh mice)

Co-housed Jackson mice (JAX coh mice)

Inducible B cell IL-27 knockout transgene (IL-27p28$^{fl/fl}$hCD20TAM^{Cre})

IL-27p28$^{fl/fl}$hCD20TAM^{Cre} transgenic mice (Cre+ mice)

Wild type littermates (WT mice)

Intraperitoneal (i.p.)

Intravenously (i.v.)

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ABSTRACT OF THE THESIS

The role of gut microbiome composition and IL-27 in regulating B cell responses to chronic viral infection

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B cells are an important facet of the immune response against chronic viral infection. In the first chapter of this thesis, we studied the role of gut microbiome composition on B cell responses to chronic lymphocytic choriomeningitis virus clone 13 (LCMV Cl13) infection in mice. We used genetically similar mice from two different vendors, Taconic Biosciences and The Jackson Laboratory, that have been established to contain dramatically different intestinal microbiome compositions. We found that mice from Taconic Biosciences differed from mice from The Jackson Laboratory in the accumulation of B cells in the blood and spleen at certain timepoints during persistent LCMV Cl13 infection. Co-housing mice from different vendors resulted in the partial transfer of B cell accumulation in the blood and spleen and also resulted in impaired viral clearance. In the second chapter, we investigated the role of late B cell-derived IL-27 on B cell responses to persistent LCMV infection in mice. We used genetic systems

to modulate the availability of the IL-27p28 subunit in an inducible manner specifically in B cells. Late deletion of B cell-derived IL-27p28 resulted in subtle increases in the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells and $IgD^{-}IgM^{-}$ class-switched B cells, which peaked by 60 days post-infection. At even later timepoints post-infection, B cell-derived IL-27p28 deletion resulted in increases in the accumulation of total B cells. Overall, our results suggest that B cell responses to persistent viral infection are modulated by gut microbiome composition and may be downregulated by B cell-derived IL-27.

Introduction

1. Chronic infections are a major global health burden

Chronic disease can arise from a plethora of infectious agent. However, viruses that can establish chronic disease pose a significant health risk considering the limitations for treatment. Approximately 38 million people across the world have Human Immunodeficiency Virus (both HIV-1 or HIV-2), 71 million people are infected with Hepatitis C virus (HCV), and 257 million people are infected with Hepatitis B virus (HBV) (World Health Organization, 2020). Many individuals that are infected with these viruses progress to severe disease pathology, which can have life-threatening consequences.

In order to control viral infections, the host must rely on a coordinated effort from both the innate and adaptive arms of its immune system. The innate immune response is an immediate reaction to pathogens and is comprised of mostly dendritic cells (DC), natural killer (NK) cells, and macrophages. These cells use pattern recognitions receptors (PRR), such as Toll-like Receptors (TLR) and Retinoic acid Inducible gene-I-like Receptors (RLR) to detect conserved motifs in the surface molecules or nucleic acids of foreign pathogens (Aoshi et al., 2011). NK cells are further regulated by expressing a variety of receptors that can either stimulate or suppress NK cell activity (Vivier et al., 2011). Upon recognition of a viral pathogen, innate cells release pro-inflammatory cytokines and anti-viral cytokines such as Type I Interferons (IFN-I) (Aichele et al., 2006; Montoya et al., 2002; Garcia-Sastre and Biron, 2006). These responses help curb viral replication and promote the development of adaptive immune responses (Schenten and Medzhitov, 2011). The adaptive immune system is composed primarily of T and B lymphocytes that develop highly specific receptors in order to

recognize foreign antigens, including those expressed by invading pathogens. Upon recognition of a viral pathogen, lymphocytes will clonally expand and develop effector functions that will be used to ultimately eliminate the virus (Mueller and Rouse, 2008). During infection, lymphocytes can differentiate into diverse subsets that each accomplish a specific function, and regulation of these subsets is crucial for maintaining an immune response that is appropriate for the pathogenic challenge and is able to avoid immune-mediated pathology in the host (Rouse and Sehrawat, 2010; Strutt et al., 2013).

Those that struggle with chronic viral infection are commonly observed to have dysregulated immune responses. For example, during chronic HIV infection, many dysfunctions have been observed in both the innate and adaptive arms of the immune system (Zuniga et al., 2015). Macrophages and monocytes have several functional defects, including compromised chemotaxis and Fc receptor function (Rosenberg and Fauci, 1989). Disease progression also correlates with the loss of clonogenic potential and activity from anti-HIV cytotoxic T lymphocytes (CTL) (Rosenberg and Fauci, 1989; Pantaleo et al., 1990). $CD4^+$ T cells, which act as the primary reservoir of HIV in the peripheral blood, exhibit a shift from a Th1 helper cell response to a Th2 helper cell response, a phenotype that is critical in the pathogenesis of HIV infection (Clerici et al., 1993). During the late stages of infection, levels of $CD4^+$ T cells face a sharp decline, leading to opportunistic infections (Mattapallil et al., 2005; Douek et al., 2009). B cells also show striking abnormalities during HIV infection, which is characterized by aberrant polyclonal activation, spontaneous proliferation *in vitro*, and hypergammaglobulinemia *in vivo* (Coffin et al. 1997; Lane et al., 1983). An increase in short-lived plasmablasts have

also been observed in the peripheral blood and the spleen in individuals with chronic HIV infection (Moir and Fauci, 2017). Similar abnormalities have been seen in innate and adaptive immune cells during chronic HBV and HCV infection as well (Tseng and Huang, 2017; Kaplan, 2015).

Viruses that can establish a persistent infection acquired the ability to subvert or take advantage of certain aspects of the immune response through coevolution with their hosts (Lucas et al., 2001). However, the precise molecular mechanisms that cause abnormalities in immune responses still largely remain unknown. *In vitro* experiments have shown how viral surface molecules may play an important role in suppressing specific immune cell subsets. The HIV glycoprotein gp120 has been shown to compromise antigen responsiveness and signal transduction by binding to both cytoplasmic and membrane-bound CD4 (Coffin et al., 1997; Weinhold et al. 1989). Other HIV viral products, such as Nef, Vpu, and Tat, have been reported to downregulate CD4 expression and suppress proliferative responses to antigen (Coffin et al. 1997; Orecchini et al., 2014). However, it is yet to be shown if these mechanisms truly play a role in HIV pathogenesis *in vivo*. Furthermore, many other immune cells from both innate and adaptive arms have their functions perturbed or suppressed even in the absence of direct HIV infection of these cells, suggesting that the mechanisms for immune suppression can go beyond interactions with viral products and can be mediated through the disruption of fundamental immune cell signaling processes (Rosenburg and Fauci, 1989a). The pathogenic mechanisms of HBV remain even more elusive. It has been demonstrated that the hepatitis B core antigen (HBcAg) can elicit strong immune responses in both a T-dependent and -independent fashion (Balmasova

et al.,2014). Studies have also shown that the hepatitis B e-antigen (HBeAg) plays an important role in developing the T-cell tolerance that ultimately leads to chronic HBV infection (Chen et al., 2005). Still, the direct immunoregulatory effects of these viral products are yet to be determined. The mechanisms that cause persistence of HCV infection, on the other hand, are much better understood. HCV has several mechanisms that allow it to downregulate the expression of antiviral type I and type III interferons. The viral proteins E2 and NS5A induce the phosphorylation of protein kinase R (PKR) and elongation initiation factor 2a, which results in the overall reduction of mRNA translation (Gale et al., 1998; Taylor et al., 1999). The HCV core protein has been shown to inhibit several factors involved in TLR3-mediated secretion of interferons, including STAT1 activation and ISGFR3 translocation into the nucleus (Zhengkun et al., 2010; Ebihara et al., 2008; Okamoto et al., 2014). Furthermore, because the viral polymerase contains no proofreading mechanism, HCV can accumulate mutations in its immunogenic epitopes, resulting in CTL escape variants (Chang et al., 1997; Erickson et al., 2001). Identifying the biological mechanisms that allow viruses to establish chronic infections is indispensable for developing effective treatments and vaccines.

2. Lymphocytic Choriomeningitis Virus as a model of chronic infection

Lymphocytic Choriomeningitis Virus (LCMV) is a non-cytolytic negative sense single strand RNA virus from the Arenaviridae family that is a natural murine pathogen (Bocharov et al., 2015). LCMV can establish an acute or chronic infection in mice depending on the strain, viral dose, and route of administration (Hotchin, 1962; Ahmed et al., 1984). For example, the Armstrong 53b strain of LCMV causes an acute infection

that results in robust responses from innate immune cells and T lymphocytes. On the other hand, intravenous inoculation of a high dose of the Clone 13 (Cl13) strain of LCMV can establish a chronic infection that leads to the impairment of responses from both the innate and adaptive arms of the immune system. Thanks to this model of chronic infection, multiple studies have identified immune cell adaptations which have also been found to manifest during chronic viral infection in humans. A prime example of this is T cell 'exhaustion', which was first described in mice and is characterized by the expression of multiple inhibitory receptors, most notably programmed cell death protein 1 (PD-1), and the progressive deterioration of CTL effector functions (Wherry et al., 2003; Wherry et al., 2007; Wherry et al., 2015). In humans, T cell 'exhaustion' has been documented in individuals infected with HIV and hepatitis, and some aspects of the T cell exhaustion state, such as upregulated PD-1 expression, are now recognized as key therapeutic targets (Fenwick et al., 2019; Ye et al., 2015). Furthermore, the LCMV mouse model of chronic infection has been used to uncover the role of the cytokine IL-10 in suppressing T cell responses and driving exhaustion (Brooks et al., 2008). Sustained and elevated levels of IL-10 have also been correlated with impaired T cell activity and enhanced viral replication during HIV infection (Brockman et al., 2009). Moreover, $CD4^+$ T cell production of IL-21 has been shown to play an important role in maintaining $CD8^+$ T cell responses during both persistent LCMV infection in mice (Elsaesser et al., 2009; Xin et al., 2015; Fröhlich et al., 2009; Yi et al., 2009) and HIV infection in humans (Chevalier et al., 2010; Parmigiani et al., 2011; Yue et al., 2010). While these studies have shed light on the inhibitory mechanisms that viruses may utilize in order to persist in the host, other studies have also revealed that these

adaptations may play a vital role in the survival of the host. For example, infection of mice that lack PD-L1 (PD-L1$^{-/-}$) with LCMV Cl13 resulted high mortality due to fatal immunopathological damage (Barber et al., 2006). Taken together, studies utilizing the LCMV model of persistent infection have revealed that chronic infection promotes an immunosuppressive environment that allows for viral tolerance in order to avoid immune-mediated pathology.

3. The B cell response and adaptations during chronic viral infection

In a typical response to acute viral infection, B cells play an essential role in fighting off the primary infection as well as in preventing secondary infection. The hallmark of B cells is their ability to produce protective antibodies. During the earliest stages of viral infection, broadly specific circulating “natural” IgM antibodies provide an immediate humoral defense that helps control initial viral replication (Kearney et al., 2015; Cyster and Allen, 2019; Ochsenbein et al., 1999). These early IgM responses are mounted by innate-like B-1 cells that primarily reside in the peritoneal and pleural cavities of the host (Grönwall et al., 2012; Savage et al., 2017). As the infection progresses and adaptive immune responses take the stage, B cells develop a robust collection of highly specific antibodies (Eisen et al., 2014). Within the secondary lymphoid organs, B cells receive help from CD4^{+} T follicular helper (Tfh) cells and form structures known as germinal centers in which they undergo a process of intense proliferation and expansion. It is within the germinal centers that B cells tailor their affinity for antigen by accumulating mutations in their B cell receptors (BCR), a phenomenon known as somatic hypermutation (Cyster and Allen, 2019; Berek and

Milstein, 1987). B cells in this stage will also switch the constant region of their receptors from IgM to IgG, IgA, or IgE, a process known as class switch recombination (Muramatsu et al., 2000). Once B cells have acquired a sufficient amount of mutations in their receptors, they will receive signals to exit the germinal centers and terminally differentiate into short-lived plasma cells (SLPC) or long-lived plasma cells (LLPC) (Palm and Henry, 2019). SLPC will immediately produce high amounts of antibodies of low affinity, while LLPC will traffic to the bone marrow and continuously secrete protective antibodies of refined affinity. Alternatively, germinal center B cells can differentiate into memory B cells (MBC) that enter the circulatory system and survey for antigen. Upon antigen recognition, MBC will efficiently mount recall responses by swiftly re-entering the germinal center reaction. In general, MBC have relatively less affinity for antigen in comparison to PC, allowing for some degree of flexibility upon developing secondary responses (Palm and Henry, 2019). The combined effort from both these effector B cell subtypes is necessary for preventing secondary infections (Akkaya et al., 2020). Aside from their ability to produce and secrete antibodies, B cells can also secrete cytokines and chemokines that regulate lymphoid tissue development and T cell responses (Shen and Fillatreau, 2015). For example, lymphotoxin α1β2 (LTα1β2) provided by B cells has been shown to play roles in lymphoid maturation and organogenesis (Ansel et al., 2000; Ngo et al., 2001; Tumanov et al., 2002) and can determine the polarization of T helper cell responses during *Heligmosomoides polygyrus* infection (León et al., 2012). Furthermore, B cells have been shown to promote inflammatory responses through the production of IFN-γ (Barr et al., 2010) or suppress inflammation through the production of IL-10 and IL-35 (Fillatreau et al., 2002;

Madan et al., 2009; Shen et al., 2014). Altogether, B cells fight against viral infections by honing antibody responses and can also perform immunomodulatory functions through the production of various cytokines.

However, during persistent viral infection, many of the classical B cell responses mentioned above are drastically altered. During the early stages of chronic LCMV infection, multiple studies have reported that antiviral B cells are largely deleted due to interferon-driven inflammation (Fallet et al., 2016; Moseman et al., 2016). The anti-viral B cell repertoire is eventually replaced by new bone marrow emigrants. Studies have noted that chronic infection also perturbs the development of virus-specific antibody responses by promoting the development of virus-unspecific antibodies, a condition known as hypergammaglobulinemia (HGG) (Greczmiel et al., 2020; Hunziker et al., 2003; De Milito et al., 2004). The emergence of virus-unspecific antibodies is driven by elevated IFN-I signaling and is accompanied by the expansion of the Tfh population (Daugan et al., 2016). Furthermore, B cell differentiation is also modified during persistent viral infection, with germinal center reactions being extensively prolonged in order to support the increased output of antibody secreting cells (ASC). Importantly, the development of neutralizing antibody (nAb) responses plays a crucial role in fighting off persistent viral infections. During LCMV infection, it has been demonstrated that the combined effort of both B and T cell responses are required to curb chronicity (Planz et al., 1997; Bergthaler et al., 2009). Taken together, the data suggest that the immune system adapts to chronic infection by suppressing cell-mediated immunity to instead rely on humoral defenses in order to control viral replication while avoiding fatal immunopathology.

4. The gut microbiome influences immune responses

The gut microbiome has been recognized to play a significant role in the development and regulation of the immune system. While the gut microbiome is conventionally considered to regulate immune homeostasis, recent studies have revealed that the gut microbiome has a significant impact on immune responses during primary infections. In one study, treatment with antibiotics in mice resulted in impaired anti-viral T cell responses during LCMV infection and increased mortality during influenza infection (Abt et al., 2012). Moreover, multiple studies have reported that the outcome of pathogenic challenge is correlated with the composition of the gut microbiome. The presence of segmented filamentous bacteria (SFB) in mice from Taconic Biosciences was shown to correlate with the presence of Th17 cells in the small intestinal lamina propria (SILP) and conferred increased resistance to the intestinal bacterial pathogen *Citrobacter rodentium* (Ivanov et al., 2009). Additionally, the presence of the commensal bacteria *Akkermansia muciniphila* and *Enterococcus hirae* were shown to enhance the efficacy of anti-PD-1-based immunotherapy against epithelial tumors (Routy et al., 2018). Taken together, the data support the notion that specific bacteria in the microbiome can confer positives outcomes to an array of diseases. However, the mechanisms by which these microbes achieve this are largely unknown.

The dialogue between the intestinal microbiome and the host immune system has been attributed to the recognition of microbe-associated molecular patterns (MAMP) by innate immune cells (Belkaid and Hand, 2014). For example, flagellin, a structural protein involved in bacterial chemotaxis, is detected by TLR5 on innate

immune cells, prompting them to release antimicrobial peptides and cytokines that maintain gut homeostasis (Uematsu et al., 2006; Uematsu et al., 2008; Kinnebrew et al., 2012). Another example is polysaccharide A, a component of the capsule of *Bacteroides fragilis* that has been shown to have immunomodulatory functions by expanding IL-10-producing regulatory T cells (Mazmanian et al., 2005; Mazmanian et al., 2008; Ramakrishna et al., 2019). Furthermore, microbial metabolites such as short chain fatty acids (SCFA) have been shown to be recognized by innate immune cells and can tune their inflammatory function (Maslowski et al., 2009). Recently, one study has shown that B cells can use SCFAs produced by commensal microbes in order to fuel energy-demanding antibody production (Kim et al., 2016). While it is clear that the gut microbiome can communicate with the host immune system using a variety of direct and indirect mechanisms, evidence has also shown that commensal microbes can influence the activity of invading viral pathogens. The same microbial products that are used to communicate with the host immune system can also be used to suppress or promote viral infections. For example, SCFAs from the culture fluids of oral bacteria have been shown to induce the synthesis of early antigens of Epstein-Barr viruses (Asai et al., 1991). In another study, bacterial lipopolysaccharide (LPS) was found to be capable of binding to and destabilizing influenza virus (Bandoro and Runstadler, 2017). Conversely, commensal microbes can also be influenced by the invading virus. While the mechanisms that allow pathogenic viruses to modulate commensal microbes is largely unclear, it has been suggested that viral infection induces microbiota dysbiosis (Li et al., 2019; Domínguez-Díaz et al., 2019). These perturbations in the microbial communities within the host have profound implications on the immune responses to

infection. Taken together, the evidence suggests that the emergence of effective anti-viral immune responses relies on a complex crosstalk between the immune system, the host microbiome, and the invading virus itself.

Here, we utilized genetically similar mice that have different intestinal microbiome compositions in order to characterize discrepancies in B cell responses to chronic viral infection due to differential microbiome composition. We found that mice from Taconic Biosciences and mice from Jackson Laboratory had different proportions and quantities of B cells in the blood during the earlier stages of chronic viral infection. We also found differences in the accumulation of B cells within the spleen at day 30 post-infection, suggesting differences in B cell differentiation. Furthermore, we found transient differences in the accumulation of class-switched B cells in the blood at days 45 and 60 post-infection. These differences in the proportions and quantities of B cells were not associated with differences in LCMV-specific IgG titers, nor were they associated with any differences in viral clearance. Co-housing these mice together resulted in the partial transfer of B cell accumulation in the blood and spleen in a biased manner. Interestingly, we found that mice from Jackson Laboratory that were co-housed with mice from Taconic Biosciences had impaired viral clearance, suggesting that partial transfer of gut microbiome composition through co-housing may promote the chronicity of viral infection.

In the second chapter, we utilized a genetic mouse model that allows B cell specific deletion of IL-27p28 in order to characterize the role of late B cell-derived IL-27 on B cell responses and viral clearance. We found that there were only subtle and

transient differences in the accumulation of B cells in the blood upon late IL-27p28 deletion in B cells. Furthermore, while viral clearance was impaired in mice that had IL-27p28 deleted in B cells, we also found that wild-type littermates also had impaired viral clearance, leaving the role of B cell-derived IL-27 in viral clearance inconclusive. In one experiment, we found that tamoxifen treated mice had significant deviations in the proportions and numbers of B cells in the blood compared to mice that never received tamoxifen, giving rise to concerns regarding our tamoxifen treatment.

Chapter 1: Characterizing B cell responses to chronic viral infection due to differences in intestinal microbiome composition

The commensal gut microbiome is known to have a profound influence not only on immune homeostasis, but also on immune response to pathogenic challenges. Multiple studies have documented that certain microbes residing in the gut can be indicative or can even confer positive outcomes to a variety of diseases. However, the role of the gut microbiome in influencing immune response to persistent viral infections is less explored. Using genetically similar mice from Taconic Biosciences and The Jackson Laboratory, which have been demonstrated to vastly differ in intestinal microbiome composition, we studied how differences in intestinal microbiome composition might result in differences in B cell responses to persistent LCMV infection in mice. We found that Taconic mice differed from Jackson mice in the accumulation of class-switched B cells in the blood during the early B cell responses to chronic infection and again during the chronic phase of LCMV infection. In the spleen, differences were also seen in the proportions of GC B cells and PC, suggesting that differences in gut microbiome composition may influence B cell differentiation in response to chronic viral infection. These differences were not associated with any differences in the titers of LCMV-specific IgG antibodies in the serum. We also observed that co-housing Jackson mice with Taconic mice resulted in Jackson mice partially recapitulating the blood and splenic B cell phenotypes of Taconic mice. In contrast to two earlier independent experiments in our lab, we were unable to see accelerated viral clearance in Taconic mice. However, we notably found that co-housed Jackson mice had significantly

impaired viral clearance. These studies underline the importance of screening the effects of microbial transfers as a therapeutic in the context of multiple disease states.

Background

The vertebrate intestine is colonized by an incredible number of bacteria spanning multiple diverse taxa. The gut provides a hospitable and nutritious environment for microbes to thrive, while in return these bacteria carry out essential metabolic functions, establishing a mutualistic symbiotic relationship between the host and resident microbial communities (Belkaid and Hand, 2014). It is widely recognized that the gut microbiome has a profound influence on the immune system. Moreover, recent studies have found that the presence of certain microbes within the gut microbiome can significantly influence the outcome of different disease states. This has proven to be true when studying immune responses in wild-type C57BL/6 (B6) mice purchased from different vendors, namely Taconic Farms (now known as Taconic Biosciences) and The Jackson Laboratory. Wild-type Taconic and Jackson B6 mice are very closely genetically related but differ in a number of genes, including the nicotinamide nucleotide transhydrogenase gene (*Nnt*) (Nicholson et al., 2010) and the Nod-like receptor pyrin domain containing 12 gene (*Nlrp12*; Ulland et al., 2016). Many studies have documented that Taconic and Jackson mice have distinct microbiomes that can result in differences in immune responses. A 2009 study by Ivanov *et al*. demonstrated that Taconic mice had an increased accumulation of Th17 helper cells in the small intestinal lamina propria in comparison to Jackson mice (Ivanov et al., 2009). While the gut microbiome compositions of Taconic and Jackson mice differed in multiple bacterial taxa, the authors of this study attributed this increased accumulation of Th17 helper cells to the presence of Segmented Filamentous Bacteria (SFB) in the gut of Taconic B6 mice. Transfer of SFB into germ free mice resulted in increased

accumulation of Th17 cells in the small intestinal and large intestinal lamina propria. Furthermore, co-housing mice from Taconic Biosciences with mice from Jackson Laboratory conferred increased resistance to the intestinal bacterial pathogen *Citrobacter rodentium* in Jackson B6 mice. However, in a separate 2015 study, Sivan *et al*. (Sivan et al., 2015) found that Jackson B6 mice exhibited slower growth rates of melanoma tumors compared to Taconic B6 mice in a melanoma tumor model. The authors attributed this improved anti-tumor response to the significantly higher presence of *Bifidobacterium* in the gut microbiome of Jackson mice (Sivan et al., 2015). Taken together, these studies highlight the importance of gut microbiome composition in shaping immune responses, but also reveal that the potential of gut microbes for boosting immune responses is highly dependent on the specific pathogenic challenge.

Our laboratory is interested in exploring the immunological effects of differential microbiome composition in Taconic and Jackson B6 mice in the context of chronic viral infection. In two early independent experiments, wild-type B6 mice from Taconic Biosciences (TAC mice) and Jackson Laboratory (JAX mice) were infected intravenously with LCMV Cl13. Most notably, we found in these early experiments that TAC mice were able to clear the virus from the serum much faster than JAX mice. Considering that antibody responses are required for the containment of LCMV persistence (Bergthaler et al., 2009), we set out to characterize antibody and B cell responses to chronic LCMV infection in TAC and JAX mice in order to determine whether B cells contribute to this accelerated viral clearance. TAC and JAX mice were purchased from the corresponding vendors and housed within our facility in the same room. After two days of acclimation, we compared the baseline accumulation of B cells

in the blood of TAC and JAX mice. One day later, we infected these mice with Cl13 and evaluated the accumulation of B cells in the blood at multiple timepoints post-infection. In order to determine whether any differences in B cell responses were transferrable, mice from both vendors were purchased, co-housed for three weeks in advance, and infected alongside single-housed mice. In evaluating the accumulation of B cells in the blood, we looked at the numbers and frequencies of total B cells, which were defined as $CD19^{+}B220^{+}$ cells (Madan et al., 2009). Within the total B cell population, we evaluated the proportions and numbers of $IgD^{+}IgM^{+}$ naïve, $IgD^{-}IgM^{+}$ non-naïve B cells, and class-switched B cells. The gating for the latter two populations was based on a recent pre-print from Dr. John Wherry's laboratory, in which $IgD^{-}IgM^{+}$ and $IgD^{-}IgM^{-}$ events were collectively referred to as 'non-naïve B cells' (Staupe et al., 2019). However, during our analysis, we noticed that $IgD^{-}IgM^{+}$ events within this gate could represent a distinct population of B cells. Therefore, for our experiments, $IgD^{-}IgM^{+}$ events were placed in their own gate, which we referred to as $IgD^{-}IgM^{+}$ non-naïve B cells, while $IgD^{-}IgM^{-}$ events were referred to as class-switched B cells (Phares et al., 2014). We also evaluated the accumulation of B cells in the spleen during the chronic stage of LCMV infection using this same gating strategy, as well as gating $CD138^{+}$ plasma cells (PC) (Madan et al., 2009), $CD38^{-}GL7^{+}$ germinal center (GC) B cells, and $CD38^{+}GL7^{-}$ memory B cells (MBC) (Staupe et al., 2019) within the class-switched B cell population.

We observed that differences in the proportions of $IgD^{-}IgM^{+}$ non-naïve and class-switched B cells in the blood of uninfected TAC and JAX mice were irreproducible in two independent experiments. During LCMV infection, we found that the accumulation of naïve, $IgD^{-}IgM^{+}$ non-naïve, and class-switched B cells in the blood of TAC mice

deviated from that in JAX mice at days 8 and 15 post-infection, which suggests differences in the kinetics of B cell activation and class-switching during the intermediate stage of LCMV infection. While we found no differences in the accumulation of B cells in the blood at day 30 post-infection, we found significant deviances when analyzing B cells in the spleen. After day 30, TAC mice had transient increases in the accumulation of class-switched B cells in the blood at days 45 and 60 post-infection, suggesting that TAC mice had enhanced class-switched B cell responses during these timepoints. By day 90, differences in B cell accumulation were no longer able to be detected. Taken together, these data indicate that differences in gut microbiome composition resulted in differences in B cell responses in the blood and spleen during chronic viral infection. However, these discrepancies were not associated with any differences in the titers of virus-specific IgG antibodies, and in contrast to early experiments, TAC mice did not exhibit accelerated viral clearance. Thus, the role of antibody responses in the improved viral clearance seen in earlier experiments remains unclear. In co-housed Taconic (TAC coh) and Jackson (JAX coh) mice, we saw that the numbers and proportions of B cells in the blood and spleen during LCMV infection were only partially transferred from TAC coh mice to JAX coh mice. JAX coh and TAC coh mice also did not have any differences in the output of anti-viral IgG. Notably, however, we found that JAX coh mice had significantly impaired viral clearance compared to all other groups, indicating that microbial transfer from TAC mice to JAX mice may actually exacerbate chronic infection.

Materials and Methods

Mice. Wild type female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and wild type female C57BL/6N mice were purchased from Taconic Biosciences (San Diego, CA). Mice purchased from Taconic Biosciences were specifically requested to come from SFB-positive barriers. Mice acquired from commercial vendors were immediately transferred to our specific-pathogen free facility, housed in the same room, and were given a 48 hour acclimation period before being used in experiments. All mouse handling was in accordance with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego (UCSD).

Virus. Viruses were grown, identified, and quantified as previously described (Ahmed et al., 1984; Borrow et al., 1995). Frozen stocks of LCMV Cl13 were propagated using baby hamster kidney (BHK) cells. BHK cells were infected with 1 x 10^6 PFU of LCMV Cl13. The BHK cells were incubated with the virus inoculum at 37^{o}C for 1.5 hr, after which the virus inoculum was replaced with fresh media and the cells were incubated at 37^{o}C for an additional 48 hours. The supernatants from the cell cultures were then harvested and spun down at 2200 rpm for 15 minutes. Viral titers were determined by titrating the clarified homogenate on Vero cell monolayers using 6-fold dilutions. 6-week old female mice were infected i.v. with 2 x 10^6 PFU of LCMV Cl13. As an acute infection control, mice were infected i.p. with 2 x 10^6 PFU of LCMV Cl13.

Blood collection and processing. Blood was collected from the facial vein using heparin-coated capillary tubes. 10 uL of blood was diluted 1:20 in FACS buffer (5% fetal bovine serum in PBS) and used for cell counting on a Guava EasyCyte Flow Cytometer. The remaining volume of blood was spun down at 10,000 rpm for 5 minutes in order to extract the serum. Red blood cells were then lysed in two rounds of ACK lysis buffer. Cells were then resuspended in FACS buffer prior to cell staining.

Spleen collection and processing. Mice were euthanized and spleens were harvested in accordance with the guidelines set by the Animal Care Program at UCSD. Spleens were perfused with 1 mL of collagenase D (Roche), then incubated at 37^{o}C for 20 minutes. Spleens were then homogenized and washed through a 100 um cell strainer. The homogenized spleens were spun down at 1500 rpm for 5 minutes at 4^{o}C. Red blood cells were lysed in ACK lysis buffer. Cells were then spun down then resuspended in RPMI complete media. Cells were counted on a Guava EasyCyte Flow Cytometer.

Flow cytometry. To identify B cells, cell suspensions were stained for 1 hour at 4^{o}C with the following antibodies purchased from eBioscience or BD Biosciences: anti-B220-PE-CF594 (RA3-6B2), -CD19-PerCP-Cy5.5 (eBio1D3), -CD138-PE (281-2), -GL7-eFluor660 (GL-7), -CD38-PE-Cy7 (90), -IgM-APC-eFluor780 (RMM-1), -IgD-BV650 (11-26c.2a), -IgG1-FITC (A85-1), -IgG2a-biotin (RMG2a-62), -streptavidin-BV605, and -PDL-1-BV421 (10F.9G2). Prior to staining for B cells, cell suspensions were stained with an amine reactive viability dye (Tonbo Biosciences; CA) in order to distinguish

dead cells from live cells. After staining, cells were fixed with 2% paraformaldehyde. Cells were acquired using the BioRad ZE5 Cell Analyzer. Flow cytometric data were analyzed with FlowJo software (TreeStar; CA).

LCMV-specific antibody ELISA. Enzyme-linked immunosorbent assays were used to determine the relative amounts of LCMV-specific antibodies in the serum, as previously described (Hammond et al., 1997; Harker et al., 2011). Antigen was prepared by propagating frozen stocks of LCMV Cl13 in BHK cells, then purifying LCMV antigen from the culture supernatant on a renografin gradient. 96-half-well plates were coated with purified antigen for at least 24 hours. After a blocking step with 1% bovine serum albumin in PBS, the LCMV-coated plates were incubated with serial dilutions of infected sera for 2 hours at room temperature. Sera was then washed off and the plates were incubated with HRP-conjugated anti-Ig specific antibodies for 1 hour at room temperature. After incubation with the secondary antibodies, a TMB substrate was added (BD Biosciences; CA), followed by the addition of 2M H_2SO_4. OD450 was taken on a spectrophotometer.

Plaque Assay. Vero cells were seeded on 6-well plates. Once cells reached 95-100-% confluency, cells were incubated with serial dilutions of infected sera for 1 hour at 37^{o}C in a 5% CO_2 incubator. Once infection was complete, the cells were overlaid with a 1:1 mixture of 2% agarose and Eagle's Minimum Essential Medium supplemented with FBS, L-glutamine, and penicillin/streptomycin. The cells were incubated with this overlay for 6 days at 37^{o}C in a 5% CO_2 incubator. After 6 days, the

cells were fixed with 10% paraformaldehyde and stained with crystal violet. Plaques were enumerated and titers were calculated as previously described (Welsh and Seedhom, 2008).

Statistical Analysis. Statistical differences were determined by Student's t-test and One-way ANOVA with Tukey's post-hoc test using GraphPad Prism 8 (Graphpad, CA). *p-val<0.05, **p-val<0.01, ***p-val<0.001, ****p-val<0.0001.

Results

No consistent differences in homeostatic B cell populations in the peripheral blood between mice from two different vendors

In order to evaluate B cell pre-infection phenotypes that may come as a result of differential gut microbiome composition, we evaluated blood B cell markers in female wild-type C57BL/6 mice purchased from Taconic Biosciences (TAC mice) and Jackson Laboratory (JAX mice) one day before infection in two independent experiments. Mice from these two vendors are genetically very similar, but vastly differ in their gut microbiome composition (Ivanov et al., 2009). In one experiment, we observed significantly higher total peripheral blood mononuclear cell (PBMC) numbers in TAC mice (Fig. 1Ai). When examining the total B cell population in this experiment, we saw that numbers and frequencies of $CD19^{+}B220^{+}$ B cells tended to be slightly lower in TAC mice compared to JAX mice, although this was not statistically significant (Fig. 1ii). We further observed that TAC mice in this experiment had significantly lower numbers and frequencies of $IgD^{+}IgM^{+}$ naïve B cells in this experiment. This was associated with significantly higher frequencies, but not numbers, of $IgD^{-}IgM^{+}$ non-naïve and $IgD^{-}IgM^{-}$ class-switched B cells in TAC mice (Fig. 1-1Aiv-v). Although these data would indicate that TAC mice have different homeostatic proportions and numbers of B cells in the blood compared to JAX mice, we found in a second independent experiment that there were no differences in any of the parameters that we evaluated (Fig. 1-1B). The conflicting data from these two experimental repeats left the role of the gut microbiome composition in regulating basal B cell accumulation in the blood of uninfected mice unclear.

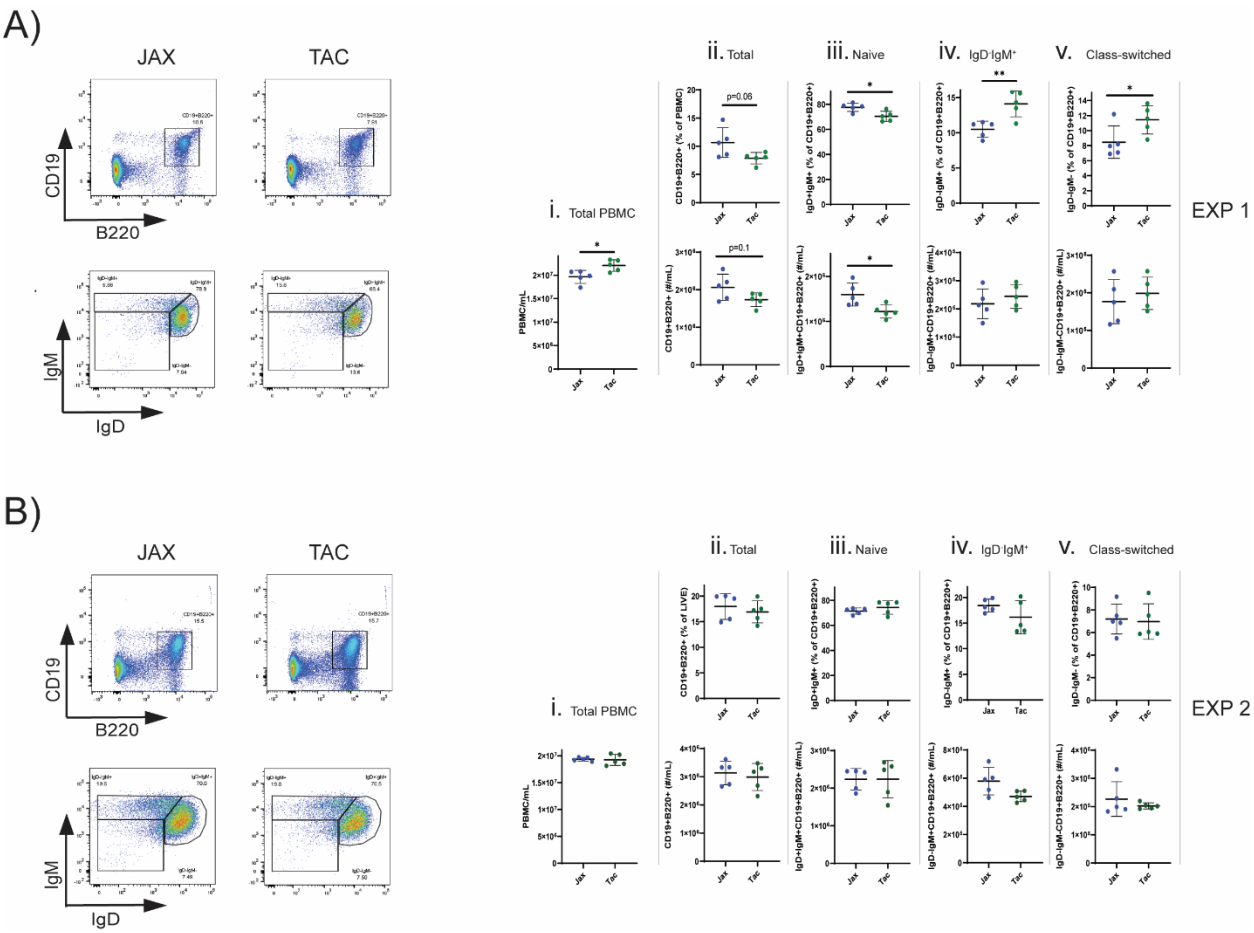


Figure 1-1. Inconsistent differences in homeostatic B cell accumulation in the peripheral blood of mice from two different vendors. 6-week old female C57BL/6 mice were purchased from The Jackson Laboratory (JAX) and Taconic Biosciences (TAC) and were immediately transferred to our housing facilities and kept in the same room. After two days of acclimation, B cells in the blood were analyzed at one day before infection with immunofluorescence staining and flow cytometry. (A-B) Representative flow cytometry gating with quantification of PBMC (i), $CD19^+B220^+$ B cells (ii), IgD^+IgM^+ naïve B cells (iii), IgD^-IgM^+ non-naïve B cells (iv), and IgD^-IgM^- class-switched B cells (v) in JAX (blue) and TAC (green) mice of two independent experiments with 5 mice/group. Averages ± SD are shown (Ai-v and Bi-v. Student's t-test (A-B); *p-val<0.05, **p-val<0.01.

Differences in the accumulation of B cells between mice purchased from two different vendors arose during the intermediate stage of chronic LCMV infection

In order to evaluate the differences in B cell responses to chronic LCMV infection between TAC and JAX mice, which differ in their gut microbiome composition (ref stated above), mice were purchased from both vendors, housed for three days in the same

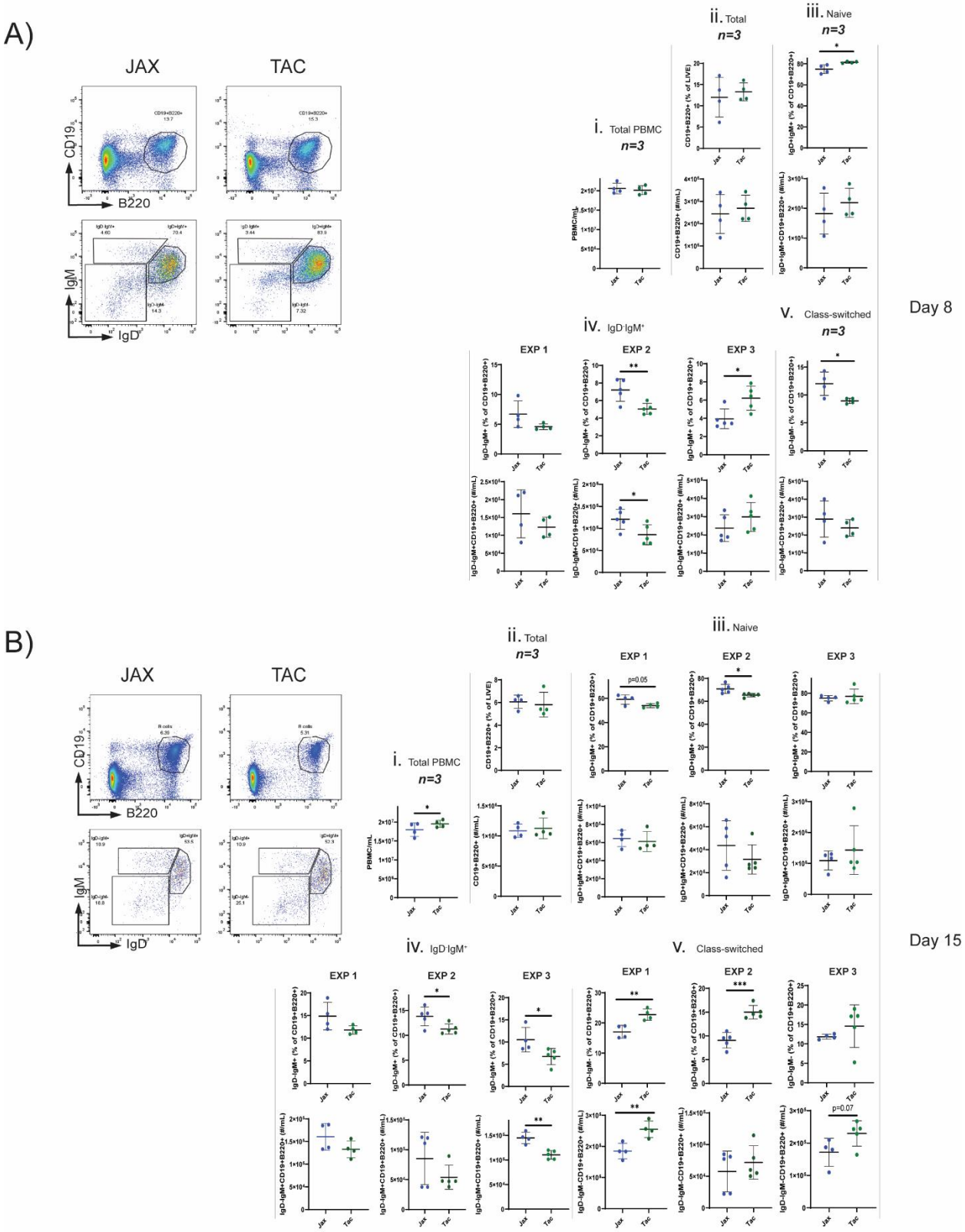
room, then were infected intravenously with 2 x 10^6 PFU of LCMV Cl13. B cell phenotypes in the blood were evaluated at days 8, 15, 30, 45, 60, and 90 post-infection using the same B cell parameters that were used at one day before infection. In three independent experiments, we analyzed B cells in the blood at days 8 and 15 post-infection. In all iterations of this experiment, we saw that at day 8 post-infection, there were no differences in blood cellularity, nor were there any significant differences in the numbers and frequencies of $CD19^+B220^+$ B cells in the blood (Fig. 1-2Ai-ii). Within the B cell population, however, we saw that TAC mice had significantly higher frequencies, but not numbers, of naïve B cells in the blood compared to JAX mice in all three experiments (Fig. 1-2Aiii). Differences in the accumulation of IgD^-IgM^+ non-naïve B cells were observed as well; however, these divergences were inconsistent in three independent experiments. In one experiment, numbers and frequencies of IgD^-IgM^+ non-naïve B cells tended to be slightly lower in TAC mice (Fig. 1-2Aiv), and in a second independent experiment, the lower trends of IgD^-IgM^+ non-naïve B cell accumulation in TAC mice were even more pronounced (Fig. 1-2Aiv). However, in a third experiment, TAC mice had significantly higher frequencies and slightly higher numbers in comparison to JAX mice (Fig. 1-2Aiv). While the differences in the accumulation of IgD^-IgM^+ non-naïve B cells were bidirectional, we observed that frequencies and numbers of IgD^-IgM^- class-switched B cells tended to be lower in TAC mice in all three experiments (Fig. 1-2Av). These data indicated that while there were no differences in the accumulation of total B cells in the blood at day 8 post-infection, a lower percentage of these B cells were class-switched in TAC mice. Although differences in the

accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells were also observed, these differences were inconsistent.

At day 15 post-infection, we observed that TAC mice had significantly higher numbers of total PBMC in two out of three experiments (Fig. 1-2Bi). However, differences in PBMC numbers at this timepoint were not associated with any significant differences in the numbers or proportions of total $CD19^{+}B220^{+}$ B cells (Fig. 1-2Bii). Upon analyzing B cell subsets within the total population at this timepoint, we observed that the frequencies of naïve B cells tended to be lower in TAC mice in 2 out of 3 experiments; however, no significant differences in naïve B cell numbers were able to be detected (Fig. 2Biii). The percentages of $IgD^{-}IgM^{+}$ non-naïve B cells also tended to be lower in TAC mice, with the numbers of these cells following a similar trend (Fig. 1-2Biv). Upon evaluating class-switched B cell accumulation, we found that TAC mice tended to have higher numbers and frequencies compared to JAX mice (Fig. 1-2Bv). This increase in the frequencies of class-switched B cells reached statistical significance in two out of three experiments, while the increase in cell numbers reached statistical significance in one experiment (Fig. 1-2Bv). These data showed that the proportions of naïve B cells in TAC mice were reduced from day 8 to 15, which coincided with an increase in the accumulation of class-switched B cells. On the other hand, JAX mice maintained trends of higher $IgD^{-}IgM^{+}$ non-naïve B cell accumulation at day 15, suggesting that a higher percentage of B cells in JAX mice had not switched their immunoglobulin isotype upon activation by this timepoint. Taken together, these data indicate that the differences between TAC and JAX mice in intestinal microbiome

composition may influence the dynamics of class-switch recombination during the intermediate stage of chronic LCMV infection.

Figure 1-2. Differences in B cell accumulation in the peripheral blood during the intermediate phase of persistent LCMV infection between mice from two different vendors. C57BL/6 mice from The Jackson Laboratory and Taconic Biosciences were infected with LCMV Cl13 and PBMC numbers and B cell accumulation in the blood were analyzed during the early B cell responses to Cl13 infection. (A-B) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 8 p.i. (A) and day 15 p.i. (B) are shown. Averages ± SD are shown (Ai-v and Bi-v). Data are representative of 1 (Aiv and Biii-v) or 3 (Ai-iii,v and Bi-ii) independent experiment(s) with 4-5 mice/group. Student's t-test; *p-val<0.05, **p-val<0.01, ***p-val<0.001.


A)
JAX
TAC
CD19
B220
IgM
IgD
i. Total PBMC
n=3
ii. Total
n=3
iii. Naive
n=3
iv. IgD-IgM+
v. Class-switched
n=3
EXP 1
EXP 2
EXP 3
Day 8
B)
JAX
TAC
CD19
B220
IgM
IgD
i. Total PBMC
n=3
ii. Total
n=3
iii. Naive
iv. IgD-IgM+
v. Class-switched
EXP 1
EXP 2
EXP 3
p=0.05
p=0.07
Day 15

Presence of class-switched B cells in the blood of mice purchased from Taconic Biosciences was transiently increased during the chronic phase of infection

In order to assess differences between TAC and JAX mice in late B cell response to chronic viral infection, we analyzed the same B cell markers in the blood at days 30, 45, 60, and 90 post-infection. In three independent experiments, we assessed the numbers and frequencies of B cells in the blood of TAC and JAX mice at 30 days post-infection. At this timepoint, we saw in all three triplicates that TAC mice continued to have significantly elevated numbers of PBMC compared to JAX mice (Fig. 1-3Ai). However, at this timepoint, there were no significant differences in any of the B cell parameters that we analyzed in all three experiments (Fig. 1-3Aii-v). These data suggested that B cell responses at day 30 post-infection were largely similar between TAC and JAX mice. In order to further explore the B cell responses in TAC and JAX mice during the late stage of chronic infection, we continued to look at the proportions and quantities of B cells in the blood at days 45 and 60 in two independent experiments. Total PBMC numbers remained elevated in TAC mice compared to JAX mice at day 45 post-infection (Fig. 1-3Bi). When analyzing the accumulation of $CD19^{+}B220^{+}$ B cells in the blood at this timepoint, we observed inconsistent trends in two independent experiments. In one experiment, the frequencies of total B cells were unchanged between TAC and JAX mice, while the numbers were significantly higher in TAC mice. (Fig. 1-3Bii, left). In another experiment, however, TAC mice had significantly lower frequencies, while the numbers were analogous between the two groups. Further analysis within the total B cell population revealed inconsistent trends in the accumulation of naïve and $IgD^{-}IgM^{+}$ non-naïve B cells as well (Fig. 1-3Biii and iv).

However, upon examining class-switched B cells at this timepoint, we found that the percentages and numbers in the blood tended to be higher in TAC mice in both experiments (Fig. 1-3Bv). These data indicated that while the trends in the accumulation of class-switched B cells at day 15 post-infection were not seen at day 30, TAC mice consistently had increased accumulation of class-switched B cells in the blood once again at day 45.

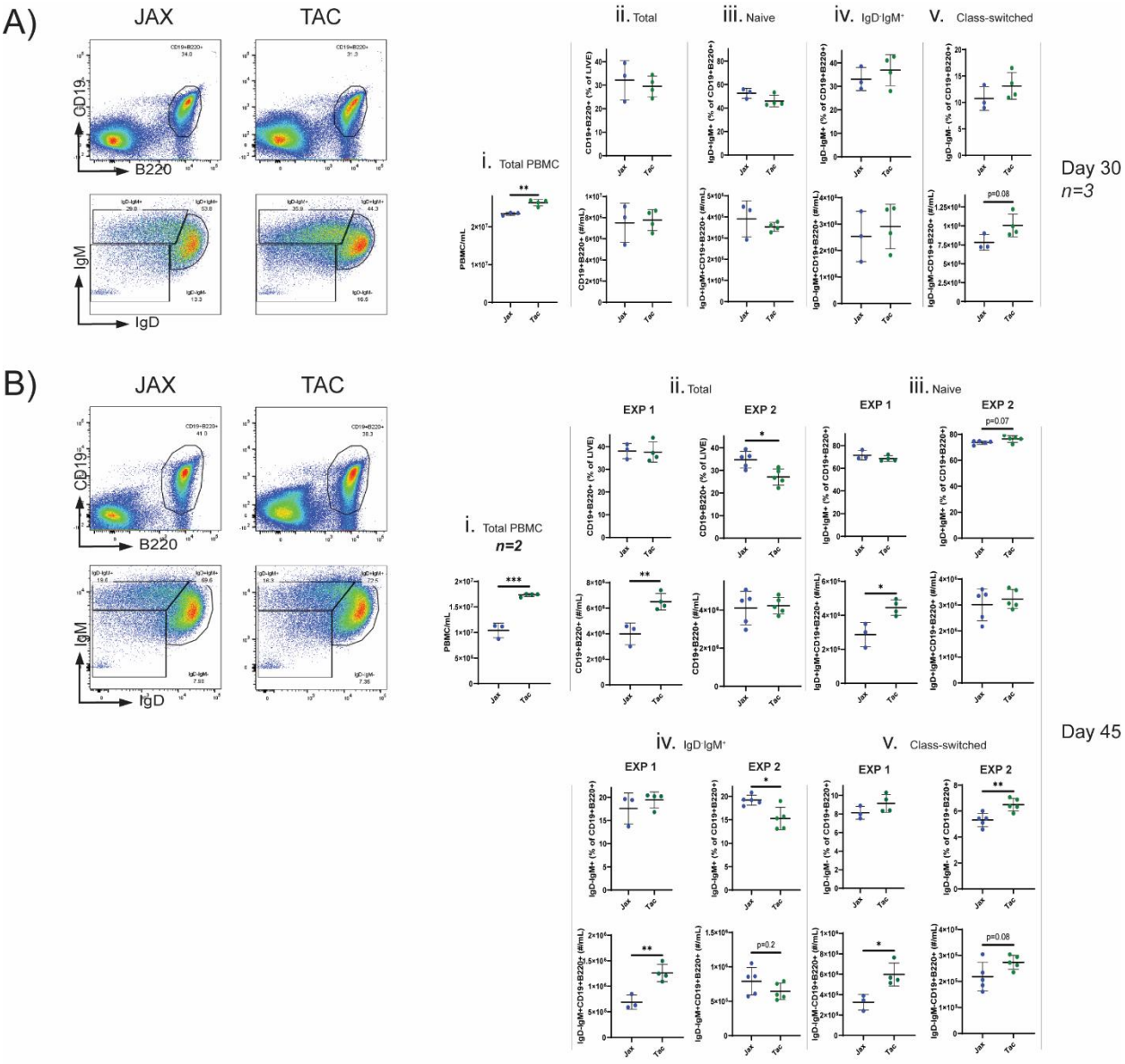


Figure 1-3. Presence of class-switched B cells in the peripheral blood was transiently increased in mice purchased from Taconic Biosciences during the chronic stage of LCMV infection (1/2). B cell accumulation in the blood of LCMV-infected JAX and TAC mice was analyzed during the chronic stage of persistent viral infection. (A-B) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 30 (A) and day 45 (B) p.i. are shown. Averages ± SD are shown (Ai-v and Bi-v). Data are representative of 1 (Bii-v), 2 (Bi), or 3 (Ai-v) independent experiment(s) with 3-5 mice/group. Student's t-test; *p-val<0.05, **p-val<0.01, ***p-val<0.001.

At day 60 post-infection, we saw that, in both experiments, numbers of PBMC were still significantly elevated in TAC mice (Fig. 1-4Ai). Furthermore, like in previous late timepoints, there were no differences in the accumulation of total B cells (Fig. 1-

4Aii), nor were there significant differences in the accumulation of naïve and $IgD^{-}IgM^{+}$ non-naïve B cells at this timepoint (Fig. 1-4Aiii-iv). Interestingly, in one experiment, we saw that TAC mice had significantly elevated numbers and frequencies of class-switched B cells at day 60 post-infection (Fig. 1-4Av), and in another independent experiment, the numbers and frequencies in TAC mice trended in a similar fashion (Fig. 1-4Av). These data showed that TAC mice continued to have a higher accumulation of class-switched B cells at day 60. We continued to evaluate the quantities and percentages of B cells in the blood at day 90 post-infection in one experiment. At this timepoint, the higher numbers of PBMC that were seen in TAC mice at previous late timepoints were still sustained (Fig. 1-4Bi). However, at this timepoint, we no longer saw significant differences in any of the B cell parameters that we analyzed (Fig. 1-4Bii-v), suggesting that B cell responses were once again similar between TAC and JAX mice by day 90 post-infection. Taken together, these data indicated that during the late stage of chronic infection, differences in gut microbiome composition led to transient differences in the quantities of B cells in the blood at days 45 and 60 post-infection. While most of these differences during these timepoints were unable to be replicated in two independent experiments, we notably saw that TAC mice consistently had increased numbers and frequencies of class-switched B cells in the blood. This finding suggested that there was an interval during the chronic stage of LCMV infection in which TAC mice had a higher proportion of B cells that had undergone class-switch

recombination, which may be explained by differences in intestinal microbiome composition.

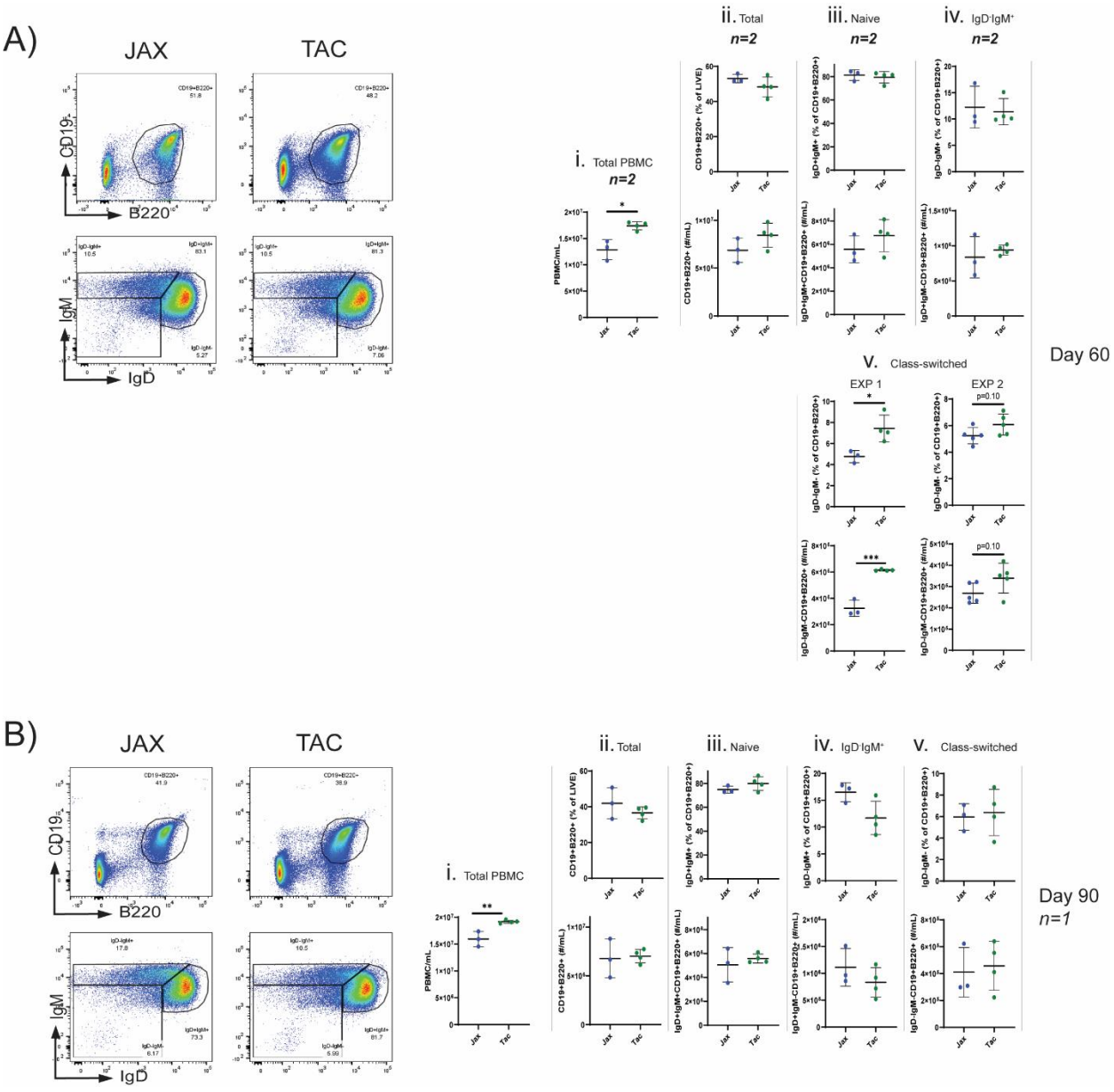


Figure 1-4. Presence of class-switched B cells in the peripheral blood was transiently increased in mice purchased from Taconic Biosciences during the chronic stage of LCMV infection (2/2). (A-B) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 60 (A) and day 90 (B) p.i. Averages ± SD are shown (Ai-v and Bi-v). Data are representative of 1 (Av, Bi-v) or 2 (Ai-iv) independent experiment(s) with 3-5 mice/group. Student's t-test; *p-val<0.05, **p-val<0.01, ***p-val<0.001.

No consistent differences in splenic B cell accumulation between mice from two different vendors during the chronic phase of LCMV infection

The differences seen in the frequencies and numbers of class-switched B cells in the blood between Cl13-infected TAC and JAX mice prompted us to investigate potential differences in B cell responses within secondary lymphoid organs. In two experiments, we looked at the quantities and proportions of B cells in the spleen at 30 days post-infection. In one out of two experiments, we saw that TAC mice had significantly lower spleen cellularity when compared to JAX mice (Fig. 1-5Ai). Upon assessing the accumulation of total B cells within the spleen in this experiment, we saw that there were no significant differences in the frequencies of $CD19^{+}B220^{+}$ B cells; however, we found that TAC mice had significantly lower numbers (Fig. 1-5Aii). Further analysis within the total B cell population revealed that while there were no differences in the percentages of naïve and $IgD^{-}IgM^{+}$ non-naïve B cells, the numbers of these cells were also significantly reduced in TAC mice (Fig. 1-5Aiii-iv). TAC mice had a significant reduction in the numbers of class-switched B cells as well, which was associated with a slight reduction in the frequencies (Fig. 1-5Av). The data from this one experiment show that the numbers, but less so the proportions, of B cells in the spleen were lower in TAC mice. In a second experimental repeat, we saw no differences in the numbers of total splenocytes (Fig. 1-5Ci). Instead, we saw a profound reduction in the numbers and frequencies of $CD19^{+}B220^{+}$ B cells in TAC mice in this experiment (Fig. 1-5Cii). Within the total splenic B cell population, TAC mice had a significant reduction in the numbers, but not frequencies, of naïve B cells (Fig. 1-5Ciii). TAC mice in this experiment also had significantly lower numbers $IgD^{-}IgM^{+}$ non-naïve B cells, which was also accompanied by

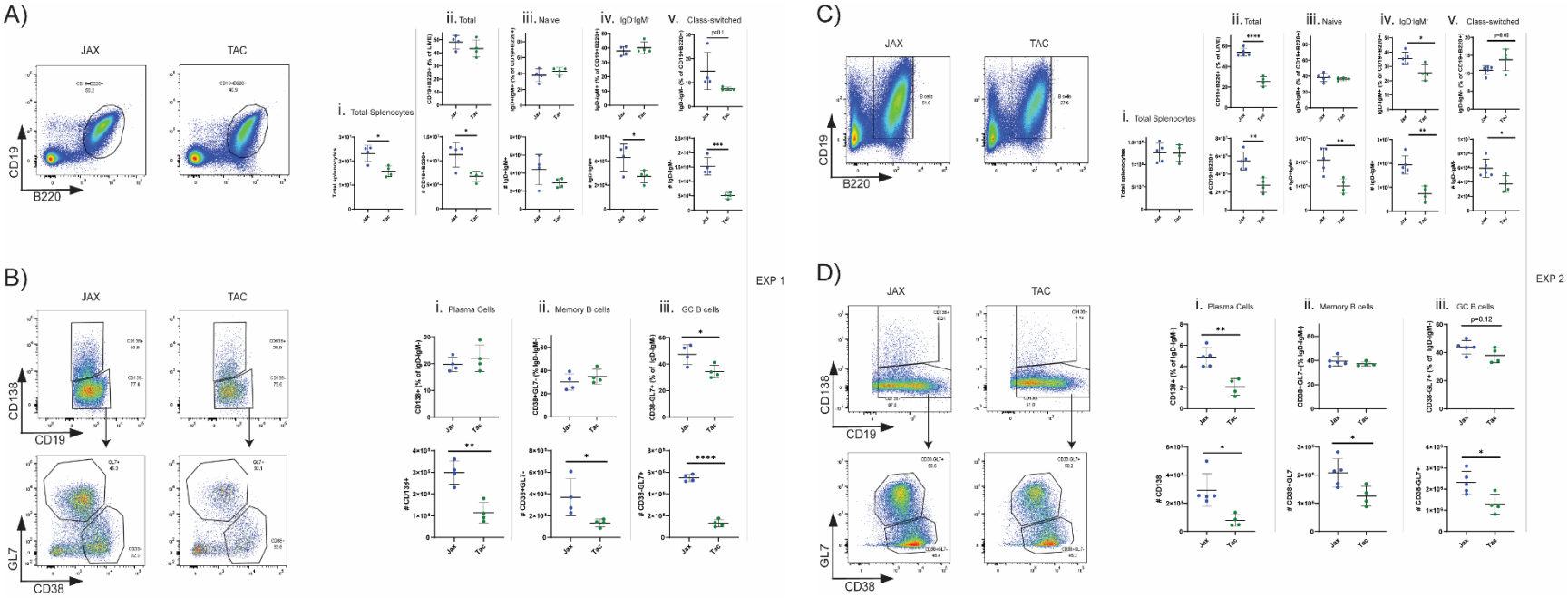
a significant reduction in the frequencies (Fig. 1-5Civ). Similar to what was seen in our first experimental repeat, TAC mice had significantly lower numbers of class-switched B cells (Fig. 1-5Cv, bottom); however, in this second experimental repeat, we noticed that the frequencies of class-switched B cells tended to be slightly higher in TAC mice (Fig. 1-5Cv, top). Taken together, these data from both these experiments showed that the quantities of total, naïve, $IgD^{-}IgM^{+}$ non-naïve, and class-switched B cells were reduced in TAC mice. However, it was not clear whether these differences in numbers were primarily caused by differences in the proportions of B cells or differences in overall spleen cellularity.

Tight regulation of B cell differentiation into germinal center (GC) B cells, memory B cells (MBC), and plasma cells (PC) is critical for the development of optimal antibody responses (Cyster and Allen, 2019). Thus, in two independent experiments, we looked at the proportions of these B cell subsets residing in the spleens of infected TAC and JAX mice at day 30 post-infection. Within the class-switched B cell population, PC were identified as $CD138^{+}$, MBC were defined as $CD38^{+}GL7^{-}$, and GC B cells were defined as $CD38^{-}GL7^{+}$ (Madan et al., 2009; Staupe et al., 2019). In one out of two experiments, we found that there was a significant decrease in the numbers of PC and MBC, although the frequencies of these cells were unchanged (Fig. 1-5Bi and ii). Interestingly, we observed that TAC mice had a significant reduction in GC B cell frequency as well as number compared to JAX mice (Fig. 1-5Biii), suggesting that TAC mice had a lower proportion of B cells within the germinal centers. In another independent repeat of this experiment, we again found that TAC mice had significantly lower numbers of GC B cells, although the differences in GC B cell frequencies were not as pronounced as in

the first experiment (Fig 1-5Diii). TAC mice also had a reduction in the numbers, but not frequencies, of MBC, similar to what was observed in the first experimental repeat (Fig. 1-5Dii). Notably, we found that TAC mice in this experiment had significant reductions in not only the numbers, but also the frequencies of PC (Fig. 1-5Di), which suggested that these mice had a lower output of PC within the spleen.

Our results showed that in one out of two experiments, TAC mice had significantly reduced total splenocytes, which in turn significantly reduced the numbers of total B cells, naïve B cells, $IgD^{-}IgM^{+}$ B cells, and class-switched B cells. Within the class-switched B cell population, numbers of MBC and PC also had similar reductions in TAC mice. Importantly, TAC mice had significant reductions in both the frequencies and numbers of GC B cells. In another independent experiment, we found no differences in spleen cellularity but found that TAC mice had significantly reduced numbers and frequencies of total B cells. This was associated with similar decreases in the numbers of naïve B cells $IgD^{-}IgM^{+}$ B cells, and class-switched B cells. TAC mice in this experiment also had reduced numbers of MBC and GC B cells, although the frequencies GC B cells between TAC and JAX mice were similar to each other in this experiment. Instead, TAC mice in this experiment exhibited a significant reduction in the frequencies and numbers of PC. Taken together, these data suggested that differences in gut microbiome composition can influence B cell differentiation within secondary lymphoid organs in response to chronic viral infection, although the mechanisms by which this occurs were unclear.

Figure 1-5. Differences in splenic B cell accumulation between mice from two different vendors were inconsistent in two independent experiments. C57BL/6 mice were infected with LCMV Cl13 and sacrificed at day 30 p.i. B cell accumulation in the spleen was analyzed with immunofluorescent staining and flow cytometry. (A and C) Two independent experiments analyzing total splenocytes (i), $CD19^{+}B220^{+}$ B cells (ii), $IgD^{+}IgM^{+}$ naïve B cells (iii), $IgD^{-}IgM^{+}$ non-naïve B cells (iv), and $IgD^{-}IgM^{-}$ class-switched B cells (v). (B and D) Two independent experiments analyzing $CD138^{+}$ plasma cells (i), $CD38^{+}GL7^{-}$ memory B cells (ii), and $CD38^{-}GL7^{+}$ GC B cells (iii) within the $IgD^{-}IgM^{-}$ class-switched B cell population. Averages ± SD are shown (Ai-v, Bi-iii, Ci-v, Di-iii). Data are representative of 1 independent experiment with 4-5 mice/group. Student's t-test; *p-val<0.05, **p-val<0.01, ***p-val<0.001, ****p-val<0.0001.


A)
JAX
TAC
CD19
B220
i. Total Splenocytes
ii. Total
iii. Naive
iv. IgD IgM+
v. Class-switched
EXP 1
C)
JAX
TAC
CD19
B220
i. Total Splenocytes
ii. Total
iii. Naive
iv. IgD IgM+
v. Class-switched
B)
JAX
TAC
CD138
CD19
GL7
CD38
i. Plasma Cells
ii. Memory B cells
iii. GC B cells
EXP 1
D)
JAX
TAC
CD138
CD19
GL7
CD38
i. Plasma Cells
ii. Memory B cells
iii. GC B cells
p=0.12
EXP 2

The role of antibody responses in the improved viral clearance seen in mice from Taconic Biosciences remained elusive

In order to confirm that TAC mice had enhanced systemic viral clearance, we quantified viral titers in the serum at days 8, 30, 45, 60, 90, and 120 post-infection. From days 8 to 60 post-infection, TAC and JAX mice had similar viral titers in the serum (Fig. 1-6Ai). At day 90, we saw that all TAC mice had cleared the virus from the serum, while virus was still able to be detected in 1 out of 3 JAX mice (Fig. 1-6Aii); however, the difference in serum viral titers between TAC and JAX mice at day 90 were not statistically significant. By day 120, all JAX mice had cleared the virus (Fig. 1-6Ai). These data showed that in contrast to 2 previous independent experiments performed in our laboratory, we did not detect enhanced systemic viral clearance in TAC mice in any of the experiments mentioned above. When assessing LCMV-specific IgG titers at day 45 post-infection, we found that there were no significant differences in the titers of total anti-viral IgG between TAC and JAX mice (Fig. 1-6B). Furthermore, there were no significant differences in the titers of anti-viral IgG1 or IgG2a (Fig. 1-6B), suggesting that the proportions of these isotypes were unchanged. These data suggested that despite seeing the aforementioned differences in the numbers and proportions of B cells in the blood and spleen during chronic LCMV infection, these differences did not impact viral clearance or anti-LCMV IgG responses. Although the lack of an improved viral clearance phenotype may correlate with the absence of differential anti-viral IgG antibody titers, it remained inconclusive whether or not anti-LCMV antibody responses played a role in the improved viral clearance seen in TAC mice during initial pilot experiments.

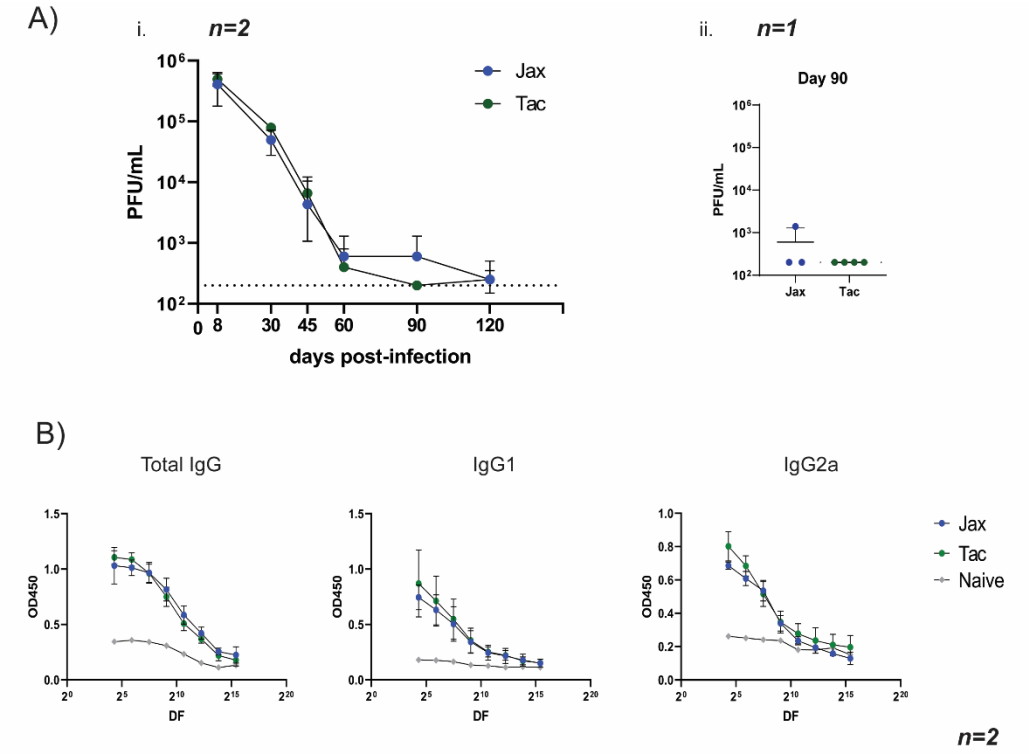


Figure 1-6. No differences in viral clearance or anti-viral IgG antibody responses between mice from two different vendors. C57BL/6 mice from The Jackson Laboratory and Taconic Biosciences were infected with LCMV Cl13 and serum was collected at days 8, 30, 45, 60, 90, and 120 post-infection. (A) Viral titers in the serum were quantified at days 8, 30, 45, 60, 90, and 120 p.i. Kinetics of viral clearance in JAX and TAC mice (i) and comparison at day 90 p.i. (ii) are shown. (B) LCMV-specific IgG, IgG1, and IgG2a antibody titers were quantified at day 45 p.i. Averages ± SD are shown (A-B). Data are representative of 2 independent experiments with 3-5 mice/group. Student's t-test.

Cohousing mice from two different vendors resulted in the transfer of homeostatic B cell accumulation in the peripheral blood

In order to directly test the role of intestinal microbiome composition in regulating homeostatic B cell accumulation, TAC and JAX mice were co-housed together for three weeks as a method of passive microbial transfer (Ericsson and Franklin, 2015). In two independent experiments, we evaluated B cell markers in the blood of co-housed TAC (TAC coh) and co-housed JAX (JAX coh) mice one day prior to infection alongside single-housed TAC and JAX mice that were purchased and housed within our facilities for two days. When evaluating overall blood cellularity, we found that, in one out of two

independent experiments, PBMC numbers were equivalent between the two co-housed groups and fell in between the numbers seen in single-housed JAX and TAC mice (Fig. 1-7Bi). Upon evaluating the accumulation of total B cells in this experiment, we found that there were no significant differences in the frequencies or numbers of $CD19^{+}B220^{+}$ B cells across all groups (Fig. 1-7Bii). Further analysis within the total B cell population revealed that, much like what was observed in TAC mice, both JAX coh and TAC coh mice had a significant reduction in the frequencies, but not numbers, of $IgD^{+}IgM^{+}$ naïve B cells compared to JAX mice in this first experimental repeat (Fig. 1-7Biii). These reductions in the frequencies of naïve B cells in co-housed mice were accompanied by increases in the frequencies of non-naïve $IgD^{-}IgM^{+}$ non-naïve B cells and $IgD^{-}IgM^{-}$ class-switched B cells, although the numbers of these cells also remained unchanged (Fig. 1-7Biv-v).

In a second experimental repeat, we observed that, much like TAC mice, both JAX coh and TAC coh mice had significantly elevated PBMC numbers compared to JAX mice (Fig. 1-7Ci). Moreover, JAX coh and TAC coh mice in this second experiment had a reduction in the frequencies, but not numbers, of $CD19^{+}B220^{+}$ B cells, which was also similar to what was observed in TAC mice (Fig. 1-7Cii). When analyzing the accumulation of IgD- and IgM-expressing subsets within the total population, we found that naïve, $IgD^{-}IgM^{+}$ non-naïve, and class-switched B cells in JAX coh mice in this experiment were largely unchanged from those in their single-housed counterparts in this experiment (Fig. 1-7Ciii-v). Of note, the frequencies and numbers of these B cell subsets in TAC coh mice slightly deviated from those in single-housed TAC mice; however, these subtle shifts were not statistically significant (Fig. 1-7Ciii-v). These

findings indicated that co-housing resulted in JAX coh mice adopting similar levels of basal B cell accumulation in the peripheral blood as that of single-housed TAC mice. Although the transfer of these phenotypes was inconsistent in two independent experiments, these data suggested that the gut microbiome composition plays a role in regulating homeostatic B cell accumulation in the peripheral blood. On the other hand, the accumulation of B cells in the blood of TAC coh mice were mostly analogous to their single-housed counterparts, suggesting that TAC mice were less impacted by the effects of co-housing.

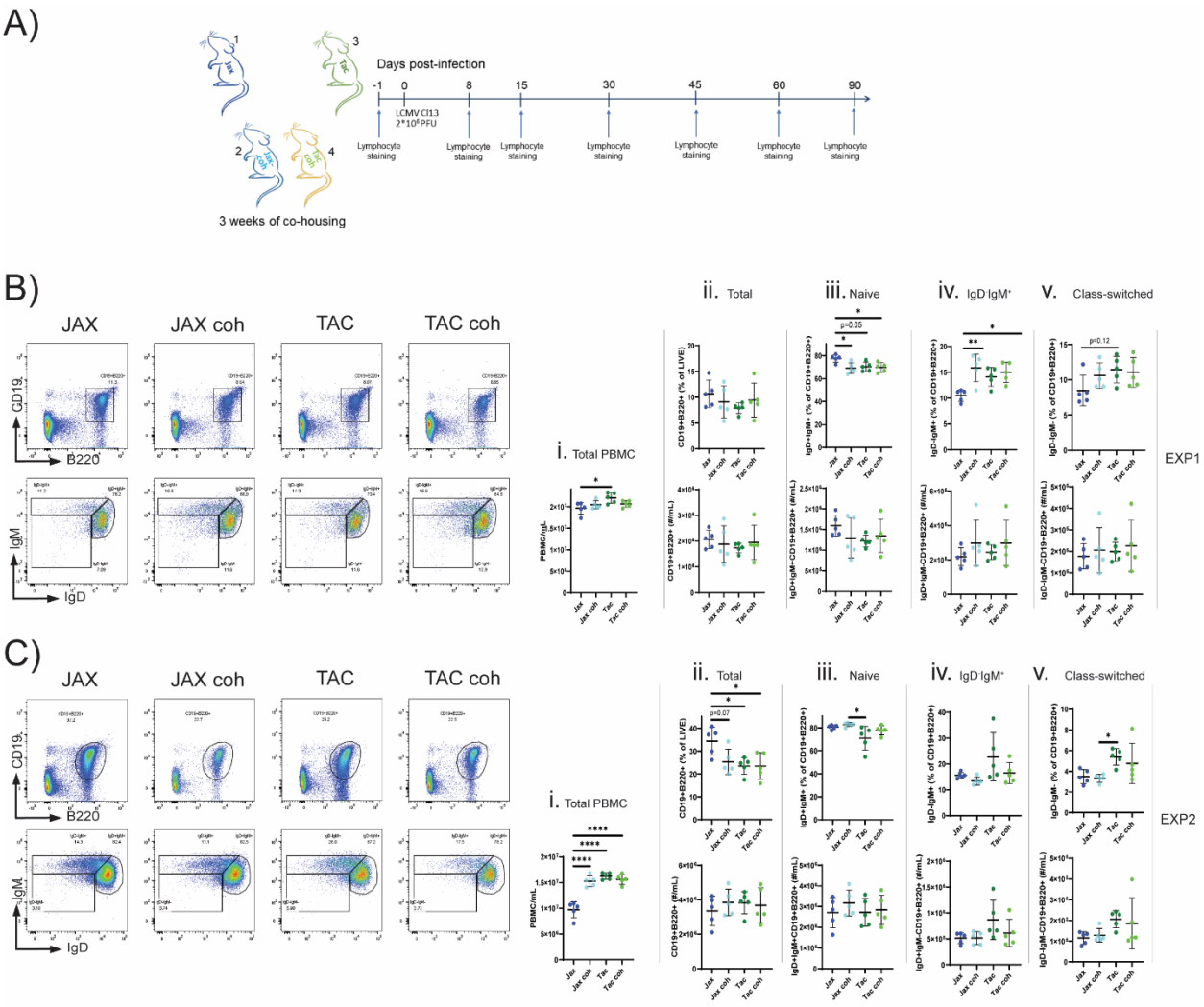


Figure 1-7. Co-housing mice from two different vendors resulted in the transfer of homeostatic B cell accumulation in the peripheral blood. (A) 3-week old C57BL/6 JAX and TAC mice were purchased and immediately transferred to our housing facilities and kept in the same room. After two days of acclimation, JAX and TAC mice were co-housed together for 3 weeks. B cell accumulation in the blood of co-housed JAX (JAX coh; 2) and co-housed TAC (TAC coh; 4), as well as in 6-week old single-housed JAX (1) and TAC (3) mice that had been acclimated in our facilities for 2 days, were analyzed at days -1, 8, 15, 30, 45, 60, and 90 p.i. (B-C) Representative flow cytometry gating and quantification of PBMC (i), $CD19^+B220^+$ B cells (ii), IgD^+IgM^+ naïve B cells (iii), IgD^-IgM^+ non-naïve B cells (iv), and IgD^-IgM^- class-switched B cells in two independent experiments. Averages ± SD are shown (Bi-v and Ci-v). Data are representative of 1 independent experiment with n=5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01, ****p-val<0.0001.

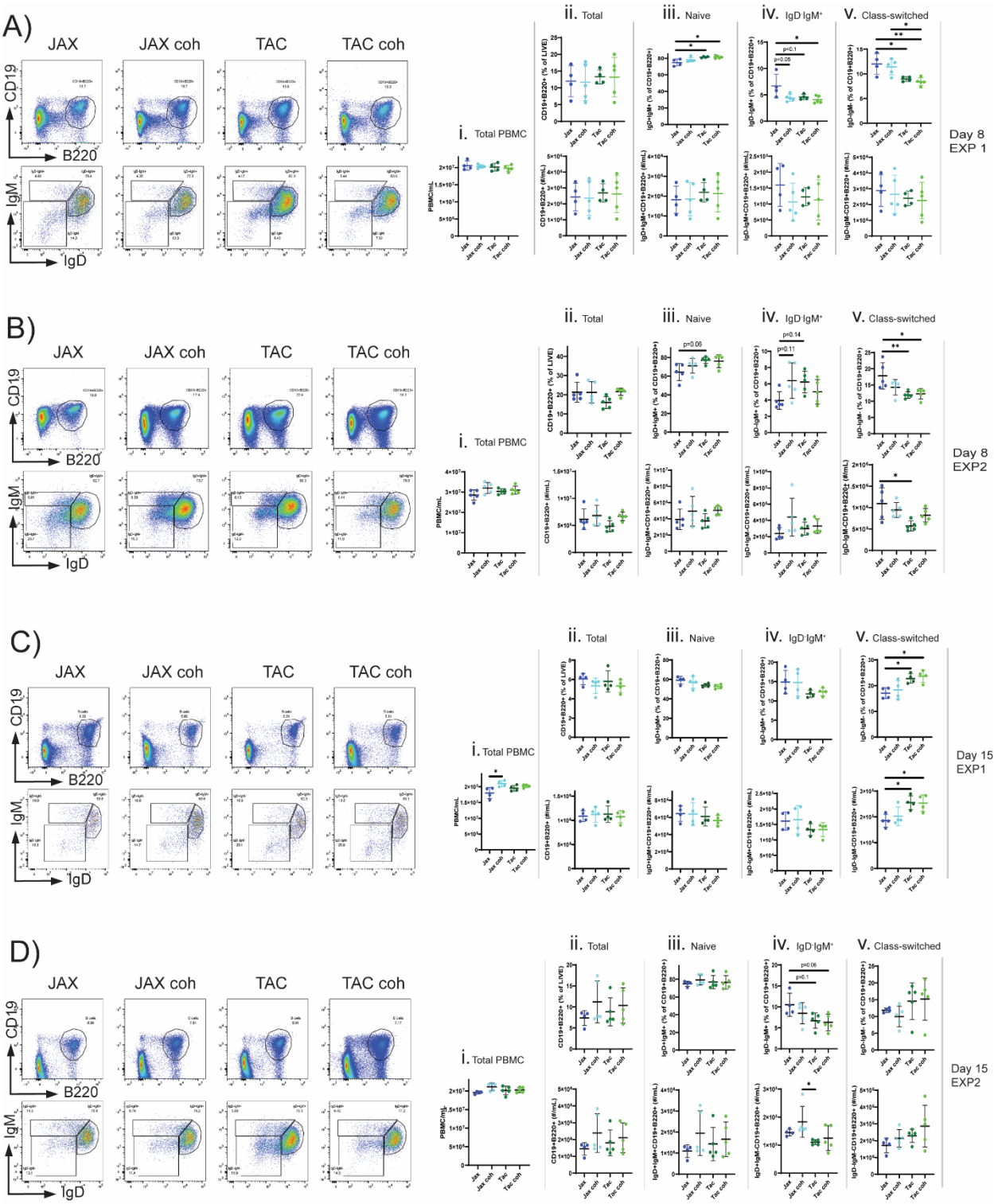
Cohousing resulted in consistent deviations in the proportions of $IgD^{-}IgM^{+}$ B cells in the peripheral blood of co-housed Jackson mice at 8 days post-infection

In order to further test the role of the gut microbiome composition in regulating B cell responses against persistent Cl13 infection, B cells in the blood of co-housed mice were analyzed at days 8, 15, 30, 45, 60, and 90 post-infection along with their single-housed counterparts utilizing the same B cell markers (Fig. 1-7A). After infection, co-housed mice remained housed together for the duration of the experiment. In two independent experiments, we evaluated the presence of B cells in the blood at days 8 and 15. At day 8 post-infection, we found that there were no significant differences in the numbers of total PBMC across all groups in both experiments (Fig. 1-8Ai and Bi), nor were there significant differences in the numbers and frequencies of $CD19^{+}B220^{+}$ B cells (Fig. 1-8Aii and Bii). When evaluating the accumulation of B cell subsets within the total population, we observed that there were no significant differences in the numbers and frequencies of naïve B cells between co-housed mice and their single-housed counterparts, although the frequencies in JAX coh mice were slightly elevated and seemed to be approaching the levels in TAC and TAC coh mice in both experiments (Fig. 1-8Aiii and Biii). Of note, the differences in naïve B cell number and frequency were insignificant between JAX coh and TAC coh mice in both experiments (Fig. 1-8Aiii and Biii). When we evaluated the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells in co-housed mice, we saw that the frequencies in both TAC coh and JAX coh mice reflected those observed in TAC mice in both experiments (Fig. 1-8Aiv and Biv, top), while the numbers of these cells were similar across all groups (Fig. 1-8Aiv and Biv, bottom). Upon examination of class-switched B cells, we saw in one experiment that JAX coh

mice had analogous numbers and frequencies when compared to single-housed JAX mice (Fig. 1-8Av). However, in another experiment, JAX coh mice were seen to have reduced frequencies, but not numbers, of class-switched B cells compared to their single-housed counterparts, although this reduction was not statistically significant (Fig. 1-8Bv). In TAC coh mice, the accumulation of class-switched B cells was largely similar to that in TAC mice in both experiments (Fig. 1-8Av and Bv). When comparing JAX coh mice to TAC coh mice, the frequencies, but not numbers, of class-switched B cells in TAC coh mice were significantly lower compared to JAX coh mice in one experiment (Fig. 1-8Av), but in another experiment, frequencies and numbers were largely similar (Fig. 1-8Bv). Taken together, these results showed that B cell responses in TAC coh mice were equivalent to the B cell responses in their single-housed counterparts at day 8 post-infection. Conversely, JAX coh mice consistently deviated from single-housed JAX mice in the proportions, but not numbers, of naïve and $IgD^{-}IgM^{+}$ B cells in a manner that reflected the proportions observed in single-housed TAC mice in both experiments, suggesting that JAX coh mice had adopted similar $IgD^{-}IgM^{+}$ non-naïve B cell responses from TAC mice. Additionally, we found that JAX coh mice had proportions of class-switched B cells that were very similar to those in TAC mice in one out of two experiments. This finding suggested that JAX coh mice may likewise adopt class-switched B cell accumulation from TAC mice, although co-housing may not necessarily result in robust changes in class-switch recombination.

Next, we evaluated B cell markers in the blood at day 15 post-infection. JAX coh mice at this timepoint had higher numbers of total PBMC compared to JAX mice, with

Figure 1-8. Co-housing resulted in consistent changes in the proportions of IgD^{-} IgM^{+} B cells in the blood at 8 days post-infection. JAX coh and TAC coh mice, as well as JAX and TAC mice, were infected with LCMV Cl13 and B cells in the blood were evaluated at days 8 and 15 post-infection. (A-B) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 8 p.i. from 2 independent experiments. (C-D) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 15 p.i. from 2 independent experiments. Averages ± SD are shown (Ai-v, Bi-v, Ci-v, Di-v). Data are representative of 1 independent experiment with 4-5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01.


A)
JAX
JAX coh
TAC
TAC coh
CD19
B220
IgM
IgD
i. Total PBMC
ii. Total
iii. Naive
iv. IgD IgM+
V. Class-switched
Day 8
EXP 1
B)
Day 8
EXP2
C)
Day 15
EXP1
D)
Day 15
EXP2

this increase reaching statistical significance in one out of two experiments (Fig. 1-8Ci and Di). We observed no significant differences in the numbers or frequencies of $CD19^{+}B220^{+}$ B cells between JAX coh mice and JAX mice in both experiments (Fig. 1-8Cii and Dii). Likewise, there were no significant differences in the numbers or frequencies of naïve B cells (Fig. 1-8Ciii and Diii). Upon evaluating $IgD^{-}IgM^{+}$ non-naïve B cell accumulation, we found in one experiment that JAX coh mice were equivalent to their single-housed counterparts (Fig. 1-8Civ). However, in another independent experimental repeat, JAX coh mice had slightly lower frequencies of $IgD^{-}IgM^{+}$ B cells compared to JAX mice but had significantly higher numbers in comparison to TAC mice (Fig. 1-8Div). Although slight variations in the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells in JAX coh mice were seen in one out of two experiments, no deviances in the accumulation of class-switched B cells could be found in both experiments (Fig. 1-8Cv and Dv). In TAC coh mice, blood cellularity and B cell accumulation in the peripheral blood were largely equivalent to those of single-housed TAC mice in both experiments. These data indicated that despite JAX coh mice having deviations in blood cellularity and $IgD^{-}IgM^{+}$ B cell accumulation in one out of two experiments, these discrepancies were irreproducible, which suggested that B cell responses in the blood of co-housed mice remained similar to B cell responses in their single-housed counterparts at day 15 post-infection.

Altogether, these data indicated that JAX mice were able to adopt the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells in the blood from TAC mice through co-housing at day 8 post-infection. The data from day 8 also indicated that co-housing could result in the transfer of class-switched B cell accumulation from TAC mice to JAX

mice at this timepoint, although this was not consistent in two independent experiments. Furthermore, the data at day 15 demonstrated that co-housing did not significantly impact B cell responses to persistent LCMV infection at this timepoint. Altogether, these findings suggested that microbial transfer through co-housing shifted B cell accumulation in the blood only within a brief window during the intermediate stage of chronic viral infection. Nonetheless, these data supported the role of the gut microbiome composition in influencing early B cell responses to persistent viral infection. Moreover, considering that there were largely no significant differences in the proportions or numbers of B cells between TAC coh and TAC mice at any of these timepoints, these data further suggested that co-housing may have impacted the presence of B cells in the peripheral blood of JAX mice at day 8 in a biased manner.

Co-housing did not significantly impact B cell accumulation in the blood at thirty days post-infection

Following day 15 post-infection, we next evaluated B cell accumulation in the blood at day 30 in two experiments. At this timepoint, total PBMC numbers in co-housed mice were largely similar to each other and fell between the numbers in TAC and JAX mice, similar to what was observed in one experiment at one day pre-infection (Fig. 1-9Ai and Bi). In evaluating B cell parameters at this timepoint, we found that there were no significant differences in the numbers or frequencies of total, naïve, or $IgD^{-}IgM^{+}$ B cells across all groups (Fig. 1-9Aii-iv and Bii-iv). Numbers and frequencies of class-switched B cells in co-housed mice were also analogous to their single-housed counterparts in two experiments (Fig. 1-9Av and Bv). These data indicate that B cell

responses to persistent viral infection in co-housed mice at day 30 were mostly unchanged.

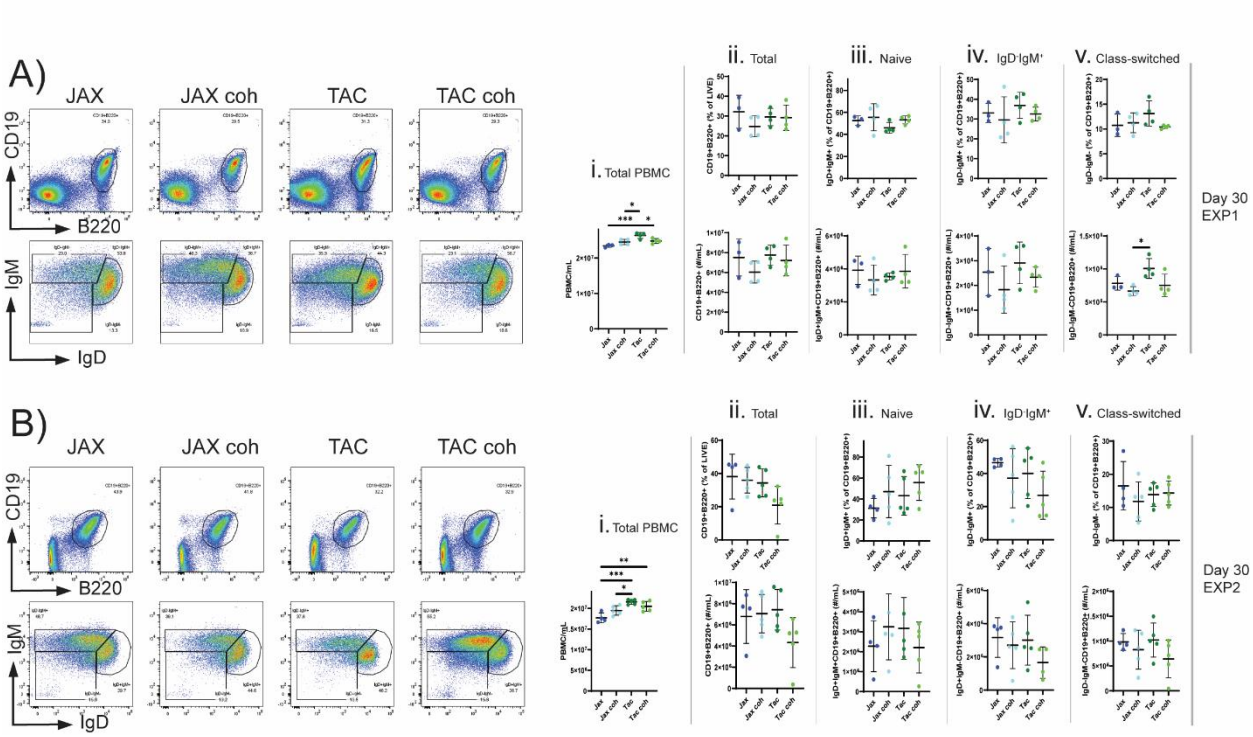


Figure 1-9. Co-housing did not significantly impact B cell accumulation in the blood at day 30 post-infection. B cell accumulation the blood of LCMV-infected JAX, TAC, JAX coh, and TAC coh mice were analyzed at day 30 p.i. (A-B) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 30 p.i. from 2 independent experiments. Averages ± SD are shown (Ai-v, Bi-v). Data are representative of 1 independent experiment with 4-5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01, ***p-val<0.001

Co-housing impacted peripheral blood cellularity and the numbers, but not proportions, of B cells in a biased manner during the chronic phase of LCMV infection

In one experiment, we analyzed the proportions and numbers of B cells in the blood of co-housed mice at days 45, 60, and 90 post-infection. At day 45, both JAX coh and TAC coh mice had significantly elevated numbers of total PBMC that were similar to the levels in TAC mice (Fig. 1-10Ai). Upon evaluating B cell accumulation at this timepoint, we observed that the frequencies of $CD19^{+}B220^{+}$ B cells were largely similar across all groups, as were the frequencies of naïve and $IgD^{-}IgM^{+}$ B cells (Fig. 1-10Aii-iv, top). However, the numbers of total B cells, as well as the numbers of naïve and IgD^{-} IgM^{+} B cell, in co-housed mice were considerably elevated compared to JAX mice and mirrored the trends in TAC mice at this timepoint (Fig. 1-10Aii-iv, bottom). The proportions of class-switched B cells were similar across all groups as well, although the percentages in TAC coh mice were slightly reduced when compared to single-housed TAC mice (Fig. 1-10Av). While quantities of class-switched B cells in co-housed mice were elevated as well, we found that the increases seen in TAC coh mice were not as pronounced as the increases seen in their single-housed counterparts (Fig. 1-10Av, bottom). These data show that similar to TAC mice, JAX coh mice had significantly higher blood cellularity and had increased numbers, but not frequencies, of total, naïve, $IgD^{-}IgM^{+}$, and class-switched B cells compared to single-housed JAX mice at this timepoint. These findings indicated that co-housing impacted the quantities of B cells in the blood of JAX coh mice at day 45 post-infection but left the proportions of these cells unchanged. On the other hand, B cell accumulation in TAC coh mice at this timepoint was very similar to that of single-housed TAC mice, which indicated that B cell

responses between these two groups were very similar at this timepoint, albeit slight differences in class-switched B cell accumulation were observed in one experiment.

The increased blood cellularity in JAX coh and TAC coh mice that was acquired at day 45 continued to be present at day 60 post-infection (Fig. 1-10Bi). When evaluating total B cell accumulation at this timepoints, we found that numbers and frequencies of $CD19^{+}B220^{+}$ B cells in JAX coh mice were slightly increased from the levels in JAX mice, although not to a significant degree (Fig. 1-10Bii). Conversely, total B cell accumulation in TAC coh mice was analogous to that in TAC mice (Fig. 1-10Bii). Within the total B cell population, we discovered that there were no significant differences in the frequencies of naïve B cells or $IgD^{-}IgM^{+}$ non-naïve B cells across all groups (Fig. 1-10Biii-iv, top). However, the numbers of these cells were slightly elevated in JAX coh mice when compared to JAX mice, following the trends that were seen in total B cell numbers at this timepoint (Fig. 1-10Biii-iv, bottom). Meanwhile, numbers of naïve B cells in TAC coh mice were slightly reduced in comparison to TAC mice and more closely resembled the levels in JAX mice, while the numbers of $IgD^{-}IgM^{+}$ B cells remained largely unchanged but had a much higher standard deviation (Fig. 1-10Biii-iv, bottom). Upon evaluating the accumulation of class-switched B cells at this timepoint, it appeared that there were no significant differences in the proportions of these cells when comparing co-housed mice to their single-housed counterparts, although frequencies in JAX coh mice tended to be slightly elevated above the frequencies in JAX mice and were approaching the levels seen in TAC and TAC coh mice (Fig. 1-10Bv, top). The numbers of class-switched B cells in JAX coh mice also tended to be elevated above those in JAX mice, while the numbers in TAC coh mice were similar to

single-housed TAC mice but again had a higher standard deviation (Fig. 1-10Bv, bottom). Taken together, these data indicate that JAX coh mice had higher trends in the numbers of total, naïve, $IgD^{-}IgM^{+}$ non-naïve, and class-switched B cells compared to JAX mice, similar to what was observed at day 45 but to a lesser degree. Furthermore, the frequencies of these B cells at day 60 post-infection were again mostly unchanged. These data demonstrate that B cell responses in the blood of JAX coh mice at day 45 and day 60 post-infection largely resembled the B cell responses in TAC mice during these timepoints. At day 90, we again saw increased blood cellularity in JAX coh and TAC coh mice (Fig. 1-10Ci), but we saw no significant differences in any of the B cell parameters that were evaluated (Fig. 1-10Cii-v).

Taken together, the data from this experiment showed that B cell accumulation in TAC coh mice did not deviate significantly from that in TAC mice. Although we saw slight deviances in class-switched B cell numbers and frequencies at day 45, these differences between TAC coh and TAC mice never reached statistical significance. However, in JAX coh mice, we saw significantly increased blood cellularity compared to JAX mice at day 45 post-infection, as well as increased numbers of total, naïve, $IgD^{-}IgM^{+}$, and class-switched B cells. By day 60, JAX coh mice continued to have elevated blood cellularity and had higher quantities of B cells in the blood compared to JAX mice, albeit these numbers appeared to be normalizing. Finally, at day 90, JAX coh mice continued to have significantly higher blood cellularity compared to JAX mice but had no discrepancies in the accumulation of B cells. These differences between JAX coh mice and JAX mice during these timepoints of chronic LCMV infection were consistent with the trends seen when comparing TAX mice to JAX mice. Considering these similarities

between JAX coh and TAC mice, these findings suggested that late B cell responses to persistent infection in TAC mice may have transferred to JAX mice through co-housing, supporting the role of the gut microbiome composition in regulating B cell responses late during chronic viral infection. Furthermore, the limited deviances in the B cell responses of TAC coh mice further suggested that co-housing impacted B cell responses in JAX coh mice in a biased manner.

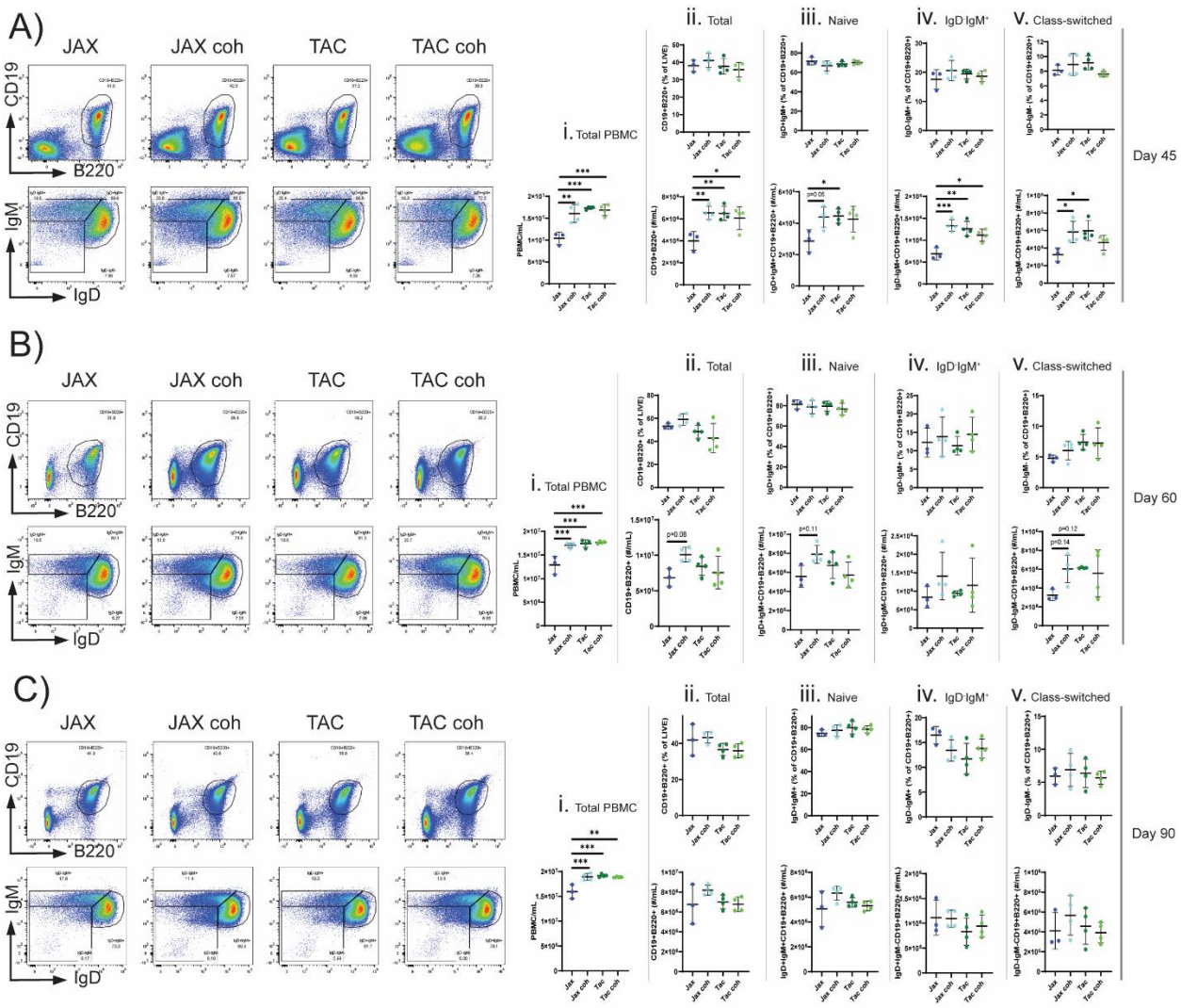


Figure 1-10. Co-housing impacted peripheral blood cellularity and the quantities, but not proportions, of blood B cells in a biased manner during the chronic stage of LCMV infection. B cell accumulation in the blood of LCMV-infected JAX, TAC, JAX coh, and TAC coh mice were analyzed during the chronic phase of LCMV infection. (A-C) Representative flow cytometry gating and quantification of PBMC and B cell parameters at day 45 (A), 60 (B), and 90 (C) p.i. Averages ± SD are shown (Ai-v, Bi-v, Ci-v). Data are representative of 1 independent experiment with 3-5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01, ***p-val<0.001

Co-housing introduced changes in splenic cellularity and in the proportions B cells during the chronic stage of LCMV infection

In order to further understand the impact of co-housing on B cell responses to persistent viral infection, we evaluated the numbers and proportions of total B cells and B cell subsets in the spleens of JAX coh and TAC coh mice. In one experiment, we found that, much like what was seen in TAC mice, both JAX coh and TAC coh mice had significantly lower numbers of total splenocytes compared to JAX mice (Fig. 1-11Ai). When evaluating the total splenic B cell population, we saw that JAX coh mice had similar frequencies of $CD19^{+}B220^{+}$ B cells in comparison to both single-housed mice; however, we saw a reduction in the frequencies of $CD19^{+}B220^{+}$ B cells in TAC coh mice, which were significantly lower when compared to the frequencies in JAX mice (Fig. 1-11Aii, top). The numbers of total B cells in JAX coh and TAC coh mice were significantly decreased in comparison to JAX mice, much like what was seen in TAC mice (Fig. 1-11Aii, bottom). Within the total B cell population, we found that JAX coh mice had similar frequencies of naïve B cells compared to both JAX and TAC mice (Fig. 1-11Aiii, top). However, frequencies of naïve B cells in TAC coh mice tended to be much higher in comparison to the three other groups (Fig. 1-11Aiii, top). The numbers of naïve B cells in JAX coh resembled the numbers in TAC mice, and despite the differences in the percentages of naïve B cells, TAC coh mice also had similar numbers as TAC mice (Fig. 1-11Aiii, bottom). When assessing the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells, we discovered that while proportions in JAX coh mice were unchanged, TAC coh mice had a significant reduction in the percentages of $IgD^{-}IgM^{+}$ B cells that was statistically significant when compared to their single-housed counterparts (Fig. 1-

11Aiv, top). Like single-housed TAC mice, numbers of $IgD^{-}IgM^{+}$ B cells in JAX coh mice were diminished compared to JAX mice, with TAC coh mice appearing to have an even greater reduction. (Fig. 1-11Aiv, bottom). Upon evaluating class-switched B cells, we found that both JAX coh and TAC coh mice had similar frequencies compared to TAC mice (Fig. 1-11Av, top). Furthermore, both co-housed mice saw significant reductions in the numbers of class-switched B cells (Fig. 1-11Av, bottom). These data indicated that JAX coh mice shared similarities with TAC mice in splenic cellularity and B cell accumulation. TAC coh mice, however, were shown to have deviations in the frequencies of total, naïve, and $IgD^{-}IgM^{+}$ activated B cells in comparison to the single-housed TAC mice.

We next evaluated the quantities and proportions of PC, MBC, and GC B cells within the class-switched B cell population in co-housed mice. PC and MBC frequencies in co-housed mice were similar to those of their single-housed counterparts (Fig. 1-11Bi and ii, top). However, both JAX coh and TAC coh mice had significantly reduced numbers of PC and MBC compared to JAX mice, mirroring the trends that were observed in TAC mice (Fig. 1-11Bi and ii, bottom). When analyzing GC B cells, we found that JAX coh mice appeared to have frequencies that closely resembled those in TAC mice (Fig. 1-11Biii, top). When compared back to single-housed JAX mice, the frequencies of GC B cells in JAX coh mice tended to be lower, although this difference was not statistically significant (Fig. 1-11Biii, top). On the other hand, TAC coh mice, like their single-housed counterparts, had significantly reduced proportions of GC B cells in comparison to JAX mice (Fig. 1-11Biii, top). Both JAX coh and TAC coh mice had a profound reduction in the numbers of GC B cells compared to JAX mice (Fig. 1-11Biii,

bottom). These data showed that the proportions of PC and MBC in the spleens of co-housed mice at day 30 post-infection were not significantly impacted by co-housing. However, co-housing had slightly modulated the frequencies of GC B cells in JAX coh mice and resulted in a remarkable reduction in the numbers of PC, MBC, and GC B cells.

Taken together, these data demonstrated that co-housing did not significantly impact the proportions of total, naïve, $IgD^{-}IgM^{+}$, and class-switched B cells in JAX coh mice. Furthermore, the proportions of PC and MBC within the class-switched B cell population in co-housed mice were also unchanged. However, we saw that JAX coh mice, like TAC mice at this timepoint, had significantly reduced numbers of total B cells, class-switched B cells, PC, and MBC. Importantly, numbers of GC B cells in JAX coh mice were also significantly diminished, with the percentages being slightly affected as well. These data suggested that splenic B cell numbers, as well as GC B cell differentiation, were modulated in JAX coh mice as a result of co-housing, which would support the role of the gut microbiome composition in regulating B cell responses to chronic viral infection in the spleen. Furthermore, while there were no significant differences in the proportions of class-switched B cells, or in the proportions of PC, MBC, and GC B cells, between TAC coh and TAC mice, there were significant deviances in the percentages of total, naïve, and $IgD^{-}IgM^{+}$ non-naive B cells. These findings suggested that while there were largely no differences in B cell accumulation in the blood of TAC coh mice, differences between TAC coh and TAC mice may be more apparent within secondary lymphoid organs.

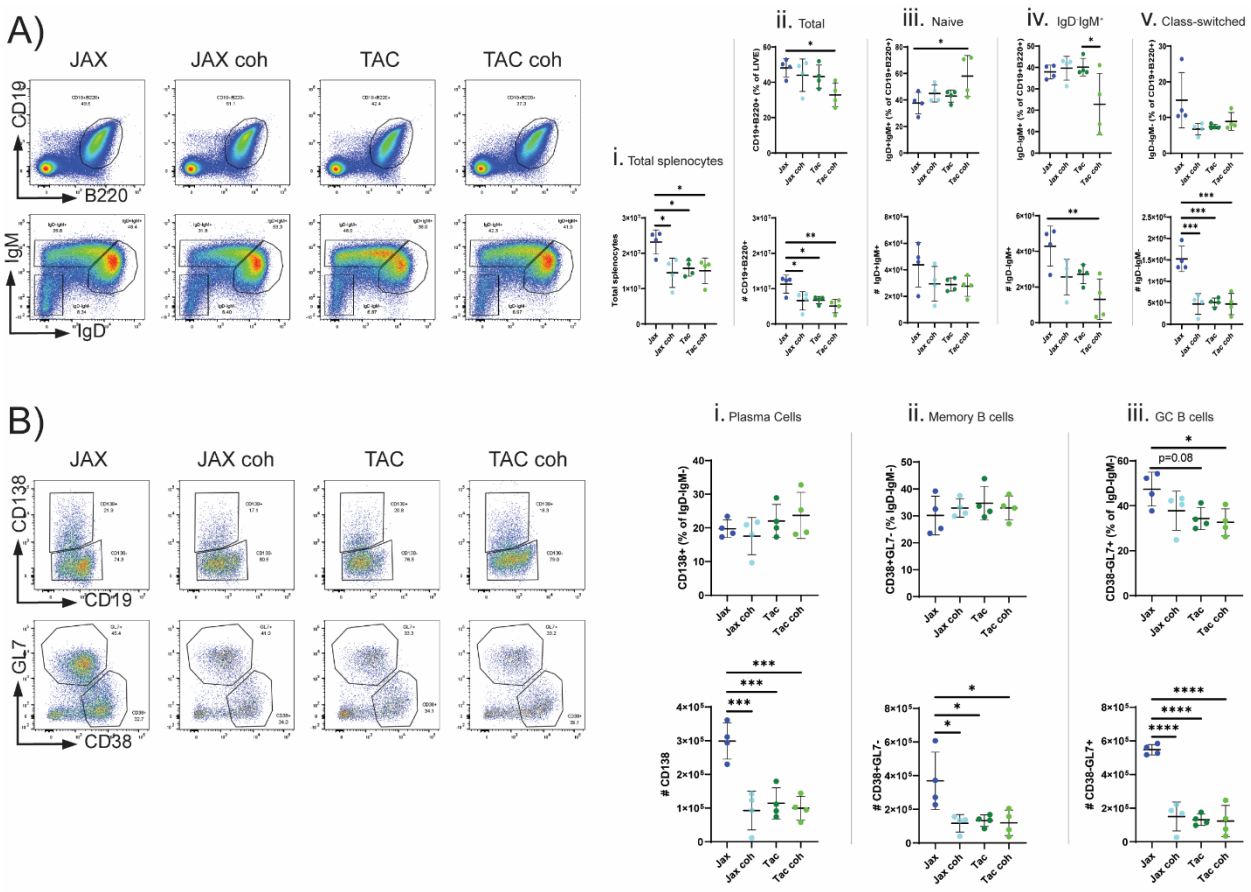


Figure 1-11. Co-housing introduced changes in splenic cellularity and in the accumulation of B cells in the spleen during the chronic phase of Cl13 infection. B cell accumulation in the spleens of Cl13-infected JAX, TAC, JAX coh, and TAC coh mice was analyzed at day 30 p.i. with immunofluorescent staining and flow cytometry. (A) Representative gating and quantification of (i), $CD19^+B220^+$ B cells (ii), IgD^+IgM^+ naïve B cells (iii), IgD^-IgM^+ non-naïve B cells (iv), and IgD^-IgM^- class-switched B cells (v). (B) Representative gating and quantification of $CD138^+$ plasma cells (i), $CD38^+GL7^-$ memory B cells (ii), and $CD38^-GL7^+$ GC B cells (iii) within the class-switched B cell population. Averages ± SD are shown (Ai-v, Bi-iii). Data are representative of 1 independent experiment with 4-5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01, ***p-val<0.001, ****p-val<0.0001.

Mice from Jackson Laboratory that were co-housed with mice from Taconic Biosciences had impaired viral clearance that was independent of antibody responses

In one experiment, we assessed viral clearance in co-housed mice by quantifying viral titers in the serum at days 8, 30, 45, 60, 90, and 120 post-infection. Surprisingly, we found that in JAX coh mice, the kinetics of viral clearance deviated from that of the other groups from days 8 to 60 post-infection (Fig. 1-12Ai). By day 90, JAX coh mice had significantly higher viral titers compared to TAC and TAC coh mice (Fig. 1-12Ai and ii), and the difference in viral titers at this timepoint between JAX coh and JAX mice was on the border of statistical significance (Fig. 1-12Aii). By day 120, viral titers in JAX coh mice were significantly elevated compared to all other groups (Fig. 1-12Aii). To determine whether this impaired viral clearance in JAX coh mice was associated with any changes in antibody responses, we quantified LCMV-specific antibody titers in the serum at day 45. We found that there were no significant differences in LCMV-specific IgG, IgG1, or IgG2a antibody titers across all groups (Fig. 1-12B). These findings demonstrated that while co-housing did not affect viral clearance in TAC coh mice, it did have a profound impact on viral clearance in JAX coh mice. Furthermore, these findings suggested that the impaired viral clearance seen in JAX coh mice was not mediated by LCMV-specific IgG responses.

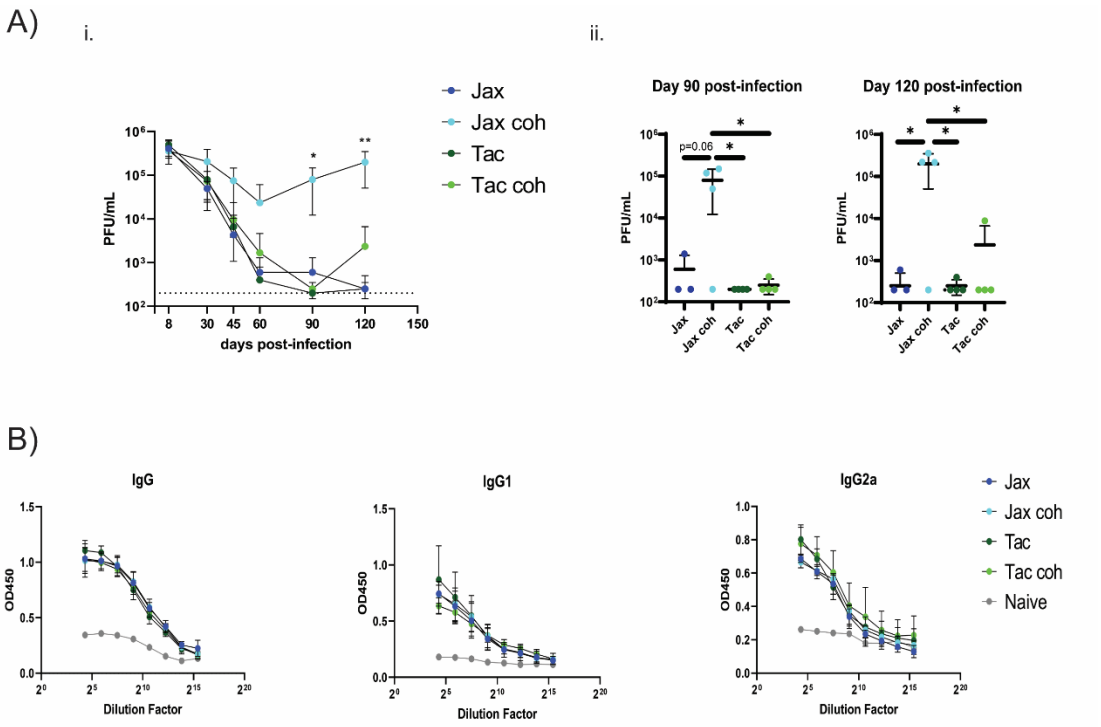


Figure 1-12. Cl-13-infected JAX coh mice had impaired viral clearance that was independent of LCMV-specific IgG antibody responses. Serum was collected from LCMV-infected JAX, TAC, JAX coh, and TAC coh mice at days 8, 30, 45, 60, 90, and 120 post-infection. (A) Viral titers in the serum were quantified at days 8, 30, 45, 60, 90, and 120 p.i. Kinetics of viral clearance (i) and comparisons at day 90 and 120 p.i. (ii) are shown. (B) LCMV-specific IgG, IgG1, and IgG2a antibody titers were quantified at day 45 p.i. Averages ± SD are shown (A-B). Data are representative of 1 independent experiment with 3-5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01

Discussion

During development, the gut microbiome shapes an immune landscape which can ultimately influence immune responses to infections. Characterizing variable immunophenotypes as a result of differential gut microbiome composition may expand our understanding of how the immune system is regulated and may further open the doors to microbe-mediated immunotherapy. In this study, we used LCMV infection in its natural rodent host to characterize differences in B cell responses to chronic viral infection between mice from Taconic Biosciences and mice from Jackson Laboratory,

which, while genetically very similar with the exception of 29 SNPs (Pettitt et al., 2009; Mekada et al., 2009; Zurita et al., 2011), greatly differ in gut microbiome composition (Rosshart et al., 2019; Ivanov et al., 2009). While TAC and JAX mice have been used before in previous studies as a model for investigating how differences in intestinal microbiome composition lead to differences in immune responses to bacterial pathogens (Ivanov et al., 2009) and melanoma tumors (Sivan et al., 2015), we are, to our knowledge, the first group to have used this approach in the context of chronic viral infection. We showed that mice from Taconic Biosciences had higher proportions of naïve B cells and lower proportions of class-switched B cells in the blood at day 8 post-infection in three independent experiments. At day 15 post-infection, we saw an increase in the accumulation of class-switched B cells in the blood of TAC mice. While differences in the proportions and numbers of B cells in the blood were not seen at day 30, we saw a transient increase in the numbers and proportions of class-switched B cells in TAC mice at days 45 and 60 post-infection. We also saw differences in the proportions and numbers of B cells in the spleen at day 30 post-infection, although these differences were divergent in two independent experiments. These data suggest that TAC mice had differences in early $IgD^{-}IgM^{+}$ B cell and class-switched B cell responses at days 8 and 15, differences in splenic B cell responses at day 30, and transient increases in the presence of class-switched B cells in the blood during the late stage of chronic infection. This may be explained by discrepancies in intestinal microbiome composition, which may differentially influence B cell responses by altering the availability of certain microbe-associated metabolites for fueling effector functions or by modulating the initial innate responses, such as the production of inflammatory or

suppressive cytokines, that would govern subsequent adaptive responses. The increased accumulation of class-switched B cells in the blood of TAC mice at days 15, 45, and 60 post-infection is consistent with published results that described increased accumulation of Tfh in systemic lymphoid tissues in an arthritis mouse model due to the transfer of TAC mice-derived SFB (Teng et al., 2016), albeit Tfh cells were not analyzed in our hands at these timepoints. Our data also showed that co-housing JAX mice with TAC mice resulted in JAX coh mice partially recapitulating the accumulation of naïve and $IgD^{-}IgM^{+}$ non-naïve B cells in the blood at day 8. We also found that JAX coh mice had increased numbers, but not frequencies, of B cells at days 45 and 60 post-infection, which was analogous to what was seen in single-housed TAC mice. Furthermore, we also saw that much like TAC mice, JAX coh mice had a significant reduction of total splenocytes and B cells and also tended to have lower frequencies of GC B cells. Despite these differences in the numbers and frequencies of B cells in the blood and the spleen, we were unable to see differences in LCMV-specific IgG titers. Furthermore, contrary to two previous independent experiments, we were not able to see accelerated viral clearance in TAC mice. However, in one experiment we saw that JAX coh mice had significantly impaired viral clearance, suggesting that transferring the gut microbiome composition of TAC mice to JAX mice may exacerbate the chronicity of LCMV infection.

We found in one experiment that homeostatic proportions of class-switched B cells in the blood were significantly higher in TAC mice. Class-switching in B cells in secondary lymphoid organs requires signals from cytokines and costimulatory molecules provided by Tfh cells (Kemeny, 2012). Although we did not evaluate Tfh cells

at this timepoint in our hands, this finding is consistent with a previous study that found that SFB from the TAC gut microbiome can promote Tfh cell differentiation in Peyer's patches (PP) and Tfh cell migration into systemic lymphoid tissues in an autoimmune arthritis mouse model (reference stated above). Future studies will need to examine if increased Tfh frequencies and numbers can be observed in secondary lymphoid organs of healthy wild-type TAC mice. Interestingly, we saw that co-housing JAX mice with TAC mice resulted in JAX coh mice recapitulating the proportions of naïve, $IgD^{-}IgM^{+}$ non-naïve cells, and class-switched B cells in the blood one day before infection, further supporting the notion that the gut microbiome content can influence the homeostatic proportions of B cells in the blood. However, we were unable to observe differences in the numbers and proportions of blood B cells in a second independent repeat of this experiment. The conflicting data may be explained by variance within the TAC gut microbiome composition. While commensal SFB have gained considerable attention for altering immune phenotypes, it is important to consider that differences in microbiome composition between TAC and JAX mice span across multiple and diverse bacterial classes (Ivanov and Littman, 2010). Considering this, it is possible that microbes other than SFB could influence B cell responses, and it is not clear whether the presence of these microbes within the gut is consistent between individual TAC mice that were bred within SFB-positive barriers. Metagenomic sequencing could be an avenue towards discerning the nuanced differences in gut microbiome composition between different cohorts of TAC mice. Considering viral clearance did not show any improvement in two experiments with differing pre-infection phenotypes, it is likely that changes in the

homoeostatic proportions and numbers of B cells in the blood before infection do not play a role in viral clearance.

At day 8 post-infection, we found that TAC mice had a significantly higher number and frequency of naïve B cells and a lower frequency of class-switched B cells in the blood compared to JAX mice. However, by day 15, TAC mice had significantly increased the number and frequency of class-switched B cells in the blood above the levels seen in JAX mice. These data suggest that there may be differences in the kinetics of class switch recombination during the intermediate phase of persistent LCMV infection. This may be explained by differences between TAC and JAX mice in the availability of Tfh cell help or the T cell cytokines that direct class-switching, such as IFN-γ and IL-4 (Snapper and Paul, 1987). Evaluation of the expression of activation-induced cytidine deaminase (AID), the principle enzyme for inducing class switch recombination (Muramatsu et al., 2000), in the B cells of TAC and JAX mice at days 8 and 15 post-infection would be needed to confirm that there were differences in the dynamics of class switch recombination. Furthermore, assessing Tfh cell accumulation and the expression of IFN-γ and IL-4 within secondary lymphoid organs would be required in order to test for differences in the signals that drive class switch recombination. Class-switched B cells in the blood in this context would most likely be circulating MBC or migrating antibody secreting cells (ASC) (Bergmann et al., 2013; Blink et al., 2005), suggesting that there are differences between TAC and JAX mice in B cell differentiation and output of these B cell subsets into the peripheral blood during these timepoints. Antibody staining for MBC and ASC in the blood followed by flow cytometry would inform us if there is indeed a difference in the proportions of these B

cell subsets between TAC and JAX mice. B cell ELISpot can also be used to determine the proportions of ASC and MBC in the blood, as previously described (Shah and Koelsch, 2015; Walsh et al., 2013). Class-switched B cells in the blood can be derived from the germinal centers, but considering that the initial antibody responses to T-dependent antigen are produced by extrafollicular plasma cells, it is possible that the class-switched B cell responses at these early timepoints were developed outside of the germinal centers (Paus et al., 2006). Evaluating B cell phenotypes within the spleen with flow cytometry and histology at these timepoints would provide more insight into potential differences between JAX and TAC mice in extrafollicular and GC B cell responses during the early development of anti-viral B cells during persistent viral infection. Another avenue to investigate differences between TAC and JAX mice during this window of early anti-viral B cell responses is early interferon signaling. Early IFN-I signaling during persistent LCMV Cl13 infection has been shown to drive the preferential differentiation of virus-specific B cells into short-lived plasmablasts (Fallet et al., 2016; Zellweger et al., 2006). Modulation of interferon responses to viral infections due to the gut microbiome have been documented in mice colonized by a human-associated gut microbe (Steed et al., 2017) as well as in other animal models (Abt et al., 2012; Yitbarek et al., 2018). Evaluating differences in IFN-I signaling between TAC and JAX mice could provide an explanation for the differences seen at days 8 and 15 and could demonstrate a mechanism through which the gut microbiome influences early B cell responses to persistent LCMV infection.

These data from days 8 and 15 post-infection fall within the window during which anti-viral B cell responses are still developing within secondary lymphoid organs

(Staupe et al., 2019; Palm and Henry, 2019). While high-affinity LCMV-neutralizing antibodies cannot yet be detected at this early point during infection, LCMV-specific but non-neutralizing antibodies can be detected and have been shown to be an essential component of the humoral response (Richter and Oxenius, 2013), suggesting that the class-switched B cells observed at days 8 and 15 may have comprised of B cells that produce LCMV-specific non-neutralizing antibodies. However, persistent LCMV infection has been shown to drive short-lived LCMV-unspecific antibody responses during the early stage of infection, a condition known as hypergammaglobulinemia (HGG) (Greczmiel et al., 2020; Daugan et al., 2016). At present, the proportion of LCMV-specific, class-switched B cells that were in the blood at days 8 and 15 is unknown. Taking this into account, it is possible that a considerable portion of the class-switched B cells in the blood observed at days 8 and 15 post-infection were not specific for LCMV. Considering that the differences in blood class-switched B cells seen at days 8 and 15 had were not associated with any enhanced viral clearance phenotype, it is possible that these differences were caused by changes in the LCMV-unspecific B cell compartment. To confirm this, staining B cells in the peripheral blood with a variety of fluorescently labelled LCMV antigens, such as a fluorescently labelled LCMV nucleoprotein (NP) or glycoprotein (GP) as previously done (Staupe et al., 2019; Schweier et al., 2019; Sommerstein et al., 2015), would help discern the proportions of LCMV-specific B cells found in the blood at these early timepoints. Furthermore, measuring titers of antibodies with specificities for LCMV-unspecific antigens, such as 2,4-dinitrophenol conjugates (e.g. NP-CGG) or hen egg lysosome (HEL) (Greczmiel et al., 2020), could be done as a way to assess differences in LCMV-unspecific B cell

responses between TAC and JAX mice. As an alternative, high-throughput sequencing of B cell receptors (BCR) (Yaari and Kleinstein, 2015) is a next generation sequencing approach that would allow us to characterize and compare the specificities of the B cell repertoire between TAC and JAX mice. Again, looking at B cell responses to IFN-I would also provide more insight, as it has been shown that IFN-I signaling during the acute phase of LCMV infection compromises specific antibody responses and drives HGG (Daugan et al., 2016). These would be steps toward uncovering whether differences in the gut microbiome composition can differentially regulate the degree of HGG during persistent viral infections.

At these timepoints, we also saw differences in the numbers and frequencies of $IgD^{-}IgM^{+}$ non-naive B cells in the blood. This population could represent antigen-experienced IgM^{+} memory B cells. While the origin, function, and longevity of IgM memory B cells are still subjects of controversy, it has been shown that IgM memory B cell responses are involved in germinal center recall responses, unlike IgG memory B cells which directly differentiate into plasma cells upon antigen re-challenge (Dogan et al., 2009; Pape et al., 2011). Additionally, unswitched memory B cells have been shown to be generated early following primary immunization in a GC-independent fashion (Taylor et al., 2012). Furthermore, IgM memory B cells generated during infection with *Ehrlichia muris* in mice were shown to play an important role in IgG recall responses, albeit these IgM memory B cells were not able to be detected in the blood in this study (Yates et al., 2013). Follow-up studies will need to investigate the potential of this $IgD^{-}IgM^{+}$ subpopulation for mounting recall responses to LCMV infection, which can be done by the adoptive transfer of these cells into healthy mice followed by LCMV

infection (Wherry et al., 2003; Zuccarino-Catania and Shlomchik, 2015). Alternatively, this $IgD^{-}IgM^{+}$ B cell population could represent IgM-expressing plasma cells, which in mice are primarily elicited by T-independent, bacteria-associated polysaccharide antigens (Blanc et al., 2016; Foote et al., 2012). Considering this, the differences in the proportions and numbers of $IgD^{-}IgM^{+}$ non-naive B cells between TAC and JAX mice may be explained by differences in the accessibility of certain microbe-associated molecular patterns and/or antigens due to differential microbiome composition. However, IgM-expressing plasma cells can also be elicited in a T-dependent manner. Following interaction with $CD4^{+}$ T cells, B cells can either enter the germinal centers to undergo class switch recombination, or they can leave the B cell follicles and differentiate directly into plasma cells with relatively weak antigen affinity (Paus et al., 2006). This decision to either differentiate into extrafollicular plasma cells or into germinal center B cells following $CD4^{+}$ T cell-mediated activation is governed by the strength of antigen recognition (Chan et al., 2009; Paus et al., 2006; Ise et al., 2018]. In conjunction with this, it has been shown that T-dependent B-cell production of antibodies is linked to microbial antigen exposure, and more recently it has been shown through a deep sequencing approach that exposure to commensal microbes shapes immunoglobulin diversity in germ free mice (Zhao and Elson, 2018; Li et al., 2020). Considering the important role of commensal microbes in shaping the primary B cell repertoire, the differences in the output of $IgD^{-}IgM^{+}$ non-naive B cells between TAC and JAX mice may be explained by differences in the primary B cell repertoire as a result of differential microbiome content, which in turn led to differences in antigen recognition strength. Follow-up studies will need to confirm that plasma cells can be found within

this population of $IgD^{-}IgM^{+}$ non-naïve B cells, which can be identified by B lymphocyte-induced maturation protein (Blimp-1), a transcriptional repressor known to be a master regulator of plasma cell differentiation (Shaffer et al., 2002; Shapiro-Shelef et al., 2003). This line of investigation could also benefit from deep sequencing of B cell populations within systemic lymphoid organs in order to evaluate differences in the immunoglobulin repertoire between TAC and JAX mice. Additionally, differences between TAC and JAX mice in antibody affinity to LCMV antigens can be tested using a surface plasmon resonance-based assay (Hearty et al., 2012).

At day 30 post-infection, we found that TAC mice had differences in the proportions and numbers of B cells in the spleen. These data suggest differences in virus-specific B cell differentiation late during chronic infection and may have implications on the dynamics of the germinal center reaction. In order to develop and refine antibody responses, B cells undergo a cyclic process in the germinal centers in which they undergo affinity maturation in the dark zones and compete for Tfh cell help in the light zones (Staupe, et al., 2019). It is in the light zone where B cells receive the signals that decide whether they will terminally differentiate into MBC or PC or re-enter the dark zone to undergo additional affinity maturation (Palm and Henry, 2019). These signals include the magnitude of BCR affinity for antigen, CD40:CD40L interactions with Tfh cells, and cytokines provided by Tfh cells such as IL-21 (Palm and Henry, 2019). It has been shown on a transcriptional level that GC B cells favor MBC and PC differentiation over dark zone re-entry during chronic LCMV infection (Staupe et al., 2019) and recent studies have documented sustained ASC output during chronic LCMV infection (Fallet et al., 2020; Kräutler et al., 2020). These differences in the proportions

of GC B cells and PC between TAC and JAX mice could be explained by differences in the signals that guide GC B cells toward terminal differentiation or away from dark zone re-entry as a result of differential microbiome composition. Future studies will need to examine differences between TAC and JAX mice in the signals that govern these responses, such as the expression of MHC class II and CD40 on the surface of B cells. Evaluating IL-21 and IL-4 secretion by Tfh cells as well as Tfh cell surface expression of CD40L and ICOS would also provide more insight into the differences between TAC and JAX mice in B cell differentiation signals. Furthermore, differences in PC and MBC precursor signatures within GC B cells—such as the expression of CCR6 and IL9R on the surface of MBC precursors and the upregulation of IRF4 in PC precursors—will have to be verified. We saw in TAC mice a reduction in the proportions of GC B cells in one experiment and a reduction in PC in another experiment, suggesting that in one experiment TAC mice had a decrease in the signals that promote dark zone re-entry, while in another experiment TAC mice had a reduction in the signals that promote plasma cell differentiation. The contrasting observations indicate that the mechanisms through which the gut microbiome influences B cell responses in the spleen remain unclear. This study would benefit from metagenomic sequencing of the TAC gut microbiome in order to evaluate variability in the microbial composition. We also saw in one experiment that TAC mice had lower numbers of total splenocytes and total B cells and in another experiment TAC mice had lower numbers and frequencies of total B cells, which may be indicative of a higher number of cells egressing from sites of development and entering circulation. This may be supported by the observation that TAC mice had elevated accumulation of class-switched B cells in the blood at days 45

and 60 post-infection. Follow up studies will have to examine the proportions and numbers of B cells in multiple immunological niches, such as the gut, blood, and bone marrow, in order to confirm this.

When co-housing JAX mice with TAC mice during chronic LCMV infection, we saw that JAX coh mice recapitulated certain aspects of the B cell response in TAC mice at days 8, 30, 45, and 60 post-infection. In both experiments, we saw that the frequencies of $IgD^{-}IgM^{+}$ non-naïve B cells in JAX coh mice at day 8 post-infection were similar to the levels seen in single-housed TAC mice. These data suggest that exposure of JAX mice to the TAC mice microbiome composition altered the generation of IgM-expressing memory B cells or IgM-expressing PC. As mentioned above, these cells can be produced outside of the germinal center, and IgM-expressing PC are produced depending on their affinity for antigen, which may be shaped by microbial exposure. Considering this, our data also suggests that co-housing may have modulated the primary B cell repertoire in JAX coh mice. Alternatively, the similarities in the frequencies of $IgD^{-}IgM^{+}$ non-naïve B cells between JAX coh and TAC mice may be explained by the acquisition of certain microbe-associated molecular patterns and/or antigens by JAX coh mice from the TAC mouse gut microbiome, leading to similarities in the formation of T-independent PC. As suggested previously, the identity of these $IgD^{-}IgM^{+}$ non-naïve B cells will need to be discerned in order to further investigate these postulates. It would also be of interest to compare the primary B cell repertoires between single-housed JAX mice and co-housed JAX mice. At this timepoint, we also saw that JAX coh mice seemed to partially recapitulate the frequencies of class-switched B cells in the blood of TAC mice in only one out of two experiments. These

data suggest that while the transfer of gut-associated microbes through co-housing may modulate antigen-specific B cell responses and class-switch recombination, these effects may not consistently appear. Considering that co-housing may result in a relatively incomplete transfer of microbes, this study may benefit from a more controlled method for microbial transfers, such as oral gavage administration (Ericsson and Franklin, 2015; Robertson et al., 2019).

During the chronic stage of infection, we saw in one experiment that the numbers of overall PBMC and the numbers of total splenocytes were transferred from TAC mice to JAX mice. Consequently, we saw that the numbers of B cells in the blood and spleen of JAX coh mice reflected the numbers seen in TAC mice. Screening B cells in the spleen for the expression of regulators of migration, such as sphingosine-1 phosphate receptor (S1Pr1) and Krüppel-like factor 2 (KLF2), may reveal a correlation between the reduction of total splenocyte numbers and the elevation of PBMC numbers Kumar and Saba, 2015; Winkelmann et al., 2011). It would be useful to analyze the proportions and numbers of other immune cell types in these tissues, such as T cells, monocytes, macrophages, and dendritic cells, in order to form a meaningful association between this phenotype and disease progression. Notably, we saw in one experiment that the majority of JAX coh mice did not clear the virus from the serum by day 120 post-infection. This prolonged chronicity of infection may be due to the differences in gut microbiome composition between co-housed mice and single-housed mice. Without antibiotics, an existing microbiota has strong resistance to colonization by new microbes (Ericsson and Franklin, 2015). Considering this, sustained co-housing may not completely transfer the microbial composition from TAC mice to JAX mice, but instead

configure a unique microbial community that is distinct from those found in single-housed TAC and JAX mice. It is possible that this unique gut microbiome composition may influence immune responses in a way that promotes viral persistence rather than control. Additional studies will need to reproduce this phenotype and compare the gut microbiome compositions between single-housed and co-housed mice in order to discern these disparities. Furthermore, other immune cells such as cytotoxic T lymphocytes in co-housed mice will need to be evaluated for their capacity to carry out effector functions during chronic LCMV infection.

Contrary to two previous experiments, we found that TAC mice in these experiments did not exhibit accelerated viral clearance. These contrasting observations invoke the issue of reproducibility of disease models due to changes in the gut microbiome composition. Several factors can influence the composition of the microbiome, such as changes in animal husbandry, changes in diet, and relocation of mice to a different housing facility (Ericsson and Franklin, 2015). Considering these factors, our studies would benefit from a thorough documentation of housing conditions as well as gut microbiome composition before infection and at multiple timepoints throughout infection as a means to control for variances in disease outcome. This can be easily done in this modern era of biological research considering the decreased cost and increased accessibility of next-generation sequencing. Though it is possible that the accelerated viral clearance seen in TAC mice in previous experiments may have been associated with enhanced anti-viral antibody responses, the data generated from these recent experiments cannot be used to support this association. Nevertheless, our recent experiments demonstrated significant differences in the numbers and proportions of B

cells at multiple timepoints during chronic LCMV infection, suggesting that though differences in B cell responses may arose due to differential microbiome composition, these differences did not impact the kinetics of viral clearance.

Using genetically similar mice from two different vendors that have been shown to vastly differ in gut microbiome composition, we investigated how variances in intestinal microbiome composition may differentially regulate B cell responses. While TAC and JAX mice have been used in previous studies that investigated differences in immune responses to intestinal bacterial pathogens and melanoma tumors, we utilized this approach to study differences in immune responses in the context of persistent viral infection. Importantly, we uncovered that TAC and JAX mice had differences in the accumulation of class-switched B cells in the blood during early B cell responses to chronic viral infection and transiently during the chronic stage of Cl13 infection. Furthermore, we found that while co-housing resulted in a limited transfer of blood B cell accumulation from TAC to JAX mice, JAX coh mice had significantly impaired viral clearance, suggesting that microbial transfer exacerbated the chronicity of Cl13 infection. These studies highlight the importance of thoroughly studying the role of microbiome composition and bacterial transfers in influencing immune responses in different disease states, as microbes that may confer positive outcomes for one pathogen may also lead to adverse consequences for another.

Chapter 2: Characterizing B cell responses to persistent viral infection following the late deletion of B cell-derived IL-27p28

IL-27 is a pleiotropic and heterodimeric cytokine of the IL-12 family that has been uncovered to perform a variety of immunoregulatory functions. Earlier studies in our laboratory have demonstrated that IL-27 plays a role in promoting the survival of virus-specific $CD4^+$ T cells and that IL-27 is transiently upregulated in B cells during the chronic stage of LCMV infection. Here we investigate the impact of B cell-derived IL-27p28 deletion on B cell accumulation in the blood late during persistent LCMV infection. In contrast to one early independent experiment, IL-27p28 deletion in B cells did not reduce the accumulation of total B cell in the blood, but instead resulted in slight increases in the accumulation of IgD^-IgM^+ non-naïve B cells and class-switched B cells that peaked at day 60 post-infection, after which we observed a slightly higher accumulation of total B cells. While mice with IL-27p28 deletion in B cells were seen to have impaired viral clearance, wild type control mice were also unable to clear the virus from the serum, and thus the role of B cell-derived IL-27 in viral control remains unclear.

Background

The effective control of viral infections is achieved through the combined effort of both the innate and adaptive arms of the immune system. However, during chronic viral infection, adaptive immune cells play an augmented role. T cells have conventionally been regarded to be the mediators of viral control by their ability to directly kill infected cells, limiting disease progression and severity (Rosendahl Huber et al., 2014). The importance of T cells has been demonstrated in patients with HIV, HBV, and HCV. Several studies have reported that the control of these viruses was correlated with robust virus-specific $CD4^+$ T cell responses capable of enhancing effector responses from virus-specific $CD8^+$ T cells (Ulsenheimer et al., 2003; Urbani et al., 2006; Rosenberg et al., 1997). More recently, the role B cells in containing viral infections has become much more appreciated. Many studies have underlined the importance of B cells in their ability to produce neutralizing antibodies (nAb) that can directly prevent cell entry and viral replication (Baumgarth, 2013; Logvinoff et al., 2004; Seiler et al., 1998; Battegay et al., 1993; Wright and Buchmeier, 1991). However, B cells are capable of antibody-independent functions, such as cytokine production, and it is less clear how these alternative functions play a role in viral immunity.

IL-27 is a pleiotropic cytokine that is a heterodimer composed of the IL27p28 and EBI3 subunits. It is a member of the IL-12 family of cytokines and signals through a heterodimeric receptor comprised of gp130 in conjunction with WSX-1 (*IL27ra*). While gp130 is ubiquitously expressed, WSX-1 is highly expressed on lymphocytes (Sprecher et al., 1998; Chen et al., 2000; Yoshida et al., 2011) and is also expressed to a lesser degree on myeloid cells (Pflanz et al., 2004). IL-27 ligation to its receptor leads to

downstream phosphorylation of STAT1 and 3 (Pflanz et al., 2004). Dendritic cells and macrophages are regarded as the predominant source of IL-27 following TLR signaling (Kamakura et al., 2006; Molle et al., 2007). While IL-27 was initially regarded to promote inflammation by mediating Th1 development (Pflanz et al., 2002; Villarino et al.,2006), it is now recognized that IL-27 also has immunosuppressive functions as well. For example, IL-27 has been shown to promote the production of IL-10 by Th1, Th2, and Th17 subsets (Awasthi et al., 2007; Stumhofer et al., 2007). The duality of IL-27 has also been illustrated in the context of cancer, in which IL-27 has been found to promote anti-tumor immunity by enhancing the development and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTL) but can also promote the expression of PDL-1 on a variety of cancer cell types as well as on myeloid cells (Salcedo et al., 2004; Carbotti et al., 2015; Horlad et al., 2016; Versteven et al., 2018). In the Zuñiga Lab, we have found a role of IL-27 in enhancing immune responses late during chronic viral infection by promoting the survival of virus-specific $CD4^+$ T cells *in vivo* and in inducing IL-21 production *ex vivo* (Harker et al., 2013). We also saw that IL-27 receptor ablation (*IL27ra*$^{-/-}$) resulted in reduced LCMV-specific IgG titers from day 30 post-infection onwards, suggesting that IL-27 receptor signaling promotes the induction of antibody responses.

While investigating the cellular source of late IL-27, we found that IL-27 expression was upregulated in B cells at day 30 post-infection. Thus, we aimed to determine the role of late B cell-derived IL-27 in the control of LCMV Cl13 infection. Using a transgenic mouse model that allows for IL27p28 deletion specifically in B cells ($IL27p28^{fl/fl}hCD20TAM^{Cre}$), we found in one experiment that B cell-specific IL-27 deletion during the late stage of chronic infection resulted in reduced accumulation of B cells in

the blood and impaired viral clearance. The results presented here are from independent repeats of this initial experiment. In these experimental replicates, we found that the accumulation of B cells in the blood were only subtly modulated, with the proportion of class-switched B cells being slightly higher at days 30-60 post-infection and the proportion of total B cells tending to be higher at days 90-120 in inducible knockout mice instead of wild-type littermates. These trends were not associated with any differences in LCMV-specific IgG titers at day 45 post-infection. While inducible knockout mice were seen to have impaired viral clearance, we also observed that WT littermates could not clear the virus by day 120 post-infection. In one experiment in which we co-housed inducible knockout mice and wild-type littermates with mice purchased from Taconic Biosciences, we saw that mice treated with tamoxifen had significantly altered accumulation of B cells in the blood compared to mice that were never administered tamoxifen, suggesting that issues with viral clearance in WT littermates may have been associated with our tamoxifen treatment.

Materials and Methods

Mice and specific deletion of *il27p28* in B cells. hCD20TAM^{Cre} mice were kindly provided by Dr. Mark Shlomchik (University of Pittsburg). hCD20TAM^{Cre} mice were crossed with IL-27p28$^{fl/fl}$ mice in order to generate IL-27p28$^{fl/fl}$hCD20TAM^{Cre} mice. IL-27p28$^{fl/fl}$ mice with or without the hCD20TAM^{Cre} allele were injected i.p. with 5 daily doses of tamoxifen (1 mg/day) at days 14-18 post-infection with LCMV Cl13. Deletion of the *il27p28* gene was verified using genotyping PCR. 6-week old wild type female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and 6-

week old wild type female C57BL/6N mice were purchased from Taconic Biosciences (San Diego, CA). Mice purchased from commercial vendors were immediately transferred to our specific pathogen free facilities and housed in the same room. Animals were given 48 hours to acclimate before being used in experiments. Mice were bred and maintained in a closed breeding facility, and mouse handling was in accordance with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego (UCSD). Genotyping primers to detect the null p28 allele following Cre-recombination: forward: CTGCAGCCAAGCTATCGAATTCCT, reverse: TGCATCACCACACTTGGCGTACTA.

Virus. 6-week old female mice were infected i.v. with 2 x 10^6 PFU of LCMV Cl13. As an acute infection control, mice were infected i.p. with 2 x 10^6 PFU of LCMV Cl13. LCMV Cl13 stocks were produced in BHK-21 cells and viral titers were determined by plaque assay on Vero cells as previously described (Ahmed et al., 1984; Borrow et al., 1995). LCMV infections were performed in BSL-2 facilities as previously described (Ahmed et al., 1984).

Blood collection and processing. Blood was collected from the facial vein using heparin-coated capillary tubes. 10 uL of blood was diluted 1:20 in FACS buffer (5% fetal bovine serum in PBS) and used for cell counting on a Guava EasyCyte Flow Cytometer. The remaining volume of blood was spun down at 10,000 rpm for 5 minutes in order to extract the serum. Red blood cells were then lysed in two rounds of ACK lysis buffer. Cells were then resuspended in FACS buffer prior to cell staining.

Flow cytometry. To identify B cells, cell suspensions were stained for 1 hour at 4^{o}C with the following antibodies purchased from ebioscience or BD biosciences: anti-B220-PE-CF594 (RA3-6B2), -CD19-PerCP-Cy5.5 (eBio1D3), -CD138-PE (281-2), -GL7-eFluor660 (GL-7), -CD38-PE-Cy7 (90), -IgM-APC-eFluor780 (RMM-1), -IgD-BV650 (11-26c.2a), -IgG1-FITC (A85-1), -IgG2a-biotin (RMG2a-62), -streptavidin-BV605, and -PDL-1-BV421 (10F.9G2). Prior to staining for B cells, cell suspensions were stained with a LIVE/DEAD cell viability dye (Tonbo Biosciences; CA) in order to distinguish dead cells from live cells. After staining, cells were fixed with 2% paraformaldehyde. Cells were acquired using a BioRad ZE5 Cell Analyzer. Staining for fluorescence activated cell sorting (FACS) was done using the following antibodies: anti-IgD-FITC (11-26c), -CD19-PE (eBio1D3), -Thy1.2-PercP-Cy5.5 (HIS51), -GR-1-PercP-Cy5.5 (RB6-8C5), -NK1.1-PercP-Cy5.5 (PK136), -B220-APC (RA3-6B2), -IgM-APC-eFluor780 (RMM-1), and CD11b-PE-Cy7 cells (M1/70). B cells were isolated on a FACSAria to >95% purity. Flow cytometric data were analyzed with FlowJo software (TreeStar; CA).

LCMV-specific antibody ELISAs. LCMV-specific ELISAs and avidity assays were done as we and others have previously described (Hammond et al., 1997; Harker et al., 2013).

Statistical Analysis. Statistical differences were determined by Student t-test or One-way ANOVA with Tukey's post-hoc test with GraphPad Prism 8 (Graphpad, CA). *p-val<0.05, **p-val<0.01, ***p-val<0.001, ****p-val<0.0001.

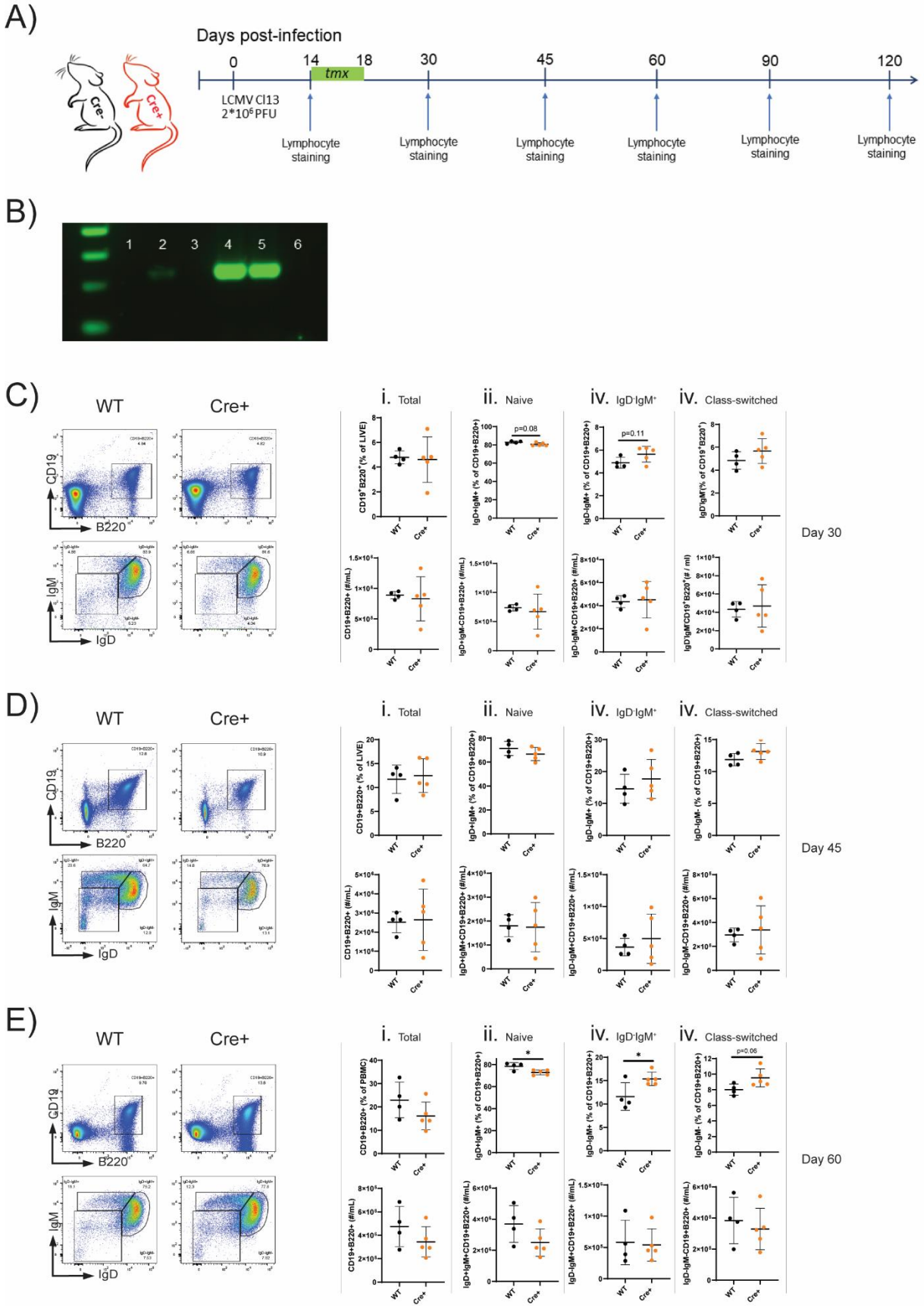
Results

Subtle shifts in B cell accumulation in the peripheral blood manifested following IL-27 deletion in B cells late during chronic infection

In one experiment, we sought to characterize the influence of B cell-derived IL-27 on B cell responses late during chronic viral infection by infecting transgenic IL27p28$^{fl/fl}$hCD20TAM^{cre} (Cre+) mice, where *il27p28* expression in B cells can be attenuated by tamoxifen treatment (Khalil et al., 2012), and littermate controls (WT) with 2 x 10^6 PFU of LCMV Cl13 intravenously (Fig. 2-1A). Cre+ mice, along with WT littermates, were administered 1 mg of tamoxifen daily from days 14 through 18 post-infection in order to abrogate B-cell derived IL-27 in Cre+ mice late in infection. At day 20 post-infection, CD11b$^+$ leukocytes, IgD$^+$IgM$^+$ naïve B cells, and IgD$^-$IgM$^-$ class-switched B cells were sorted from the blood of both Cre+ and WT mice and *il27p28* deletion was verified by a PCR for the *il27p28 null* gene, which would only produce a detectable signal upon the deletion of *il27p28* (Fig. 2-1B). In order to evaluate late B cell phenotypes as a result of *il27p28* deletion, we assessed the proportions and numbers of B cells in the blood at days 30, 45, 60, 90, and 120 post-infection. We found that at day 30, there were no significant differences in the numbers or frequencies of total CD19$^+$B220$^+$ B cells in the blood (Fig. 2-1Ci). Within the total B cell population, the frequencies, but not numbers, of naïve B cells had a slight tendency to be lower in Cre+ mice (Fig. 2-1Cii). This coincided with subtle elevations in the frequencies of IgD$^-$IgM$^+$ and class-switched B cells, although the numbers of these cells were largely unchanged from those of WT littermates (Fig. 2-1Ciii-iv). Similar trends in the accumulation of total B cells and B cell subsets were seen at day 45 post-infection (Fig. 2-1D). However, by

day 60, frequencies and numbers of $CD19^{+}B220^{+}$ B cells were slightly reduced in Cre+ mice (Fig. 2-1Ei). Furthermore, Cre+ mice had a significant reduction in the percentages of naïve B cells (Fig. 2-1Eii, top), which was associated with a significant increase in the percentages of $IgD^{-}IgM^{+}$ non-naïve B cells and slightly elevated frequencies of class-switched B cells (Fig. 2-1Eiii-iv, top). While significant differences were seen in the proportions of these cells, differences in cell numbers were only modest (Fig. 2-1Eii-iv). Taken together, these data indicated that the deletion of *il27p28* in B cells late during chronic infection primarily resulted in subtle increases in the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells and class-switched B cells at days 30 and 45 post-infection, and by day 60, these increases became more apparent.

Figure 2-1. IL-27p28 deletion in B cells late during chronic viral infection resulted in slightly increased accumulation of $IgD^{-}IgM^{+}$ non-naïve and $IgD^{-}IgM^{-}$ class-switched B cells in the blood at days 30-60 post-infection. (A) IL-$27p28^{fl/fl}$h$CD20TAM^{Cre}$ mice and WT littermates were infected with LCMV Cl13. IL-27p28 deletion in B cells was induced with 5 daily doses of tamoxifen at days 14 through 18. B cell accumulation in the blood was analyzed at days 30, 45, 60, 90, and 120 post-infection (p.i.). (B) WT $CD11b^{+}$ cells (1), Cre+ $CD11b^{+}$ cells (2), WT $IgD^{+}IgM^{+}$ naïve B cells (3), Cre+ $IgD^{+}IgM^{+}$ naïve B cells (4), Cre+ $IgD^{-}IgM^{-}$ class-switched B cells (5), and WT $IgD^{-}IgM^{-}$ class-switched B cells (6) were FACS purified from the blood at day 20 p.i. and IL-27p28 deletion was verified with a PCR that amplified the *il27p28 null* gene. (C-E) Representative flow cytometry gating plots and quantification of $CD19^{+}B220^{+}$ B cells (i), $IgD^{+}IgM^{+}$ naïve B cells (ii), $IgD^{-}IgM^{+}$ non-naïve B cells (iii), and $IgD^{-}IgM^{-}$ class-switched B cells (iv) at days 30 (C), 45 (D), and 60 (E) p.i. Averages ± SD are shown (Ci-iv, Di-iv, Ei-iv). Data are representative of one independent experiment with 4-5 mice/group. Student's t-test; *p-val<0.05.


A)
Days post-infection
0
14
18
30
45
60
90
120
tmx
LCMV Cl13
2*10⁶ PFU
Lymphocyte staining
Cre-
Cre+
B)
1
2
3
4
5
6
C)
WT
Cre+
CD19
B220
IgM
IgD
i. Total
ii. Naive
iv. IgD⁻IgM⁺
iv. Class-switched
p=0.08
p=0.11
Day 30
D)
Day 45
E)
p=0.06
Day 60

By day 90 post-infection, we found that the numbers and frequencies of $CD19^+B220^+$ B cells in the blood were increased in Cre+ mice slightly above the levels in WT littermates (Fig. 2-2Ai). Further analysis within the total B cell population revealed that Cre+ mice had significantly elevated frequencies of naïve B cells, with numbers being slightly elevated as well (Fig. 2-2Aii). However, this increase in the accumulation of naïve B cells was not accompanied by any considerable reductions in the accumulation of IgD^-IgM^+ non-naïve B cells or class-switched B cells (Fig. 2-2Aiii-iv). At day 120 post-infection, Cre+ mice still maintained higher trends of numbers and frequencies of total B cells, albeit these were much more variable at this timepoint (Fig. 2-2Bi). However, at this timepoint, we observed no differences in the percentages of naïve, IgD^-IgM^+, or class-switched B cells, although the numbers of these cells were slightly higher in Cre+ mice (Fig. 2-2Bii-iv). The data from these timepoints indicated that the trends seen at days 30 to 60 post-infection of elevated IgD^-IgM^+ and class-switched B cell accumulation in Cre+ mice were no longer observed. Instead, we found that Cre+ mice at days 90 and 120 had slightly increased the accumulation of total B cells, while proportions of IgD^-IgM^+ non-naïve and class-switched B cells were largely unchanged from those of WT littermates. These data suggested that the total B cell accumulation in the peripheral blood was slightly enhanced at days 90 and 120 as a result of late IL-27p28 deletion in B cells in this experiment.

Taken together, these data show that late IL-27p28 deletion in B cells impacted B cell responses in the blood by subtly augmenting the output of IgD^-IgM^+ non-naïve B cells and class-switched B cells at days 30 to 60 post-infection and by slightly increasing the bulk accumulation of B cells at days 90 and 120. These findings would

suggest that B cell-derived IL-27p28 acts as a negative regulator of B cell accumulation, activation, and class-switching during the late phase of chronic viral infection.

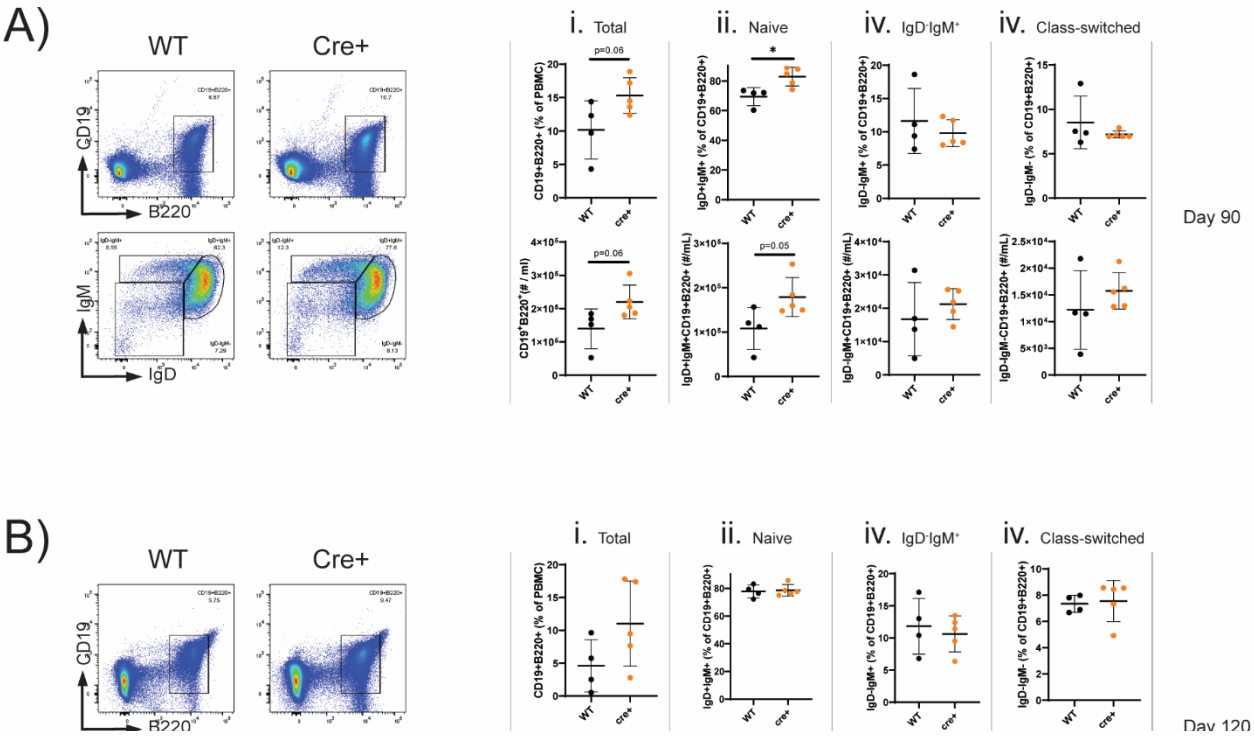


Figure 2-2. IL-27p28 deletion in B cells late during chronic viral infection resulted in slightly higher accumulation of total B cells in the blood at days 90 and 120 post-infection. (A-B) Representative gating plots and quantification of B cell parameters at days 90 (A) and 120 (B) p.i. Averages ± SD are shown (Ai-iv and Bi-iv). Data are representative of one independent experiment with 4-5 mice/group. Student's t-test; *p-val<0.05.

The role of late B cell-derived IL-27 deletion in viral clearance remained inconclusive

To assess whether late IL-27p28 deletion in B cells impacted viral clearance, we quantified viral titers in the serum at days 30, 45, 60, 90, and 120 post-infection. At day 30, we saw that there were largely no differences in viral titers between Cre+ and WT littermates, though it appeared that two out of four WT mice had considerably lower titers (Fig. 2-3A, D30). At day 45, we saw a slight trend in which all WT mice had reduced viral titers, while only one out of five Cre+ mice had reduced titers (Fig. 2-3A, D45). We saw this trend continue at day 60, at which point one out of four WT mice had

viral titers that were below the limit of detection, while viral titers in Cre+ remained elevated (Fig. 2-3A, D60). By day 90, we saw that viral titers in the three WT mice that had not yet cleared the virus by day 60 had elevated back to the levels seen at day 30 (Fig. 23A, D90). At day 120 post-infection, we saw viral titers were still above the limit of detection in these three WT mice, which indicated that LCMV was still present in the serum of these mice (Fig. 2-3A, D120). Viral titers in Cre+ mice at days 90 and 120 largely remained unchanged from those seen at day 60. Taken together, these data showed that Cre+ mice had severely impaired viral clearance. However, contrary to previous experiments, we saw that most WT littermates were unable to clear the virus as well. Because of this finding, the role of late B cell-derived IL-27 in viral clearance remained unclear. Furthermore, it still remained unclear whether the differential accumulation of B cells in the blood of Cre+ late during chronic infection associated with the impaired viral clearance. Considering that viral titers in WT mice tended to be lower at day 45, we assessed anti-LCMV IgG responses in the serum of both Cre+ and WT littermates at this timepoint. We found that there were no differences in the titers of total IgG, IgG1, and IgG2a between Cre+ and WT mice (Fig. 2-3B). While titers of anti-LCMV IgG2b tended to be slightly higher in Cre+ mice, this difference was not statistically significant (Fig. 2-3B). We also found that there were no differences in IgG avidity (Fig. 2-3B). These data indicated that the differences in viral titers at day 45 were not due to significant differences in anti-LCMV IgG responses between Cre+ mice and WT littermates.

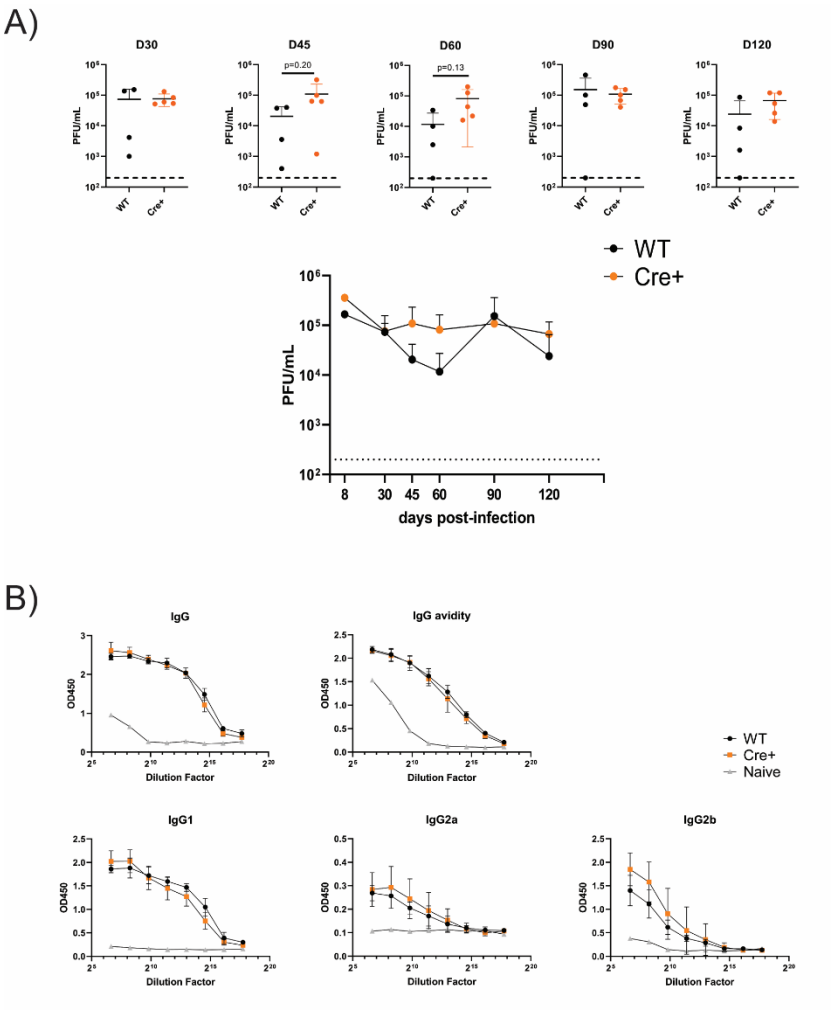


Figure 2-3. IL-28p28$^{fl/fl}$hCD20TAM^{Cre} mice and WT littermates had no differences in viral clearance or LCMV-specific IgG antibody responses. Serum was collected from LCMV-infected inducible knockout mice and wild type littermates at days 8, 30, 45, 60, 90, and 120 p.i. (A) Viral titers in the serum were quantified at days 8, 30, 45, 60, 90, 120 p.i. (B) LCMV-specific IgG, IgG1, IgG2a, and IgG2b antibody titers and IgG avidity were quantified at day 45 post-infection. Averages ± SD are shown. Data are representative of 1 independent experiment with 4-5 mice/group. Student's t-test.

B cell accumulation in the blood of co-housed IL27p28$^{fl/fl}$hCD20TAM^{Cre} mice and wild-type littermates were equivalent before the deletion of late B cell-derived IL-27

Due to the impaired viral clearance seen in WT littermates, we were not able to form any conclusions regarding the role of late B cell-derived IL-27 in viral clearance. Considering the accelerated viral clearance we saw in TAC mice during early experiments, we asked whether introducing the TAC mouse microbiome into WT

littermates would enhance viral clearance in these control mice. In one experiment, Cre+ mice and WT littermates were co-housed with TAC mice for three weeks prior to infection with 2 x 10^6 PFU of LCMV Cl13, after which these mice were separated from co-housing. The same tamoxifen treatment was administered to both Cre+ and WT mice at days 14 through 18 in order to induce *il27p28* deletion specifically in B cells late during infection (Fig. 2-4A). In order to investigate any differences between co-housed Cre+ and WT littermates before infection, we evaluated the proportions and numbers of B cells in the peripheral blood one day before infection. At this timepoint, both Cre+ and WT littermates were wild type, as tamoxifen had not yet been administered. We found that uninfected, wild type Cre+ mice had significantly higher numbers of PBMC compared to uninfected WT controls (Fig. 2-4Bi). However, this difference in blood cellularity was not linked to any differences in any of the B cell parameters that we analyzed (Fig. 2-4Bii-v). These data indicated that co-housed wild type Cre+ mice had significantly higher homeostatic numbers of PBMC but had no differences in basal B cell accumulation compared to WT littermates.

In order to investigate whether co-housed Cre+ and WT mice had any differences in B cell responses to LCMV infection before the administration of tamoxifen, we analyzed the proportions and numbers of B cells in the blood at 8 days post-infection. At this timepoint, we saw that there were no differences in the numbers of PBMC between Cre+ and WT mice (Fig. 2-4Ci). Furthermore, we observed that co-housed Cre+ and WT littermates had similar levels of $CD19^+B220^+$ B cell numbers and frequencies, although tended to be slightly lower in Cre+ mice (Fig. 2-4Cii), and within the total B cell population we found no differences in the accumulation of naïve, IgD^-

IgM^{+}, or class-switched B cells (Fig. 2-4Ciii-v). These data suggested that wild type co-housed Cre+ mice and WT littermates had similar B cell responses at day 8 post-infection. Taken together, these data confirmed that there were no significant differences between co-housed Cre+ and WT in B cell accumulation in the peripheral blood before the late deletion of *il27p28* in B cells.

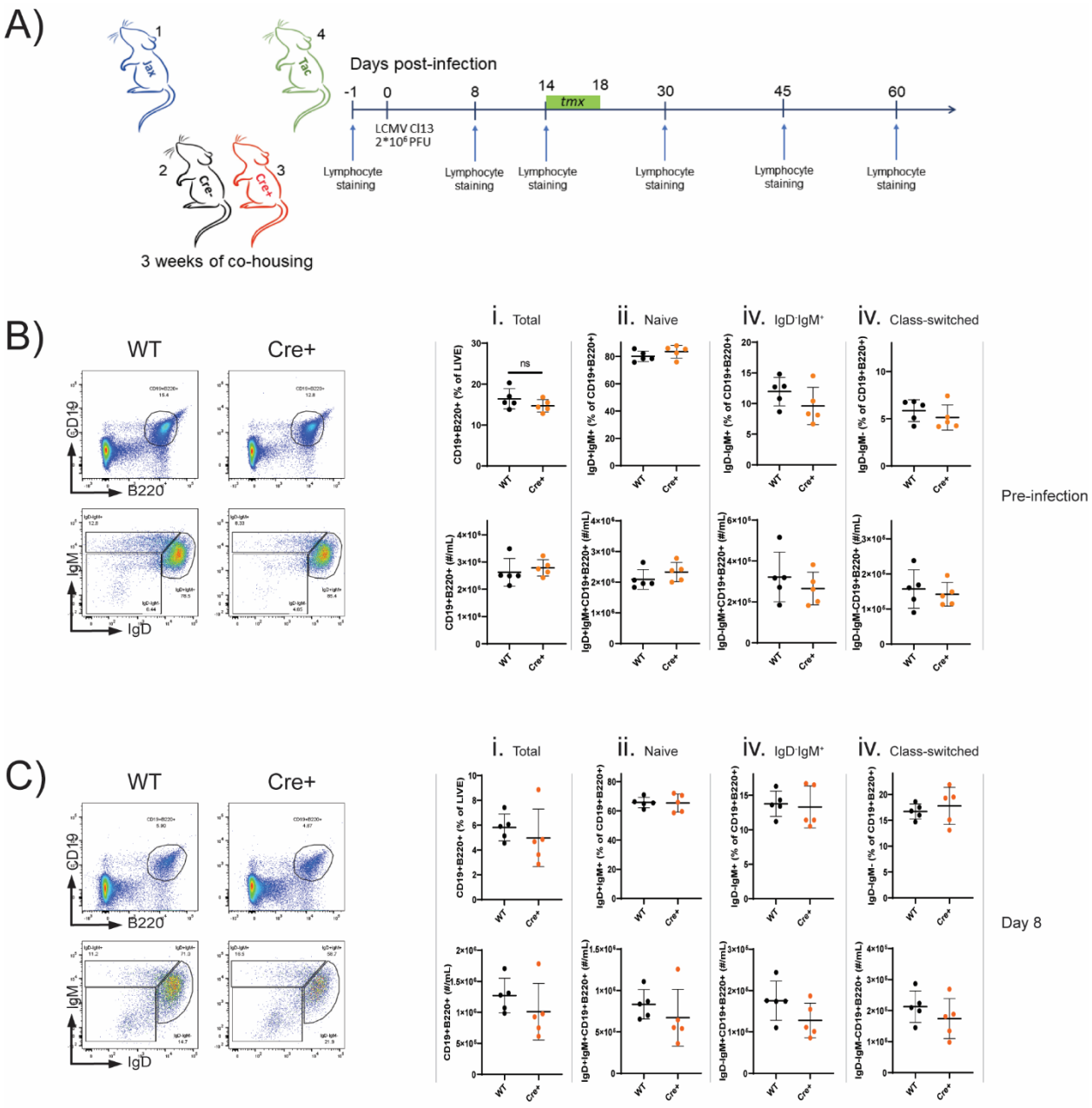


Figure 2-4. No differences in blood B cell accumulation between Cre+ mice and WT littermates were observed before tamoxifen treatment. (A) IL-27p28$^{fl/fl}$hCD20TAM^{Cre} mice and WT littermates were co-housed with mice from Taconic Biosciences for three weeks, after which mice were separated and blood B cell phenotypes in single-housed JAX (1), co-housed Cre+ (2), co-housed WT (3), and single-housed TAC (4) mice were evaluated at days -1, 8, 30, 45, and 60 p.i. B cell IL-27p28 deletion was induced by administering tamoxifen at days 14-18 p.i. (B) Blood B cell accumulation was evaluated one day before infection with LCMV Cl13. Representative gating and quantification of B cell parameters are shown. (C) After Cl13 infection, B cell percentages and numbers in the blood were evaluated at day 8 p.i. Averages ± SD are shown (Bi-iv and Ci-iv). Data are representative of 1 independent experiment with 5 mice/group. Student t-test.

B cell accumulation in the blood of co-housed IL27p28$^{fl/fl}$hCD20TAM^{Cre} mice and wild-type littermates treated with tamoxifen were largely similar after the deletion of B-cell derived IL-27 and differed from that of untreated single-housed mice from commercial vendors

After tamoxifen treatment at days 14 through 18 post-infection, we evaluated blood B cell phenotypes in co-housed Cre+ and WT mice, as well as in single-housed TAC and JAX mice, at days 30, 45, and 60 post-infection. At day 30 post-infection of this experiment, we saw that co-housed Cre+ mice had slightly higher numbers of PBMC compared to WT littermates, although not to a significant degree (Fig. 2-5Ai). We further observed that CD19^{+}B220^{+} B cell accumulation in the blood of Cre+ mice tended to be slightly lower, but this difference was only modest (Fig. 2-5Aii). Within the total B cell population, we saw that Cre+ mice and WT mice had comparable numbers and frequencies of naïve, IgD^{-}IgM^{+} non-naive, and class-switched B cells (Fig. 2-5Aiii-v). These data indicate that there were no significant differences in the quantities or proportions of B cells in the blood between co-housed Cre+ and WT mice at day 30 post-infection, 12 days after *il27p28* was deleted in B cells. Surprisingly, however, we noticed that the quantities and proportions of B cells in Cre+ and WT mice significantly differed from those in single-housed TAC and JAX mice that were never treated with tamoxifen. Total B cell accumulation in Cre+ and WT littermates was significantly reduced compared to untreated single-housed mice (Fig. 2-5Aii). Further analysis within the total B cell population revealed that Cre+ and WT mice had a significant increase in the frequencies of naïve B cells compared to TAC and JAX mice and a significant reduction in the numbers, with the magnitude of this reduction being even greater in

Cre+ mice (Fig. 2-5Aiii). Additionally, this increase in the percentages of naïve B cells was associated with a significant decrease in the accumulation of $IgD^{-}IgM^{+}$ B cells (Fig. 2-5Aiv). However, we found that while numbers of class-switched B cells were also significantly reduced in Cre+ and WT mice, there were no significant differences in the proportions of these cells (Fig. 2-5Av). These data suggested that while there were no significant differences in B cell responses between co-housed Cre+ and WT littermates at day 30 post-infection, these tamoxifen-treated mice had dramatic differences in B cell accumulation in the blood compared to single-housed untreated TAC and JAX mice at 12 days after tamoxifen treatment.

At day 45 post-infection, we found that while there were still no significant differences between Cre+ and WT mice in any of the B cell parameters that we analyzed, Cre+ and WT mice still had marked differences in the numbers and proportions of B cells in comparison to untreated TAC and JAX mice. Much like what was seen at day 30, Cre+ and WT mice had significantly reduced numbers and frequencies of total B cells, although these had slightly increased above the levels at day 30 (Fig. 2-5Bii). However, frequencies of naïve B cells in tamoxifen-treated mice at this timepoint were significantly lower compared to untreated TAC mice, as untreated mice had elevated the proportions of these cells (Fig. 2-5Biii, top). Nevertheless, numbers of naïve B cells were still significantly lower in Cre+ and WT mice, although they had slightly increased from day 30 (Fig. 2-5Biii, bottom). Frequencies of $IgD^{-}IgM^{+}$ non-naïve B cells at this timepoint were similar to those of untreated mice, although the numbers of these cells were still significantly reduced (Fig. 2-5Biv, bottom). Interestingly, we found that the percentages of class-switched B cells in Cre+ and WT

mice at day 45 were significantly higher than those seen in TAC and JAX mice, and there were no significant differences in class-switched B cell numbers across all groups (Fig. 2-5Bv). These data showed that tamoxifen-treated Cre+ and WT mice continued to have no significant differences in the quantities or proportions of B cells in the blood at day 45 post-infection. Furthermore, the levels of these parameters in Cre+ and WT mice had shifted from day 30 and indicated an increased output of class-switched B cells. However, Cre+ and WT littermates at this timepoint continued to have marked differences in the numbers and frequencies of B cells compared to TAC and JAX mice, which further suggested that tamoxifen treatment had profoundly altered B cell responses in co-housed Cre+ and WT mice.

At day 60 post-infection, we again found no significant differences in total PBMC number or blood B cell accumulation between Cre+ and WT mice (Fig. 1C). Compared to TAC and JAX mice, the numbers and frequencies of $CD19^{+}B220^{+}$ B cells in Cre+ and WT mice were still reduced, but these differences at this timepoint were not as robust as we saw at days 30 and 45 (Fig. 2-5Cii). There were no differences in the frequencies of naïve B cells between tamoxifen-treated and -untreated mice, although both Cre+ and WT mice still had significantly reduced numbers compared to TAC and JAX mice (Fig. 2-5Ciii). We further found no differences in the accumulation of $IgD^{-}IgM^{+}$ non-naïve B cells between treated and untreated mice at this timepoint (Fig. 2-5Civ). Finally, when evaluating class-switched B cells, we saw that Cre+ and WT mice continued to have higher frequencies of these cells compared to untreated TAC and JAX mice, while the numbers remained unchanged across all groups (Fig. 2-5Cv). These data indicated that B cell responses were still largely equivalent between co-housed Cre+ and WT mice at

day 60 post-infection, and while Cre+ and WT mice at day 60 were still had significant differences in B cell accumulation compared to untreated TAC and JAX mice, the magnitude of these differences seemed to be considerably lower at this timepoint.

Altogether, these data suggested that deletion of B cell IL-27p28 production in Cre+ mice co-housed with wild type mice from Taconic Biosciences had no effects on the proportions and quantities of B cells in the blood compared to co-housed WT littermates. Considering the differences seen in tamoxifen-treated Cre+ and WT littermates in comparison to untreated TAC and JAX mice, these data further suggest that tamoxifen treated mice had B cell responses that were distinct from B cell responses in non-treated mice.

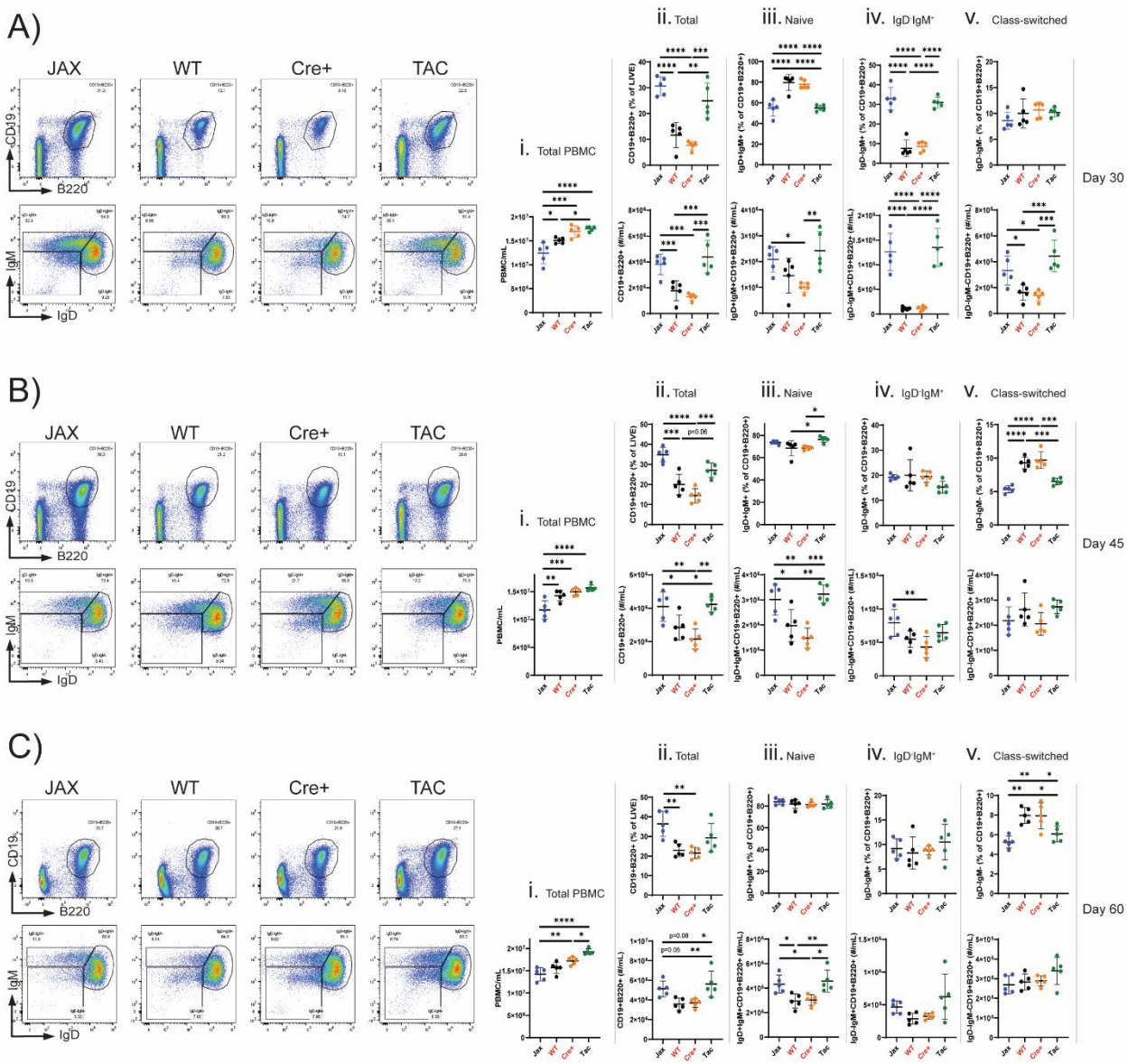


Figure 2-5. **Blood B cell accumulation was similar between tamoxifen-treated transgenic and WT mice and dramatically differed from that of untreated single-housed mice during the late phase of LCMV infection.** (A-C) Blood B cell numbers and frequencies in Cl13-infected co-housed tamoxifen-treated mice, as well as in single-housed untreated mice from commercial vendors, were analyzed at days 30 (A), 45 (B), and 60 (C) p.i. Tamoxifen-treated mice are indicated in red text. Representative flow cytometry gating plots and quantification of B cell parameters are shown. Averages ± SD are shown (Ai-v, Bi-v, and Ci-v). Data are representative of 1 independent experiment with 5 mice/group. One-way ANOVA and Tukey's post-hoc test; *p-val<0.05, **p-val<0.01, ***p-val<0.001, ****p-val<0.0001.

Co-housing IL27p28$^{fl/fl}$hCD20TAM^{Cre} mice and wild-type littermates with mice purchased from Taconic Biosciences did not impact viral clearance or LCMV-specific IgG antibody responses

To evaluate viral clearance in co-housed Cre+ mice and WT littermates, we looked at viral titers in the serum at days 45 and 60. We saw that at day 45, Cre+ mice had significantly higher viral titers compared to WT mice (Fig. 2-6A, D45). This finding recapitulated the trend in viral clearance that was seen between single-housed Cre+ and WT mice at this timepoint (Fig. 2-6A, D45). By day 60, however, we saw that there were no differences in viral titers in the serum (Fig. 2-6A, D60). These findings indicated that co-housing Cre+ and WT mice with TAC mice did not rectify the impaired viral clearance that was seen in the wild type controls. When evaluating LCMV-specific IgG titers at day 45, we again found that there were no differences in the titers of total IgG, IgG1, or IgG2a, which showed that the difference in viral titers at day 45 was not associated with any differences in LCMV-specific IgG responses (Fig. 2-6B). Taken together, these suggested that, despite co-housing, Cre+ and WT mice still had similar antibody responses and still had impaired viral clearance at day 60, a timepoint at which LCMV is typically cleared from the serum by wild type C57BL/6 mice (Wherry et al., 2003).

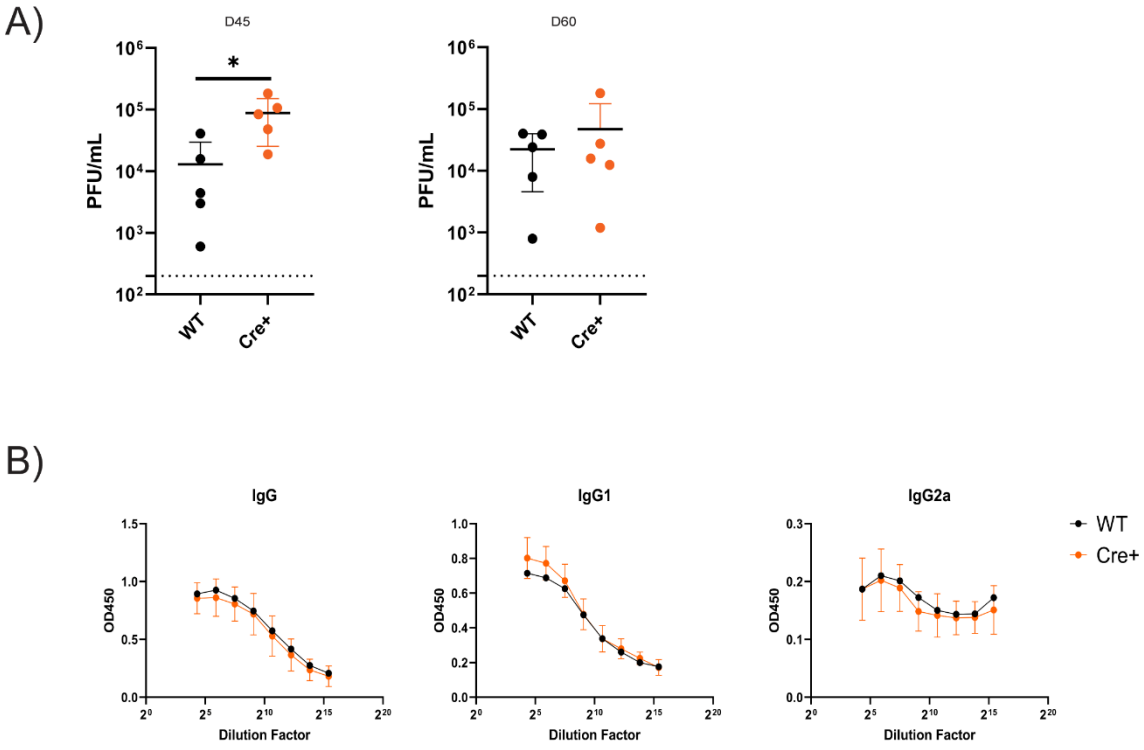


Figure 2-6. Co-housing WT control mice with mice from Taconic Biosciences did not ensure viral clearance. (A) Viral titers in the serum of Cl13-infected transgenic mice and WT littermates that were co-housed with mice from Taconic Biosciences were quantified at day 45 and day 60 p.i. (B) LCMV-specific IgG antibody titers in the serum were measured at day 45 p.i. Averages ± SD are shown. Data are representative of 1 independent experiment with 5 mice/group. Student t-test; *p-val<0.05.

Discussion

IL-27 is an immunomodulatory cytokine that is known to have both inflammatory and immunosuppressive effects. Previous studies in our lab have shown that direct IL-27 signaling promotes the accumulation of virus-specific $CD4^+$ T cells late during chronic viral infection and drives their production of IL-21. While investigating the source of late IL-27, we found that B cells upregulate their expression of IL-27 at day 30 post-infection. In this study, we examined the role of late B cell-derived IL-27 production late during chronic infection by using LCMV Cl13 infection in transgenic $IL27p28^{fl/fl}hCD20TAM^{Cre}$ inducible knockout mice that delete IL27p28 expression specifically in B cells. We report that in one experiment, late B cell-derived IL-27p28 deletion modulated the numbers and proportions of B cells in the blood during the late stage of chronic infection. Contrary to one previous independent experiment, late IL27p28 deletion did not reduce the numbers and frequencies of total B cells in the blood throughout the late stage of chronic infection, but instead resulted in a higher output of IgD^-IgM^+ non-naïve and class-switched B cells that peaked at day 60, and at even later timepoints resulted in subtle increases in the accumulation of total B cells at days 90 and 120 post-infection. We also saw that late IL-27 deletion did not impact the titers of LCMV-specific IgG antibodies in the serum at day 45 post-infection. These findings are in contrast with previous studies in our lab which found a role of IL-27 in promoting the survival of virus-specific $CD4^+$ T cells and their production of IL-21 (Harker et al., 2013). While we saw that mice with B cell-derived IL-27 deletion were unable to clear the virus from the serum by day 120 post-infection, we also noticed that wild-type littermates were also unable to clear the virus by this timepoint. Co-housing

inducible knockout mice and wild-type littermates with mice from Taconic Biosciences, which have dramatically different intestinal microbiome composition, did not change B cell phenotypes in the blood or the outcome of infection, despite observing that the TAC mouse microbiome accelerates viral clearance in wild type C57BL/6 mice. These data show that late IL-27 deletion in B cells did not substantially alter the accumulation of B cells in the blood in response to chronic viral infection. The role of late B cell-derived IL-27 in viral clearance remains unclear.

While differences in the accumulation of B cells in the blood at days 30 and 45 were subtle, we saw that the proportions of $IgD^{-}IgM^{+}$ non-naïve and class-switched B cells at day 60 were significantly higher in Cre+ mice. Furthermore, the proportions of class-switched B cells in Cre+ mice at this timepoint also tended to be higher. These findings may indicate that B cell-derived IL27p28 deletion resulted in increased activation of B cells, further supporting the role of IL-27 in regulating B cell responses. This would be consistent with published results demonstrating that the overexpression of IL27p28 resulted in a reduction of GC B cells in a murine model of chronic *Toxoplasma gondii* infection (Park et al., 2019). Considering this, these data suggest that IL-27 may regulate B cell responses during chronic LCMV infection in a suppressive manner. In conjunction with our observations at day 60 post-infection, the accumulation of total B cells tended to be higher in the blood of Cre+ at days 90 and 120. Additional studies will need to evaluate GC B cell responses as well as the accumulation of MBC and PC in the spleen of Cre+ mice in order to further investigate how IL-27 regulates B cell responses. Furthermore, it would be of interest to evaluate the expression of B cell activation markers, such as MHC-II molecules and CD86

(Marshall-Clarke et al., 2003), in order to confirm that B cell activation is elevated in Cre+ mice. This study may also benefit from assessing the features of Tfh that are involved in B cell activation, such as ICOS and CD40L expression and IL-21 and IL-4 secretion. Although we saw no differences in the titers of LCMV-specific IgG in this experiment, deep sequencing of antibodies and B cell receptors would offer a more thorough evaluation of antibody responses in Cre+ mice and would help answer whether increases in B cell activation are associated with differences in affinity maturation and somatic hypermutation.

In addressing the impaired viral clearance seen in WT littermates, we postulated that this dysfunction may be associated with the gut microbiome. Therefore, in another independent experiment we co-housed Cre+ mice and WT littermates with mice from Taconic Biosciences, which we have shown to exhibit accelerated viral clearance during chronic LCMV Cl13 infection (data not published). In this experiment, we were not able to see the trends in the accumulation of blood B cells that were seen in the previous experiment. Although the co-housing factor of this experiment may invalidate direct comparisons between the two experiments, these data may suggest that the changes in blood B cell accumulation in Cre+ mice are not reproducible. A second independent experimental repeat using single-housed Cre+ and WT littermates would need to be done in order to confirm this. Our findings also show that co-housing did not improve viral clearance in WT mice, which may suggest that the issues in viral clearance seen in WT mice were not related to the gut microbiome. However, shotgun metagenomics sequencing may be required to confirm that the gut microbiome composition in Cre+ and WT mice substantially differ from that in mice purchased from commercial vendors.

Surprisingly, we saw in this experiment that both Cre+ and WT mice had significant differences in the accumulation of B cells in the blood compared to mice that were never treated with tamoxifen. Considering that Cre-induced recombination was absent in WT mice, these observations invoke concerns regarding our tamoxifen treatment. While tamoxifen has been widely used to induce tissue- and cell-specific genetic recombination through the CreERT-loxP system, it has been reported that tamoxifen treatment can result in substantial gastric toxicity (Huh et al., 2012). Furthermore, it is important to note that studies from the Shlomchik lab, from which hCD20TAM^{Cre} mice were originally generated, have used 3 daily doses of tamoxifen in order to achieve cre-recombination in these transgenic mice as opposed to 5 daily doses (Khalil et al., 2012). Taking these points into consideration, it is likely that the tamoxifen treatment we use to induce IL-27 deletion in B cells needs to be adjusted. Follow up studies will first need to confirm that the differences seen in the accumulation of B cells in the blood of tamoxifen-treated mice can be reproduced in another independent experiment using our current tamoxifen treatment, including vehicle controls.

While the IL-27p28 subunit was initially deleted in these experiments in order to investigate the role of IL-27 in regulating B cell responses to chronic viral infection, it is important to consider that p28 can exert biological activities independently of EBI3 (Kourko et al., 2019). Indeed, p28 is also known as the cytokine IL-30, and, like IL27, is a pleiotropic cytokine that has been shown to be exhibit various immunoregulatory functions on multiple immune cells. Initially, p28 was demonstrated to exhibit similar functions as IL-27 in suppressing the development of Th17 cells, though to a lesser

extent (Stumhofer et al., 2006). However, additional studies have revealed that p28 can perform functions that are distinct from IL-27. In one study, it was reported that p28 can act as an antagonist of IL-6 and IL-27 signaling through gp130 (Stumhofer et al., 2010). Furthermore, in this same study, the authors found that p28 prevented IL-10 production in $CD4^+$ T cells in the presence of TGF-β, IL-6, and IL-27 *in vitro* and that intrinsic overexpression of p28 in B cells of a transgenic mouse model led to defective development of antibody secreting cells (ASC) and germinal centers following primary immunization with T-dependent antigens. While it is now clear that p28 can fulfill biological functions independently of EBI3, the relationship between IL-27 and its p28 subunit is even further complicated by the fact that p28 can have binding partners other than EBI3, such as cytokine-like factor 1 (CLF), an EBI3 homolog (Crabé et al., 2009). The same group that discovered the p28/CLF binding complex also found in another study that p28/CLF can promote B cell proliferation and plasma cell differentiation *in vitro* and can elevate the production of antigen-specific IgG antibodies in response to primary immunization *in vivo* (Tormo et al., 2013). In our experiment, the increases in the accumulation of IgD^-IgM^+ non-naïve and class-switched B cells at day 60 as well as the higher trends of B cell accumulation at days 90 and 120 in Cre+ mice may be due to the loss of p28 activity itself and not necessarily due to the loss of IL-27 activity. In future experiments, it will be important to distinguish between IL-27 and its p28 subunit in order to more accurately elucidate the role of B cell-derived IL-27, which may be accomplished through the utilization of both our B cell IL-27p28 knockout model and a B cell IL-27p28 and EBI3 double knockout model.

By using a transgenic mouse line that specifically deletes IL-27p28 in B cells, our study found that late B cell-derived IL27p28 deletion during chronic LCMV infection resulted in slightly higher outputs of $IgD^{-}IgM^{+}$ non-naïve B cells and class-switched B cells, and in even later timepoints resulted in a slightly higher accumulation of total B cells compared to wild type littermates. However, because wild type controls in this experiment were shown to have impaired viral clearance, the role of B cell-derived IL-27 in viral clearance remains elusive. Comparison of tamoxifen-treated mice to mice that never received tamoxifen revealed stark differences in the accumulation of total B cells as well as in the proportions of subsets within this population. Thus, our tamoxifen treatment may need to be reevaluated.

Conclusion

Chronic viral infections result in distinct adaptations in both innate and adaptive immune cells. These adaptations arise due to viral mechanisms that promote the suppression or dysregulation of the effector functions that are typically used to resolve acute infections (Zuniga et al., 2015; Virgin et al., 2009). Recently, it has been appreciated that the host gut microbiome has a substantial influence on immune responses during viral infections in both humans and mice (Li et al., 2019; Ericsson and Franklin, 2015). Several studies have reported that specific microbes can confer positive outcomes of infection and treatment (Ivanov et al., 2009; Sivan et al., 2015; Routy et al., 2018). Thus, studying variances in immune responses as a result of differential microbiome composition may lead to a more robust understanding of the interactions between the immune system and the host microbiome and may potentiate effective microbe-mediated strategies for treating chronically infected individuals.

In this study, we characterized B cell responses to chronic viral infection in mice purchased from two different vendors that are known to have differing microbiome compositions. Mice from Taconic Biosciences (TAC mice) have been reported to contain segmented filamentous bacteria in their gut microbiomes, which have been shown to be associated with immune responses to bacterial infections and melanoma tumors that are distinct from immune responses in mice from Jackson Laboratory (JAX mice). However, differences between TAC and JAX mice in the immune responses to chronic viral infection are less characterized. In early experiments in our lab, we found that TAC mice were able to clear LCMV from the serum much earlier than JAX mice. Our aim in this study was to evaluate how B cell and antibody responses might

contribute to this accelerated viral clearance. We report that TAC and JAX mice had significant differences in the accumulation of B cells in the blood and in the spleen during chronic LCMV infection. However, in contrast to what was seen in earlier experiments, TAC mice in these recent experiments did not exhibit accelerated viral nor differences in anti-viral antibody titers despite having these differences. When co-housing mice from these two vendors, we found that the accumulation of B cells in the blood and spleen were modulated in co-housed JAX mice (JAX coh mice) in a way that resembled what was seen TAC mice. Surprisingly, we found that JAX coh mice had significantly impaired viral clearance, suggesting that partial transfer of the microbiome composition of TAC mice to JAX mice may promote viral persistence. Future studies will need to replicate this phenotype seen in JAX coh mice and would benefit from more thorough metagenomic screening and documentation as a means to control for variances in gut microbiome composition.

In a separate study, we also evaluated the role of late B cell-derived IL-27 on B cell responses during chronic viral infection. IL-27 is a pleiotropic cytokine that has been reported to have roles in both inflammation and immunosuppression. Earlier studies conducted in our laboratory have reported that IL-27 signaling promotes the survival of LCMV-specific $CD4^+$ T cells. Furthermore, by using a transgenic mouse model that conditionally deletes the IL-27p28 subunit specifically in B cells, we have found in one experiment that late deletion of B cell-derived IL-27p28 during chronic LCMV infection reduced the accumulation of total B cells in the blood and led to impaired viral clearance. Our aim in this current study was to assess whether these phenotypes are reproducible. We report that late IL-27 deletion in B cells during chronic viral infection in

these recent experiments did not result in reduced accumulation of total B cells in the blood, but rather slightly increased the accumulation of class-switched and total B cells. These data would support the role of IL-27 as a suppressor of B cell responses. Confoundingly, we found that both Cre+ and WT littermates had impaired viral clearance. In another experiment, we initially co-housed both Cre+ and WT mice with TAC mice as an attempt to rectify the impaired viral clearance in WT littermates. However, we saw that both tamoxifen-treated Cre+ and WT mice had profound differences in the accumulation of B cells in the blood compared to single-housed mice that were never given tamoxifen. Cre+ mice and WT littermates in this co-housing experiment were again unable to clear the virus from the serum. Future studies will need to confirm that these differences in Cre+ and WT mice are indeed mediated by tamoxifen and will have to adjust the tamoxifen treatment accordingly.

Multiple studies have reported a relationship between IL-27 and the microbiome. In one study, the authors reported that modulation of the microbiome with antibiotics significantly reduced the production of IL-27 in $CD11b^{+}CD11c^{+}$ cells in the blood (Gülden et al., 2017). In another study, the investigators demonstrated that IL-27 served a protective role during colitis-associated cancer by suppressing the production of anti-inflammatory cytokines in intestinal epithelial cells (Cui et al., 2017). Considering the correlations that have been made between IL-27 and the microbiome in immune regulation, it is possible that IL-27-microbe interactions may play a role in regulating immune responses during chronic viral infection. Investigating the relationship between IL-27 and the gut microbiome during LCMV infection in mice would be a means to

elucidating a potential mechanism through which the gut microbiome influences immune responses during chronic viral infection.

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