

THESIS

LONGITUDINAL CHARACTERIZATION OF PERIPHERAL PRION PATHOGENESIS IN
SYRIAN HAMSTERS EXPERIMENTALLY INOCULATED WITH HAMSTER-ADAPTED
TRANSMISSIBLE MINK ENCEPHALOPATHY

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ABSTRACT

LONGITUDINAL CHARACTERIZATION OF PERIPHERAL PRION PATHOGENESIS IN SYRIAN HAMSTERS EXPERIMENTALLY INOCULATED WITH HAMSTER-ADAPTED TRANSMISSIBLE MINK ENCEPHALOPATHY

Transmissible Spongiform Encephalopathies (TSEs) are a group of infectious, neurodegenerative diseases affecting animals, including humans. Caused by misfolded proteins called prions, TSEs are characterized by a long, asymptomatic incubation period that eventually cause neuronal death and progressive neurodegeneration. Transmissible Mink Encephalopathy (TME) is a rare TSE of mink. Syrian hamsters are susceptible to infection with TME, serving as a natural system of infection. Hyper strain (HY) of hamster-adapted TME is of particular interest due to its rapid spread into the lymphoreticular system (LRS) and blood, enabling us to study peripheral accumulation and spread of prions. Real-time quaking-induced conversion (RT-QuIC) is a rapid and sensitive assay used to detect prions, and has been used to investigate prion pathogenesis. In this study, we hypothesize that RT-QuIC can be used to detect splenic prions longitudinally throughout HY-TME infection, including during the asymptomatic phase of disease. Sixty male Syrian hamsters were extranasally (EN)-inoculated with HY-TME. We collected their spleen, blood, brain, and other tissues at early-, mid-, and late-disease course. Our study shows evidence for the temporal accumulation of prions both within the spleen and brain throughout disease. We detected RT-QuIC seeding activity in spleen and brain tissue harvested from HY-TME-inoculated hamsters in late disease, between 155 and 169 (terminal) days PI. Titers in the spleen ranged from 10^{-2} to 10^{-7} . Titers in the brain ranged from 10^{-3} to 10^{-5} .

Additionally, we detected RT-QuIC seeding activity in the spleen of a clinical hamster before it was detectable in the brain, supporting previous work showing that the spleen is a site of peripheral prion accumulation before neuroinvasion. Our work validates the Syrian hamster, EN-inoculated with HY strain of hamster-adapted TME, as a system to evaluate peripheral prion burden within the LRS.

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INTRODUCTION: Transmissible Spongiform Encephalopathies

Transmissible Spongiform Encephalopathies (TSEs) are a group of infectious, neurodegenerative diseases affecting animals, including humans (Beekes and McBride 2007; Aguilar-Calvo, 2015). Notable diseases caused by prions include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, transmissible mink encephalopathy (TME) in mink, as well as Kuru, Creutzfeld-Jakob's disease (CJD), and variant Creutzfeld-Jakob's disease (vCJD) in humans (Bessen and Marsh 1992; Beekes and McBride 2007; Shikiya et al. 2017). Although these diseases share similar pathogenesises resulting in fatal neurodegeneration, the incubation period, pathology, organs affected, transmission routes, and potential for inter-species transmission vary between specific prion strains and hosts.

Some prion diseases, such as scrapie in sheep and goats, have been identified since the 1700s (Colby and Prusiner 2011), while others such as BSE have arisen into prominence within the last few decades (Gonzales et al. 2014). It was hypothesized that scrapie was caused by a slow virus due to its transmissibility, long incubation period, and infectivity after filtration (Belay and Schonberger 2005). Through efforts to characterize scrapie, it was found to be resistant to mechanisms that typically denature viruses (Aguilar-Calvo et al. 2015) leading to the discovery that the scrapie agent lacks both viral particles and nucleic acids and is associated only with a misfolded protein.

TSEs, or prion diseases, are characterized by long, asymptomatic incubation periods that eventually cause neuronal death and progressive neurodegeneration (Bessen and Marsh 1992; Beekes and McBride 2007; Murphey 2013; Shikiya et al. 2017). Although prions are

transmissible through a variety of infectious tissues (brain, uterus, placenta, spleen) and bodily secretions (blood, urine, feces, saliva, and reproductive fluids) (Büeler et al. 1993; Belay and Schonberger 2005; Seeger 2005; Haley et al. 2011; Mathiason et al. 2006; Aguilar-Calvo et al. 2015; Nalls and McNulty et al. 2017), TSEs may also be genetically acquired, or arise through spontaneous mutation (Colby and Prusiner 2011; Mathiason, 2015). Prions are highly infectious, and persist for long periods in the environment (Hamir et al. 2004; Georgsson et al. 2006).

PrP and The Prion Hypothesis

In 1966, Alper et al. postulated that: 1) scrapie is not caused by a virus, and 2) it can replicate without nucleic acids (Alper et al. 1966). Based upon this work, in 1967 Griffith suggested a pathway of protein self-replication, hypothesizing that scrapie can arise from a host gene despite its apparently infectious nature (Griffith 1967). In 1982, Prusiner et al. published (Prusiner et al. 1982) that a protein is associated with scrapie infectivity, and coined the name “prion”: proteinaceous infectious particle. Prusiner and Bolton et al. (Bolton and Prusiner 1982) purified the scrapie agent and demonstrated that it is resistant to Proteinase K (PK) treatment, as well as standard disinfectants and sterilization techniques (Colby and Prusiner 2011).

Subsequent investigations identified the normal cellular protein PrP (PrP^C) in 1982 (Bolton and Prusiner 1982; Aguzzi and Heikenwalder 2006; Beekes and McBride 2007). Encoded by the *Prnp* gene, PrP^C (or prion protein) is highly conserved and is expressed by most mammalian cells (Colby and Prusiner 2011). This GPI-anchored protein is found in highest concentrations on neuronal cells (especially neuron synaptic membranes and astrocytes) (Tripathi and Singh 2016) and cells in the lymphoreticular system. Lower concentrations of PrP^C have been identified in other tissues such as kidney, skeletal muscle, heart, and within the blood

in lymphocytes, B cells, platelets, and follicular dendritic cells (Aguzzi, 2006; Mathiason et al. 2010; Aguilar-Calvo et al. 2015; Bradford et al. 2017; Giles et al. 2017).

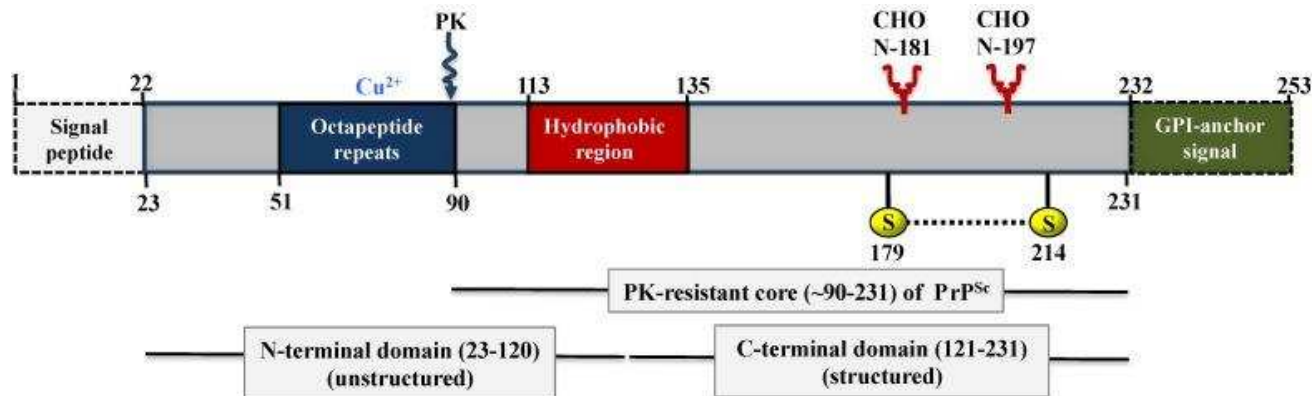


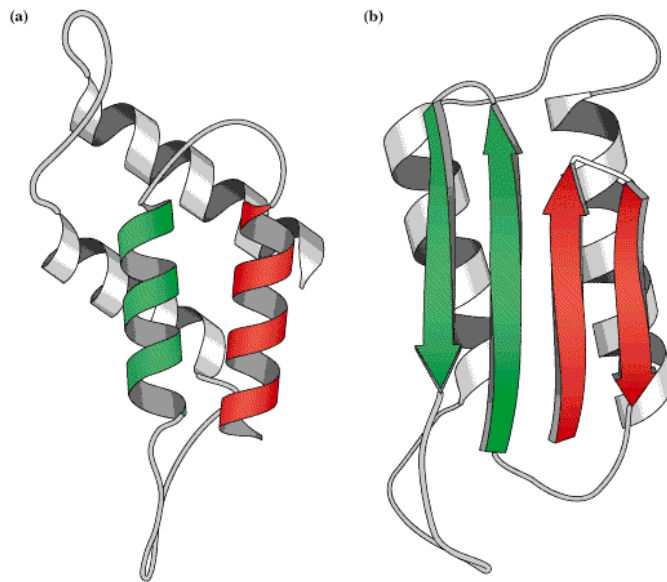
Figure 1. *Prnp* Structure. The OR region lies between nucleotides 51-90. The hydrophobic region lies between nucleotides 113-135. The PK resistant core is located approximately between nucleotides 90-231 of PrP^{Sc}. (Acevedo-Morantes and Wille 2014)

Although PrP functions as part of the cell membrane, perhaps as cell surface scaffold protein (Linden 2017), its exact role is not clearly understood. Only one mammal has been found in nature lacking PrP^C, a Norwegian dairy goat with an early truncating mutation of *Prnp* (Benestad et al. 2012), suggesting that PrP^C plays a role in mammalian cell function. In 2016, Küffer reported that PrP may function to protect myelination of neuronal cells as part of the G protein-coupled receptor of Schwann cells (Küffer et al. 2016). Other proposed functions include a role in copper metabolism (Kubosaki et al. 2003; Alfaidy et al. 2013). Transgenic mice lacking the *Prnp* gene (which therefore lack PrP^C) do not develop TSE infection, proving that PrP^C, whatever its function, is required for prion formation (Büeler et al. 1993; Linden 2017). The development of *Prnp* knockout mice (Büeler et al. 1993) and transgenic mice expressing species-specific PrP^C (Colby and Prusiner 2011) has revolutionized the way prions are studied.

PrP^C is composed of three α -helices and two β -sheets, with a long flexible amino-terminal tail. The N-terminal portion of PrP^C contains two conserved regions (figure 1): five

repeats of octameric amino acid sequence (OR) that connect the second β -sheet to the third α -helix, and which may aid with copper binding and pathogenesis; and a downstream “hydrophobic core” preceded by a hydrophilic “charge cluster”. Per the ‘Protein-only Model’ of prion propagation, the normal PrP^{C} isoform undergoes transformation into the abnormal prion, PrP^{Sc} (figure 2). PrP^{Sc} then recruits more PrP^{C} to convert to the aberrant isoform in a templating chain-reaction (Collinge 1999; Colby and Prusiner 2011; Aguilar-Calvo et al. 2015).

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Figure 2. Structure of PrP^{C} (a) and PrP^{Sc} (b). Note that PrP^{Sc} conforms into β -sheets.

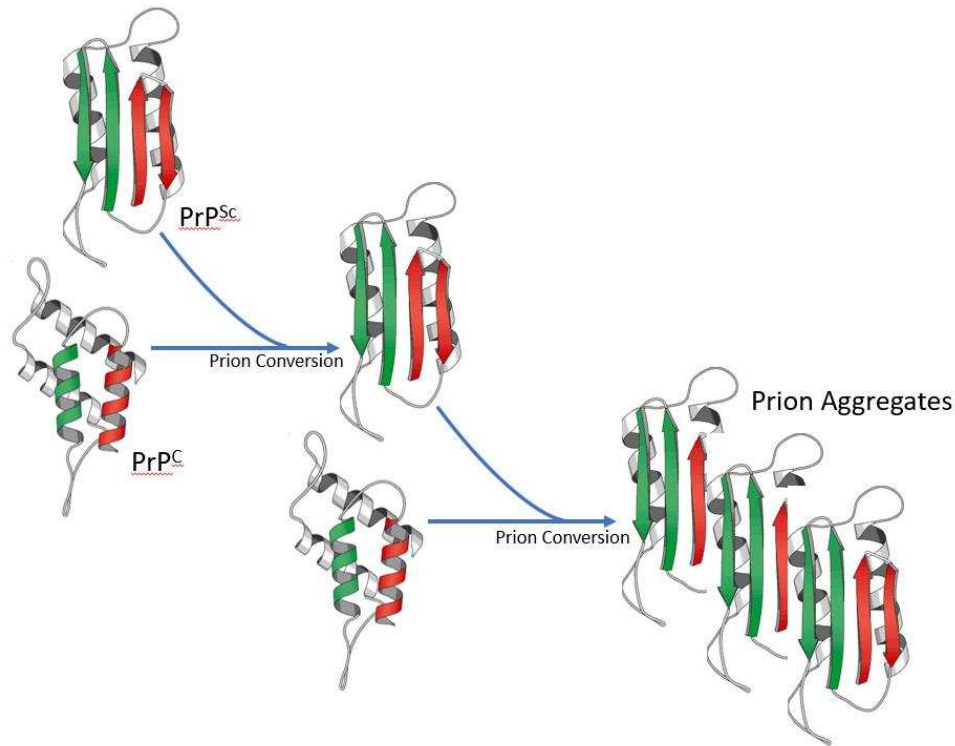


Figure 3. Prion Conversion and Aggregation. PrP^C conversion to PrP^{Sc}, forming prion aggregation. PrP^{Sc} encounters PrP^C and induces conformational change into PrP^{Sc} isoform, which accumulate into plaques.

The PK-resistant core of PrP^{Sc} resides between amino acid residues 134 and 231 (Aguzzi and Heikenwalder 2006). PrP^{Sc} conforms into β -sheets which assemble into aggregates, fibrils, and “plaques” (Sigurdson, 2011; Prusiner 2013) (figure 3). Formation of “spongiform” lesions appear as vacuoles, or holes, within the brain tissue, are the crux of the name, *spongiform encephalopathy*, and are responsible for glial cell death and subsequent neurodegeneration. Interestingly, PrP^C can fold into different PrP^{Sc} conformers, and various mixtures of conformers may form different prion strains and disease phenotypes (Colby and Prusiner 2011; Sigurdson et al. 2011; Shikiya et al. 2017). To date, however, the exact mechanisms of PrP^C misfolding and prion formation are still unknown.

Prion disease in humans

Humans are susceptible to prion diseases, which may arise spontaneously or from infection (Prusiner 2013; Giles et al. 2017). Greater than 40 genetic mutations are associated with the known human heritable prion diseases (Colby and Prusiner 2011). The most well-known genetic and spontaneous human TSE is Creutzfeldt-Jakob's disease (CJD) (Collinge 1999). CJD in humans may arise through spontaneous mutation of *Prnp* (85-90% of cases) or iatrogenic exposure to the CJD agent (2-5% of cases) (Mabbot, 2006; Chen and Dong 2016). CJD, like all prion diseases, has a long “silent” incubation phase (Belay and Schonberger 2005) followed by progressive dementia, memory loss, personality changes, and other behavior associated with incoordination and involuntary movements.

Variant CJD (vCJD), another human TSE, is a zoonotic disease that researchers believe was transmitted from cattle to humans by oral ingestion of beef products contaminated with BSE prions (described in animal section below) (Hill, 1997). vCJD was first identified in 1996 (Priola 1996), several years after the onset of the UK outbreak of BSE. Despite how widespread the BSE outbreak was, and the number of infected cattle and potentially-contaminated products, comparatively few people (less than 200 worldwide) have been diagnosed with vCJD (Belay and Schonberger 2005). However, an analysis of lymphoid tissue collected from the general population in the affected area of the UK suggests that 1 in 2000 people harbor a silent prion infection (Gill, 2013). Onset of clinical signs of vCJD cause much earlier onset of clinical symptoms but results in similar progressive neurodegeneration (Belay and Schonberger 2005).

Humans are also susceptible to a rare infectious form of TSE called Kuru (Collinge 1999) that might have originated from a spontaneous mutation of *Prnp* in one individual. This disease was transmitted among the Fore people of Papua New Guinea, who consumed infected human

brain and other organs during ritualistic funeral cannibalism (Zigas and Gajusek 1957).

Traditionally during this rite, women and sometimes children (but never males over six years old) consume the brain of the deceased, therefore Kuru primarily affects females (Whitfield et al. 2008). With the decline of cannibalistic practices, Kuru is largely considered a disease of historical significance; the last death from Kuru was in 2005, although with an incubation time of 2 - 50 years, cases may still arise (Alpers 2005).

Scrapie

Scrapie, a TSE of sheep and goats, was first recorded in 1732 (Liberski et al. 2012; Gonzales et al. 2014). Endemic now in most of the world, sheep producers have drastically reduced disease prevalence within flocks by breeding sheep that are genotypically resistant to scrapie (Cook et al. 2016). As with all prion diseases, scrapie has a long asymptomatic incubation phase of 2-5 years; once infected, sheep survive from 6 months to two years. Clinical signs include behavioral changes, incoordination, weight loss or emaciation, and pruritus leading to wool loss—carrying an additional economic impact (Aguilar-Calvo et al. 2015). Scrapie can persist in the environment for up to 16 years (Georgsson et al. 2006) while remaining infective—a leading factor in its transmission via direct contact.

Atypical scrapie cases are rare compared to classical scrapie (Benestad et al. 2003). Prion deposition in the brain between atypical and classical strains vary; additionally, animals affected with atypical scrapie, although they also become ataxic and lose body condition, display clinical symptoms at an older age (Benestad et al. 2008). Only one case of atypical scrapie transmission into the natural small ruminant host has been reported in the literature, and cases primarily occur

as a single animal within a flock, suggesting a spontaneous mutation origin (Benestad et al. 2003; Benestad et al. 2008).

Bovine spongiform encephalopathy

BSE (“mad cow disease”) has infected cattle worldwide (Belay and Schonberger 2005; Aguilar-Calvo et al. 2015). Its origins are currently unknown (Smith, 2003). Affected cattle can remain asymptomatic for up to two years, and display symptoms similar to scrapie-infected sheep: hair loss, wasting, and incoordination, but also aggression and hyper-reactivity. An epidemic of BSE in the UK was thought to have been perpetuated by cattle ingesting meat and bone meal generated from the carcasses of BSE-infected cattle (Smith, 2003). BSE successfully crossed the species barrier into the human population, causing vCJD in humans (Collinge 1999; Murphy, 2013). It also jumped into cats, causing Feline Spongiform Encephalopathy (FSE) (Collinge et al. 1996), and it is the likely origin of Transmissible Mink Encephalopathy in mink (Baron et al. 2007).

Cross-species transmission events likely occurred through the ingestion of infected beef products (Smith, 2003). The widespread BSE outbreak in the UK in the late 1980s has profoundly impacted animal agriculture practices worldwide, stimulating a variety of legislation regulating animal feeds, banning suspect animals from entering the human food chain, increasing surveillance of BSE, and prohibiting meat from cattle over 30 months old to enter the human food chain (Smith, 2003).

Chronic wasting disease

Chronic wasting disease (CWD) is the TSE of captive and free-ranging cervids including white-tailed deer, mule deer, elk, moose, and—most recently—reindeer (Williams and Young 1980; Tamguney et al. 2009; Haley et al. 2011; Aguilar-Calvo et al. 2015; Benestad et al. 2016) (figure 4). CWD was first detected in Colorado in the late 1960s. The origin of CWD may have been a sporadic event in cervids or a cross species transmission from scrapie-infected sheep, although the exact origin is unknown (Williams and Young 1980; Belay and Schonberger 2005). In the past 60 years, CWD has rapidly spread throughout the western United States and Canada, was accidentally imported to South Korea, and was found in Norway in 2016 (Benestad et al. 2016).

Transmission is primarily horizontal through direct contact with contaminated tissues and bodily secretions (urine, blood, saliva, and feces) (Mathiason et al. 2006; Haley et al. 2011) or contaminated environments (Miller et al. 2004; Mathiason et al. 2009). While less studied, CWD prion transmission has also been demonstrated to occur from mother-to-offspring (Bessen et al. 2011; Nalls et al. 2013; Nalls and McNulty et al. 2017). Transmission between susceptible cervid species is very efficient, yet there is no current evidence suggesting that CWD has been transmitted to humans. The zoonotic potential of CWD remains a question of high importance when considering the popularity of cervid hunting and product consumption (Belay et al. 2004).

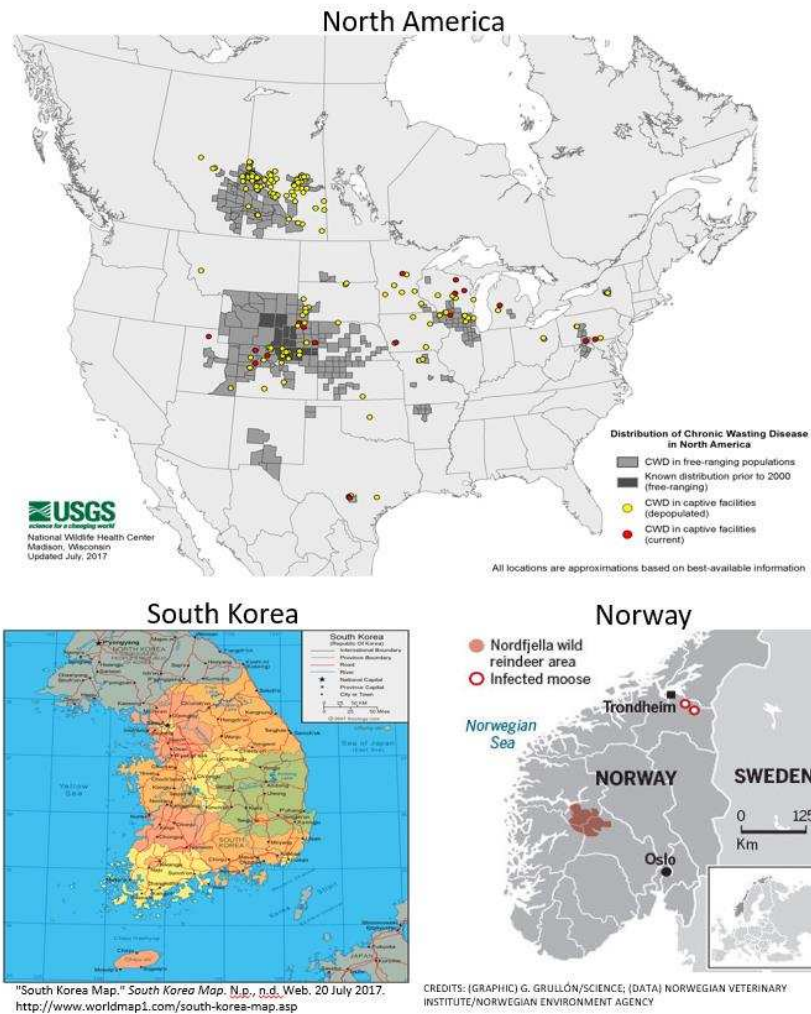


Figure 4. Map of CWD Spread. Map depicts North America, South Korea, and Norway. Dark gray areas of North American map denote distribution of CWD. On the Norwegian map, red circles denote where CWD was found.

Transmissible mink encephalopathy

TME is a rare TSE, first seen in ranch-raised mink in Wisconsin (Hartsough and Burger 1965). During natural disease infection, after an incubation of 8-12 months (Marsh 1972), the animals displayed progressive behavioral changes (lack of grooming and lost cleanliness) indicating neurodegeneration leading to difficulty eating, incoordination, hyper-aggression, and hyper-excitement. This initial outbreak had 100% mortality in mink, and caused “scrapie-like” neurodegeneration with spongiform changes in the brain. The outbreak was thought to be caused by ingestion of feed contaminated by “scrapie-like particles”, and believed at that time to be linked to scrapie (Marsh, 1969). However, current cross-species transmission studies suggest that TME arose from L-type BSE (Baron et al. 2007). TME has been passaged into a variety of animals including Syrian golden hamsters (Marsh, 1969). These hamsters have become a natural system for TME infection.

Hamster-adapted prion strains

Strains of L-form BSE, scrapie, and TME have all been experimentally inoculated into Syrian golden hamsters. Upon inoculation, these strains result in varied tissue tropisms and incubation times within the hamster (Ayers et al. 2008; Shikiya et al. 2010).

Upon three serial passage into hamsters, each passage with sequentially shortening incubation times, TME begins to manifest two different phenotypes: Hyper (HY) and Drowsy (DY) strain (Bessen and Marsh 1992; McKenzie et al. 1996). These strains contain structural and biochemical differences (Bessen and Marsh 1992). Evidence suggests that although both forms appear to have been present in wild type TME infection in mink, mink are susceptible only to

back-passages of DY. This indicates the DY strain was the major component of TME infection in natural infection (Bessen and Marsh 1992).

These two disease phenotypes, or strains, have different PrP^{Sc} conformations, and are named after differences in their clinical symptoms (McKenzie et al. 1996; Bartz et al. 2005; Mabbot, 2006; Ayers et al. 2008).

- HY presentation resembles that of hamster-adapted scrapie strain 263K. Hamsters become hyper-excitabile and hyper-aggressive, and develop cerebellar ataxia as well as other brain lesions. They also demonstrate the wasting typical of TSEs, but do not lose their appetites. Upon additional passages of HY TME, brain lesion intensity decreases.
- DY hamsters become lethargic, difficult to rouse, lack coordination without cerebral ataxia, and do not become hyper-excitabile. Unlike other TSEs, DY TME does not require lymphoid neuroinvasion before spreading to the brain, and does not result in clinical infection when inoculated intraperitoneally.

HY- vs DY-TME

Regardless of how it is inoculated, HY-TME has a consistently shorter incubation time than DY. The incubation period for HY-TME extranasally (EN)-inoculated hamsters is 194 ± 43 days, whereas incubation time is greater than 650 days in DY-inoculated hamsters. Most importantly, HY-TME prions spread not only to the nervous system, but also propagate within the lymphoreticular system (LRS) (Shikiya et al. 2017). DY prions can cross mucosal epithelia into the LRS, but it does not propagate in LRS tissues (Bartz et al. 2005; Shikiya et al. 2017).

Hamsters inoculated with HY-TME have microvacuolization of the cerebellar cortex, brain stem, gray matter of cerebellum, and mild spongiform degeneration of thalamus (Bessen and

Marsh 1992; Liberiski, 2009). DY causes less vacuolar change in the brainstem and cerebellum, but more intense pathology of the cerebral cortex, and focal degeneration of the pyramidal layer of the hippocampus (the latter does not occur in HY).

Although their primary amino acid sequence is the same, HY and DY possess strain-specific migration patterns due to differences in PK sensitivity and conformational stability (differing α -helical and β -sheet content), which may be due to posttranslational modifications (Bessen and Marsh 1992). Consistent with these changes are differences in PrP^{Sc} formation and rate of spread (Mulcahy and Bessen 2003; Liberski et al. 2009). HY has a higher rate of PrP^{Sc} formation and spread than DY, which may explain its increased rate of deposition within the brain (Ayers et al. 2008) and the decreased incubation period. Co-infection of these two strains reveal that DY can increase incubation time of, or even block, HY strain (and hamster-adapted strain of scrapie, 263K) (Shutt, 2008). Researchers suggest this may be due to the first-inoculated strain pre-emptively filling prion replication sites or using conversion factors before the second-inoculated strain reaches them (Shikiya et al. 2010).

Syrian hamster as a system for prion pathogenesis

The hamster is an ideal system for the study of prion pathogenesis. It is an obligate nasal breather, and will immediately inhale a drop of brain homogenate (inoculum) placed beneath the nostril (Kincaid et al. 2012). EN inoculation is 10-100x more efficient than PO inoculation, and mimics a more natural route of infection when compared with intracranial injection (Clouse et al. 2015). PrP^{Sc} deposition (called PrP^{RES}) can be found in the LRS of HY EN-inoculated hamsters by 14 weeks PI (Kincaid and Bartz 2007). Additionally, outbred hamster strains can be used for

these studies rather than special transgenic animals, hamsters as relatively easy to house and handle, and they are well characterized in lab animal literature.

Neuroinvasion and peripheral prions

During most TSE infections, prions accumulate in the LRS (including spleen) before traveling to neural tissues and the brain (Aguzzi and Heikenwalder 2006; Mabbott and MacPherson 2006; Kim et al. 2016; Titlow et al. 2016). However, it is also suspected that some TSE strains (i.e. DY-TME, BSE) may be unable to establish, or do not require, propagation within LRS tissues (Wells 2003; Shikiya et al. 2017).

The spleen is composed of discrete nodules of white pulp surrounded by red pulp, encapsulated by the stroma (Bacha 2006) (figure 5). The white pulp is formed by two regions: the primary follicle, containing dense fields of lymphocytes such as follicular dendritic cells (FDCs), unstimulated B cells, and macrophages; and the periarteriolar lymphoid sheath (PALS). The PALS surrounds the primary follicle and merges into the red pulp, but also contains T cells and macrophages (McGovern et al. 2004).

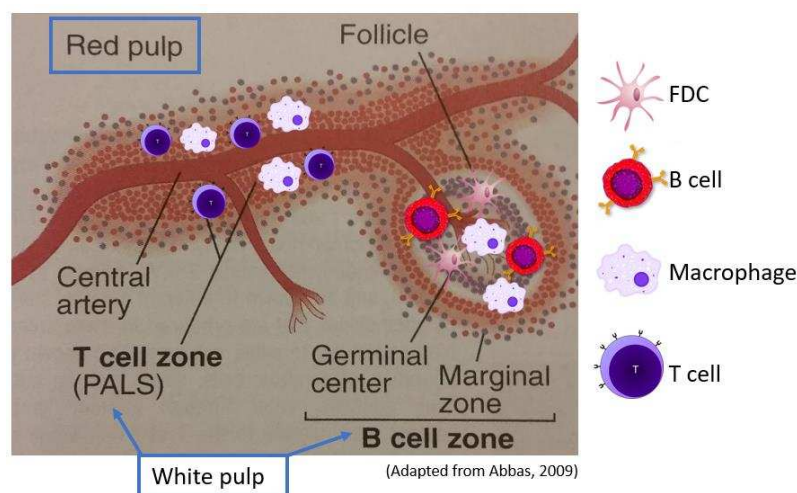


Figure 5. Diagram of the spleen, differentiating red pulp from white pulp. The white pulp is comprised of lymphoid follicles (containing undifferentiated B cells, FDCs, and macrophages) and PALS (containing T cells and macrophages).

In lymphoid tissues, PrP^{Sc} accumulates within macrophages, on follicular dendritic cells populating lymphoid germinal centers, and within B cell follicles (Bradford et al. 2017). B cells are important for their role in stimulating and maintaining FDC differentiation by producing the cytokines lymphotoxin $\alpha_1\beta_2$ and tumor necrosis factor (TNF) α . These two cell types have a synergistic effect: while B cells secrete cytokines that aid in FDC differentiation, FDCs promote affinity maturation of B cells (Kaesler et al. 2001).

B cells and Follicular Dendritic Cells

Mice without B cells, or the cytokines lymphotoxin $\alpha_1\beta_2$ and (TNF) α , lack FDCs, delaying neuroinvasion by inhibiting TSE accumulation in lymphoid tissues (Mabbot, 2006). Although the mechanism by which prions localize to lymphoid tissues is currently unknown, animals lacking components of complement (C1q, C2, C3, factor B) impairs TSE accumulation in the spleen (Mabbot, 2001). This suggests that one or more of these complement components targets and binds TSE prions, creating a complex that is then captured by the FDC (Klein et al. 2001; Mabbott, 2001). Alternatively, it is possible that PrP^C receptors on the FDC cell surface bind PrP^{Sc} (Mabbot, 2001).

Upon infection with scrapie in a mouse model, several abnormalities occur within the spleen that cause increased B cell proliferation and maturation. These alterations, in turn, change immune complex binding mechanisms within the spleen, facilitating trapping of PrP^{Sc} (McGovern et al. 2004). This promotion of B cells during prion infection suggests that these cells play an important role in prion pathogenesis.

Transferring wild type bone marrow to Tg *Prnp*^{-/-} mice restores the ability of the spleen to accumulate high titers of prions, yet does not kindle neuroinvasion (Blatter et al. 1997; Aguzzi

and Heikenwalder 2006). This suggests that while blood cells such as B cells, T cells, macrophages, and FDCs aid in transporting PrP^{Sc} to lymphoid organs for replication, they may not be the primary reservoir for neuroinvasion (Shikiya et al. 2017). Additionally, further thymectomy and T lymphocyte knockout studies demonstrate that T lymphocytes *alone* do not affect scrapie infectivity of the spleen (Klein et al. 2001), whereas a deficiency in B cells has a much more profound effect. This is likely due to their role in differentiation of FDCs (Mabbot, 2001).

The spleen's role in neuroinvasion

Mice intra-cranially inoculated with spleen homogenate harvested from early (4 days PI) and late stage scrapie-infected mice conferred prion infectivity (Büeler et al. 1993). This suggests that prions reach and possibly begin accumulating within the spleen as early as four days post-infection, and remain there throughout infection. The incubation period of scrapie is prolonged in splenectomized mice, suggesting that the spleen is an important site of neuroinvasion (Kimberlin and Walker 1979; Kimberlin and Walker 1989), in addition to facilitating prion accumulation (Shikiya et al. 2017). However, prion strain and host species likely affect the spleen's role in peripheral prion pathogenesis, as it has also been shown that splenectomy does not affect incubation times in scrapie-inoculated Syrian hamsters and CJD-inoculated mice (Aguzzi and Heikenwalder 2006).

After peripheral (outside nervous system) inoculation, prions cross mucous membranes into associated lymphoid tissues. Once in the LRS, they disseminate and accumulate into draining regional lymph nodes before spreading to the spleen and distant lymph nodes through the lymphatics—or possibly the blood stream (Kimberlin and Walker 1979; Kincaid and Bartz

2007). After establishing infection in the spleen, as occurs with Syrian hamsters EN-inoculated with HY-TME, prions propagate through the peripheral nervous system and enter the central nervous system via synaptic retrograde transport (Bartz et al. 2002; Shikiya et al. 2017) (figure 6).

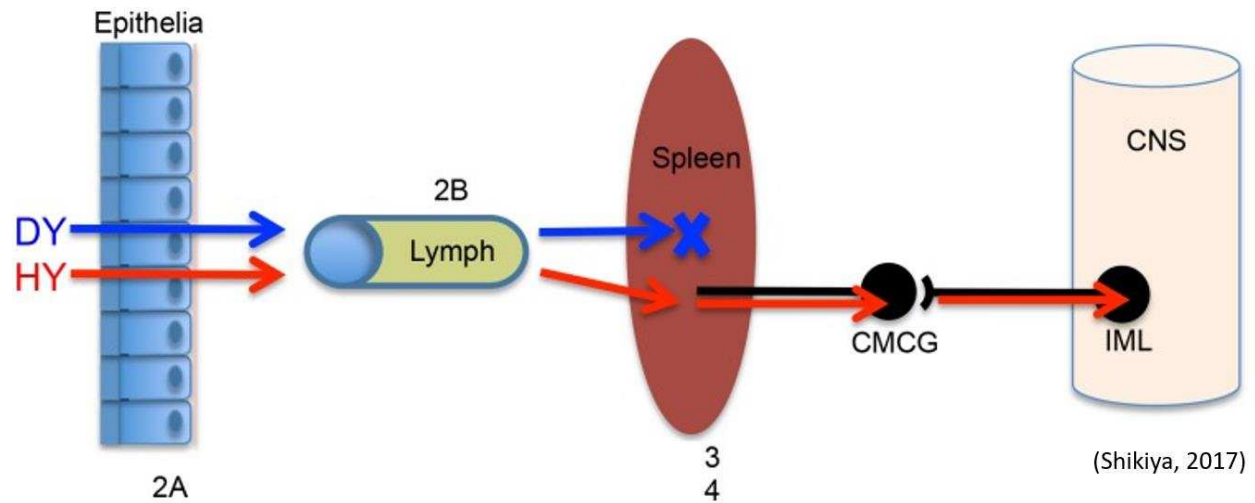


Figure 6. Spleen Role in Neuroinvasion. Denoting HY- and DY-TME prion infiltration of mucosal epithelia. Prions enter the LRS and travel to the spleen. From there, HY-TME enters the peripheral neural system and travels to the central nervous system.

In summary, the evidence clearly suggests that with certain prion strains and host species, the spleen and various blood cells are important components in the progression of prions into the nervous system, affecting prion pathogenesis and neuroinvasion. Thus, the ability to detect prions within the spleen, especially in the asymptomatic stage, is vital in diagnosing or even treating prion infection.

In addition to its roles in the lymphatics and aiding the immune system, the spleen also plays a role in the circulatory system by filtering out—and even maturing new—red blood cells. B cells and FDCs from the spleen circulate through the body within the blood stream, suggesting the possibility of hematogenous spread, or blood-borne prion pathogenesis.

Blood-borne prions

After the BSE epidemic of cattle in the UK in the late 1980s, resulting in vCJD in humans, investigators learned that vCJD was transmissible between humans (Brown et al. 1998; Belay and Schonberger 2005; Murphy 2013). The first cases of human-to-human vCJD transmission via blood transfusion were reported in 2004 from a blood donation was made in 1996, three years before the donor developed symptoms of vCJD (Peden et al. 2004; Llewelyn et al. 2004; Head et al. 2009). Three of four blood recipients, who were all methionine homozygous at codon 129 of *Prnp*, developed clinical signs of vCJD; the fourth recipient (who was heterozygous at codon 129) never developed clinical symptoms and died of unrelated causes, but did possess prion deposition within the spleen and cervical lymph node (Head et al. 2009; Diack et al. 2014). The UK outbreak of BSE and subsequent cross-species transmission into humans triggered the beginning of a paradigm shift: the consideration of blood as a player in prion pathogenesis.

Blood is comprised of several fractions: red blood cells, which carry oxygen to the body; platelets, which aid in coagulation of blood; white blood cells, a variety of cell types which carry out functions of the immune system; and plasma, which forms the liquid milieu through which the blood cells flow. When centrifuged, whole blood can be separated into fractions with plasma and platelet-rich plasma (PRP) resting atop a “buffy coat” of white blood cells and granulocytes, which rests atop the red blood cells (figure 7).

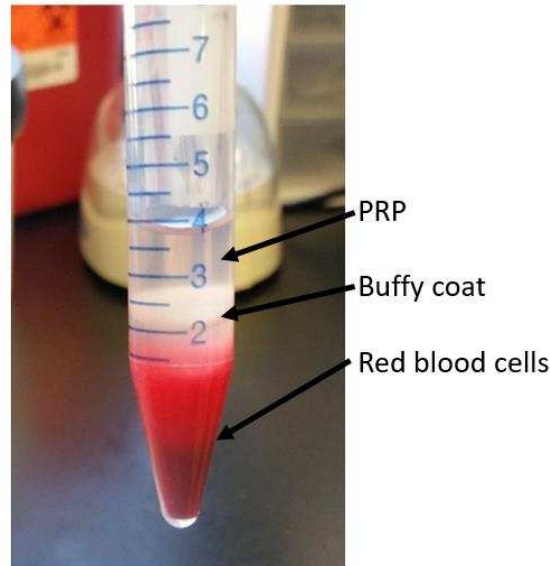


Figure 7. Whole blood components separated by centrifugation into platelet-rich plasma (PRP), the buffy coat, and red blood cells.

Prions associated with whole blood fractions

Mouse bioassays examining prion infectivity present within the cellular buffy coat and plasma fractions demonstrated that the buffy coat conferred more infectivity than plasma, but that they were both infectious (Brown et al. 1999). In 2002, researchers determined that the blood of both BSE and scrapie-infected sheep conferred infectivity to transfused sheep (Hunter et al. 2002), supporting evidence that vCJD might be transmissible between humans via blood transfusions. Further studies examined whether blood from sheep infected with either BSE or scrapie could transmit prions to recipient sheep (Edwards et al. 2010). They found that both whole blood and buffy coat transfusions conferred infectivity in both clinical and preclinical donors. The more progressed a donor's disease course was when the blood was collected, the more infective the sample became to the recipient. Studies of CWD infection in white-tailed deer lend further support for the presence of blood-borne prions in preclinical and clinical donors (Mathiason et al. 2006). Further studies proved that leukoreduction, reducing the number of white blood cells, eliminated

42% of TSE infectivity from the whole blood. These studies confirmed that components of buffy coat contain prion infectivity and that leukoreduction does not decrease the prion transmission within the plasma fraction, which may account for 40% of blood infectivity (Gregori et al. 2004).

Prions associated with certain cell types

We (Mathiason et al. 2010) revealed that B cells and platelets harvested from TSE-infected hosts harbor prion infectivity. We further detected prions in the blood of TME-infected hamsters and CWD-infected cervids throughout the entire disease course (Elder et al. 2015), establishing the presence of a prionemia throughout the entirety of disease. Further support for cell-specific infectivity is evidenced in scrapie, where B cells, monocytes, T lymphocytes, and platelet-rich plasma harbor prions (Edwards et al. 2010; Dassanayake et al. 2016). Additionally, scrapie prions accumulate within macrophages in hamster Peyer's patches, sheep B-cell follicles, and mouse spleens, as well as in macrophages within the tonsils of mule deer infected with CWD (Mabbot 2006). Whether these macrophages are attempting to clear infection or are participating in pathogenesis of the disease remains unclear (Mabbot 2006; Mathiason et al. 2010).

CD4 and CD8 T cells of scrapie-infected mice have infiltrated the blood-brain barrier into the hippocampus, cortex, and cerebellum after intracranial (85 days PI), intraperitoneal (185 days PI), and oral inoculation (282 days PI) (Lewicki et al. 2002). This phenomenon has also been found in CNS parenchyma of 5/6 patients with clinical CJD (Lewicki et al. 2002). This suggests a mechanism for prion transmission from the blood, through the blood-brain barrier, to the brain.

We know that prions are present in the blood throughout the entire disease course. Yet are blood cells simply harboring prions, bathing lymphoreticular and nervous tissue with prions? Or are various blood cells sites of amplification of prion infection? Are cell types such as macrophages

attempting to phagocytize and sequester prions? In 2010, Panigaj determined that the amount of PrP^C present on blood cells varies by species (Panigaj et al. 2010). If PrP^C is present, the potential for amplification exists. What role do blood cells play in prion pathogenesis? How many asymptomatic humans and animals are unknowing carriers and shedders of prions, and how might this impact cross-species spread?

Before we can answer these questions, we need to understand at the more fundamental level: which blood cell types are associated with prions, and do these populations change over the disease course?

CHAPTER ONE: Longitudinal Detection of Prions within the Spleen of Syrian Hamsters Infected with Hamster-adapted Transmissible Mink Encephalopathy

Hypothesis

- We hypothesize that the spleen is a site of prion accumulation beginning acutely, and that prions within the spleen will be detectible throughout the entire disease course.
- Aim: To determine the temporal presence of prions within the spleen of hamster-adapted-HY TME-inoculated hamsters. We will examine spleen tissue harvested from HY-inoculated hamsters minutes post infection through terminal TSE disease for prions using IOB-RT-QuIC.

Introduction

Transmissible Spongiform Encephalopathies (TSEs) are a group of infectious, neurodegenerative diseases affecting humans and animals (Beekes and McBride 2007; Aguilar-Calvo et al. 2015). Caused by misfolded proteins called prions, TSEs are characterized by long, asymptomatic incubation periods that eventually cause neuronal death and progressive neurodegeneration (Bessen and Marsh 1992; Beekes and McBride 2007; Shikiya et al. 2017). Notable diseases caused by prions include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, transmissible mink encephalopathy (TME) in mink, as well as Kuru and Creutzfeld-Jakob's disease (CJD) in humans (Bessen and Marsh 1992; Beekes and McBride 2007; Shikiya et al. 2017).

TME is a rare TSE first recorded in 1947 by Hartsough and Burger after an outbreak occurred on a mink farm in Wisconsin (1965). Over 2-8 weeks, the animals displayed behavioral

changes (lack of grooming and cleanliness) as well as difficulty eating, incoordination, hyper-aggression, and hyper-excitement (Marsh 1972). The Syrian hamster is susceptible to extranasal (EN) inoculation of TME, and serves as a natural system of this infection (Kincaid et al. 2012). Hamsters inoculated with the hyper (HY) strain of TME demonstrate clinical signs and wasting typical of TSEs, but do not lose their appetites (Bessen and Marsh 1992). HY-TME infectivity has been detected in the nervous system, as well as the lymphoreticular system (LRS) including the spleen, the skeletal muscle, nasal secretions, and blood (Shikiya et al. 2017).

The spleen is an important site of peripheral neuroinvasion of many strains of prions (Kimberlin and Walker 1979; Kimberlin and Walker 1989; Blatter et al. 1997). Once infection is established in the spleen, prions propagate through the peripheral nervous system and enter the central nervous system via retrograde axonal transport (Bartz et al. 2005; Shikiya et al. 2017).

This study sought to characterize the temporal change of splenic prions in HY-TME-inoculated Syrian hamsters using real-time quaking-induced conversion (RT-QuIC). Because prions have previously been detected in the spleen as early as 14 weeks post EN-inoculation (Kincaid and Bartz 2007), and because of previous work establishing a prionemia throughout the entire disease course in this animal system (Elder et al. 2013), we hypothesized that prions would be detectable in the spleen with RT-QuIC throughout the entire disease course. We also sought to evaluate whether prion deposition within the spleen changed throughout the disease course, as well as compare the detectability of immunohistochemistry (IHC) to RT-QuIC. We hypothesized that RT-QuIC would have earlier detection of prion positivity than IHC.

Materials and methods

Animal Use: This study was approved by the IACUC of Colorado State University (#16-6983A). Male Syrian Golden Hamsters (age 8-10 weeks) were purchased from Envigo and housed in an AAALAC International-accredited animal facility. Hamsters acclimated for 1 week after their arrival at the facility and were housed under standard conditions. Hamsters were pair housed (Allentown, Allentown, NJ) with enrichment including cardboard tubes. They had ad libitum access to irradiated pelleted feed (no. 2918, Harlan Teklad) and sterile water. All cages contained sterilized aspen bedding (Harlan Teklad Sani-chip 7090) to a depth of ¼ in. Room environments were maintained at $21.7 \pm 1.7^{\circ}\text{C}$, 20% - 40% humidity, and 12:12-h light:dark cycle.

Study Design (Fig. 8): Sixty male Syrian Golden hamsters were placed into one of two cohorts: Cohort 1: n=30 TME+ inoculates, and Cohort 2: n=30 TME- sham inoculates. These cohorts were further divided into 10 groups (n= 3/cohort) by terminal time point: un-inoculated control, 15 minutes post inoculation (PI), 30 minutes PI, 60 minutes PI, 24 hrs PI, 48 hours PI, 60 days PI, 90 days PI, 155 days PI, and clinical (euthanized at 169 days and 238 days PI).



Figure 8. Study design. 60 Syrian hamsters were EN-inoculated with sham or TME+ brain homogenate. At selected time points, tissues were collected and analyzed by RT-QuIC or IHC.

Inoculation: The hamsters were lightly anesthetized with isoflurane and placed in dorsal recumbency. Once anesthetized, 5uL of brain homogenate (10% weight/volume (w/v) containing $10^{6.8}$ intracerebral 50% lethal doses per ml of HY-TME agent, or 10% w/v sham brain homogenate) was placed inferior to each nostril. After inhaling the homogenate, the hamsters recovered from anesthesia in their home cage. At the designated time point, or if the hamsters became clinical for disease, the hamsters were deeply anesthetized with isoflurane prior to humane euthanasia via terminal cardiocentesis for tissue collection. During tissue collection, we were careful to decrease the opportunity for prion contamination between tissues by using new single-use forceps and blades to handle each separate tissue.

Tissue Preparation: Sections of spleen and other tissues (including brain, spinal cord, blood, lymph nodes, intestinal tract, and vital organs such as lung, liver, and heart) were harvested from each hamster and prepared as fixed or frozen samples. *Fixed tissue:* Each tissue was placed in McLean's paraformaldehyde-lysine-periodate (PLP) fixative for two days followed by storage in phosphate buffered saline stock solution (PBS) (1.37M NaCl, 0.027M KCl, and 0.119M phosphates) (Fisher BioReagents) diluted 1:10 in deionized water and filter sterilized. These fixed samples of spleen were trimmed, embedded in paraffin, and 5 μ m sections were mounted onto slides for IHC analysis. *Fresh tissue:* Tissue sections of spleen and brain (hind brain/cerebellum and brain stem) were placed in separate 1.8 mL cryovials (Genesee Scientific) and frozen at -80°C for RT-QuIC analysis. Tissues were removed from -80°C , thawed, and homogenized to 10% w/v in filter-sterilized PBS for analysis with RT-QuIC.

Iron Oxide Beads (IOB) and Control Preparation: *Spleen samples- iron oxide bead (IOB) preparation:* Samples were prepared with IOB as previously described (Henderson et al. 2013; Denkers et al. 2016). Briefly, 50 μ L of 10% w/v spleen sample homogenate was serially diluted in 450 μ L RT-QuIC PBS (130 mM NaCl, 7 mM Na₂HPO₄·7H₂O, 3 mM NaH₂PO₄·1H₂O) from 10⁻² through 10⁻⁸. Two (2) μ L of superparamagnetic iron oxides beads (Bangs Laboratories, Indiana) per sample were washed two times 1:1 with RT-QuIC PBS and 2 μ L added to each sample. Each sample was incubated at room temperature using an end-over-end rotator for 30 minutes. Samples were placed on a magnet particle separator (MPS) (Pure Biotech, New Jersey) and allowed to sit for one minute before the supernatant was removed. The sample was removed from the magnet and re-suspended into 10 μ L of 0.1% sodium dodecyl sulphate (SDS), then briefly vortexed to mix. *TME+ and sham brain preparation:* 5 μ L of 10% w/v brain homogenate from TME+ and sham-inoculated hamsters was serially diluted in 45 μ L of SDS from 10⁻² through 10⁻⁵, and briefly vortexed to mix

Recombinant PrP (rPrP) protein: Truncated recombinant Syrian hamster PrP (SHrPrP) (aa 90-231) was produced as previously described (Henderson et al. 2013), and used as the PrP^C “seed” for RT-QuIC. In summary, 1 liter of LB containing Auto Induction™ supplements (EMD Biosciences) was incubated overnight with SHrPrP-expressing *E. coli* (Rosetta strain) and harvested at an OD (600 nm) of ~3. Bacterial cells were lysed (Bug Buster™ reagent with Lysonase™ [EMD Biosciences]). Inclusion bodies (IB) were collected after centrifugation of the lysate at 15,000 $\times$ g for 15 minutes. IB pellets were washed twice and stored at -80°C until purification. For purification, IB pellets were re-suspended in 8 M guanidine hydrochloride (GuHCl) in 100 mM NaPO₄ and 10 mM Tris [pH 8.0], centrifuged at 15,000 $\times$ g for 15 minutes,

and added to a Super Flow Ni-NTA resin (Qiagen) pre-equilibrated with denature buffer (6.0 M GuHCl, 100 mM NaPO₄, 10 mM Tris [pH 8.0]). The SHrPrP within the resin incubated by rotation at room temperature for 45 minutes before being added to an XK FPLC column (GE Healthcare). SHrPrP was refolded on a column using a linear refolding gradient of denature buffer to refold buffer (100 mM NaPO₄, 10 mM Tris [pH 8.0]), 240 mL at 0.75 mL/min. SHrPrP was then eluted with a linear gradient of refold buffer to elution buffer (100 mM NaPO₄, 10 mM Tris [pH 8.0], 500 mM imidazole [pH 5.5]), 100 mL at 2.0 mL/min. The fraction was pooled, dialyzed in two changes of 4.0 I dialysis buffer (20 mM NaPO₄ pH 5.5 [CSU], 10 mM NaPO₄ [pH] 5.8 [RML]), and adjusted to ~4.0 mg/mL.

RT-QuIC: Real-time quaking induced conversion (RT-QuIC) was initiated as previously described (Henderson et al. 2015; Denkers et al. 2016; Nalls and McNulty et al. 2017). Hamster or deer brain tissues of known TSE status were prepared and run as plate controls for every experiment. Two (2) μ L of each IOB-treated spleen sample was mixed with 98 μ L of “RT-QuIC buffer” (130 mM NaCl, 1 mM EDTA, 10 mM thioflavin T [ThT], 0.1 mg/ml SHrPrP substrate) and distributed into each well of a 96-well plate (Nalgene Nunc). Plates containing four or five replicates of each sample (for a total of 8-12 replicates total per sample) were loaded into BMG FLUOstar Omega microplate reader. For a total of 250 cycles, the thermocycler shook each sample for one minute, every other minute for 14 minutes, at 700rpm. After each cycle, the ThT fluorescence (excitation 450 nm and emission 480 nm) was read and recorded. Total reaction time was 62 hours at 42°C. Data was reported as the rate (1/time) that fluorescence crossed the positivity threshold (greater than five times the standard deviation of plate average fluorescence). Data were processed using Microsoft Excel (Microsoft Inc) and GraphPad Prism. TME+ samples

were compared to shams with a Mann-Whitney unpaired two-tailed T test. The confidence interval for statistical significance was 95%.

IHC: Slides were heated in a 65°C incubator for 30 minutes, and were then immersed twice in xylene for 10 minutes/each before being rehydrated through graded alcohols, 5 minutes each. Slides were rinsed under running tap water for 10 minutes before being placed in PK buffer (1.0 µg/mL PK in CaCl / Tris buffer [1.0 mM CaCl₂ / 50 mM Tris buffer]) for 30 minutes at 37°C. Slides were placed in tris-biffered saline (TBS) twice for 5 minutes (with rocking), then processed through antigen retrieval (“The Retriever” [Aptum Biologics]) in 10 mM EDTA [final pH 6.0]. Upon removal from antigen retrieval slides were placed in TBS wash (5 min) prior to immersion in 88% formic acid for five minutes and a final TBS wash (5 min). Tissue sections were circled with Dako DAP pen before being quenched in 3% H₂O₂ in MeOH for 30 minutes at room temperature. An additional wash in TNT buffer (0.1M Tris [pH 7.5] + 0.15M NaCl + 0.05% Tween-20), twice for 5 minutes/each, was followed by TNB blocking buffer (Cold Spring Harbor) for 30 minutes at room temperature. Excess block was tapped off, and slides were incubated with anti-prion 3F4 antibody at 1:600 (Chemicon, Temecula, CA) diluted in TNT buffer, overnight at 4°C.

All remaining incubations occurred at room temperature, with two TNT washes between each step. Slides were incubated in secondary biotinylated horse anti-mouse antibody at 1:600 (Vector Laboratories, Burlingame, CA) diluted in TNT buffer for 30 minutes, followed by amplification with avidin/biotin complex (ABC) at 1:600 (Vector Laboratories, Burlingame, CA) for 20 minutes and aminoethyl carbazole (AEC) (Abcam) for 6 minutes. Finally, slides were counterstained with hematoxylin (Sigma) for 30 secs, rinsed with tap water twice for 30 secs,

and incubated with bluing reagent for two minutes. Slides were rinsed again with tap water, cover-slipped, and observed under magnification.

Results

Clinical disease: Tissues were collected at the designated time points during early, mid, or late-disease. Beginning at 120 days, we monitored hamsters three days per week for clinical signs. These signs include cerebellar ataxia, and hyperreactivity. One of three (n=1/3) hamsters within the 155 days PI cohort was noted to be clinical at 153 days PI. This hamster demonstrated moderate proprioceptive defects while walking as well as hyperreactivity and cerebellar ataxia. Two (n=2/3) hamsters were noted to be mildly clinical at 163 days PI and were collected with their sham counterparts at 169 days PI. Both had mild hyperreactivity and mild cerebellar ataxia. The remaining hamster (n=1/3) did not show signs of clinical TSE and was collected with his sham counterpart at 238 days PI.

RT-QuIC: We performed an initial RT-QuIC screening for prion positivity by evaluating all sample spleens at a dilution of 10^{-2} with iron oxide beads as previously described (Denkers et al. 2016). Only reactions with a resulting fluorescence greater than the mean initial fluorescence plus five standard deviations were considered positive (~5,000—11,000 fluorescence units). In this initial screening, spleen from all study animals were evaluated. Tissues from time and age-matched sham-inoculates were collected, processed, and evaluated with each HY-inoculated counterpart. Each tissue was assayed to collect 8-10 replicates/tissue/hamster (n= 3 hamsters / time point). In the early-disease time points (15 minutes, 30 minutes, 60 minutes, 24 hours, and 48 hours PI) we detected little seeding activity (positivity) within the samples (Figure 9).

Seeding activity was demonstrated in 1/24 replicates at 15 and 30 minutes PI, 2/24 replicates at 60 minutes PI, 0/24 at 24 hours, and 2/24 replicates at 48 hours PI. In the mid-disease time points (60 days and 90 days PI; n=3 hamsters/time point) we detected increased positivity in several sample replicates (3/24 total replicates at 60 days and 90 days PI), but it was not statistically significant. Statistically significant positivity was detected in the late-disease hamsters (n= 3/5), within the 155 and 169 days PI groups (21/24 total replicates at 155 days PI, and 13/20 total replicates at 169 days PI). The final hamster, remaining asymptomatic through 238 days PI (n=1), remained free of RT-QuIC seeding activity.

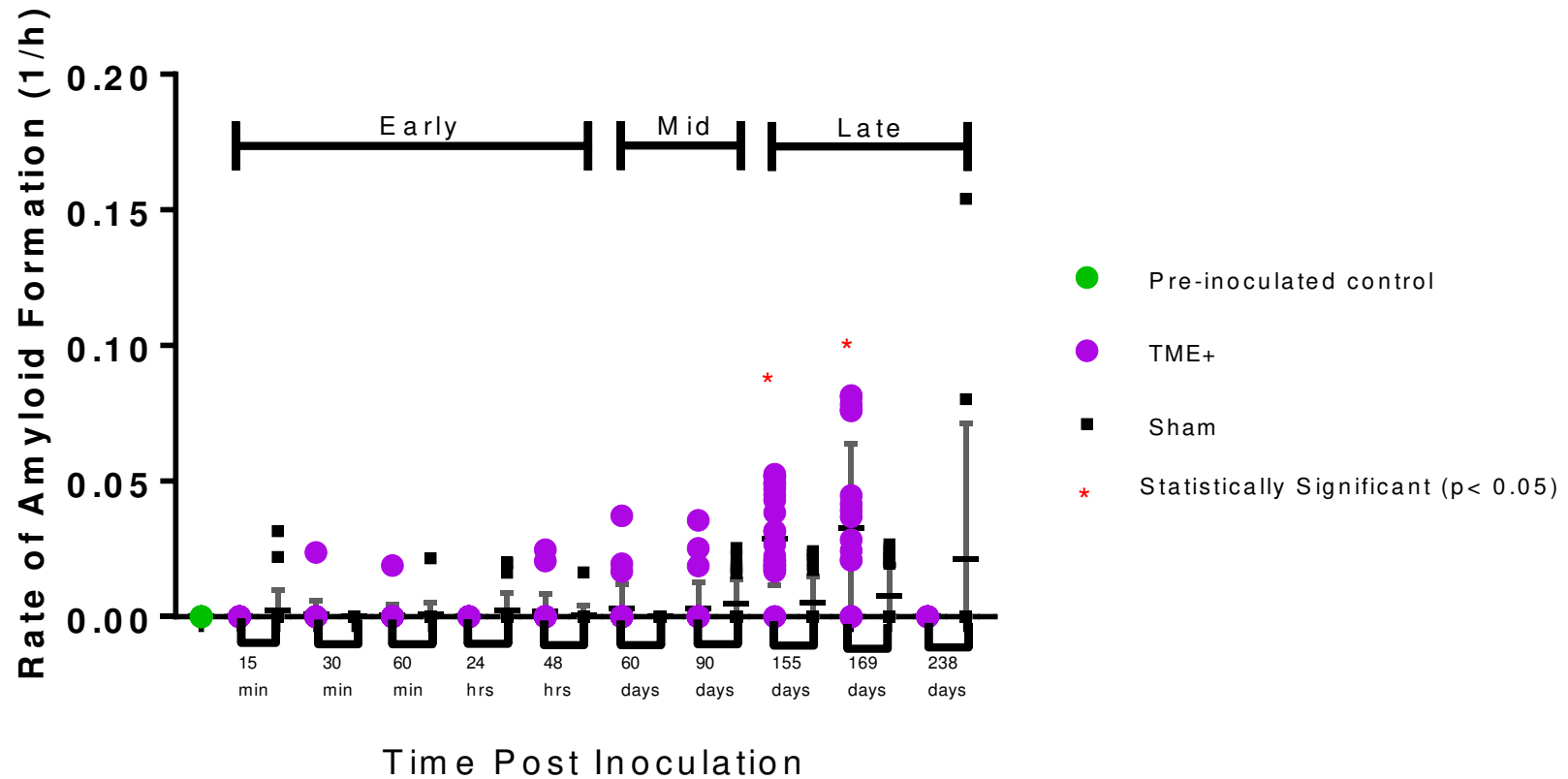


Figure 9. Longitudinal RT-QuIC seeding activity in spleen (10^{-2}) of hamster-adapted HY-TME EN-inoculated hamsters. Spleens from sham-inoculated hamsters are shown with black squares. Spleens from hamster-adapted HY TME-inoculated hamsters shown with purple circles. Pooled HY-TME+ inoculated replicates for each time point were compared to pooled sham replicates within each time point. * denotes statistical significant ($p < 0.05$) of the HY-TME-inoculated spleen replicates from paired age- and time-matched sham. All statistically significant differences in positivity occur in the late disease course at 155 days PI ($p = < 0.0001$) and 169 days PI ($p = 0.0061$). We ran 8-10 replicates of each sample. Gray bars represent standard deviation.

Spleen: After determining the temporal prion detection profile in longitudinal spleen samples at 10^{-2} , we sought to further evaluate the prion titer in each spleen by dilutional titration (10^{-3} through 10^{-8}), and analyzed ten replicates of each dilution/hamster.

When pooling the RT-QuIC replicates of all hamsters for each time point, we show prion seeding activity at 155 days PI (dilution 10^{-5}), and at 169 days PI (dilutions 10^{-3} through 10^{-7}) (Figure 10, A). Statistical significant seeding activity was not demonstrated in hamsters euthanized prior to 155 days PI. When evaluating each hamster separately (Figure 11, A) statistical significant prion detection was evident in 2/3 hamsters euthanized at 155 days PI cohort: in hamster #1, asymptomatic, we detected positivity at 10^{-7} ; in hamster #2, asymptomatic, negative RT-QuIC; and in hamster #3 showing advanced clinical symptoms (hyperreactivity, cerebellar ataxia, and incoordination), we detected positive seeding activity in 10^{-3} through 10^{-6} .

In the hamsters within the later clinical disease timepoint (169 days PI), 2/3 showed statistically significant prion positivity: both hamsters were euthanized at 169 days PI and presented with mild clinical signs of TSE disease. In hamster #1, exhibiting mild clinical symptoms (hyperreactivity and mild incoordination), we detected positivity in dilutions 10^{-3} through 10^{-6} ; and in hamster #2, with milder clinical symptoms (hyperreactivity), we detected positivity of 10^{-5} through 10^{-7} . Hamster #3 did not show signs of TSE disease by 238 days PI, was euthanized and did not demonstrate RT-QuIC prion seeding activity.

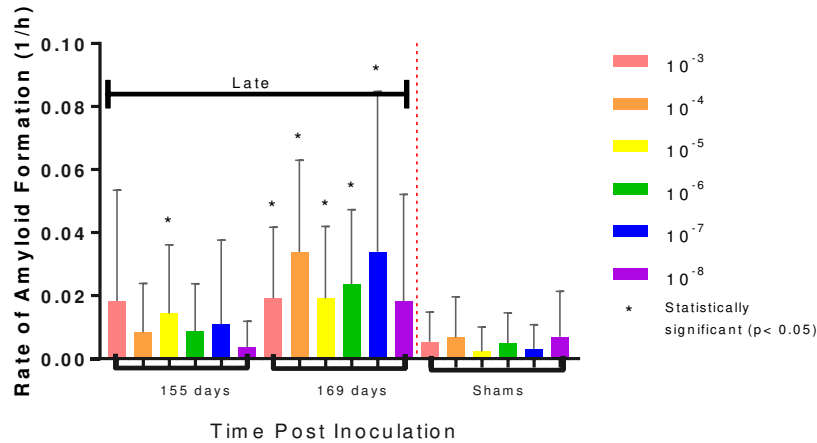
Brain: We evaluated the hind brain of mid- (90 days PI) and late- (155 days and 169 days PI) disease hamsters for prion positivity (Figure 10, B). In the 90-day PI cohort (n=6 [3 extra hamsters were inoculated for this time point in case of unrelated losses over time]) we evaluated

four replicates of each dilution for a total of 24 replicates at each dilution; in the 155 days PI cohort (n=3) and 169 days PI (n=2) we evaluated ten replicates of each dilution from each hamster. Positive and negative control brain homogenates of known TSE status were assayed for comparison.

We detected prion positivity in the brain tissue of HY-inoculated hamsters at 155 days PI (dilution 10^{-3}) and at 169 days PI (dilutions 10^{-3} and 10^{-4}). Statistical seeding activity was not demonstrated in hamsters euthanized at 90 days PI. Individually (Figure 11, B), statistical seeding activity was detected in 1/3 hamsters collected at 155 days PI. Hamsters #1 and #2, both asymptomatic, demonstrated no statistical seeding activity, but hamster #3, with moderate clinical signs (hyperreactivity, incoordination, cerebellar ataxia), showed seeding activity at 10^{-3} through 10^{-5} .

At 169-days PI, 1/2 hamsters showed statistical seeding activity: Hamster #1, mildly clinical (hyperreactivity and mild incoordination), at 10^{-3} through 10^{-5} . Hamster #2, mildly clinical (hyperreactivity), did not demonstrate statistical seeding activity.

A) Spleen



B) Brain

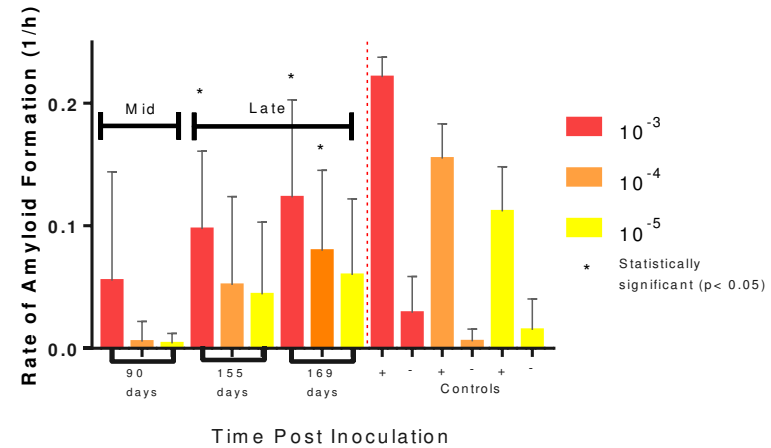


Figure 10. RT-QuIC titration of prion seeding activity in spleen and brain harvested from mid and late stage HY-TME EN-inoculated hamsters. A) Spleen dilutions (10^{-3} through 10^{-8}) from late disease course hamsters, evaluated by RT-QuIC (mean values presented). The graph represents dilutions of TME+ inoculated hamster spleen, pooled at each time point, compared with the pooled spleens of sham-inoculates for each time point. Prion seeding activity is detected at 155 days PI, dilution 10^{-5} ($p = 0.0008$); 169 days PI dilutions 10^{-3} through 10^{-7} ($p = 0.0036, <0.0001, <0.0001, <0.0001, \text{ and } 0.0005$, respectively). Data represents 10-12 replicates/sample from each hamster (155 days, $n=3$; 169 days, $n=2$), with IOB preparation. B) Hind brain dilutions (10^{-3} through 10^{-5}) from mid- and late- disease course hamsters, evaluated by RT-QuIC (mean values presented). The graph represents dilutions of TME+ inoculated hamster brain, pooled at each time point, compared with positive and negative brain controls from CWD-inoculated white-tailed deer. We demonstrate prion seeding activity at 155 days PI at dilution of 10^{-3} ($p = 0.0016$), and 169 days PI at dilutions 10^{-3} and 10^{-4} ($p = 0.0026$ and 0.0036 , respectively). * denotes statistical significance from TME-inoculated vs paired age- and time-matched sham-inoculates ($p < 0.05$). Data represents four replicates of each dilution from each hamster ($n=6$) at 90 days PI; at 155 ($n=3$) and 169 days ($n=2$), data represents 10 replicates of each dilution from each hamster. Gray bars represent standard deviation.

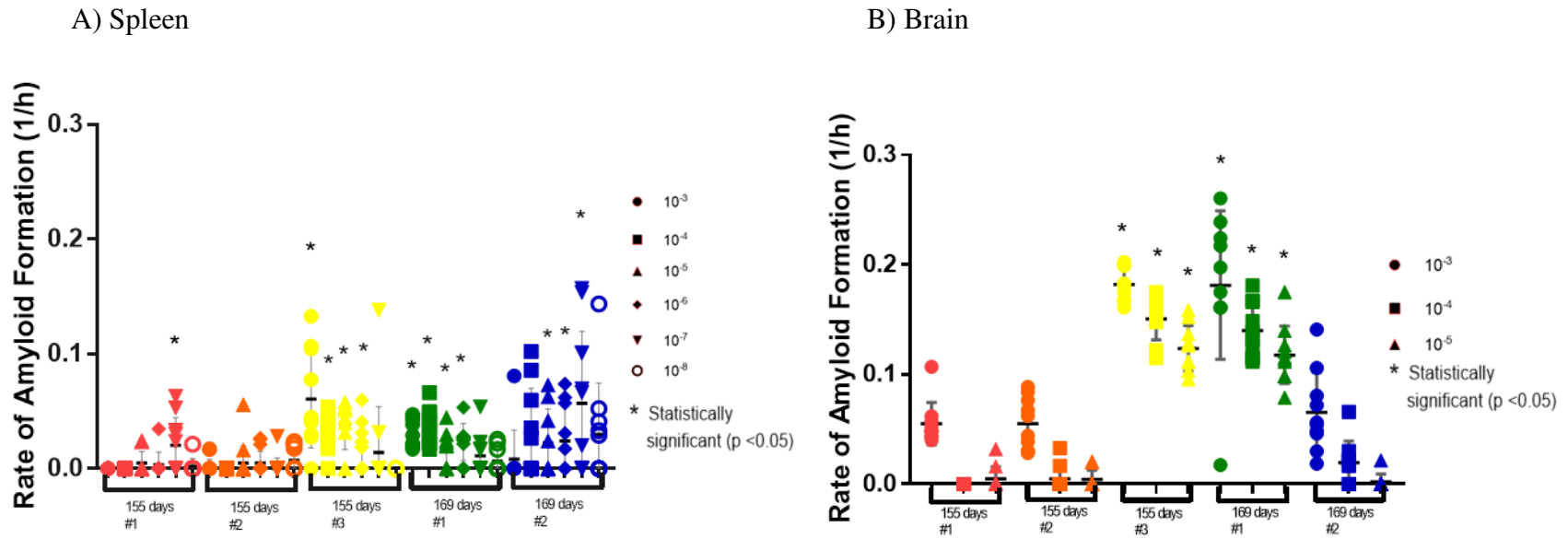


Figure 11. RT-QuIC titration of prion seeding activity in spleen and brain of individual HY-TME EN-inoculated hamsters. The graphs represent RT-QuIC dilution series of individual spleen and brain homogenates during late stage disease in TME-inoculated hamsters. A) Spleen dilutions (10^{-3} through 10^{-8}). Prion seeding activity is demonstrated at 155-day PI: hamster #1, 10^{-7} ($p = 0.005$); hamster #2, no significant positivity; hamster #3 10^{-3} ($p < 0.0001$), 10^{-4} ($p < 0.0001$), 10^{-5} ($p < 0.0001$), and 10^{-6} ($p = 0.0084$). 169-day PI: hamster #1 showed seeding activity at 10^{-3} ($p < 0.0001$), 10^{-4} ($p < 0.0001$), 10^{-5} ($p = 0.0009$), and 10^{-6} ($p = 0.0007$); hamster #2 demonstrates seeding activity at 10^{-5} ($p = 0.0053$), 10^{-6} ($p = 0.0423$), and 10^{-7} ($p = 0.0018$). B) Brain dilutions (10^{-3} through 10^{-5}). Prion seeding activity is demonstrated at: 155-day PI, hamster #1 and #2, no significant positivity; hamster #3 showed positivity from 10^{-3} through 10^{-5} ($p = 0.002$, 0.002 , and 0.002 respectively). 169-day PI, hamster #1 showed positivity from 10^{-3} through 10^{-5} ($p = 0.0023$, 0.002 , and 0.002 respectively); hamster #2 showed no significant positivity. * denotes statistical difference of the TME-inoculated spleen replicates from the paired age- and time-matched shams ($p < 0.05$). The black dash denotes the median rate. Data represents ten replicates/dilution for each hamster and tissue type. Gray bars represent standard deviation.

Discussion

In some prion strains (HY-TME, vCJD, scrapie) the spleen is a vital site of prion accumulation prior to neuroinvasion (Aguzzi and Heikenwalder 2006; Mabbott and MacPherson 2006; Kim et al. 2016; Titlow et al. 2016). In these strains, prions begin to accumulate in the spleen early during infection, and remain throughout the disease course (Büeler et al. 1993; Aguzzi, 2006; Shikiya et al. 2017). The ability to detect prions within the spleen, before an animal becomes symptomatic for disease, aids in disease diagnosis and evaluating prion accumulation and pathogenesis, and provides opportunity to block or potentially treat the infection before it reaches the nervous system. Therefore, we sought to determine longitudinal detection of prion seeding activity (prion positivity) with RT-QuIC, a novel method for this evaluation.

RT-QuIC detection of HY-TME within EN-inoculated Syrian hamster spleen: RT-QuIC is a sensitive and rapid assay for prion detection (Henderson et al. 2015; Orrú et al. 2015). We hypothesized that prions would be detectable within the spleen of HY-TME inoculated hamsters throughout the entire disease course if assessed by the highly sensitive amplification methodology, real time quaking-induced (RT-QuIC). We evaluated spleens throughout disease progression, with several early-, mid-, and late-disease time points. The early time points spanned from 15 minutes to two days PI, the mid-disease time points were 60 and 90 days, and the late-disease time points were between 155 days and clinical (169 days).

We detected prions within the spleen in the late stage of disease, beginning 155 days PI. 2/3 hamsters at this time point were asymptomatic at harvest, though they ultimately showed positivity within the spleen. The third hamster was beginning to show clinical signs at 153 days

PI and was harvest at 155 days PI. We decided to wait until the remaining three hamsters became clinical before collecting their tissues; two of these hamsters were noted to be mildly clinical at 163 days, and were euthanized for tissue collection at 169 days post infection. We detected positivity in the spleen of both (2 of 2) of these symptomatic hamsters. The final hamster was not clinical at 238 days PI and showed no seeding activity within its tissues.

Previous studies show the presence of scrapie prion infectivity in the spleen as early as four days PI (Beuler, 1993), and that prions accumulate in the spleen over time (Eklund, 1967; Rubenstein et al. 1987). Additionally, using conventional immunohistochemistry (IHC), prion deposition can be detected in the spleen of the EN HY hamster system by 14 weeks PI (Kincaid and Bartz 2007). Studies using this animal system demonstrated prions within the blood minutes after inoculation (Elder et al. 2015) with RT-QuIC. Because RT-QuIC is a more sensitive assay than IHC (Hoover et al. 2015), we expected to detect positive seeding activity in the spleen throughout the disease course.

Thus, our data contrasts other studies evaluating prions in the LRS in this hamster system. In white-tailed deer orally inoculated with CWD, the LRS (spleen and peripheral lymph nodes) contained prion deposition (via IHC) and prion seeding activity (RT-QuIC) early in the disease course (three months PI), before any evidence of nervous system invasion (Hoover et al. 2017). Interestingly, in two mouse-adapted scrapie strains, RML and ME7, seeding activity was detected in the spleen in early infection, 30 days PI (Shi et al. 2013). The seeding activity decreased from 30-90 days PI before increasing during mid-to-late disease stages (90-150 days PI) (Shi et al. 2013). This indicates that in earlier stages of disease, the splenic prion accumulation in HY-TME-inoculated hamsters may be lower than the detection threshold of our assay, but accumulates over time to detectable levels later in the disease process.

These data do not support our hypothesis that prions would be detectable throughout the entirety of disease. However, evaluating detection of prions in the hamster-adapted HY-TME Syrian hamster system with RT-QuIC is novel. Continued optimization of our assay may lead to earlier detection of prions within the spleen.

Prion accumulation in the spleen over time: Inoculation via peripheral routes leads to rapid prion trafficking across mucous membranes and entrance to the LRS (Kincaid and Bartz 2007). Previous studies demonstrate that prions accumulate in cells associated with the white pulp (lymphoid follicles and PALS) of the spleen (Bradford et al. 2017), and from there can enter the peripheral nervous system and eventually the central nervous system (Bartz et al. 2002; Shikiya et al. 2017). The importance of the spleen in this peripheral neuroinvasion can vary based on prion strain and host. In the Syrian hamster, EN-inoculation of HY-TME leads to IHC detectable prion deposition in the spleen by 14 weeks PI (Kincaid and Bartz 2007). This deposition is restricted to few lymphoid follicles within the white pulp.

Our data show evidence of prion accumulation within the spleen over time. We detected increased seeding activity in the spleen between 155-169 days PI, with higher prion burdens within the spleen of hamsters exhibiting clinical signs of TSE disease. Scrapie studies in mice (Eklund, 1967), hamsters, and sheep (Rubenstein et al. 1987) show that prions reach the spleen and begin to accumulate within it early in the disease before clinical signs are present (Eklund, 1967). Although we only detected positivity in late-disease animals, our data support a pattern of temporal prion accumulation in the spleen within HY-TME EN-inoculated hamsters.

Using the methods outlined in previous studies (Hoover et al. 2015), we determined the LD₅₀/g spleen detected with our assay by associating seeding rates of our spleen samples to the

seeding rates of an equivalent amount of brain, and then converted that brain equivalent into LD₅₀/gram. To do this, we associated with the seeding rates of our brain inoculum to the amount of brain present by extrapolating a semi-fit log line from the linear range of brain dilutions 10⁻⁵ to 10⁻⁸ (500 ng to 0.5 ng brain) (figure 11). Because our spleen samples were run under the same conditions as the brain, the equation of this line ($y = m \log(x) + b$) was used to relate y (the seeding rate of a spleen dilution) to x (equivalent brain (ng)). We know the LD₅₀ of our brain inoculum (10^{5.8}/g), and converted our solved brain equivalents (ng) to an LD₅₀/g spleen. Thus, the average amyloid seeding rates of our spleen dilutions at 10⁻³ contains ~2333 LD₅₀/g spleen (~10^{3.37} LD₅₀/g), which is two-fold less than the brain inoculum. We detected four-fold less than this in our spleen dilutions at 10⁻⁷.

The spleen develops fewer numbers of scrapie-associated fibrils (SAFs) compared to the brain, suggesting there is less prion burden within the spleen (Rubenstein et al. 1987; Rubenstein et al. 1991). Therefore, we next assessed brain tissue for prions by RT-QuIC.

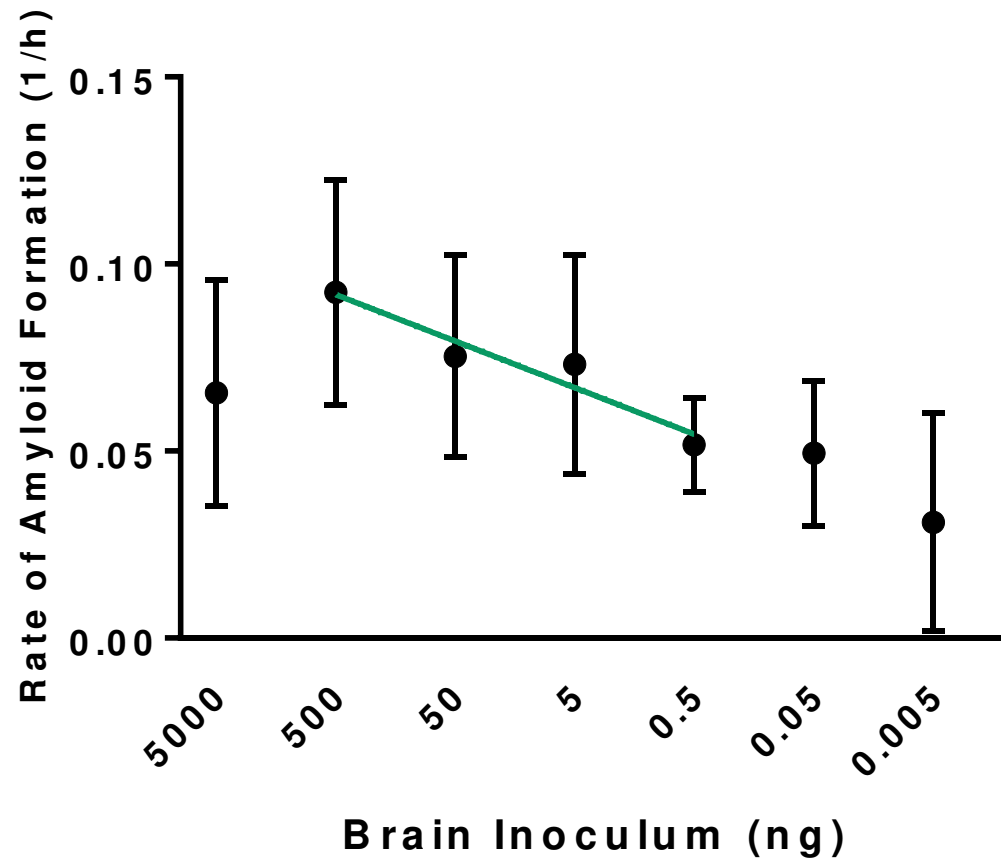


Figure 11. RT-QuIC Quantification of TME+ Brain Sample. RT-QuIC rates of a dilutional series of TME+ brain homogenate (inoculum) containing $10^{5.8}$ IC LD₅₀ /g. The solid green line represents a semi-fit log line extrapolated from the linear range (500 ng to 0.5 ng) and relates the amyloid formation rate (y) to amount brain present in the sample (ng) (x). Because brain and spleen were run under the same conditions, the equation of this line, $y = 0.01244 \log(x) + 0.05825$, was used to quantify the brain equivalent (ng) from a spleen seeding rate, which was then used to estimate LD₅₀.

Detection of prions in brain over time: RT-QuIC is commonly used to evaluate the presence of prions within the brain (Wilham et al. 2010; Shi et al. 2013; Orrú et al. 2015, Masujin et al. 2016), and can detect seeding activity as early as 90 days PI in mouse-adapted scrapie strains (Shi et al. 2013). In addition to evaluating the spleens of our study cohort animals, we wanted to compare seeding activity (positivity) in the brain to the spleen. Because TSEs ultimately accumulate in the brain to form a spongiform encephalopathy, we suspected that we would be able to detect prions within the brain tissue at mid- or late- disease.

We found positivity in the hind brain tissue (homogenates of cerebellum and brainstem) of 2/5 hamsters at late stage disease (155 - 169 days PI), when 3/5 animals were showing mild-to-moderate clinical signs of TSE disease. Seeding activity was detected in spleen in concentrations as low as 10^{-7} (~4.35 – 5.8 pg protein, $\sim 3.28 \times 10^{-4}$ LD₅₀ infectious doses) and in the brain at 10^{-5} (7.5 – 10 ng protein, ~133 LD₅₀ infectious doses). We did not evaluate brain dilutions higher than 10^{-5} . Additionally, prions were demonstrated in the spleen of one hamster (169 days PI hamster #2) that did not have detectable levels of seeding activity within the brain, providing supporting evidence of temporal splenic accumulation proceeding prion entry into nervous tissue (Shikiya et al. 2017).

Deposition of PrP^{Sc} in the spleen: Our collaborators have previously demonstrated IHC prion deposition within the splenic follicles of hamsters EN inoculated with HY-TME as early as 14 weeks PI (Kincaid and Bartz 2007). We were not able to visualize PrP^{RES} deposition within the splenic follicles, despite expecting to find this deposition in our late disease cohorts. In CWD, prions are detectable within the spleen and other LRS tissues by three months PI (Hoover et al. 2017). Prion deposition was found, post-mortem, in spleen and cervical lymph node of an

asymptomatic human infected with vCJD from a contaminated blood transfusion (Head et al. 2009; Diack et al. 2014). In scrapie, 36% of a dairy goat herd culled for scrapie surveillance possessed preclinical prion deposition, 94% of which was found within the LRS (Gonzales et al. 2009). This same study found that if an animal possessed more wide-spread prion deposition in the LRS, it increased the chances of prions accumulating within the brain (Gonzales et al. 2009). In BSE, prion deposition in the LRS is restricted to the gut-associated lymphoid tissue such as the Peyer's Patches, but has been found as early as three months PI (Kaatz et al. 2012).

We suspect that difference in tissue preservation may also impact IHC staining; our collaborators fix tissues with PLP for 5-7 hours before transferring tissues into 70% ethanol, whereas our lab fixes tissues in PLP for 2 days before transferring tissues into PBS. Additionally, because we did detect prion seeding activity in late-disease spleens with the more sensitive RT-QuIC assay, levels of prion accumulation within the spleen may have been present but below our level to detect with IHC.

Conclusions

Ultimately, our data supports previous work showing that the spleen is a site of prion accumulation, and validates the Syrian hamster as a system for peripheral prion infection when EN-inoculated with hamster-adapted HY-TME. It is important to understand the spleen's role in peripheral prion pathogenesis because this organ aids several major body systems: the circulatory system, LRS, and both the adaptive and cell-mediated arms of the immune system (Bacha 2006). Its ability to accumulate prions is reliant on several blood cell types (B cells, FDCs, and macrophages) (Bradford et al. 2017), which in turn can harbor prions (Mathiason et al. 2010; Edwards et al. 2010; Dassanayake et al. 2016) and have the capability to travel beyond the spleen

to the rest of the body. We know that prions can infiltrate the peripheral nervous system after accumulating within the spleen, but the role of the spleen in blood-borne prion pathogenesis, if any, remains unclear.

CHAPTER TWO: Blood-borne Prion Pathogenesis of Syrian Hamsters Experimentally Inoculated with HY-TME

Hypothesis

- We hypothesize that various blood cell phenotypes play a role in prion pathogenesis, and that these populations may change over the disease course.
- Aim: To determine which blood cell phenotypes are associated with or traffick prions in the hamster-adapted-HY TME system. We will examine PBMC, platelets, CD4+ T cells, CD8+ T cells, B cells, pan-granulocytes, pan-lymphocytes, and CD16+ cells harvested from whole blood of HY-inoculated hamsters minutes post infection through terminal TSE disease for prions using MACS cells sorting, flow cytometry, and RT-QuIC.

Introduction

The UK outbreak of BSE and subsequent cross-species transmission into humans triggered the consideration of blood as a player in prion pathogenesis. Researchers soon determined that the buffy coat and plasma could transmit prions (Hunter et al. 2002; Edwards et al. 2010), and that leukoreduction, which may account for 40% of blood infectivity, does not decrease the prion transmission within the plasma fraction (Gregori et al. 2004). The first cases of human-to-human vCJD transmission via blood transfusion were reported in 2004 from a blood donation that was made in 1996, three years before the donor developed symptoms of vCJD (Peden et al. 2004; Llewelyn et al. 2004; Head et al. 2009). Studies of CWD infection in white-tailed deer lend further support for the presence of blood-borne prions in preclinical and clinical donors (Mathiason et al. 2006).

We (Mathiason et al. 2010) revealed that B cells and platelets harvested from TSE-infected hosts harbor prion infectivity. We further detected prions in the blood of TME-infected hamsters and CWD-infected cervids throughout the entire disease course (Elder et al. 2015). Further support for cell-specific infectivity is evidenced in scrapie, where B cells, monocytes, T lymphocytes, and platelet-rich plasma harbor prions (Edwards et al. 2010; Dassanayake et al 2016). After peripheral inoculation, CD4 and CD8 T cells of scrapie-infected mice have infiltrated the blood-brain barrier into the hippocampus, cortex, and cerebellum (Lewicki et al. 2002), suggesting a mechanism for prion transmission from the blood, through the blood-brain barrier, to the brain.

To investigate the role various blood cell phenotypes play over the longitudinal course of prion disease, we collected blood from sham or HY-TME EM-inoculated hamsters described in chapter one of this thesis.

Materials and Methods

Blood collection: Blood was collected from hamsters inoculated in chapter one of this thesis prior to euthanasia via terminal anesthesia and cardiocentesis as approved by CSU IACUC (#16-6983A). We collected 4-6 mL of whole blood into a 12 cc syringe (with 22 gauge needle) pre-filled with 200 units heparin. Note that the older, larger hamsters (later in disease course) generally yielded more blood than the younger hamsters in more acute phases of disease. Hamsters were immediately euthanized by administration of 0.5 mL pentobarbitol euthanasia solution through the same needle as blood collection.

After gently inverting the syringe to mix the heparin with the blood, approximately 100 μ L of blood was transferred into Terumo™ Capiject™ brick red blood collection tube (Fisher Scientific) for CBC analysis at the Colorado State Diagnostic Lab in Fort Collins, CO; ~100 μ L

whole blood was saved into a 1 mL cryovial; the remaining blood (3-5 ml) was transferred into a 15 mL polypropylene conical tube (Falcon) and placed on ice before processing.

Blood was immediately processed for Magnetic Cell Sorting (MCS) and Flow Cytometry (FACS). Heparinized whole blood (3-4 mL) was centrifuged at $1000\times g$ for 15 minutes at 4°C . The platelet-rich plasma and buffy coat were drawn from the top of the resulting cell gradient, respectively, and immediately processed.

Plasma Processing: The plasma was centrifuged at $1000\times g$ for 15 minutes at room temperature to isolate platelets. The platelets were incubated with RBC lysing buffer (155 mM NH_4Cl , 12 mM NaHCO_3 , 0.1 mM EDTA) and washed twice with FACS buffer ($1\times$ PBS [130 mM NaCl , 7 mM $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$, 3 mM $\text{NaH}_2\text{PO}_4\cdot 1\text{H}_2\text{O}$], 1% fetal bovine serum [FBS, Gibco], 10 mM EDTA). Between each wash, the platelets were centrifuged at $1000\times g$ for 5 minutes at room temperature. The final platelet-rich cell pellet was re-suspended in 500 μL FACS buffer. This suspension was counted with Countess™ Cell Counter (Fisher Scientific), and viability percentage and total number of live cells per mL recorded (Appendix C). The platelets and frozen at -80°C for later RT-QuIC analysis.

Buffy Coat Processing: The buffy coat was diluted 1:1 with $1\times$ tissue culture PBS (tcPBS) (1:10 Dulbecco's Phosphate Buffered Saline [Sigma] in Sigma Water, filtered sterilized). Room temperature Histopaque 1083 (Sigma Aldrich) was slowly layered beneath the diluted whole blood using a 3mL syringe and 18g needle. This suspension was centrifuged at $1000\times g$ at room temperature for 15 minutes on low brake. The top supernatant was discarded and the cell band collected. The cell band was incubated with RBC lysing buffer and twice with FACS buffer following the same protocol as the plasma processing. The resulting cell pellet was

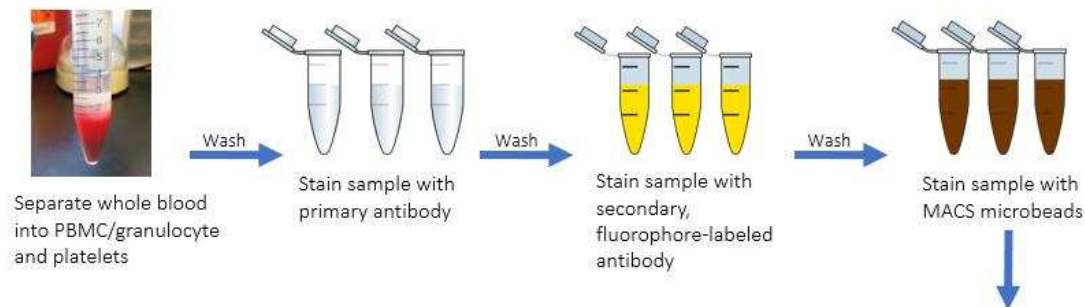
re-suspended in 1000 μ L FACS buffer for immediate cell staining and sorting, 100 μ L of which was saved and stored at -80°C for further analysis.

Cell Staining: During cell staining (Figure 12), all centrifugations occurred at 1000 $\times$ g for five minutes at room temperature, and all washes were performed twice with 0.5 mL FACS buffer, with supernatant removed via pipetting. 100 μ L of the PBMC cell suspension (1/10 of collected cells, an average of 58×10^6 cells, see table in Appendix C) was placed in a 2 mL eppendorf tube containing 14 μ L FBS (Gibco), 100 μ L FACS buffer, and 1 μ g of the specific cell phenotype primary antibody of interest (Table 1). The cells were incubated for 30 minutes in the dark at 4°C. The cells were then centrifuged and washed before the resulting cell pellet was re-suspended with 100 μ L of the appropriate secondary antibody (Table 2), at 1:4000 diluted in PBS, and allowed to incubate for 30 minutes in the dark at 4°C. Cells were centrifuged and washed before incubating with the secondary antibodies, at 1:4000 diluted in PBS, for 15 minutes in the dark at 4°C. Cells were washed again before the resulting pellet was re-suspended in 100 μ L MACS buffer (final concentration 0.5% BSA, Miltenyi Biotec) mixed with 20 μ L Anti-Cy5/Anti-Alexa Fluor 647 Microbeads (Miltenyi Biotec). These magnetic beads are specific for the fluorophore Alexa 647 or PE/Cy5, and bind to the secondary antibody attached to our cells of interest. The beads incubated with the cell suspension for 10 minutes in the dark at 4°C. Each sample was then washed and the cell pellet was re-suspended in 1000 μ L of MACS buffer for MACS cell sorting.

Miltenyi MACS sorting: Positive cell sorting was performed per MACS protocol (Miltenyi Biotec) using LS, anti-Alexa Fluor 647 columns. Briefly, each column was placed on

the MACS magnet and flushed with 2 mL of cold MACS buffer. Each sample was then passed through the column four times (eluate collected to re-run over the column), with cells of interest remaining bound to the column via the magnet. The column was flushed with 1.5 mL MACS buffer to remove all unlabeled cells (cell populations not of interest). The column was removed from the magnet, and 1 mL of MACS buffer was used to flush the sample from the column. The resulting 1 mL isolated cell populations were placed in flow cytometry tubes for FACS analysis to determine purity of samples.

Flow cytometric (FACS) analysis: Samples were run on a Gallios flow cytometer (Beckman Coulter) gated on hamster PBMCs and granulocytes, and set to a channel that collected light emission of the fluorophore of the secondary antibody (either Alexa Fluor 647 or PE/Cy5—see Table 2). Forward and side scatter information was collected in addition to fluorophore detection. Samples collected for 120 seconds or until 10,000 cells were analyzed. Unstained cell controls from each hamster were run with the samples from every hamster. Using FlowJo software (LLC and Illumina), the unstained control was used to set a threshold for fluorophore-positive emission; fluorophore-positive cells were our cell population of interest. This data was used to determine the percentage of cells of interest in the sample (“isolate purity”).



Positive-Selection MACS Sorting

1. Place LS columns on magnet
2. Flush column with MACS buffer
3. Run sample over column (save eluate to run over column 3x more)
4. Remove column from magnet
5. Flush sample from column into flow cytometry tube

The illustration shows a hand placing a sample tube into a MACS Multi Stand. The stand has four orange sample tubes and one purple elution tube. To the left, a small inset shows the steps of the MACS sorting process: 1. Placing the LS column on the magnet, 2. Flushing the column with MACS buffer, 3. Running the sample over the column, 4. Removing the column from the magnet, and 5. Flushing the sample into a flow cytometry tube.

<http://www.miltenyibiotec.com>

Figure 12. Diagram of Cell Staining and MACS sorting. Whole blood was processed via centrifugation and Histopaque gradient into platelet-rich plasma and PBMC/granulocyte components. Aliquots of PBMC/granulocytes were stained consecutively with primary specific cell-type antibody, secondary, fluorophore-labeled antibody, and MACS microbeads. Samples were then run four times over the MACS magnetic column for positive cell selection. Samples were removed from the column and flushed into a flow cytometry tube for FACS analysis.

Cell Counts: Before storage at -80°C for later analysis by RT-QuIC, 5 µL of each purified population, as well as the PBMC and platelet isolates, was gently pipetted onto unifrost plus microscope slides (VWR) and stained with Shandon™ KwikDiff stain (Fisher Scientific) for future cytological analysis. These samples were counted with the Countess™ Cell Counter (Fisher Scientific), recording viability percentage and total number of live cells per mL.

Results

CBC: CBC data (Appendix A) was analyzed in Excel (Microsoft, Inc) with unpaired T-tests between sham and hamster adapted HY-TME EN-inoculated hamster cohorts. No trends or statistically significant differences in the cell profiles between the TME+ and sham groups were noted (figure 15). Our results support the current dogma that prion infections are not inflammatory due to being disorders of self-proteins.

Isolated populations: Across the experimental time points, the Pan-Lymphocyte antibody conjugated to Alexa Fluor 647 bound most strongly to the cell samples and provided the highest purity after MACS sorting (up to 96%). Average purities (Appendix B): CD4: 52.09%; CD8: 54.57%; B cell: 53.28%; Lymphocyte: 72.92%; CD16: 58.04%. *Cell counts* (Appendix C): The average total cell counts for PBMC fraction was: 5.8×10^8 cells/mL; platelets: 4.24×10^8 cells/mL; and isolated lymphocytes: 2.6×10^7 cells/mL. *Cytology:* PBMC isolates had the best visualization after KwikDiff staining, likely due to the increased cell count associated with this fraction. The platelet and other isolated populations had very low cellularity with pale staining and were difficult to visualize. PBMC fractions consisted of uniform cells with a single, dense, large nucleus and minimal cytoplasm, consistent with PBMCs (Figure 13).

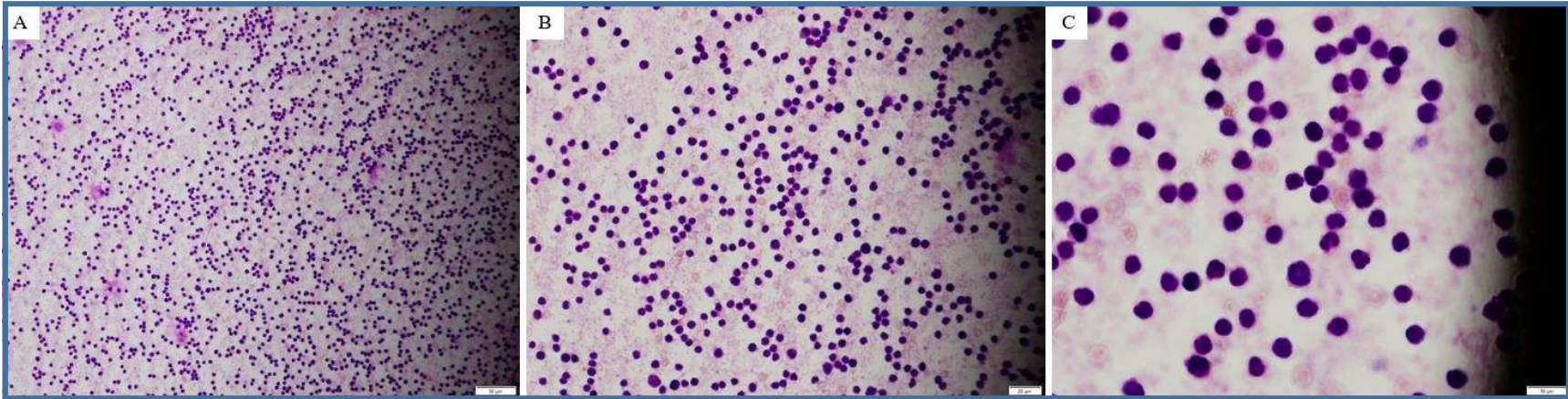


Figure 13. Cytology of PBMC. The data shows representative cytology of peripheral blood mononuclear cells (PBMCs). A) 50 μm . Highly cellular sample. Primarily small, with some large, basophilic cells with a large, dense nucleus and minimal cytoplasm, on an eosinophilic background. B) 20 μm . Few cells with segmented nucleus (granulocytes) are present among the PBMCs. C) 10 μm . Majority of cells are $\sim 10 \mu\text{m}$ diameter and have single, dense, dark nucleus with minimal eosinophilic cytoplasm (lymphocytes). Few granulocytes (neutrophils and rare basophil) are present. Few pale eosinophilic cells with no nucleus (red blood cells) present in sample.

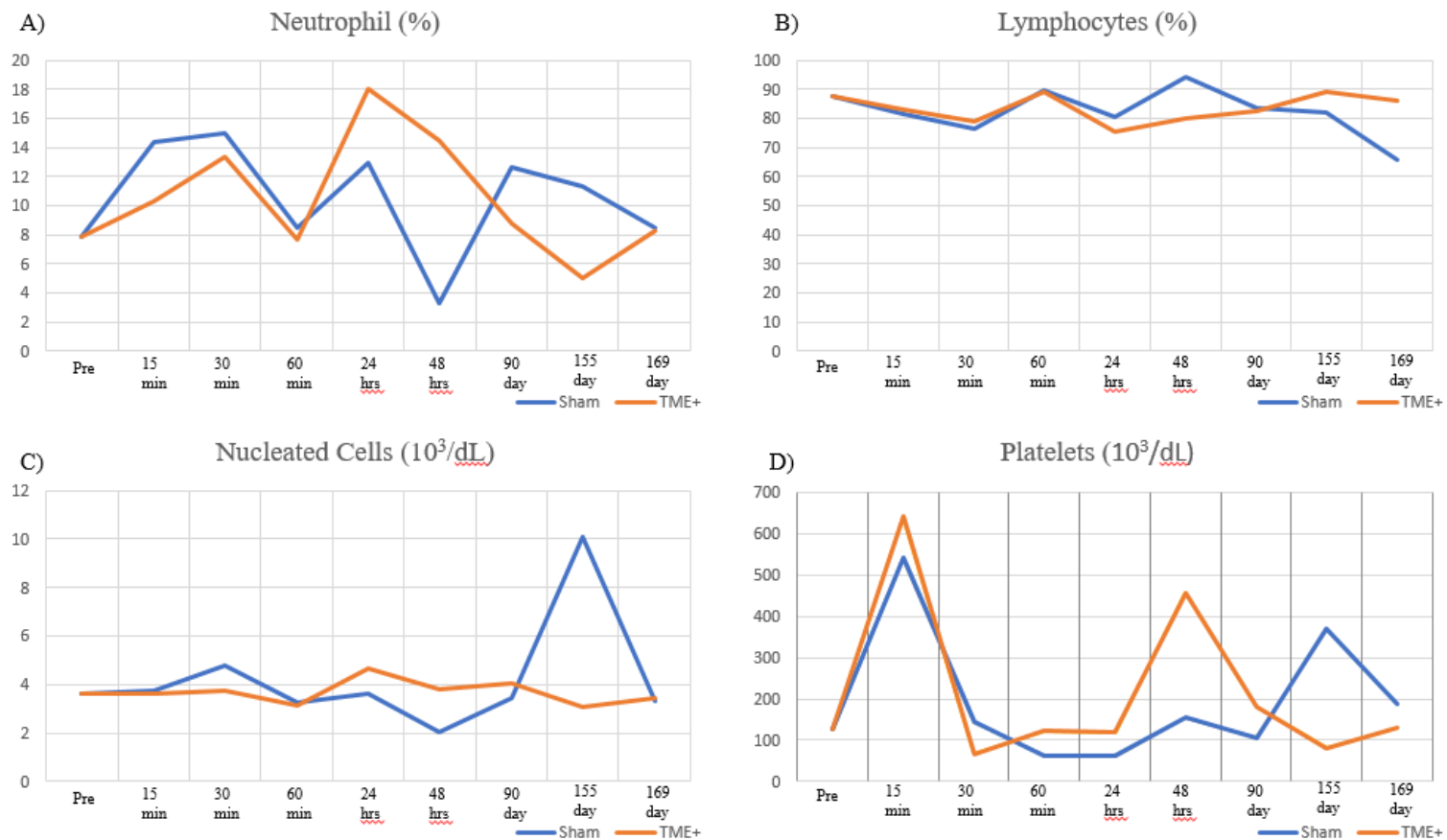


Figure 14. Longitudinal Neutrophil, Lymphocyte, Nucleated Cell, and Platelet Trends. Data represents averages of selected trends from the complete blood count profile over time, including A) neutrophil percentage, B) total lymphocyte percentage, C) nucleated cells ($10^3/\text{dL}$) and, D) total platelets ($10^3/\text{dL}$). CBC values from the HY-TME EN-inoculated hamsters trend with their sham EN-inoculated counterparts. There was no statistical difference between the HY-TME and sham-inoculated groups.

Table 1			ANTIBODY CHART	
Antibody	Staining For	Subclass	Secondary Antibody Used	Vendor / Host
HAB1A	CD4+ cells	IgG1	F(ab') ₂ -Goat anti-Mouse IgM Cross Absorbed Secondary Antibody (Thermo Fisher Scientific #31186) pre-conjugated to Alexa Fluor 647	WSU Monoclonal Antibody Center (S-GT2002) Host: Mouse
MRC OX-8	CD8+ cells	IgG1	F(ab') ₂ -Goat anti-Mouse IgM Cross Absorbed Secondary Antibody (Thermo Fisher Scientific #31186) pre-conjugated to Alexa Fluor 647	Thermo Fisher Scientific (MA1-70003) Host: Mouse
HASA7A	B Cells	IgG1	F(ab') ₂ -Goat anti-Mouse IgM Cross Absorbed Secondary Antibody (Thermo Fisher Scientific #31186) pre-conjugated to Alexa Fluor 647	WSU Monoclonal Antibody Center (HM2007) Host: Mouse
HASA26B	Pan Granulocyte	IgM	Goat Anti-Mouse IgM H&L (Abcam #9167) conjugated to Lightning-Link® PE/Cy5 (Innova Biosciences)	WSU Monoclonal Antibody Center (HM2013) Host: Mouse
HAB6B	Pan Lymphocyte	IgG2a	F(ab') ₂ -Goat anti-Mouse IgM Cross Absorbed Secondary Antibody (Thermo Fisher Scientific #31186) pre-conjugated to Alexa Fluor 647	WSU Monoclonal Antibody Center (HM2009) Host: Mouse
ASH 1975	CD16+ cells	IgG2a	F(ab') ₂ -Goat anti-Mouse IgM Cross Absorbed Secondary Antibody (Thermo Fisher Scientific #31186) pre-conjugated to Alexa Fluor 647	Thermo Fisher Scientific (MA1-7633) Host: Mouse

Discussion

Blood cells associated with prions circulate throughout the body (Mathiason et al. 2010; Edwards et al. 2010; Dassanayake et al. 2016), and could contribute to the spread of prions through the body, and as little as 100 μ L whole blood can transmit scrapie from an asymptomatic donor to a recipient sheep (Douet et al. 2016). RT-QuIC is a sensitive and rapid assay for prion detection (Orrú et al. 2015), which we have previously used to demonstrate a prionemia within whole blood of our hamster system throughout disease course (Elder et al. 2015). In this study we sought to isolate blood cell types of interest from whole blood of hamsters EN-inoculated with HY-TME, and investigate the role these cell phenotypes play over the longitudinal course of prion disease using IOB-RT-QuIC.

Using centrifugation and MACS cell sorting (Miltenyi Biotec), we sorted whole blood into PBMC, platelets, CD4+ T cell, CD8+ T cell, B cell, Granulocyte, Lymphocyte, and CD16+ (natural killer cells, polymorphonuclear leukocytes, monocytes, and macrophages) populations. We used flow cytometry to evaluate the purity of isolated populations, and saved these isolates for future analysis with RT-QuIC. The Pan-Lymphocyte population sorted with the highest purity, and is our prime candidate for future analysis with RT-QuIC seeding assay, along with the PBMC and platelet fractions. This analysis will enable us to learn which cell phenotypes are positively associated with prions in our model, but not whether prion amplification is occurring within these cells.

We have shown that B cells from CWD-positive white-tail deer are infective in mouse bioassay (Mathiason et al. 2010). B cells, as well as FDCs, macrophages, and T cells, are closely associated with the white pulp of the spleen (Bradford et al. 2017). Not only can they harbor prions (and do, as demonstrated in WTD and Scrapie) (Mathiason et al. 2010; Edwards et al.

2010; Dassanayake et al. 2016) they may be able to enter the bloodstream, contributing to blood-borne prion pathogenesis.

It is important to determine the role these blood cell types play during prion infection (whether simply harboring prions, or amplifying them) to understand how blood-borne prions impact the infectivity and spread of TSEs, and the interplay between these cells and other sites of peripheral prion accumulation such as the spleen.

FUTURE DIRECTIONS

In future studies, we would like to examine the blood components that we collected from these hamsters (whole blood, PBMCs, platelets, CD4+ cells, CD8+ cells, B cells, lymphocytes, granulocytes, and CD16+ cells) for a similar temporal pattern associated with prions throughout the disease course.

Looking back

- Although spleen is often run at a dilution of 10^{-2} (a 10% dilution of the 10% homogenate) both with and without iron oxide beads, I suspect that when there is a heavier burden of prion within the spleen, the ability of RT-QuIC to detect at 10^{-2} becomes inconsistent. It may be most efficient to run spleen samples at a further dilution of 10^{-3} ; this has the added benefit of using less sample to run more plates, and the samples can be stored at 10^{-2} in PBS.
- IOB samples require a **lot** of sample, about 5x more than running NAPTA. For my experiment, I would add 50ul of 10% homogenate into 450 μ L of PBS. However, I detected positivity in the spleen with adding 25 μ L homogenate into 475 μ L, and 10uL homogene into 490uL PBS. These lower concentrations decrease detectability but use less sample.
- We may have seen variability in spleen positivity with RT-QuIC based upon the composition of each homogenate. When our collaborators have found prion positivity in spleens (Kincaid and Bartz 2007), there were few positive follicles per spleen section. The spleen homogenates are made with the entire saved spleen

section, however variability in how spleens were cut during initial tissue collection, or when pipetting from the homogenate tube, may mean that some samples are richer in follicles (and therefore, positivity) than others. Additionally, when saving tissue for later analysis, I would recommend freezing the largest sections possible for later use in RT-QuIC homogenates. Central sections, closer to the arteries, may yield more white pulp and therefore more positivity.

- In the blood-borne section of this project, isolate purity is a primary concern. We may have seen such low purities do to the nature of the MACS beads and the overall lack of hamster-specific reagents. In this project, the blood cell isolation required several steps of incubation: incubate the cells with a primary antibody, then a secondary antibody attached to a fluorophore (Alexa 647 or PE/Cy5), and finally with the MACS beads that bind to Alexa 647 and PE/Cy5. The cell sorting columns would bind with the tagged fluorophore, rather than primarily with the cell type of interest. Therefore, there is opportunity in each step to lose purity or suffer decreased binding. In future, if a sorting mechanism could directly target each hamster cell type without requiring the additional incubation steps and reagents, I think the purity of the isolates could be vastly improved.
- Additionally, due to the nature of this sorting system, each isolate was tagged to the same fluorophore and sorted individually. If columns could detect the specific cell types or if different fluorophores could be used, the whole blood or PBMCs might be able to be run over a series of subsequent columns.
- The Pan Granulocyte conjugated to PE/Cy5 does work well on its own, but does not interact with the MACS columns well. The average isolate purity was ~70%.

In future studies, it may be beneficial to carefully collect the granulocyte layer (beneath the PBMC layer and above the RBCs layer after gradient separation), rather than trying to sort them out of PBMCs using the antibody.

- The way our fixed tissues were collected works well for RT-QuIC, but potentially affected how well the spleens stained with IHC. If IHC is the primary assay in future studies, altering preservation protocol to mirror our collaborators (using PLP followed by 70% ethanol) may result in cleaner, more appropriate IHC staining.

Heading forward

- For this project, spleens were run with RT-QuIC using iron oxide beads. However preliminary work reveals that NAPTA precipitating the spleen samples also can increase detection. It could be interesting to repeat our spleen scans with NAPTA instead of IOB in search of increased detectability at earlier timepoints. Additionally, collecting the spleen near arteries to target white pulp, or specifically targeted splenic PBMCs, may yield better detection.
- Taken what we learned about isolating blood cells, I would like to see this project repeated with a different isolation methodology. Cervidized or Scrapie Tg mice will be beneficial to this end, because more reagents are available for mice rather than hamsters.
- I would save pure plasma rather than spinning down and trying to isolate platelets (the cell pellet formed from hamsters is negligible compared to that formed from cervids). However, I did save platelets, PBMCs, and whole blood from every

hamster (except not whole blood from the 90 day hamsters) that could be evaluated with RT-QuIC going forward.

- We saved almost every tissue from the hamster, and these valuable tissues (such as brain, peyer's patches, submandibular lymph nodes, intestines, etc) are available for further explorations with RT-QuIC and other assays.
- Although late disease brains were analyzed with RT-QuIC both with and without beads, few replicates of these samples were run. In future, more replicates, as well as samples from mid-disease and early-disease hamsters, should be analyzed.

REFERENCES

- Abbas, Abul K., and Andrew H. Lichtman. "Introduction to the Immune System." *Basic Immunology Functions and Disorders of the Immune System*. Philadelphia: Saunders, 2009. 15. Print.
- Acevedo-Morantes, Claudia, and Holger Wille. "The Structure of Human Prions: From Biology to Structural Models — Considerations and Pitfalls." *Viruses* 6.10 (2014): 3875-892. Print.
- Aguilar-Calvo, Patricia, Consolación García, Juan Carlos Espinosa, Olivier Andreoletti, and Juan María Torres. "Prion and Prion-like Diseases in Animals." *Virus Research* 207 (2015): 82-93. Print.
- Aguzzi, Adriano, and Mathias Heikenwalder. "Pathogenesis of Prion Diseases: Current Status and Future Outlook." *Nature Reviews Microbiology* 4.10 (2006): 765-75. Print.
- Alfaidy, Nadia, Sylvain Chauvet, Sandrine Donadio-Andrei, Aude Salomon, Yasmina Saoudi, Pierre Richaud, Catherine Aude-Garcia, Pascale Hoffmann, Annie Andrieux, Jean-Marc Moulis, Jean-Jacques Feige, and Mohamed Benharouga. "Prion Protein Expression and Functional Importance in Developmental Angiogenesis: Role in Oxidative Stress and Copper Homeostasis." *Antioxidants & Redox Signaling* 18.4 (2013): 400-11. Print.
- Alper, Tikvah, D.a. Haig, and M.c. Clarke. "The Exceptionally Small Size of the Scrapie Agent." *Biochemical and Biophysical Research Communications* 22.3 (1966): 278-84. Print.
- Alpers, M. P. "The Epidemiology of Kuru: Monitoring the Epidemic from Its Peak to Its End." *Philosophical Transactions of the Royal Society B: Biological Sciences* 363.1510 (2008): 3707-713. Print.

- Ayers, J. I., A. E. Kincaid, and J. C. Bartz. "Prion Strain Targeting Independent of Strain-Specific Neuronal Tropism." *Journal of Virology* 83.1 (2008): 81-87. Print.
- Bacha, Linda M. "Lymphatic System." *Color Atlas of Veterinary Histology*. By William J. Bacha. 2nd ed. Ames, Iowa: Blackwell, 2006. 69-71. Print.
- Baron, Thierry, Anna Bencsik, Anne-Gaëlle Biacabe, Eric Morignat, and Richard A. Bessen. "Phenotypic Similarity of Transmissible Mink Encephalopathy in Cattle and L-type Bovine Spongiform Encephalopathy in a Mouse Model." *Emerging Infectious Diseases* 13.12 (2007): 1887-894. Print.
- Bartz, J. C., R. A. Bessen, D. McKenzie, R. F. Marsh, and J. M. Aiken. "Adaptation and Selection of Prion Protein Strain Conformations following Interspecies Transmission of Transmissible Mink Encephalopathy." *Journal of Virology* 74.12 (2000): 5542-547. Print.
- Bartz, J. C., C. Dejoia, T. Tucker, A. E. Kincaid, and R. A. Bessen. "Extraneural Prion Neuroinvasion without Lymphoreticular System Infection." *Journal of Virology* 79.18 (2005): 11858-1863. Print.
- Bartz, J. C., A. E. Kincaid, and R. A. Bessen. "Retrograde Transport of Transmissible Mink Encephalopathy within Descending Motor Tracts." *Journal of Virology* 76.11 (2002): 5759-768. Print.
- Beekes, Michael, and Patricia A. McBride. "The Spread of Prions through the Body in Naturally Acquired Transmissible Spongiform Encephalopathies." *FEBS Journal* 274.3 (2007): 588-605. Print.
- Belay, Ermias D., Ryan A. Maddox, Elizabeth S. Williams, Michael W. Miller, Pierluigi Gambetti, and Lawrence B. Schonberger. "Chronic Wasting Disease and Potential Transmission to Humans." *Emerging Infectious Diseases* 10.6 (2004): 977-84. Print.

- Belay, Ermias D., and Lawrence B. Schonberger. "The Public Health Impact Of Prion Diseases." *Annual Review of Public Health* 26.1 (2005): 191-212. Print.
- Benestad, S. L., P. Sarradin, B. Thu, J. Schonheit, M. A. Tranulis, and B. Bratberg. "Cases of Scrapie with Unusual Features in Norway and Designation of a New Type, Nor98." *Veterinary Record* 153.7 (2003): 202-08. Print.
- Benestad, Sylvie L., Jean-Noël Arsac, Wilfred Goldmann, and Maria Nafstad. "Atypical/Nor98 Scrapie: Properties of the Agent, Genetics, and Epidemiology." *Veterinary Research* 39.4 (2008): 19. Web.
- Benestad, Sylvie L., et al. "Healthy Goats Naturally Devoid of Prion Protein." *Veterinary Research*, vol. 43, no. 1, 18 Dec. 2012.
- Benestad, Sylvie L., Gordon Mitchell, Marion Simmons, Bjørnar Ytrehus, and Turid Vikøren. "First Case of Chronic Wasting Disease in Europe in a Norwegian Free-ranging Reindeer." *Veterinary Research* 47.1 (2016). Print.
- Bessen, Richard A., and Richard F. Marsh. "Biochemical and Physical Properties of the Prion Protein from Two Strains of Transmissible Mink Encephalopathy Agent." *Journal of Virology* 4th ser. 66 (1992): 2096-101. Print.
- Bessen, R. A., and R. F. Marsh. "Identification of Two Biologically Distinct Strains of Transmissible Mink Encephalopathy in Hamsters." *Journal of General Virology* 73.2 (1992): 329-34. Print.
- Bessen, Richard A., Cameron J. Robinson, Davis M. Seelig, Christopher P. Watschke, Diana Lowe, Harold Shearin, Scott Martinka, and Alex M. Babcock. "Transmission of Chronic Wasting Disease Identifies a Prion Strain Causing Cachexia and Heart Infection in Hamsters." *PLoS ONE* 6.12 (2011). Print.

- Blatter, Thomas, Sebastian Bradner, Alex J. Raeber, Michael A. Klein, Till Voigtlander, Charles Weissmann, and Adriano Aguzzi. "PrP-expressing Tissue Required for Transfer of Scrapie Infectivity from Spleen to Brain." *Nature* 389 (1997): 69-73. Print.
- Bolton, D., M. Mckinley, and S. Prusiner. "Identification of a Protein That Purifies with the Scrapie Prion." *Science* 218.4579 (1982): 1309-311. Print.
- Bougard, D., J.-P. Brandel, M. Belondrade, V. Beringue, C. Segarra, H. Fleury, J.-L. Laplanche, C. Mayran, S. Nicot, A. Green, A. Welaratne, D. Narbey, C. Fournier-Wirth, R. Knight, R. Will, P. Tiberghien, S. Hai K, and J. Coste. "Detection of Prions in the Plasma of Presymptomatic and Symptomatic Patients with Variant Creutzfeldt-Jakob Disease." *Science Translational Medicine* 8.370 (2016). Print.
- Bradford, B. M., Reizis, B., & Mabbott, N. A. (2017, 03). Oral Prion Disease Pathogenesis Is Impeded in the Specific Absence of CXCR5-Expressing Dendritic Cells. *Journal of Virology*, 91(10). doi:10.1128/jvi.00124-17
- Brown, P., L. Cervenáková, L.m. Mcshane, P. Barber, R. Rubenstein, and W.n. Drohan. "Further Studies of Blood Infectivity in an Experimental Model of Transmissible Spongiform Encephalopathy, with an Explanation of Why Blood Components Do Not Transmit Creutzfeldt-Jakob Disease in Humans." *Transfusion* 39.11-12 (1999): 1169-178. Print.
- Brown, P., Rg Rohwer, Bc Dunstan, C. Macauley, Dc Gajdusek, and Wn Drohan. "The Distribution of Infectivity in Blood Components and Plasma Derivatives in Experimental Models of Transmissible Spongiform Encephalopathy." *Transfusion* 38.9 (1998): 810-16. Print.

- Büeler, H., Aguzzi, A., Sailer, A., Greiner, R., Autenried, P., Aguet, M., & Weissmann, C. (1993, 07). Mice devoid of PrP are resistant to scrapie. *Cell*, 73(7), 1339-1347. doi:10.1016/0092-8674(93)90360-3
- Chen, Cao, and Xiao-Ping Dong. "Epidemiological Characteristics of Human Prion Diseases." *Infectious Diseases of Poverty* 5.1 (2016): n. pag. Web.
- Clouse, Melissa D., Ronald A. Shikiya, Jason C. Bartz, and Anthony E. Kincaid. "Nasal Associated Lymphoid Tissue of the Syrian Golden Hamster Expresses High Levels of PrPC." *Plos One* 10.2 (2015).
- Colby, D. W., and S. B. Prusiner. "Prions." *Cold Spring Harbor Perspectives in Biology* 3.1 (2011). Print.
- Collinge, John, Katie C. L. Sidle, Julie Meads, James Ironside, and Andrew F. Hill. "Molecular Analysis of Prion Strain Variation and the Aetiology of 'new Variant' CJD." *Nature* 383.6602 (1996): 685-90. Print.
- Collinge, John. "Variant Creutzfeldt-Jakob Disease." *The Lancet* 354.9175 (1999): 317-23. Print.
- Cook, R., J. Bingham, A. Besier, C. Bayley, M. Hawes, P. Shearer, M. Yamada, J. Bergfeld, D. Williams, and D. Middleton. "Atypical Scrapie in Australia." *Australian Veterinary Journal* 94.12 (2016): 452-55. Print.
- Dassanayake, Rohana P., Sally A. Madsen-Bouterse, Thomas C. Truscott, Dongyue Zhuang, Michelle R. Mousel, William C. Davis, and David A. Schneider. "Classical Scrapie Prions Are Associated with Peripheral Blood Monocytes and T-lymphocytes from Naturally Infected Sheep." *BMC Veterinary Research* 12.1 (2016).
- Denkers, Nathaniel D., Davin M. Henderson, Candace K. Mathiason, and Edward A. Hoover. "Enhanced Prion Detection in Biological Samples by Magnetic Particle Extraction and

- Real-time Quaking-induced Conversion." *Journal of General Virology* 97.8 (2016): 2023-029. Print.
- Diack, Abigail B., Mark W. Head, Sandra Mccutcheon, Aileen Boyle, Richard Knight, James W. Ironside, Jean C. Manson, and Robert G. Will. "Variant CJD." *Prion* 8,4 (2014): 2886-95. Web.
- Douet, Jean-Yves, et al. "Mononucleated Blood Cell Populations Display Different Abilities To Transmit Prion Disease by the Transfusion Route." *Journal of Virology*, vol. 90, ser. 2016, pp. 3439–3445. 2016. Edwards, Jane C., S. Jo Moore, Jeremy A. Hawthorn, Michael H. Neale, and Linda A. Terry. "PrPSc Is Associated with B Cells in the Blood of Scrapie-infected Sheep." *Virology* 405.1 (2010): 110-19. Print.
- Eklund, C. M., R. C. Kennedy, and W. J. Hadlow. "Pathogenesis of Scrapie Virus Infection in the Mouse." *Journal of Infectious Diseases* 117.1 (1967): 15-22. Print.
- Elder, Alan M., Davin M. Henderson, Amy V. Nalls, Jason M. Wilham, Byron W. Caughey, Edward A. Hoover, Anthony E. Kincaid, Jason C. Bartz, and Candace K. Mathiason. "In Vitro Detection of Prionemia in TSE-Infected Cervids and Hamsters." *PLoS ONE* 8.11 (2013). Print.
- Garrett, Reginald H. *Biochemistry*. 2nd ed. N.p.: Saunders, 1999. Print.
- Georgsson, G., S. Sigurdarson, and P. Brown. "Infectious Agent of Sheep Scrapie May Persist in the Environment for at Least 16 Years." *Journal of General Virology* 87.12 (2006): 3737-740. *PubMed*. Web. 14 July 2017.
- Giles, Kurt, Steven H. Olson, and Stanley B. Prusiner. "Developing Therapeutics for PrP Prion Diseases." *Cold Spring Harbor Perspectives in Medicine* 7.4 (2017).

González, L., J.I. Pitarch, S. Martin, L. Thurston, H. Simmons, C. Acín, and M. Jeffrey.

"Influence of Polymorphisms in the Prion Protein Gene on the Pathogenesis and Neuropathological Phenotype of Sheep Scrapie after Oral Infection." *Journal of Comparative Pathology* 150.1 (2014): 57-70. Print.

González, Lorenzo, Stuart Martin, Sílvia Sisó, Timm Konold, Angel Ortiz-Peláez, Laura Phelan, Wilfred Goldmann, Paula Stewart, Ginny Saunders, Otto Windl, Martin Jeffrey, Stephen A.c. Hawkins, Michael Dawson, and James Hope. "High Prevalence of Scrapie in a Dairy Goat Herd: Tissue Distribution of Disease-associated PrP and Effect OfPRNPgenotype and Age." *Veterinary Research* 40.6 (2009): 65. Print.

Gregori, Luisa, Nancy Mccombe, Douglas Palmer, Paul Birch, Samuel O. Sowemimo-Coker, Antonio Giulivi, and Robert G. Rohwer. "Effectiveness of Leucoreduction for Removal of Infectivity of Transmissible Spongiform Encephalopathies from Blood." *The Lancet* 364.9433 (2004): 529-31. Print.

Griffith, J. S. "Nature of the Scrapie Agent: Self-replication and Scrapie." *Nature* 215.5105 (1967): 1043-044. Print.

Haley, N. J., C. K. Mathiason, S. Carver, M. Zabel, G. C. Telling, and E. A. Hoover. "Detection of Chronic Wasting Disease Prions in Salivary, Urinary, and Intestinal Tissues of Deer: Potential Mechanisms of Prion Shedding and Transmission." *Journal of Virology* 85.13 (2011): 6309-318. Print.

Hamir, A. N., R. A. Kunkle, J. M. Miller, and S. M. Hall. "Abnormal Prion Protein in Ectopic Lymphoid Tissue in a Kidney of an Asymptomatic White-tailed Deer Experimentally Inoculated with the Agent of Chronic Wasting Disease." *Veterinary Pathology* 43.3 (2006): 367-69. Print.

- Hartsough, G. R., and D. Burger. "Encephalopathy of Mink: I. Epizootiologic and Clinical Observations." *Journal of Infectious Diseases* 115.4 (1965): 387-92. Print.
- Head, M. W., H. M. Yull, D. L. Ritchie, M. T. Bishop, and J. W. Ironside. "Pathological Investigation of the First Blood Donor and Recipient Pair Linked by Transfusion-associated Variant Creutzfeldt-Jakob Disease Transmission." *Neuropathology and Applied Neurobiology* 35.4 (2009): 433-36. Web.
- Henderson, D. M., K. A. Davenport, N. J. Haley, N. D. Denkers, C. K. Mathiason, and E. A. Hoover. "Quantitative Assessment of Prion Infectivity in Tissues and Body Fluids by Real-time Quaking-induced Conversion." *Journal of General Virology* 96.Pt_1 (2014): 210-19. Print.
- Henderson, Davin M., Matteo Manca, Nicholas J. Haley, Nathaniel D. Denkers, Amy V. Nalls, Candace K. Mathiason, Byron Caughey, and Edward A. Hoover. "Rapid Antemortem Detection of CWD Prions in Deer Saliva." *PLoS ONE* 8.9 (2013). Hill, Andrew F., Melanie Desbruslais, Susan Joiner, Katie C. L. Sidle, Ian Gowland, and John Collinge. "The Same Prion Strain Causes VCJD and BSE." *Nature* 389 (1997): 448-50. Print.
- Hoover, Clare E., Kristen A. Davenport, Davin M. Henderson, Laura A. Pulscher, Candace K. Mathiason, Mark D. Zabel, and Edward A. Hoover. "Detection and Quantification of CWD Prions in Fixed Paraffin Embedded Tissues by Real-Time Quaking-Induced Conversion." *Scientific Reports* 6.1 (2016). Print.
- Hoover, Clare E., Kristen A. Davenport, Davin M. Henderson, Nathaniel D. Denkers, Candace K. Mathiason, Claudio Soto, Mark D. Zabel, and Edward A. Hoover. "Pathways of Prion Spread during Early Chronic Wasting Disease in Deer." *Journal of Virology* 91.10 (2017). Print.

- Hunter, Nora, Calum Mackenzie, Fiona Houston, David Parnham, Samantha Eaton, Angela Chong, Sandra Mccutcheon, and James Foster. "Transmission of Prion Diseases by Blood Transfusion." *Journal of General Virology* 83.11 (2002): 2897-905. Print.
- Kaatz, Martin, Christine Fast, Ute Ziegler, Anne Balkema-Buschmann, Bärbel Hammerschmidt, Markus Keller, Anja Oelschlegel, Leila McIntyre, and Martin H. Groschup. "Spread of Classic BSE Prions from the Gut via the Peripheral Nervous System to the Brain." *The American Journal of Pathology* 181.2 (2012): 515-24. Print.
- Kaesler, P. S., M. A. Klein, P. Schwarz, and A. Aguzzi. "Efficient Lymphoreticular Prion Propagation Requires PrP^c in Stromal and Hematopoietic Cells." *Journal of Virology* 75.15 (2001): 7097-106. Kim, S., Han, S., Lee, H. S., Kim, Y., Choi, E., & Kim, M. (2016, 08). Impaired spleen structure and chemokine expression in ME7 scrapie-infected mice. *Immunobiology*, 221(8), 871-878. doi:10.1016/j.imbio.2016.03.008
- Kim, Soochan, Sinsuk Han, Ye Eun Lee, Woong-Jae Jung, Hyung Soo Lee, Yong-Sun Kim, Eun-Kyoung Choi, and Mi-Yeon Kim. "Prion Protein-deficient Mice Exhibit Decreased CD4 T and LT α Cell Numbers and Impaired Spleen Structure." *Immunobiology* 221.1 (2016): 94-102.
- Kimberlin, R.h., and C.a. Walker. "Pathogenesis of Mouse Scrapie: Dynamics of Agent Replication in Spleen, Spinal Cord and Brain after Infection by Different Routes." *Journal of Comparative Pathology* 89.4 (1979): 551-62. Print.
- Kimberlin, Richard H., and Carol A. Walker. "The Role of the Spleen in the Neuroinvasion of Scrapie in Mice." *Virus Research* 12.3 (1989): 201-11. Print.
- Kincaid, A. E., and J. C. Bartz. "The Nasal Cavity Is a Route for Prion Infection in Hamsters." *Journal of Virology* 81.9 (2007): 4482-491. Print.

- Kincaid, A. E., K. F. Hudson, M. W. Richey, and J. C. Bartz. "Rapid Transepithelial Transport of Prions following Inhalation." *Journal of Virology* 86.23 (2012): 12731-2740. Print.
- Klein, Michael A., Pascal S. Kaeser, Petra Schwarz, Heiko Weyd, Ioannis Xenarios, Rolf M. Zinkernagel, Michael C. Carroll, J. Sjef Verbeek, Marina Botto, Mark J. Walport, Hector Molina, Ulrich Kalinke, Hans Acha-Obbea, and Adriano Aguzzi. "Complement Facilitates Early Prion Pathogenesis." *Nature Medicine* 4th ser. 7 (2001): 488-92. Print.
- Kubosaki, Atsutaka, Yukiko Nishimura-Nasu, Takuya Nishimura, Seiichi Yusa, Akikazu Sakudo, Keiichi Saeki, Yoshitsugu Matsumoto, Shigeyoshi Itoharu, and Takashi Onodera. "Expression of Normal Cellular Prion Protein (PrP^c) on T Lymphocytes and the Effect of Copper Ion: Analysis by Wild-type and Prion Protein Gene-deficient Mice." *Biochemical and Biophysical Research Communications* 307.4 (2003): 810-13. Print.
- Küffer, Alexander, Asvin K. K. Lakkaraju, Amit Mogha, Sarah C. Petersen, Kristina Airich, Cédric Doucerain, Rajlakshmi Marpakwar, Pamela Bakirci, Assunta Senatore, Arnaud Monnard, Carmen Schiavi, Mario Nuvolone, Bianka Grosshans, Simone Hornemann, Frederic Bassilana, Kelly R. Monk, and Adriano Aguzzi. "The Prion Protein Is an Agonistic Ligand of the G Protein-coupled Receptor Adgrg6." *Nature* 536.7617 (2016): 464-68. Print.
- Lewicki, H., A. Tishon, D. Homann, H. Mazarguil, F. Laval, V. C. Asensio, I. L. Campbell, S. Dearmond, B. Coon, C. Teng, J. E. Gairin, and M. B. A. Oldstone. "T Cells Infiltrate the Brain in Murine and Human Transmissible Spongiform Encephalopathies." *Journal of Virology* 77.6 (2003): 3799-808. Print.

- Liberski, Pawel P., Beata Sikorska, Don Guiryo, and Richard A. Bessen. "Transmissible Mink Encephalopathy – Review of the Etiology of a Rare Prion Disease." *Folia Neuropathol* 2nd ser. 74 (2009): 195-204. Print.
- Liberski, Pawel P. "Historical Overview of Prion Diseases: A View from Afar." *Folia Neuropathologica* 1st ser. 50 (2012): 1-12. Print.
- Linden, Rafael. "The Biological Function of the Prion Protein: A Cell Surface Scaffold of Signaling Modules." *Frontiers in Molecular Neuroscience* 10 (2017). Print.
- Llewellyn, C., Hewitt, P., Knight, R., Amar, K., Cousens, S., Mackenzie, J., & Will, R. (2004, 02). Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *The Lancet*, 363(9407), 417-421. doi:10.1016/s0140-6736(04)15486-x
- Mabbott, Neil A., and G. Gordon Macpherson. "Prions and Their Lethal Journey to the Brain." *Nature Reviews Microbiology* 4.3 (2006): 201-11. Print.
- Mabbott, Neil A., and Moira E. Bruce. "The Immunobiology of TSE Diseases." *Journal of General Virology* 82.10 (2001): 2307-318. Print.
- Mabbott, Neil A., Moira E. Bruce, Marina Botto, Mark J. Walport, and Nark B. Pepys. "Temporary Depletion of Complement Component C3 or Genetic Deficiency of C1q Significantly Delays Onset of Scrapie." *Nature Medicine* 7th ser. 7 (2001): 485-87. Print.
- Marsh, R. F., D. Burger, R. Eckroade, G. M. Zu, and R. P. Hanson. "A Preliminary Report on the Experimental Host Range of the Transmissible Mink Encephalopathy Agent." *Journal of Infectious Diseases* 120.6 (1969): 713-19.
- Marsh, R. F., and R. P. Hanson. "Physical and Chemical Properties of the Transmissible Mink Encephalopathy Agent." *Journal of Virology* 3.2 (1969): 176-80. Print.

- Marsh, R. F. "Animal Model of Human Disease: Kuru, Creutzfeldt-Jakob Disease (slow Virus Infections). Animal Model: Transmissible Mink Encephalopathy, Scrapie-like Disease of Mink." *American Journal of Pathology* 69.1 (1972): 209-12. Print.
- Masujin, Kentaro, Christina D. Orrú, Kohtaro Miyazawa, Bradley R. Groveman, Lynne D. Raymond, Andrew G. Hughson, and Byron Caughey. "Correction for Masujin Et Al., Detection of Atypical H-Type Bovine Spongiform Encephalopathy and Discrimination of Bovine Prion Strains by Real-Time Quaking-Induced Conversion." *Journal of Clinical Microbiology* 54.5 (2016): 1407. Print.
- Mathiason, C. K., J. Hayes-Klug, S. A. Hays, J. Powers, D. A. Osborn, S. J. Dahmes, K. V. Miller, R. J. Warren, G. L. Mason, G. C. Telling, A. J. Young, and E. A. Hoover. "B Cells and Platelets Harbor Prion Infectivity in the Blood of Deer Infected with Chronic Wasting Disease." *Journal of Virology* 84.10 (2010): 5097-107. Print.
- Mathiason, C. K., J. G. Powers, S. J. Dahmes, D. A. Osborn, K. V. Miller, R. J. Warren, G. L. Mason, S. A. Hays, J. Hayes-Klug, D. M. Seelig, M. A. Wild, L. L. Wolfe, T. R. Spraker, M. W. Miller, C. J. Sigurdson, G. C. Telling, and E. A. Hoover. "Infectious Prions in the Saliva and Blood of Deer with Chronic Wasting Disease." *Science* 314.5796 (2006): 133-36. Print.
- Mathiason, Candace K., Sheila A. Hays, Jenny Powers, Jeanette Hayes-Klug, Julia Langenberg, Sallie J. Dahmes, David A. Osborn, Karl V. Miller, Robert J. Warren, Gary L. Mason, and Edward A. Hoover. "Infectious Prions in Pre-Clinical Deer and Transmission of Chronic Wasting Disease Solely by Environmental Exposure." *PLoS ONE* 4.6 (2009). Print.
- Mathiason, Candace K. "Silent Prions and Covert Prion Transmission." *PLOS Pathogens* 11.12 (2015). Print.

- McGovern, G., K.I Brown, M.E Bruce, and M. Jeffrey. "Murine Scrapie Infection Causes an Abnormal Germinal Centre Reaction in the Spleen." *Journal of Comparative Pathology* 130.2-3 (2004): 181-94.
- McKenzie, D., J.C. Bartz, and R.F. Marsh. "Transmissible Mink Encephalopathy." *Seminars in Virology* 7.3 (1996): 201-06. Print.
- Miller, Michael W., Elizabeth S. Williams, N. Thompson Hobbs, and Lisa L. Wolfe. "Environmental Sources of Prion Transmission in Mule Deer." *Emerging Infectious Diseases* 10.6 (2004): 1003-006. Print.
- Mulcahy, Ellyn R., and Richard A. Bessen. "Strain-specific Kinetics of Prion Protein Formation in Vitro and in Vivo." *Journal of Biological Chemistry* 279.3 (2003): 1643-649. Print.
- Murphy, W.G. "Lessons from the Response to the Threat of Transfusion-transmitted VCJD in Ireland." *Transfusion Clinique Et Biologique* 20.4 (2013): 416-21. Print.
- Nalls, Amy V., Erin McNulty, Clare E. Hoover, Laura A. Pulscher, Edward A. Hoover, and Candace K. Mathiason. "Infectious Prions in the Pregnancy Microenvironment of Chronic Wasting Disease-Infected Reeves' Muntjac Deer." *Journal of Virology* 91.15 (2017). Print.
- Nalls, Amy V., Erin McNulty, Jenny Powers, Davis M. Seelig, Clare Hoover, Nicholas J. Haley, Jeanette Hayes-Klug, Kelly Anderson, Paula Stewart, Wilfred Goldmann, Edward A. Hoover, and Candace K. Mathiason. "Mother to Offspring Transmission of Chronic Wasting Disease in Reeves' Muntjac Deer." *PLoS ONE* 8.8 (2013). Print.
- Orrù, Christina D., Alessandra Favole, Cristiano Corona, Maria Mazza, Matteo Manca, Bradley R. Groveman, Andrew G. Hughson, Pier Luigi Acutis, Maria Caramelli, Gianluigi Zanusso, Cristina Casalone, and Byron Caughey. "Detection and Discrimination of

Classical and Atypical L-Type Bovine Spongiform Encephalopathy by Real-Time Quaking-Induced Conversion." *Journal of Clinical Microbiology* 53.4 (2015): 1115-120. Print.

Panigaj, M., Brouckova, A., Glierova, H., Dvorakova, E., Simak, J., Vostal, J. G., & Holada, K. (2010, 11). Underestimation of the expression of cellular prion protein on human red blood cells. *Transfusion*, 51(5), 1012-1021. doi:10.1111/j.1537-2995.2010.02924.x

Peden, Alexander H., Mark W. Head, L. Ritchie Diane, E. Bell Jeanne, and W. Ironside James. "Preclinical VCJD after Blood Transfusion in a PRNP Codon 129 Heterozygous Patient." *The Lancet* 364.9433 (2004): 527-29. Print.

Priola, Suzette A. "Similar Protein Signatures for BSE and VCJD." *Nature Medicine* 2.12 (1996): 1303-304.

Prusiner, S. "Novel Proteinaceous Infectious Particles Cause Scrapie." *Science* 216.4542 (1982): 136-44. Print.

Prusiner, Stanley B. "Biology and Genetics of Prions Causing Neurodegeneration." *Annual Review of Genetics* 47.1 (2013): 601-23. Print.

Prusiner, Stanley B., David C. Bolton, Darlene F. Groth, Karen A. Bowman, S. Patricia Cochran, and Michael P. McKinley. "Further Purification and Characterization of Scrapie Prions." *Biochemistry* 21.26 (1982): 6942-950.

Rubenstein, R., P. A. Merz, R. J. Kascsak, R. I. Carp, C. L. Scalici, C. L. Fama, and H. M. Wisniewski. "Detection of Scrapie-Associated Fibrils (SAF) and SAF Proteins from Scrapie-Affected Sheep." *Journal of Infectious Diseases* 156.1 (1987): 36-42. Print.

- Rubenstein, R., P. A. Merz, R. J. Kascsak, C. L. Scalici, M. C. Papini, R. I. Carp, and R. H. Kimberlin. "Scrapie-Infected Spleens: Analysis of Infectivity, Scrapie-Associated Fibrils, and Protease-Resistant Proteins." *Journal of Infectious Diseases* 164.1 (1991): 29-35. Print.
- Schutt, Charles R., and Jason C. Bartz. "Prion Interference with Multiple Prion Isolates." *Prion* 2.2 (2008): 61-63. Print.
- Seeger, H. "Coincident Scrapie Infection and Nephritis Lead to Urinary Prion Excretion." *Science* 310.5746 (2005): 324-26. Print.
- Shi, Song, Gerda Mitteregger-Kretzschmar, Armin Giese, and Hans A. Kretzschmar. "Establishing Quantitative Real-time Quaking-induced Conversion (qRT-QuIC) for Highly Sensitive Detection and Quantification of PrPSc in Prion-infected Tissues." *Acta Neuropathologica Communications* 1.1 (2013): 44. Print.
- Shikiya, R. A., J. I. Ayers, C. R. Schutt, A. E. Kincaid, and J. C. Bartz. "Coinfecting Prion Strains Compete for a Limiting Cellular Resource." *Journal of Virology* 84.11 (2010): 5706-714.
- Shikiya, Ronald A., Katie A. Langenfeld, Thomas E. Eckland, Jonathan Trinh, Sara A. M. Holec, Candace K. Mathiason, Anthony E. Kincaid, and Jason C. Bartz. "PrPSc Formation and Clearance as Determinants of Prion Tropism." *PLOS Pathogens* 13.3 (2017). Print.
- Sigurdson, Christina J., Jason C. Bartz, and K. Peter R. Nilsson. "Tracking Protein Aggregate Interactions." *Prion* 5.2 (2011): 52-55.
- Smith, P. G. "Bovine Spongiform Encephalopathy (BSE) and Its Epidemiology." *British Medical Bulletin* 66.1 (2003): 185-98. Print.
- Tamguney, G., M. W. Miller, K. Giles, A. Lemus, D. V. Glidden, S. J. Dearmond, and S. B. Prusiner. "Transmission of Scrapie and Sheep-passaged Bovine Spongiform

- Encephalopathy Prions to Transgenic Mice Expressing Elk Prion Protein." *Journal of General Virology* 90.4 (2009): 1035-047. Print.
- Titlow, William B., Muhammad Waqas, Jihyun Lee, Jae Youl Cho, Sang Yeol Lee, Dae-Hwan Kim, and Chongsuk Ryou. "Effect of Polylysine on Scrapie Prion Protein Propagation in Spleen during Asymptomatic Stage of Experimental Prion Disease in Mice." *Journal of Microbiology and Biotechnology* 26.9 (2016): 1657-660. Print.
- Tripathi, Ajai K., and Neena Singh. "Prion Protein-Hemin Interaction Upregulates Hemoglobin Synthesis: Implications for Cerebral Hemorrhage and Sporadic Creutzfeldt-Jakob Disease." *Journal of Alzheimer's Disease* 51.1 (2016): 107-21.
- Wells, G.a.h. "Pathogenesis of BSE." *Veterinary Research Communications* 27 (2003): 25-28. Print.
- Whitfield, J. T., W. H. Pako, J. Collinge, and M. P. Alpers. "Mortuary Rites of the South Fore and Kuru." *Philosophical Transactions of the Royal Society B: Biological Sciences* 363.1510 (2008): 3721-724. Print.
- Wilham, Jason M., Christina D. Orrú, Richard A. Bessen, Ryuichiro Atarashi, Kazunori Sano, Brent Race, Kimberly D. Meade-White, Lara M. Taubner, Andrew Timmes, and Byron Caughey. "Rapid End-Point Quantitation of Prion Seeding Activity with Sensitivity Comparable to Bioassays." *PLoS Pathogens* 6.12 (2010). Print.
- Williams, E. S., and S. Young. "Chronic Wasting Disease Of Captive Mule Deer: A Spongiform Encephalopathy." *Journal of Wildlife Diseases* 16.1 (1980): 89-98.
- Zigas, V., and D. C. Gajusek. "Kuru: Clinical Study of a New Syndrome Resembling Paralysis Agitans in Natives of the Eastern Highlands of Australian New Guinea." *Medical Journal of Australia* 2.21 (1957): 745-54. Print.

APPENDIX A

Cell Blood Count Profiles

PRE	1	2	3	4	5	6
Hgb (G/dl)	11	12.4	13.6	12.2	11.7	12.9
Hgb (cell) (G/dl)	10.9	12.2	13.1	12.1	11.4	12.9
Hct (%)	33	36	39	35	33	37
RBC (10 ⁶ /ul)	5.88	6.53	7.13	6.64	6.07	6.97
MCV (fl)	56	55	54	52	55	53
RDW (%)	12.9	12.6	13.1	12.5	13.7	12.7
MCHC (G/dl)	34	34	35	35	35	35
CHCM (G/dl)	33	34	34	35	34	35
Retic %auto (%)	2.6	2.9	2.7	2.5	2.3	2.8
Retic (10 ³ /ul)	154.7	191.4	194.5	166.1	142.2	197.1
Ch-retic (mg/dl)	20.5	20.8	20.6	20.5	20.5	20.3
mcv-retic (fl)	97	65	64	63	64	63
Platelets (10 ³ /ul)	26	94	105	304	182	42
MPV (fl)	8.1	7.9	8.7	8	9.7	8.4
Nucleated cells (10 ³ /dl)	2	3.6	4.6	4.1	4.3	3
Bands# (10 ³ /dl)	0	0	0	0	0	0
Bands %	0	0	0	0	0	0
Neut # (10 ³ /dl)	0.3	0.1	0.1	0.3	0.6	0.2
Neut %	14	4	3	7	13	6
Lymph # (10 ³ /dl)	1.6	3.3	4.4	3.7	3.6	2.6
Lymph %	80	91	95	91	83	85
Mono # (10 ³ /dl)	0.1	0.2	0.1	0.1	0.1	0.2
Mono %	6	5	2	2	2	2
Eos # (10 ³ /dl)					0.1	
Eos %					2	
nRBC %						
Clumped platelets (mIU/ml)	few	many	few	mod	few	mod
Neut# auto (10 ³ /ul)	0.1	0.2	0.8	0.6	0.4	0.2
Reactive lymphs (nmol/L)						
Polychromasia (nmol/L)						
nRBC# (mIU/ml)						
plasma protein (G/dl)						
15 minutes	-1	-2	-3	1	2	3
Hgb (G/dl)	12.7	12.8	11.6	12.9	12.8	12.4
Hgb (cell) (G/dl)	12.6	12.4	11.4	12.4	12.6	12
Hct (%)	37	36	33	37	36	36
RBC (10 ⁶ /ul)	6.85	6.74	6.17	7.04	6.91	6.81
MCV (fl)	54	54	53	52	52	52
RDW (%)	12.7	12.9	12.7	12.7	13.7	13.4
MCHC (G/dl)	35	35	35	35	35	35
CHCM (G/dl)	34	34	35	34	35	34
Retic %auto (%)	2.6	2.2	2.2	2.4	3.4	3.1
Retic (10 ³ /ul)	178	148.1	136.9	171	234	212.7
Ch-retic (mg/dl)	20.5	20.6	20.6	20.8	20.9	20.7
mcv-retic (fl)	62	63	62	63	64	63
Platelets (10 ³ /ul)	715	425	487	642	587	697
MPV (fl)	10.4	10	10.1	9.7	10.5	9.4
Nucleated cells (10 ³ /dl)	5.5	3	2.8	3.3	3.4	4.2
Bands# (10 ³ /dl)	0	0	0	0	0	0
Bands %	0	0	0	0	0	0
Neut # (10 ³ /dl)	1.1	0.2	0.4	0.3	0.6	0.2
Neut %	20	7	16	9	18	4
Lymph # (10 ³ /dl)	4	2.7	2.3	3	2.4	3.7
Lymph %	72	91	81	90	70	89
Mono # (10 ³ /dl)	0.3	0	0.1	0	0.3	0.3
Mono %	6	1	2		9	6
Eos # (10 ³ /dl)	0.1				0.1	
Eos %	1	1	1	1	3	1
nRBC %	0.1					
Clumped platelets (mIU/ml)	mod	mod	many	mod	many	many
Neut# auto (10 ³ /ul)	0.9	0.2	0.1	0.3	0.5	0.6
Reactive lymphs (nmol/L)						
Polychromasia (nmol/L)		slight		slight		slight
nRBC# (mIU/ml)						
plasma protein (G/dl)						
Notes			few mast cells seen		few mast cells seen	

30 minutes	-1	-2	-3	1	2	3
Hgb (G/dl)	13	13.1	13.6	10.3	12.7	9.5
Hgb (cell) (G/dl)	13	12.9	13.1	10.4	12.1	9.4
Hct (%)	38	38	40	30	37	28
RBC (10^6/ul)	7.19	7.02	7.34	5.45	6.57	4.79
MCV (fl)	53	54	54	56	56	58
RDW (%)	12.3	12.4	13.1	13.4	12.2	12.7
MCHC (G/dl)	34	35	34	34	34	34
CHCM (G/dl)	34	34	33	34	33	34
Retic %auto (%)	2.2	2.1	4.1	2.7	2	2.4
Retic (10^3/ul)	156.9	148.5	304.4	148.9	130.3	113.3
Ch-retic (mg/dl)	19.9	20.5	20.9	21.5	20.5	21.4
mcv-retic (fl)	62	63	66	69	64	67
Platelets (10^3/ul)	88	299	45	74	34	93
MPV (fl)	9.4	10.2	13.2	8.8	10.3	10.3
Nucleated cells (10^3/dl)	5.7	4.8	3.8	4.9	3.5	2.9
Bands# (10^3/dl)	0.1	0	0	0	0	0
Bands %	1	0	0	0	0	0
Neut # (10^3/dl)	1.1	1	0.2	1.4	0.1	0.2
Neut %	19	20	6	29	3	8
Lymph # (10^3/dl)	4.2	3.5	3.2	3.1	3.2	2.3
Lymph %	73	73	84	64	92	81
Mono # (10^3/dl)	0.3	0.2	0.2	0.2	0.2	0.2
Mono %	5	4	6	5	5	8
Eos # (10^3/dl)	0.1	0.1	0.2			0.1
Eos %	2	3	4	1		2
nRBC %				1		1
Clumped platelets (mIU/ml)	many	many	many	many	many	
Neut# auto (10^3/ul)	1	0.4	0.1	0.2	0.2	0.2
Reactive lymphs (nmol/L)						
Polychromasia (nmol/L)				slight		
nRBC# (mIU/ml)						
plasma protein (G/dl)	3.5	3.8	4.1	3.3	3.5	2.5

60 minutes	-1	-2	-3	1	2	3
Hgb (G/dl)	13.3		10.8	13.5	13.1	15.6
Hgb (cell) (G/dl)	13.1		10.7	13.8	13	15.6
Hct (%)	38		31	39	37	46
RBC (10^6/ul)	6.94		5.76	7.44	7.01	8.63
MCV (fl)	54		53	53	53	53
RDW (%)	12.9		12.7	12.9	12.3	12.8
MCHC (G/dl)	35		35	35	35	34
CHCM (G/dl)	35		35	35	35	34
Retic %auto (%)	3.6		2.3	2.5	2.2	1.9
Retic (10^3/ul)	351.7		133	185.8	155.5	162.9
Ch-retic (mg/dl)	21.2		20.5	20.8	20.6	20.1
mcv-retic (fl)	65		63	65	62	62
Platelets (10^3/ul)	69		56	147	90	129
MPV (fl)	9.2		10.7	8.6	9.8	9.1
Nucleated cells (10^3/dl)	3.1		3.4	3.4	2.8	3.2
Bands# (10^3/dl)	0		0	0	0	0
Bands %	0		0	0	0	0
Neut # (10^3/dl)	0.2		0.4	0.5	0.2	0
Neut %	5		12	15	7	1
Lymph # (10^3/dl)	2.9		3	2.8	2.5	3.1
Lymph %	92		87	82	90	96
Mono # (10^3/dl)	0		0	0.1	0.1	0.1
Mono %				2	2	2
Eos # (10^3/dl)	0.1					
Eos %	3		1	1	1	1
nRBC %						
Clumped platelets (mIU/ml)	many		mod	mod	mod	mod
Neut# auto (10^3/ul)	0.3		0.4	0.9	0.3	0.7
Reactive lymphs (nmol/L)	mod		mod	slight	mod	mod
Polychromasia (nmol/L)	slight		slight	mod	slight	slight
	noted on feathered edge	perform CBC due to clot in tube	seen on feathered edge	noted on feathered edge	noted on feathered edge	

24 hours	-1	-2	-3	1	2	3
Hgb (G/dl)	11.5	12.4	12	13.6	13.6	13
Hgb (cell) (G/dl)	11.3	12.5	11.9	13.5	13.5	12.9
Hct (%)	32	36	35	39	40	38
RBC (10 ⁶ /ul)	6.11	6.63	6.37	7.09	7.23	6.9
MCV (fl)	53	54	55	56	55	55
RDW (%)	12.9	12.7	13.4	13.7	13.3	12.9
MCHC (G/dl)	35	35	34	34	34	34
CHCM (G/dl)	35	35	34	34	34	34
Retic %auto (%)	2.2	2.1	2.3	2.8	2.3	2
Retic (10 ³ /ul)	136.9	136.3	144.1	197.6	164.2	140.1
Ch-retic (mg/dl)	20.7	20.7	20.7	20.9	20.9	20.5
mcv-retic (fl)	63	64	66	66	66	66
Platelets (10 ³ /ul)	66	67	49	67	180	107
MPV (fl)	11.2	8.8	9.4	11.2	8.7	9.4
Nucleated cells (10 ³ /dl)	3.6	3	4.2	3.8	4.7	5.4
Bands# (10 ³ /dl)	0	0	0	0	0	0
Bands %	0	0	0	0	0	0
Neut # (10 ³ /dl)	0.3	0.3	0.4	0.2	0.8	1.6
Neut %	18	11	10	6	18	30
Lymph # (10 ³ /dl)	2.8	2.3	3.7	3.4	3.4	3.5
Lymph %	77	76	89	89	73	64
Mono # (10 ³ /dl)	0.2	0.3	0	0.2	0.2	0.3
Mono %	5	20		4	5	5
Eos # (10 ³ /dl)		0.1			0.2	0.1
Eos %		2	1	1	4	1
nRBC %		1				
Clumped platelets (mIU/ml)	many	many	few	many	many	many
Neut# auto (10 ³ /ul)	0.4	0.2	0.3	0.2	1	1.1
Reactive lymphs (nmol/L)	few		slight	slight	slight	few
Polychromasia (nmol/L)	slight		few	few	few	slight
Notes	few mast cells on feathered edge	few mast cells on feathered edge	few mast cells on feathered edge		1 mast cell noted on feathered edge	few mast cells on feathered edge

48 hours	-1	-2	-3	1	2	3
Hgb (G/dl)	11.7	12.2	10.9	11.8		11.9
Hgb (cell) (G/dl)	11.6	12	10.8	11.6		11.7
Hct (%)	34	36	32	34		35
RBC (10 ⁶ /ul)	6.09	6.48	5.87	6.46		6.68
MCV (fl)	56	56	54	53		53
RDW (%)	12.9	12.4	12.1	12.3		12.1
MCHC (G/dl)	34	34	34	34		34
CHCM (G/dl)	34	33	34	34		33
Retic %auto (%)	2.8	2.3	2.5	2.5		2.3
Retic (10 ³ /ul)	168.9	151.8	144.9	160		153
Ch-retic (mg/dl)	20.6	20.5	20.4	20.1		19.6
mcv-retic (fl)	64	64	64	62		61
Platelets (10 ³ /ul)	87	287	89	494		421
MPV (fl)	8.2	8.6	10.8	8.8		9.1
Nucleated cells (10 ³ /dl)	1.8	2.7	1.7	4.4		3.2
Bands# (10 ³ /dl)	0	0	0	0		0
Bands %	0	0	0	0		0
Neut # (10 ³ /dl)	0	0.2	0	1.1		0.1
Neut %	1	7	2	25		4
Lymph # (10 ³ /dl)	1.7	2.4	1.6	3		2.9
Lymph %	97	88	97	69		91
Mono # (10 ³ /dl)	0	0.1	0	0.2		0.1
Mono %	1	4		4		3
Eos # (10 ³ /dl)				0.1		0.1
Eos %	1	1	1	2		2
nRBC %						
Clumped platelets (mIU/ml)	few	many	few	many		many
Neut# auto (10 ³ /ul)	0.1	0.1	0	0.3		0.3
Reactive lymphs (nmol/L)	mod	mod	mid			mod
Polychromasia (nmol/L)	slight	mod	slight	slight		slight
Notes	few mast cells noted	few mast cells present		slight numerous platelet clumps	clotted	

60 days	-1	-2	-3	1	2	3
Hgb (G/dl)	13.3	13	13	14.1	14.4	13.3
Hgb (cell) (G/dl)	12.9	12.7	13.1	14	14	13
Hct (%)	38	38	37	40	41	38
RBC (10 ⁶ /ul)	7.3	7.34	7.08	7.56	7.46	7.35
MCV (fl)	52	51	53	53	55	52
RDW (%)	12.7	12.3	13.2	13.4	14.3	12.2
MCHC (G/dl)	35	35	35	35	35	35
CHCM (G/dl)	34	34	35	35	34	34
Retic %auto (%)	1.5	1.1	1.7	1.3	2.2	1.6
Retic (10 ³ /ul)	108.2	83	123.2	99.3	167.5	120.4
Ch-retic (mg/dl)	20.1	19.7	20.8	20.5	21.4	19.8
mcv-retic (fl)	62	60	63	63	66	61
Platelets (10 ³ /ul)	271	104	430	123	373	606
MPV (fl)	11.5	9.1	10.5	8.2	11.4	10.5
Nucleated cells (10 ³ /dl)	3.7	3.8	3.8	4.2	4.6	4
Bands# (10 ³ /dl)	0	0	0	0	0	0
Bands %	0	0	0	0	0	0
Neut # (10 ³ /dl)	0.1	0.1	0.2	0.2	0	0
Neut %	3	2	4	5	1	0
Lymph # (10 ³ /dl)	3.5	3.4	3.6	4	96	4
Lymph %	94	89	96	95	4.4	99
Mono # (10 ³ /dl)	0.1	0	0	0	2	
Mono %	2	2			0.1	
Eos # (10 ³ /dl)		0.3			1	1
Eos %	1	8				
nRBC %						
Clumped platelets (mlU/ml) few			absent			
Neut# auto (10 ³ /ul)	0.5	0.6	0.5	0.7	0.6	0.4
mod						
noted on						
feathered						
edge						
Notes		mast cells	mast cells			
		observed	observed			

90 days	1	2	3	4	5	6 Positive:	1	2	3	4	5	6
Hgb (G/dl)	14.2	12.9	15.1	14.6	15	13.6	15.2	14.4	11	12.5	14	
Hgb (cell) (G/dl)	14	12.6	15.1	14.2	15	13.3	14.8	14.1	11	12.9	14	
Hct (%)	41	37	43	41.7	44	39	44	42	33	38	43	
RBC (10 ⁶ /ul)	7.48	6.85	8.13	7.82	7.9	7.13	8.08	7.7	6.1	6.9	7.7	
MCV (fl)	55	54	53	53.4	56	55	54	54	54	55	56	
RDW (%)	12.9	13.4	13.4	13.5	14	13.3	13.1	13.2	14	14.3	13	
MCHC (G/dl)	35	35	35	35.1	35	35	34	34	34	33	33	
CHCM (G/dl)	34	34	35	34.1	33	34	34	34	33	34	33	
Retic %auto (%)	1.5	1.6	1.5	1.3	1.4	1.5	1.6	2	1.7	1.8	1.4	
Retic (10 ³ /ul)	115	110	120	105	110	108	131	154	106	122	110	
Ch-retic (mg/dl)	20.7	20.4	20.4	20.4	21	20.8	20.4	20.2	20	20.3	20	
mcv-retic (fl)	65	66	63	65.2	66	66	64	66	67	66	66	
Platelets (10 ³ /ul)	66	83	37	37	250	162	42	44	212	145	468	
MPV (fl)	10.5	10.8	8.8	9	11	7.4	9.1	9.5	13	11.4	11	
Nucleated cells (10 ³ /dl)	3.9	3	3.7	3.11	3.8	3	4.4	4.3	2.9	3.8	4.9	
Bands# (10 ³ /dl)	0	0	0	0	0	0	0	0	0	0	0	
Bands %	0	0	0	0	0	0	0	0	0	0	0	
Neut # (10 ³ /dl)	0.2	0.5	0.7	0.2	0.3	0.3	0.2	0.5	0.5	0.2	0.2	
Neut %	4	17	20	8	16	11	4	12	18	6	4	
Lymph # (10 ³ /dl)	3.6	2.4	2.9	2.7	3	2.6	4.1	3.1	1.9	3.5	4.5	
Lymph %	92	79	78	87	80	86	93	73	64	92	92	
Mono # (10 ³ /dl)	0.1	0	0	0.1	0	0.1	0	0.2	0.3	0.1	0.1	
Mono %	2	1		3	1	2		5	10	2	2	
Eos # (10 ³ /dl)		0.1	0.1	0.1	0.1		0.1	0.3	0.2		0.1	
Eos %	1	3	2	2	3	1	3	7	6		2	
nRBC %	1							3	2			
Clumped platelets (mlU/ml)	many	many	many	many	many	many	many	many	many	many	mod	
Neut# auto (10 ³ /ul)	0.2	0.2	0.4	0.2	0.7	0.3	0.2	0.2	0.3	0.3	0.8	
Reactive lymphs (nmol/L)	slight	mod	mod		mod	mod	mod					
Polychromasia (nmol/L)	mod	slight	slight			slight	slight					
nRBC# (mlU/ml)								0.1	0.1			
plasma protein (G/dl)												

155 days	-1	-2	-3	1	2	3
Hgb (G/dl)	13.4	13.3	13.1	12.7	13.8	13.2
Hgb (cell) (G/dl)	12.8	13.3	12.9	12.4	13.3	12.9
Hct (%)	38	38	37	36	40	38
RBC (10^6/ul)	6.95	7.04	6.71	6.84	7.25	6.88
MCV (fl)	54	54	55	52	54	55
RDW (%)	13.2	12.9	12.7	13.4	12.8	13.1
MCHC (G/dl)	35	36	35	36	35	35
CHCM (G/dl)	34	35	35	35	34	34
Retic %auto (%)	2	1.8	1.6	1.9	1.8	1.9
Retic (10^3/ul)	135.7	126.4	108.5	126.7	127.1	133.3
Ch-retic (mg/dl)	20.8	20.7	21.1	20.5	20.7	20.8
mcv-retic (fl)	65	64	64	62	63	63
Platelets (10^3/ul)	145	445	516	97	64	81
MPV (fl)	12.3	10.6	10.4	9.1	9.8	12.6
Nucleated cells (10^3/dl)	3.2	24	3	2.7	2.6	3.9
Bands# (10^3/dl)	0	0	0	0	0	0
Bands %	0	0	0	0	0	0
Neut # (10^3/dl)	0.3	0.2	0.5	0.1	0.2	0.2
Neut %	10	8	16	4	6	5
Lymph # (10^3/dl)	2.7	2.2	2.2	2.4	2.4	3.4
Lymph %	84	90	72	88	93	87
Mono # (10^3/dl)	0.1	0	0.2	0.1	0	0.1
Mono %	3	1	7	4	2	3
Eos # (10^3/dl)	0.1		0.2	0.1		0.2
Eos %	3	1	5	3		4
nRBC %				1		1
Clumped platelets (mIU/ml)	many	many	many	many	mod	mod
Neut# auto (10^3/ul)	0.7	0.4	0.4	0.2	0.7	0.5
Reactive lymphs (nmol/L)	mod	mod	mod			
Polychromasia (nmol/L)	slight	slight	slight			
Notes	rare mast cell noted	few mast cells noted	few mast cells notes			clinical 153d

Late Disease	155d -3	169d -4	169d -5	238d -6	155d 3	169d 4	169d 5	238d 6
Hgb (G/dl)	13.1	13.4	14	12.9	13.2	14	13.6	13.8
Hgb (cell) (G/dl)	12.9	12.7	13.8	12.1	12.9	13.3	13.4	12.2
Hct (%)	37	38	40	36	38	40	38	39
RBC (10^6/ul)	6.71	7.03	7.43	6.86	6.88	7.48	7.23	7.35
MCV (fl)	55	53	53	52	55	53	53	53
RDW (%)	12.7	12.7	12.6	12.3	13.1	12.3	12.3	12.5
MCHC (G/dl)	35	36	35	36	35	35	36	35
CHCM (G/dl)	35	34	35	34	34	35	35	34
Retic %auto (%)	1.6	1.5	1.7	1.6	1.9	1.4	1.3	1.5
Retic (10^3/ul)	108.5	103.6	127.5	112.5	133.3	106.8	95.5	106.7
Ch-retic (mg/dl)	21.1	20.4	20.7	20	20.8	20.2	20.3	20.2
mcv-retic (fl)	64	62	62	62	63	61	61	62
Platelets (10^3/ul)	516	111	65	60	81	54	73	313
MPV (fl)	10.4	7.9	9.8	13.8	12.6	9.6	13.6	10.9
Nucleated cells (10^3/dl)	3	2.9	4.2	3.2	3.9	3.2	3.2	3.5
Bands# (10^3/dl)	0	0	0	0.2	0	0	0	0
Bands %	0	0	0	6	0	0	0	0
Neut # (10^3/dl)	0.5	0.4	0.2	0	0.2	0.2	0.3	0.5
Neut %	16	14	4	0	5	5	9	14
Lymph # (10^3/dl)	2.2	2.2	3.9	0.6	3.4	2.9	2.8	2.8
Lymph %	72	77	94	20	87	90	88	79
Mono # (10^3/dl)	0.2	0.1	0.1	2.2	0.1	0.1	0	0.2
Mono %	7	2	2	68	3	2	1	7
Eos # (10^3/dl)	0.2	0.2		0.1	0.2	0.1	0.1	
Eos %	5	6		2	4	3	2	
nRBC %				0.1	1			
Clumped platelets (mIU/ml)	many	few	Mod		4 mod	mod	many	
Neut# auto (10^3/ul)		0.4	0.4	1	0.3	0.5	0.6	0.2
Reactive lymphs (nmol/L)	mod	mod		many			mod	
Polychromasia (nmol/L)	slight	slight		slight				
Notes	few mast cells notes		mast cells outside of		clinical 153d	outside counting area	on feathered edge	

APPENDIX B

Isolated Cell Population Purities

Timepoint	Pre												
	<u>TME+ 1</u>		<u>TME+ 2</u>		<u>TME+ 3</u>								
	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>							
	B cell	3.91	B cell	46.7	B cell	11.1							
	CD4	6.42	CD4	43	CD4	19.7							
	CD 8	9.79	CD 8	37.3	CD 8	13.8							
	CD16	9.22	CD16	45.3	CD16	16.7							
	Pan Lympho	29.6	Pan Lympho	47.5	Pan Lympho	16.7							
Timepoint	15 min												
	<u>TME+ 1</u>		<u>TME+ 2</u>		<u>TME+ 3</u>		<u>Sham 1</u>		<u>Sham 2</u>		<u>Sham 3</u>		
	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	
	B cell	67.2	B cell	56.3	B cell	63.4	B cell	57.9	B cell	65.2	B cell	27.7	
	CD4	55.3	CD4	47.9	CD4	70.1	CD4	27.9	CD4	66.9	CD4	49.4	
	CD 8	61.2	CD 8	57.7	CD 8	73.1	CD 8	31.3	CD 8	62.9	CD 8	45.8	
	CD16	66.5	CD16	62.2	CD16	79	CD16	31.2	CD16	63.6	CD16	33.1	
	Pan Lympho	71.9	Pan Lympho	75.3	Pan Lympho	89.4	Pan Lympho	55.2	Pan Lympho	77.7	Pan Lympho	49.6	
	Timepoint	30 min											
		<u>TME+ 1</u>		<u>TME+ 2</u>		<u>TME+ 3</u>		<u>Sham 1</u>		<u>Sham 2</u>		<u>Sham 3</u>	
		<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>
		B cell	75.1	B cell	87.3	B cell	71.6	B cell	91.1	B cell	89.2	B cell	45.6
CD4		71.3	CD4	86.1	CD4	73	CD4	88.9	CD4	84.7	CD4	37.9	
CD 8		81.5	CD 8	97	CD 8	70.3	CD 8	88.1	CD 8	84.3	CD 8	34.8	
CD16		75.8	CD16	88.7	CD16	67.9	CD16	88.6	CD16	82.6	CD16	34.7	
Pan Lympho		77.1	Pan Lympho	97.4	Pan Lympho	81.2	Pan Lympho	97	Pan Lympho	95	Pan Lympho	75	
Timepoint		60 min											
		<u>TME+ 1</u>		<u>TME+ 2</u>		<u>TME+ 3</u>		<u>Sham 1</u>		<u>Sham 2</u>		<u>Sham 3</u>	
		<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>
		B cell	68.7	B cell	82	B cell	84.6	B cell	71.6	B cell	58.4	B cell	45.2
	CD4	63	CD4	77.9	CD4	91	CD4	60.4	CD4	55.4	CD4	50.8	
	CD 8	63.5	CD 8	80.3	CD 8	90.5	CD 8	60.6	CD 8	53.8	CD 8	52.5	
	CD16	58.5	CD16	89.7	CD16	89.9	CD16	71.7	CD16	67.7	CD16	58.4	
	Pan Lympho	91.7	Pan Lympho	5.12	Pan Lympho	96.6	Pan Lympho	89.6	Pan Lympho	91.7	Pan Lympho	83.7	
	Timepoint	24 hours											
		<u>TME+ 1</u>		<u>TME+ 2</u>		<u>TME+ 3</u>		<u>Sham 1</u>		<u>Sham 2</u>		<u>Sham 3</u>	
		<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>
		B cell	23.8	B cell	71.2	B cell	64.5	B cell	60.5	B cell	48.4	B cell	41.2
CD4		30.2	CD4	67.3	CD4	70.5	CD4	62.5	CD4	61.7	CD4	48.1	
CD 8		29.2	CD 8	75.5	CD 8	83.6	CD 8	63.9	CD 8	97.1	CD 8	62.8	
CD16		25.2	CD16	78.6	CD16	81.7	CD16	60.2	CD16	66.9	CD16	60.6	
Pan Lympho		28.8	Pan Lympho	85.5	Pan Lympho	87.6	Pan Lympho	69.8	Pan Lympho	76.8	Pan Lympho	65.1	
Timepoint		48 hours											
		<u>TME+</u>		<u>TME+</u>		<u>TME+</u>		<u>Sham</u>		<u>Sham</u>		<u>Sham</u>	
		<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>	<i>Sample</i>	<i>% Purity</i>
		B cell	55.2	B cell	61.9	B cell	52.7	B cell	4.3	B cell	40	B cell	29.8
	CD4	50.8	CD4	69.5	CD4	64.8	CD4	0.72	CD4	50.7	CD4	18.8	
	CD 8	19.8	CD 8	69.2	CD 8	66.6	CD 8	0.7	CD 8	50.4	CD 8	57.8	
	CD16	51.5	CD16	65.8	CD16	74.8	CD16	1.41	CD16	47.1	CD16	30.7	
	Pan Lympho	71.1	Pan Lympho	70.5	Pan Lympho	80.2	Pan Lympho	40.4	Pan Lympho	57.7	Pan Lympho	68.4	

Timepoint	60 days											
	TME+		TME+		TME+		Sham		Sham		Sham	
	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity
	B cell	79.2	B cell	36.2	B cell	56.8	B cell	54.2	B cell	68.4	B cell	47.3
	CD4	79.8	CD4	34.3	CD4	61.9	CD4	44.6	CD4	61.1	CD4	63
	CD 8	74	CD 8	34.6	CD 8	61.4	CD 8	43.4	CD 8	65.4	CD 8	48.7
	CD16	83.3	CD16	30.3	CD16	64.2	CD16	49.5	CD16	72.1	CD16	64.6
	Pan Lympho	96.2	Pan Lympho	43.6	Pan Lympho	85.9	Pan Lympho	73.9	Pan Lympho	87.7	Pan Lympho	77.8
Timepoint	90 days											
	TME+ 1		TME+ 2		TME+ 3		Sham 1		Sham 2		Sham 3	
	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity
	B cell	83	B cell	78	B cell	51.4	B cell	68.6	B cell	40.9	B cell	81.8
	CD4	85.5	CD4	81.2	CD4	77.5	CD4	51	CD4	22.9	CD4	84.2
	CD 8	88.4	CD 8	79.1	CD 8	80.3	CD 8	34.6	CD 8	33.2	CD 8	77.4
	CD16	90.9	CD16	85.2	CD16	85.7	CD16	45.4	CD16	34.5	CD16	59.4
	Pan Lympho	95.4	Pan Lympho	93.7	Pan Lympho	84.9	Pan Lympho	67.5	Pan Lympho	59.5	Pan Lympho	76.3
Timepoint	155 days											
	TME+ 1		TME+ 2		TME+ 3		Sham 1		Sham 2		Sham 3	
	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity
	B cell	25.8	B cell	11.9	B cell	7.91	B cell	83.5	B cell	68.3	B cell	74.4
	CD4	2.36	CD4	7.15	CD4	3.65	CD4	91.6	CD4	66.6	CD4	80.1
	CD 8	5.28	CD 8	13.9	CD 8	7.81	CD 8	92.5	CD 8	72.3	CD 8	83.1
	CD16	11.7	CD16	26.3	CD16	20	CD16	91.7	CD16	75.4	CD16	81.9
	Pan Lympho	81.7	Pan Lympho	89.6	Pan Lympho	75.3	Pan Lympho	83.7	Pan Lympho	83.5	Pan Lympho	86
Timepoint	169 days											
	TME+ 4		TME+ 5		Sham 4		Sham 5					
	Sample	% Purity	Sample	% Purity	Sample	% Purity	Sample	% Purity				
	B cell	42.9	B cell	54.4	B cell	75.4	B cell	67.3				
	CD4	27.3	CD4	72.3	CD4	72.1	CD4	66.4				
	CD 8	29.8	CD 8	84.8	CD 8	76.5	CD 8	76.7				
	CD16	22	CD16	81.3	CD16	76.2	CD16	82.9				
	Pan Lympho	24.4	Pan Lympho	90.9	Pan Lympho	84.2	Pan Lympho	89.1				
Timepoint	238 days											
	TME+ 6		Sham 6									
	Sample	% Purity	Sample	% Purity								
	B cell	25.8	B cell	58.9								
	CD4	31	CD4	32.7								
	CD 8	35.3	CD 8	37.2								
	CD16	81.4	CD16	41.4								
	Pan Lympho	96	Pan Lympho	86.8								

APPENDIX C

Cell Count and Percent Viability

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
Pre H- #1						Pre H+ #1					
CD4	0.46	0.07	14	23000000	3500000	CD4	0.47	0.07	14	23500000	3500000
CD8	0.42	0.01	1	21000000	500000	CD8	0.57	0.11	18	28500000	5500000
B cell	0.49	0.08	16	24500000	4000000	B cell	0.54	0.07	13	27000000	3500000
Granulocyte	0.64	0.05	7	32000000	2500000	Granulocyte	0.48	0.08	17	24000000	4000000
Lymphocyte	0.42	0.05	11	21000000	2500000	Lymphocyte	0.46	0.04	9	23000000	2000000
CD16	0.39	0.06	14	19500000	3000000	CD16	0.33	0.08	23	16500000	4000000
PBMC	10			500000000	0	PBMC	10			500000000	0
Platelet	0.46	0.04	8	23000000	2000000	Platelet	0.47	0.06	12	23500000	3000000
Pre H- #2						Pre H+ #2					
CD4	0.64	0.01	11	23500000	500000	CD4	0.45	0.03	7	22500000	1500000
CD8	0.47	0.08	17	22000000	4000000	CD8	0.38	0.05	12	19000000	2500000
B cell	0.44	0.06	14	22000000	3000000	B cell	0.35	0.04	10	17500000	2000000
Granulocyte	0.44	0.02	3	31500000	1000000	Granulocyte	0.49	0.17	35	24500000	8500000
Lymphocyte	0.63	0.16	26	20500000	8000000	Lymphocyte	0.45	0.06	13	22500000	3000000
CD16	0.41	0.07	16	500000	3500000	CD16	0.5	0.04	8	25000000	2000000
PBMC	0.01			7500000	0	PBMC	10			500000000	0
Platelet	0.15	0.07	14	#REF!	3500000	Platelet	0.33	0.02	6	16500000	1000000
Pre H- #3						Pre H+ #3					
CD4	0.43	0.04	8	21500000	2000000	CD4	0.72	0.15	20	36000000	7500000
CD8	0.52	0.04	7	26000000	2000000	CD8	0.59	0.05	8	29500000	2500000
B cell	0.5	0.6	19	25000000	30000000	B cell	0.62	0.03	5	31000000	1500000
Granulocyte	0.51	0	14	25500000	0	Granulocyte	0.66	0.21	32	33000000	10500000
Lymphocyte	0.6	0.8	13	30000000	40000000	Lymphocyte	0.45	0.1	22	22500000	5000000
CD16	0.56	0.05	9	28000000	2500000	CD16	0.45	0.08	17	22500000	4000000
PBMC	10			500000000	0	PBMC	2	0.12	6	100000000	6000000
Platelet	0.44	0.07	10	22000000	3500000	Platelet	0.4	0.04	8	20000000	2000000

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
15min H- #1						15min H+ #1					
CD4	0.01			500000	0	CD4	0.01	0.01	100	500000	500000
CD8	1.2	0.47	39	6000000	2350000	CD8	0.01	0.01	0	500000	500000
B cell	0.05	0.02	33	2500000	1000000	B cell	0.01			500000	0
Granulocyte	0.01	0.01	50	500000	500000	Granulocyte	0.01	0.02	57	500000	1000000
Lymphocyte	0.03	0.01	20	1500000	500000	Lymphocyte	0.02	0.02	75	1000000	1000000
CD16	0.01		0	500000	0	CD16	0.01			500000	0
PBMC	0.1	0.09	65	5000000	4500000	PBMC	13	0.05	40	65000000	2500000
Platelet	0.01	0.01	100	500000	500000	Platelet	0.02	0.01	67	1000000	500000
15min H- #2						15min H+ #2					
CD4	0.02	0.01	50	1000000	500000	CD4	0.01			500000	0
CD8	0.02		0	1000000	0	CD8	0.01	0.01	100	500000	500000
B cell	0.05	0.03	56	2500000	1500000	B cell	0.02	0.02	100	1000000	1000000
Granulocyte	0.07	0.01	15	3500000	500000	Granulocyte	0.01			500000	0
Lymphocyte	0.02	0.01	67	1000000	500000	Lymphocyte	0.01		0	500000	0
CD16	0.01		0	500000	0	CD16	0.03	0.02	80	1500000	1000000
PBMC	0.38	0.18	46	19000000	9000000	PBMC	0.31	0.18	56	15500000	9000000
Platelet	0.01	0.01	100	500000	500000	Platelet	0.01	0.01	50	500000	500000
15min H- #3						15min H+ #3					
CD4	0.01			500000	0	CD4	0.01			500000	0
CD8	0.01		0	500000	0	CD8	0.01		0	500000	0
B cell	0.02	0.01	33	1000000	500000	B cell	0.01		0	500000	0
Granulocyte	0.01		0	500000	0	Granulocyte	0.01			500000	0
Lymphocyte	0.33	0.25	76	16500000	12500000	Lymphocyte	0.02		0	1000000	0
CD16	0.01		0	500000	0	CD16	0.01		0	500000	0
PBMC	0.07	0.02	31	3500000	1000000	PBMC	0.06	0.02	27	3000000	1000000
Platelet	0.02	0.02	100	1000000	1000000	Platelet	0.02	0.01	25	1000000	500000
30min H- #1						30min H+ #1					
CD4	0.01	0		500000	0	CD4	0.01	0.01	100	500000	500000
CD8	1.2	0.47	39	6000000	2350000	CD8	0.01	0.01	0	500000	500000
B cell	0.05	0.02	33	2500000	1000000	B cell	0.01	0	0	500000	0
Granulocyte	0.01	0.01	50	500000	500000	Granulocyte	0.04	0.02	57	2000000	1000000
Lymphocyte	0.03	0.01	20	1500000	500000	Lymphocyte	0.02	0.02	0	1000000	1000000
CD16	0.01	0	0	500000	0	CD16	0.01	0	75	500000	0
PBMC	0.1	0.09	65	5000000	4500000	PBMC	0.13	0.05	40	6500000	2500000
Platelet	0.01	0.01	100	500000	500000	Platelet	0.02	0.01	67	1000000	500000
30min H- #2						30min H+ #2					
CD4	0.02	0.01	50	1000000	500000	CD4	0.01	0	0	500000	0
CD8	0.02	0	0	1000000	0	CD8	0.01	0.01	100	500000	500000
B cell	0.05	0.03	56	2500000	1500000	B cell	0.02	0.02	100	1000000	1000000
Granulocyte	0.07	0.01	15	3500000	500000	Granulocyte	0.04	0	0	2000000	0
Lymphocyte	0.02	0.01	67	1000000	500000	Lymphocyte	0.01	0	0	500000	0
CD16	0.01	0	0	500000	0	CD16	0.03	0.02	80	1500000	1000000
PBMC	0.38	0.18	16	19000000	9000000	PBMC	0.31	0.18	56	15500000	9000000
Platelet	0.01	0.01	100	500000	500000	Platelet	0.01	0.01	50	500000	500000
30min H- #3						30min H+ #3					
CD4	0.01	0		500000	0	CD4	0.01	0	0	500000	0
CD8	0.01	0	0	500000	0	CD8	0.01	0	0	500000	0
B cell	0.02	0.01	33	1000000	500000	B cell	0.01	0	0	500000	0
Granulocyte	0.01	0	0	500000	0	Granulocyte	0.01	0	0	500000	0
Lymphocyte	0.33	0.25	76	16500000	12500000	Lymphocyte	0.02	0	0	1000000	0
CD16	0.01	0	0	500000	0	CD16	0.01	0	0	500000	0
PBMC	0.07	0.02	31	3500000	1000000	PBMC	0.06	0.02	27	3000000	1000000
Platelet	0.02	0.02	100	1000000	1000000	Platelet	0.02	0.01	25	1000000	500000

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
60min H- #1						60min H+ #1					
CD4	0.02	0.01	25	1000000	500000	CD4	0.02	0.01	67	1000000	500000
CD8	0.01			500000	0	CD8	0.01	0.01	50	500000	500000
B cell	0.01	0.01	50	500000	500000	B cell	0.02	0.01	50	1000000	500000
Granulocyte	0.01	0.01	100	500000	500000	Granulocyte	0.01			500000	0
Lymphocyte	0.02	0.02	75	1000000	1000000	Lymphocyte	0.02	0.01	50	1000000	500000
CD16	0.01		0	500000	0	CD16	0.02	0.01	25	1000000	500000
PBMC	0.34	0.15	43	17000000	7500000	PBMC	0.13	0.03	23	6500000	1500000
Platelet	0.1	0.04	37	5000000	2000000	Platelet	0.03	0	0	1500000	0
60min H- #2						60min H+ #2					
CD4	0.01		0	500000	0	CD4	0.01			500000	0
CD8	0.02		0	1000000	0	CD8	0.03		0	1500000	0
B cell	0.01		100	500000	0	B cell	0.01			500000	0
Granulocyte	0.03	0.01	40	1500000	500000	Granulocyte	0.01			500000	0
Lymphocyte	0.03	0.01	40	1500000	500000	Lymphocyte	0.01		0	500000	0
CD16	0.02		0	1000000	0	CD16	0.01		50	500000	0
PBMC	0.2	0.04	18	10000000	2000000	PBMC	0.07	0.02	29	3500000	1000000
Platelet	0.01	0	0	500000	0	Platelet	0.09	0.04	39	4500000	2000000
60min H- #3						60min H+ #3					
CD4	0.05	0.01	11	2500000	500000	CD4	0.02		0	1000000	0
CD8	0.01		0	500000	0	CD8	0.02		0	1000000	0
B cell	0.01		0	500000	0	B cell	0.01		50	500000	0
Granulocyte	0.04	0.03	71	2000000	1500000	Granulocyte	0.02		0	1000000	0
Lymphocyte	0.06	0.02	36	3000000	1000000	Lymphocyte	0.05	0.02	30	2500000	1000000
CD16	0.02	0.01	33	1000000	500000	CD16	0.03	0.01	20	1500000	500000
PBMC	0.13	0.04	31	6500000	2000000	PBMC	0.13	0.07	50	6500000	3500000
Platelet	0.03	0.03	83	1500000	1500000	Platelet	0.02		0	1000000	0

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
24 hr H- #1						24 hr H+ #1					
CD4	0.01		0	500000	0	CD4	0.02		0	1000000	0
CD8	0.01		0	500000	0	CD8	0.01		0	500000	0
B cell	0.65	0.22	34	32500000	11000000	B cell	0.02		0	1000000	0
Granulocyte	0.23	0	0	11500000	0	Granulocyte	0.01			500000	0
Lymphocyte	0.01	0.01	5	500000	500000	Lymphocyte	0.01		0	500000	0
CD16	0.01		0	500000	0	CD16	0.02		0	1000000	0
PBMC	0.02	0.01	33	1000000	500000	PBMC	0.03	0	0	1500000	0
Platelet	0.29	0.01	3	14500000	500000	Platelet	0.01	0.01	100	500000	500000
24 hr H- #2						24 hr H+ #2					
CD4	3.9	1.2	32	195000000	60000000	CD4	0.02	0.01	33	1000000	500000
CD8	1.2	0.43	35	60000000	21500000	CD8	0.02	0.01	67	1000000	500000
B cell	0.05	0.01	10	2500000	500000	B cell	0.01	0.01	50	500000	500000
Granulocyte	0.02	0.01	25	1000000	500000	Granulocyte	0.04	0.01	14	2000000	500000
Lymphocyte	0.01		50	500000	0	Lymphocyte	0.04	0.03	71	2000000	1500000
CD16	0.02	0.01	50	1000000	500000	CD16	0.01	0	0	500000	0
PBMC	0.21	0.06	26	10500000	3000000	PBMC	0.73	0.32	44	36500000	16000000
Platelet	0.01			500000	0	Platelet	0.03	0.03	0	1500000	1500000
24 hr H- #3						24 hr H+ #3					
CD4	5.4	0.76	14	270000000	38000000	CD4	0.02		0	1000000	0
CD8	0.03	0.01	20	1500000	500000	CD8	0.02	0.02	75	1000000	1000000
B cell	0.45	0.02	4	22500000	1000000	B cell	0.01		50	500000	0
Granulocyte	0.04	0.01	29	2000000	500000	Granulocyte	0.01			500000	0
Lymphocyte	0.41	0.04	9	20500000	2000000	Lymphocyte	0.04	0.01	14	2000000	500000
CD16	0.05		0	2500000	0	CD16	0.03	0.01	40	1500000	500000
PBMC	0.42	0.06	13	21000000	3000000	PBMC	0.27	0.1	36	13500000	5000000
Platelet	0.01		0	500000	0	Platelet	0.01	0.01	0	500000	500000
Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
48hr H-1						48hr H+1					
CD4	0.03	0.01	20	1500000	500000	CD4	0.02	0.01	33	1000000	500000
CD8	0.02	0.01	50	1000000	500000	CD8	0.01	0.01	100	500000	500000
B cell	0.61	0.19	31	30500000	9500000	B cell	0.12	0.1	87	6000000	5000000
Granulocyte	0.3	0.13	42	15000000	6500000	Granulocyte	0.01			500000	0
Lymphocyte	0.01	0	0	500000	0	Lymphocyte	0.02	0.01	33	1000000	500000
CD16	0.01	0.01	50	500000	500000	CD16	0.01	0	0	500000	0
PBMC	0.64	0.35	55	32000000	17500000	PBMC	1.4	1	71	70000000	50000000
Platelet	0.07	0.05	64	3500000	2500000	Platelet	0.02	0.01	25	1000000	500000
48hr H-1						48hr H+5					
CD4	0.01	0		500000	0	CD4	0.01			500000	0
CD8	0.57	0.14	24	28500000	7000000	CD8	0.25	0.01	2	12500000	500000
B cell	0.06	0.01	9	3000000	500000	B cell	0.29	0.13	45	14500000	6500000
Granulocyte	0.02	0.01	67	1000000	500000	Granulocyte	0.04	0	0	2000000	0
Lymphocyte	0.31	0.03	10	15500000	1500000	Lymphocyte	0.02	0.02	100	1000000	1000000
CD16	0.02	0.01	50	1000000	500000	CD16	0.02	0.01	33	1000000	500000
PBMC	1.9	0.98	50	95000000	49000000	PBMC	0.31	0.22	72	15500000	11000000
Platelet	0.24	0.21	85	12000000	10500000	Platelet	0.01	0.01	100	500000	500000
48hr H-3						48hr H+6					
CD4	0.04	0.02	50	2000000	1000000	CD4	0.03	0	0	1500000	0
CD8	0.55	0.08	15	27500000	4000000	CD8	0.01	0	0	500000	0
B cell	0.57	0.05	8	28500000	2500000	B cell	0.02	0	0	1000000	0
Granulocyte	0.01	0.01	100	500000	500000	Granulocyte	0.33	0.05	14	16500000	2500000
Lymphocyte	0.04	0.02	30	2000000	1000000	Lymphocyte	0.04	0.02	43	2000000	1000000
CD16	0.04	0.01	14	2000000	500000	CD16	0.01	0	0	500000	0
PBMC	0.14	0.1	74	7000000	5000000	PBMC	2.2	1.2	55	110000000	60000000
Platelet	0.01	0.01	100	500000	500000	Platelet	0.01			500000	0

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
60day H- #1						60day H+ #1					
CD4	0.35	0.22	62	17500000	11000000	CD4	0.38	0.14	36	19000000	7000000
CD8	0.2	0.08	41	10000000	4000000	CD8	0.23	0.16	67	11500000	8000000
B cell	0.42	0.36	86	21000000	18000000	B cell	0.45	0.25	56	22500000	12500000
Granulocyte	0.16	0.08	50	8000000	4000000	Granulocyte	0.17	0.04	21	8500000	2000000
Lymphocyte	0.49	0.06	12	24500000	3000000	Lymphocyte	0.35	0.21	60	17500000	10500000
CD16	0.01	0	0	500000	0	CD16	0.02	0	0	1000000	0
PBMC	5.8	2.5	43	290000000	125000000	PBMC	0.04	0.01	29	2000000	500000
Platelet	0.01	0	0	500000	0	Platelet	0.51	0.34	66	25500000	17000000
60day H- #2						60day H+ #2					
CD4	0.13	0.09	72	6500000	4500000	CD4	0.22	0.09	42	11000000	4500000
CD8	0.21	0.12	56	10500000	6000000	CD8	0.25	0.14	66	12500000	7000000
B cell	0.32	0.17	52	16000000	8500000	B cell	0.48	0.34	70	24000000	17000000
Granulocyte	0.33	0.2	61	16500000	10000000	Granulocyte	0.01	0	0	500000	0
Lymphocyte	0.04	0.04	100	2000000	2000000	Lymphocyte	0.33	0.17	52	16500000	8500000
CD16	0.32	0.13	41	16000000	6500000	CD16	0.02	0.01	67	1000000	500000
PBMC	0.03	0.01	33	1500000	500000	PBMC	0.77	0.38	50	38500000	19000000
Platelet	0.69	0.34	49	34500000	17000000	Platelet	0.01	0	0	500000	0
60day H- #3						60day H+ #3					
CD4	0.48	0.27	55	24000000	13500000	CD4	0.22	0.13	60	11000000	6500000
CD8	0.01	0.01	100	500000	500000	CD8	0.14	0.07	48	7000000	3500000
B cell	0.34	0.16	67	17000000	8000000	B cell	0.33	0.18	55	16500000	9000000
Granulocyte	0.01	0	0	500000	0	Granulocyte	0.3	0.19	63	15000000	9500000
Lymphocyte	0.34	0.22	64	17000000	11000000	Lymphocyte	0.24	0.08	32	12000000	4000000
CD16	0.01	0	0	500000	0	CD16	0.27	0.14	51	13500000	7000000
PBMC	0.94	0.42	45	47000000	21000000	PBMC	0.01	0	0	500000	0
Platelet	0.01	0	0	500000	0	Platelet	0.67	0.46	69	33500000	23000000
Sample	Total Cell (x10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
90 day H- #1						90 day H- #4					
CD4	29.5	0.8	3	29500000	800000	CD4	11	20	18	11000000	20000000
CD8	30.5	1.3	4	30500000	1300000	CD8	0.3	0.46	15	15000000	23000000
B cell	24.5	2.05	8	24500000	2050000	B cell	0.32	0.54	17	16000000	27000000
Granulocyte	2.8	1.6	6	2800000	1600000	Granulocyte	0.26	0.47	18	13000000	23500000
Lymphocyte	30.5	1.9	6	30500000	1900000	Lymphocyte	0.29	0.39	14	14500000	19500000
CD16	28	2.45	9	28000000	2450000	CD16	0.3	0.46	15	15000000	23000000
PBMC	210	23.5	11	210000000	23500000	PBMC	165	29	18	8250000000	1450000000
Platelet	115	11	9	115000000	11000000	Platelet	165	20.5	12	8250000000	1025000000
90 day H- #2						90 day H- #5					
CD4	19.5	2.4	12	19500000	2400000	CD4	0.38	0.51	14	19000000	25500000
CD8	28.5	1.8	6	28500000	1800000	CD8	0.34	0.41	12	17000000	20500000
B cell	15.5	2.1	14	15500000	2100000	B cell	0.3	0.53	18	15000000	26500000
Granulocyte	19.5	2	10	19500000	2000000	Granulocyte	0.24	0.28	12	12000000	14000000
Lymphocyte	24.5	3.25	13	24500000	3250000	Lymphocyte	0.31	0.45	14	15500000	22500000
CD16	24	0.2	8	24000000	200000	CD16	0.33	0.53	16	16500000	26500000
PBMC	255	37.5	15	255000000	37500000	PBMC	245	30	12	12250000000	1500000000
Platelet	165	13	8	165000000	13000000	Platelet	155	21	13	7750000000	1050000000
90 day H- #3						90 day H- #6					
CD4	19	2.35	12	19000000	2350000	CD4	0.24	0.45	19	12000000	22500000
CD8	17	1.85	11	17000000	1850000	CD8	0.27	0.72	26	13500000	36000000
B cell	13.5	1.85	13	13500000	1850000	B cell	0.4	0.15	4	20000000	7500000
Granulocyte	17.5	2.35	13	17500000	2350000	Granulocyte	0.27	0.35	13	13500000	17500000
Lymphocyte	15	2.9	19	15000000	2900000	Lymphocyte	0.31	0.24	22	15500000	12000000
CD16	21	2.7	13	21000000	2700000	CD16	0.21	0.42	20	10500000	21000000
PBMC	240	29	12	240000000	29000000	PBMC	215	24.5	11	10750000000	12250000000
Platelet	200	25.5	13	200000000	25500000	Platelet	150	28	19	7500000000	1400000000

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
90day H+ #1						90day H+ #4					
CD4	2.9	0.54	18	145000000	27000000	CD4	4	0.54	14	200000000	27000000
CD8	2.9	0.8	27	145000000	40000000	CD8	4.8	0.54	11	240000000	27000000
B cell	3.6	0.48	13	180000000	24000000	B cell	3.1	0.21	7	155000000	10500000
Granulocyte	2.5	0.73	29	125000000	36500000	Granulocyte	4.4	0.25	6	220000000	12500000
Lymphocyte	3.6	0.38	11	180000000	19000000	Lymphocyte	4.1	0.45	11	205000000	22500000
CD16	2.1	0.4	19	105000000	20000000	CD16	3.5	0.2	6	175000000	10000000
PBMC	3.7	0.46	12	185000000	23000000	PBMC	3.6	0.99	28	180000000	49500000
Platelet	4.8	0.37	8	240000000	18500000	Platelet	4.3	0.39	9	215000000	19500000
90day H+ #2						90day H+ #5					
CD4	4.4	0.49	11	220000000	24500000	CD4	1.2	0.6	5	60000000	30000000
CD8	3.6	0.44	12	180000000	22000000	CD8	2.5	0.76	30	125000000	38000000
B cell	0.27	0	0	13500000	0	B cell	3.8	0.38	10	190000000	19000000
Granulocyte	3.1	0.99	32	155000000	49500000	Granulocyte	4.6	0.32	7	230000000	16000000
Lymphocyte	4.4	0.45	20	220000000	22500000	Lymphocyte	4.2	0.46	11	210000000	23000000
CD16	0.2	0.3	15	10000000	15000000	CD16	4	0.44	11	200000000	22000000
PBMC	4.3	0.64	15	215000000	32000000	PBMC	3.5	0.43	12	175000000	21500000
Platelet	3.7	0.36	10	185000000	18000000	Platelet	5.3	0.57	11	265000000	28500000
90day H+ #3						90day H+ #6					
CD4	3.9	0.39	10	195000000	19500000	CD4	0.46	0.31	68	23000000	15500000
CD8	2.9	0.47	16	145000000	23500000	CD8	3.2	0.78	24	160000000	39000000
B cell	3.5	0.67	20	175000000	33500000	B cell	3.7	0.54	14	185000000	27000000
Granulocyte	3.3	0.1	31	165000000	5000000	Granulocyte	0.21	0.2	10	10500000	10000000
Lymphocyte	3.3	0.52	16	165000000	26000000	Lymphocyte	4.7	0.54	12	235000000	27000000
CD16	3.3	0.11	33	165000000	5500000	CD16	3.9	0.67	17	195000000	33500000
PBMC	4.1	0.57	14	205000000	28500000	PBMC	4	0.74	18	200000000	37000000
Platelet	4.7	0.42	9	235000000	21000000	Platelet	3.1	0.41	13	155000000	20500000
Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
155day H-1						155day H+1					
CD4	0.02		0	1000000	0	CD4	0.22	0.03	12	11000000	1500000
CD8	0.02		0	1000000	0	CD8	0.22	0.02	9	11000000	1000000
B cell	0.03	0.01	40	1500000	500000	B cell	0.2	0.06	28	10000000	3000000
Granulocyte	0.02	0.01	50	1000000	500000	Granulocyte	0.22	0.09	42	11000000	4500000
Lymphocyte	0.02	0	0	1000000	0	Lymphocyte	0.09	0.07	76	4500000	3500000
CD16	0.01	0	0	500000	0	CD16	0.01			500000	0
PBMC	0.64	0.4	63	32000000	20000000	PBMC	0.25	0.2	80	12500000	10000000
Platelet	0.01	0.01	100	500000	500000	Platelet	0.01			500000	0
155day H-1						155day H+5					
CD4	0.03	0.01	40	1500000	500000	CD4	0.01			500000	0
CD8	0.02	0.02	75	1000000	1000000	CD8	0.02	0.02	100	1000000	1000000
B cell	0.02	0.08	41	1000000	4000000	B cell	0.01			500000	0
Granulocyte	0.01	0	0	500000	0	Granulocyte	0.01			500000	0
Lymphocyte	0.03	0.02	80	1500000	1000000	Lymphocyte	0.01	0	0	500000	0
CD16	0.01	0	0	500000	0	CD16	0.01	0	0	500000	0
PBMC	0.44	0.3	67	22000000	15000000	PBMC	0.32	0.28	86	16000000	14000000
Platelet	0.03	0.01	33	1500000	500000	Platelet	0.07	0.05	77	3500000	2500000
155day H-3						155day H+6					
CD4	0.02	0	0	1000000	0	CD4	0.01	0	0	500000	0
CD8	0.02	0.01	50	1000000	500000	CD8	0.02	0.01	67	1000000	500000
B cell	0.02	0	0	1000000	0	B cell	0.16	0.08	52	8000000	4000000
Granulocyte	0.04	0.01	13	2000000	500000	Granulocyte	0.01			500000	0
Lymphocyte	0.03	0.02	60	1500000	1000000	Lymphocyte	0.03	0.01	20	1500000	500000
CD16	0.07	0.06	92	3500000	3000000	CD16	0.01			500000	0
PBMC	0.39	0.27	69	19500000	13500000	PBMC	0.35	0.27	77	17500000	13500000
Platelet	0.02	0.01	33	1000000	500000	Platelet	0.1	0.1	95	5000000	5000000

Sample	Total Cell (x 10 ⁶)	Live Cell	% Viability	Total	Live	Sample	Total Cell	Live Cell	% Viability	Total	Live
169d -4						169d +4					
CD4	0.01	0.01	100	500000	500000	CD4	0.03	0.03	100	1500000	1500000
CD8	0.01		0	500000	0	CD8	0.01			500000	0
B cell	0.01		50	500000	0	B cell	0.05	0.03	50	2500000	1500000
Granulocyte	0.02		0	1000000	0	Granulocyte	0.02	0	0	1000000	0
Lymphocyte	0.12	0.02	13	6000000	1000000	Lymphocyte	0.01	0.01	50	500000	500000
CD16	0.01	0.01	50	500000	500000	CD16	0.01			500000	0
PBMC	0.45	0.32	71	22500000	16000000	PBMC	1.4	1.1	77	70000000	55000000
Platelet	0.02	0	0	1000000	0	Platelet	0.01	0.01	100	500000	500000
169d -5						169d +5					
CD4	0.03	0.02	50	1500000	1000000	CD4	0.02	0.01	33	1000000	500000
CD8	0.36	0.01	3	18000000	500000	CD8	0.01	0.01	100	500000	500000
B cell	0.05	0.04	78	2500000	2000000	B cell	0.01		0	500000	0
Granulocyte	0.05	0	0	2500000	0	Granulocyte	0.03	0.01	17	1500000	500000
Lymphocyte	0.03	0.02	60	1500000	1000000	Lymphocyte	0.01		50	500000	0
CD16	0.01	0.01	29	500000	500000	CD16	0.02	0.01	33	1000000	500000
PBMC	1.7	0.96	57	85000000	48000000	PBMC	0.14	0.01	93	7000000	500000
Platelet	0.02	0	0	1000000	0	Platelet	0.02	0.01	67	1000000	500000
238d -6						238d +6					
CD4	0.32	0.16	48	16000000	8000000	CD4	0.01		0	500000	0
CD8	0.09	0.05	59	4500000	2500000	CD8	0.19	0.17	92	9500000	8500000
B cell	0.01	0	0	500000	0	B cell	0.09	0.05	59	4500000	2500000
Granulocyte	0.07	0.04	57	3500000	2000000	Granulocyte	0.01		0	500000	0
Lymphocyte	0.14	0.1	70	7000000	5000000	Lymphocyte	0.03	0.02	60	1500000	1000000
CD16	0.03	0.03	100	1500000	1500000	CD16	0.06	0.06	92	3000000	3000000
PBMC	0.48	0.16	33	24000000	8000000	PBMC	0.27	0.18	66	13500000	9000000
Platelet	0.05	0.05	9	2500000	2500000	Platelet	0.06	0.04	64	3000000	2000000