ABSTRACT

FOXP3 EXPRESSION AS A MARKER FOR SUPPRESSIVE IMMUNE CELLS IN THE GASTROINTESTINAL TRACT OF HIBERNATORS

By Elizabeth S. Weir

Hibernating mammals rely on stored fat instead of ingested food as a fuel source during the winter. This prolonged fast affects various systems and causes them to make changes to survive the long hibernation season. One system that is greatly affected is the gastrointestinal (GI) tract, which shrinks in mass and loses some of its important barrier functions. The GI tract is home to a large number of immune cells in all mammals. The number of GI immune cells increases during hibernation, which would be a sign of inflammation in other animals. Hibernators, however, show no other signs of inflammatory disease. A possible explanation for this is that within the increased numbers of immune cells in gut of hibernators there are increased numbers of suppressive immune cells. The main type of suppressive immune cell is the regulatory T-cell (Treg). Tregs suppress other immune cells and prevent autoimmune disease. All Tregs express the protein FoxP3, which is a transcription factor responsible for the development and function of Tregs**. I hypothesized that the increased numbers of immune cells in the small intestine of hibernators do not induce an inflammatory phenotype due to a larger percentage of FoxP3$^+$ regulatory T-cells (Tregs) among them.** In order to test this, I isolated intraepithelial lymphocytes and lamina propria leukocytes from small intestine of summer and hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) and analyzed them for FoxP3 expression using flow cytometry. I found general increases in FoxP3$^+$ for LPL cells during hibernation compared to summer. Some variability exists in the hibernating animals based on season. CD3$^+$FoxP3$^+$ T-cells did not vary significantly between summer and hibernators, but a significant population of CD3^{lo}FoxP3$^+$ or CD3$^-$FoxP3$^+$ cells were found in hibernators. These data suggest seasonal shifts in the intestinal immune phenotype of hibernators. qPCR results of the colon showed that FoxP3 RNA expression was not different between summer and either hibernating state, but late season hibernators had significantly more FoxP3 transcript than early season hibernators. The expression of FoxP3 RNA in the ileum did not vary significantly between summer and hibernators. These data indicate changes in leukocyte populations in the gastrointestinal tract of hibernating ground squirrels. Identification of seasonal changes in intestinal Treg populations will lead to future studies of how squirrels control immune activity during hibernation and could help identify the overactive components of the GI tract immune system in patients on total parenteral nutrition.

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A Thesis Submitted
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science - Biology

at

The University of Wisconsin Oshkosh
Oshkosh WI 54901-8621

October 2015

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10/23/2015 Date Approved

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10/23/15 Date Approved

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10/23/2015 Date Approved

DEAN OF GRADUATE STUDIES

12/16/2015
Date Approved

FORMAT APPROVAL

10/19/15
Date Approved

ACKNOWLEDGEMENTS

Many people have helped me throughout my graduate career and without them I would have not accomplished my goals. First and foremost, I would like to thank my advisor, Dr. Courtney Kurtz and my committee members Dr. Lisa Dorn and Dr. David Hall from Lawrence University. With their expertise and guidance, I was able to improve and become a better researcher and immunologist. My fellow researchers and friends were essential for helping me complete this project: I would like to thank Ryan Sprenger, Samantha Mitchell, Katelyn Malcheski, and Jeff Farvour for helping with discussion and other aspects of the project. I would also like to thank all the professors of the department of Biology for their help and guidance throughout the years. I am very grateful to the members of Science Outreach for giving a fun and fabulous opportunity teaching children. In addition, my experience through my graduate career would not have been possible without the support of my parents and siblings. I would like to say a special thank you to my husband George Weir for supporting me throughout my education and having faith in me, throughout the good times and the stressful times.

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CHAPTER 1: INTRODUCTION

THIRTEEN-LINED GROUND SQUIRREL

The thirteen-lined ground squirrel, *Ictidomys tridecemlineatus,* is a small (110-270g) hibernating rodent native to south central Canada and central United States. This small mammal is a tan/golden color with seven dark stripes separated by 6 lighter ones occasionally broken into spots down its back. Thirteen-lined ground squirrels are about 10 inches, including the 3-inch tail, and usually weigh about 110 to 270 g depending on the season. Ground squirrels are known to live alone in burrows, but in the same vicinity as other ground squirrels. They have a wide ranging diet, including seeds, leaves and grasses, fruits, insects, and eggs. Thirteen-lined ground squirrels hibernate annually for about 5-8 months of the year in order to avoid harsh winter conditions and limited food availability. After hibernation, mating occurs in early spring, and pups are born about a month later.

Thirteen-lined ground squirrels make good laboratory animal models because they are small, easily caught and maintained in captivity, and hibernate reliably in captive settings. These rodents also exist in relatively high density in close proximity to humans. They require similar nutrition to other laboratory rodents and can act as model for diseases and hazards that threaten other mammalian species. They are unique from other common laboratory rodents,

such as rats and mice, in that they hibernate. Thus, they represent an excellent rodent model for who wish to study the physiology of hibernation.

HIBERNATION

Hibernation is a survival strategy designed to conserve energy during harsh conditions. Before entering hibernation, thirteen-lined ground squirrels become hyperphagic, storing much of their ingested energy as white adipose tissue (fat). White adipose tissue is a good source of usable energy and thirteen-lined ground squirrels need to have enough energy to survive hibernation. During hibernation, thirteen-lined ground squirrels enter a metabolically-depressed state known as torpor. Torpor is a state of decreased physiological activity that reduces body temperature to about 1-2°C above ambient temperature, reduces heart rate to 3-4 beats per minute and respiratory rate to 4-6 breaths per minute (Carey et al, 2003). Torpor is broken up by bursts of metabolic activity known as interbout arousals (IBA). Interbout arousals restore body temperature, metabolic, respiratory, and heart rate to euthermic levels (Prendergast et al, 2002).

Although thirteen-lined ground squirrels store energy before hibernation, they could not survive if their metabolic rate did not decrease significantly. Metabolic rate during torpor is reduced to about 1% of active rate (Carey et al, 2003). This metabolic depression reduces the amount of energy needed and allows them to stretch their energy reserves to last the entire hibernation season. Inadequate fat stores or using too much energy by arousing more often

for longer periods can be fatal to hibernators, as evidenced in bats suffering from white-nose syndrome (Ben-Hamo et al, 2013). Protein synthesis is also severely depressed during torpor, about 0.13–0.5% of euthermic (normal) values, but is fully restored during the interbout arousal (Carey et al, 2003). Not only is protein synthesis depressed, but the overall rates of gene transcription and translation are depressed during torpor. It is known that within the small intestinal epithelial cells DNA synthesis is suppressed to about 4% of rates in active animals (Carey et al, 2003). One other important cellular function that is halted by torpor is mitosis, or cell division. Within the intestinal epithelial cells, cell division is stopped in the G2 or late S phases during torpor (Chang et al, 2005). During interbout arousals, gene transcription, translation, and mitosis all resume at close to normal rates.

Up to 80% of the energy stored for the hibernation season is consumed during interbout arousals. The significance for interbout arousals is still unknown, but there are many hypotheses. These hypotheses include the need to eliminate metabolic waste, readjust neural circuits, eliminate sleep debt, or restore immune system function (Carey et al, 2003). Elimination of metabolic waste is important because waste products are produced during torpor, but are not eliminated from the body. The readjustment of neural circuits is considered a strong hypothesis because during torpor thirteen-lined ground squirrels have a low body temperature which slows action potentials in neurons and reduced

the efficacy of synaptic transmission. This means that the neuron signals aren't reaching their targets. Elimination of sleep debt is a concern because while the ground squirrel appears to be sleeping during torpor, electroencephalograms (EEGs) have shown that there is no rapid eye movement (REM) sleep. After the ground squirrel arouses, it enters REM sleep before re-entering torpor (Walker et al, 1977). Lastly, interbout arousals are thought to allow the immune system to combat pathogens that were introduced during the preceding torpor bout (Prendergast et al, 2002). Since the number of circulating immune cells is significantly decreased during torpor and fully restored during interbout arousals, the body needs the arousals to mount an immune response and clear any infection.

There are many hypotheses for the induction of torpor, although it is likely a combination of multiple factors. In some species, there is evidence that circadian rhythms play an important role in the induction of torpor. During hibernation, the thirteen-lined ground squirrel is fasting, which alters nutrient status. This nutrient status can be determined by looking at the ratio of AMP to ATP and NAD^+ to NADH (Serkova et al, 2007). For example, the concentration of NAD^+ in the liver of thirteen-lined ground squirrels entering torpor is on average 2-3 times higher than in active ground squirrels (Serkova et al, 2007). Fasting increases both ratios, which reduces cellular nutrient status. This is thought to lead other physiological systems to respond by lowering metabolism, although the exact mechanism is unknown. Another possible signal triggering the

entrance into torpor is low levels of circulating leptin. Leptin is a hormone produced by adipose cells to regulate the amount of fat stored in the body. It is essentially in charge of both the sensation of hunger and adjusting energy expenditures. The sensation of hunger is inhibited when the amount of circulating leptin reaches a certain level. In a study done on mice, those that had high levels of leptin did not go into torpor while low leptin level mice entered into torpor (Gavrilova et al, 1999). Whatever the cues are that induce the entrance into torpor, and therefore hibernation, it is clear that this dramatic shift in physiology has significant effects on several organ systems.

GI TRACT OF HIBERNATORS

The gastrointestinal tract (GI) undergoes significant fluctuations in structure and function as ground squirrels transition into and out of the hibernation season. When hibernators go months without eating, their gut lining shrinks and the GI tract loses mass. The villus height shrinks during hibernation, increases as animals prepare to emerge in the spring, is greatest in the summer, and decreases from summer to fall (Carey, 1990). During torpor, the GI tract lining (i.e., the mucosa) shrinks, causing some dysfunction in the nutrient transport systems across the small intestine. For instance, solute transport is suppressed at cold body temperatures (~4-8°C), but when the intestine is rewarmed nutrient and electrolyte transport rates are restored to normal levels (Carey, 1990). These findings are consistent with the effects of temperature on

enzyme activity rates (i.e. kinetics). This also suggests preservation of these transport pathways during hibernation, when the animals are not eating, because active transport of these nutrients is still functional when the animals undergo an interbout arousal. Hibernation appears to have little to no effect on the sodium-glucose transporter, membrane-bound hydrolytic enzymes, or intestinal alkaline phosphatase, with regard to messenger RNA (mRNA), protein abundance, or specific activity (Carey, 1990). While hibernation negatively affects some pathways, it enhances sugar and amino acid absorption in the gut when these are normalized to mucosal mass or protein. Thus, digestive function is maintained and able to resume upon rewarming, even though the total intestinal tissue mass is reduced during harsh winter months (Carey, 1990).

Although the atrophy of the gut mucosa lead to significantly reduced energy expenditures, a benefit to hibernators on a limited energy "budget", it also causes increased permeability and weakening of the epithelial barrier. This potentially allows commensal bacteria and their products to move into sterile body tissue (Carey et all, 2012a). In most mammals, such an invasion would lead to an immune response and, most likely, to damaging inflammation. The intestinal tract contains a large immune population in all mammals. This specialized immune cell population has different strategies and responses compared to the general systemic immune population when it comes to interactions with bacteria and other invaders. Interestingly, the systemic and

intestinal immune populations of hibernators both undergo dramatic, yet different, changes during the hibernation season.

IMMUNE SYSTEM OF HIBERNATORS

Relatively few studies have examined the effects of hibernation has on the immune system. It has been known for many years that during torpor the number of circulating leukocytes decreases by ~90% (Boyer & Barnes, 1999; Bouma et al, 2011). This reduction affects granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes. During interbout arousals, the number of neutrophils and monocytes is restored to normal, but the number of lymphocytes increases to only about 50% of the normal numbers (Boyer & Barnes, 1999; Bouma et al, 2011). This reduction in immune cells during torpor leads to an impaired systemic immune system during hibernation.

The functions of both the innate and adaptive immune systems decrease during hibernation. The innate, or non-specific, immune system is the body's first line of defense against invading organisms and viruses. It begins reacting as soon as the foreign antigen enters the body. During torpor, injection of lipopolysaccharides (LPS) a product of gram-negative bacteria, which would normally elicit an innate immune response, has no effect on immune activity in golden-mantled ground squirrels (*Spermophilus lateralis*) (Prendergast et al, 2002). Upon arousal however, the animals had higher body temperatures than

non-LPS “infected” golden-mantled ground squirrels and did not enter torpor until the LPS “infection” was cleared, demonstrating a delayed immune response during torpor (Prendergast et al, 2002).

Another important aspect of innate immunity is the complement system. This system uses a series of preexisting proteins for protection from pathogens rather than utilizing cells that require activation and energy in order to properly function. Throughout the hibernation season, the classical pathway of the serum complement system remained functional, although at a significantly reduced level (Maniero, 2002). Another important player in the innate immune system is a group of cells called macrophages. Macrophages are phagocytic cells derived from monocytes that have moved into peripheral tissue. Macrophages are known to secrete tumor necrosis factor (TNF). TNF is a pro-inflammatory cytokine that regulates the synthesis of some known cytokines. Macrophages are able to bind to LPS and consequently initiate an immune response. At low temperatures, macrophages from ground squirrels are able to bind LPS as well as at higher euthermic temperatures, indicating that macrophages have the ability to bind LPS over a wide temperature range (Maniero, 2005). This is helpful to the immune protection of hibernating mammals as it allows some innate function to continue even at the cold body temperatures of torpor. Although low temperatures do not affect macrophages ability to bind to LPS, it appears that macrophages of torpid hibernators are not eliminating LPS as rapidly as summer ground squirrels (Prendergast et al, 2002). This will require

them to spend more time in the arousal state to eliminate the LPS and therefore use more energy. Overall, some innate immune function is maintained during hibernation, even at the low body temperatures of the torpid state.

Adaptive, or acquired, immunity is the other main branch of the immune system and is extremely specific, although it takes several weeks to fully develop after exposure. An extremely important aspect of adaptive immunity is the production of memory cells, which create long-term protection against re-exposure to the same invader. During hibernation, the antigen-specific response of the adaptive immune system is extremely delayed which could be due to a reduced production of antibodies during torpor and arousal. Similar results were reported in an *in vivo* study using thirteen-lined ground squirrels that were injected with foreign antigen, lipopolysaccharide (LPS) (Maniero, 2005). The squirrels had a delayed reaction for the duration of torpor. Though these data suggest an overall suppression of adaptive immune responses during torpor, the presence or absence of a response seems to depend on the antigen used. A study in ground squirrels demonstrated that the humoral immune response to a T-cell independent type 2 antigen, such as the carbohydrate ficoll, is impaired during hibernation while the response to a T-cell dependent antigen, such as ovalbumin, is maintained (Bouma et al, 2013). T-cells, like macrophages are known to secrete TNF. Unlike macrophages, the T-cells ability to produce TNF remained unchanged during hibernation compared to summer and interbout arousal states (Novoselova et al, 2000). Together, these studies suggest that the systemic

immune system is unable to mount an effective response against many foreign infectious antigens during torpor, but during interbout arousal the immune system is brought back to full restoration.

Although the overall number of circulating lymphocytes decreases during hibernation, the numbers within the GI tract rise. The GI tract contains the largest mucosal surface in the body and, as such, is exposed to a huge number and vast array of commensal bacteria and dietary antigens (Carey, Pike et al, 2012; Carey Walters et al, 2012). These mostly harmless antigens have to be tolerated, while invading pathogens must be identified and attacked. Because of its important role as a barrier, immune cells, such as dendritic cells and lymphocytes, are found in close contact with the intestinal epithelium as well as in the underlying lamina propria.

Immune cells found within the intestinal epithelium are known as intraepithelial lymphocytes (IELs) and are the first line of defense in the intestinal immune system. Phenotypically, IELs are T-cells, but unlike other T-cells, IELs are not antigen-specific, release cytokines immediately upon stimulation, and have cytotoxic activity (Chang et al, 2005). High levels of IELs generally indicate ongoing inflammation within the mucosa or, more specifically, inflammation within the gut-associated lymphoid tissue (GALT).

Immune cells, called lamina propria leukocytes (LPLs), are also found within the lamina propria, a thin layer of loose connective tissue that lies beneath the epithelium. LPLs are mainly T and B cells, antigen-specific immune

cells that can activate other cells, including macrophages and eosinophils (Sun et al, 2007). Another main component in controlling the microbial communities of the intestine is immunoglobulin A (IgA). IgA is a class of antibody produced by LPL B cells and can attach to bacteria, preventing them from penetrating the epithelial barrier.

In the 13-lined ground squirrel, the total number of IEL and LPL increases about three-fold during hibernation compared with summer (Kurtz & Carey, 2007). Both B and T-cells increased and IgA levels increased as well, especially during torpor. Within the hibernation season, IEL numbers were higher in torpor compared to interbout arousals, but LPL numbers were not different between these states. The percentage of IEL and LPL cells expressing activation markers differed. For IEL, the combined hibernator group was lower compared to summer whereas for LPL interbout arousal and combined hibernator groups were greater than summer (Kurtz & Carey, 2007). This suggests that the LPL of hibernators are of an activated phenotype that can undergo mitosis and secrete cytokines. Among the IELs, the percentage of T-cells was higher in summer and torpid squirrels compared to interbout arousals. CD8α and CD4 co-receptor expression on T-cells was higher in summer than in either hibernating state (Kurtz & Carey, 2007). The percentage of T-cells in the LPL compartment was lower, but the percentage of B cells was higher in hibernator groups compared with summer squirrels (Kurtz & Carey, 2007). The percentage of $CD8\alpha^+$ LPL was

unaltered by hibernation, but the percentage of $CD4^+$ LPL was greater during torpor and interbout arousals compared with summer (Kurtz & Carey, 2007).

Dramatic increases in immune cells, such as that seen in the intestine during hibernation, would generally be associated with inflammation and disease. In hibernators, however, no other signs of inflammation (thickness, redness, bleeding) are seen and the animals show no signs of disease. This large immune population is significant for several reasons. First, such a large population of cells would require a great deal of energy to produce and maintain. Given that the purpose of hibernation is as an energy-saving strategy, it can be assumed that these cells serve an important purpose for the animal. Second, the large increase in immune cells in the small intestine stands in stark contrast to the dramatic decrease in immune cells circulating in the bloodstream during torpor. It is possible that these cells are present in the intestine to act as sentinels, ready to respond to any invaders that may penetrate the leaky intestinal barrier. If so, however, this sets up a potentially dangerous and life-threatening situation for the animal because an invasion by bacteria or their products could lead to damaging inflammation. One potential solution to this problem would be to increase the number of anti-inflammatory or suppressive immune cells in the intestine of hibernators.

NATURAL REGULATORY T-CELLS (nTregs)

Regulatory T-cells (Tregs) are the major suppressive immune cell type. Natural Tregs (nTregs) are "born" in bone marrow, but develop within the thymus. In order to develop correctly, nTregs require higher interactions between their T-cell receptors (TCR) and major histocompatibility complex II (MHCII) on thymus cells (Huang et al, 2014). As a result of their "training" in the thymus, nTregs learn to determine which antigens will be tolerated (not mount an immune response) and which antigens will cause an immune reaction. From this training evolves their main function—the suppression of inflammation and prevention of autoimmune disease.

Tregs suppress inflammation by inhibiting or "calming down" many different types of immune cells from both the innate and adaptive immune branches. They accomplish this by secreting immunosuppressive cytokines or metabolites and/or expressing various molecules on their cell surface. Interleukin 2 (IL-2) receptors (also called CD25) are highly expressed on the cell surface of nTregs (Kurtz et al, 2011). IL-2 is an important cytokine that stimulates the activation and proliferation of naïve T-cells. When IL-2 is produced by activated T-cells in the presence of nTreg, the IL-2 receptors on the nTregs are thought to bind to the IL-2 thereby preventing it from binding to and activating naïve T-cells (Sakaguchi et al, 2009). Another molecule produced by nTregs is adenosine. Extracellular adenosine is a physiological negative regulator, or in other words, it is an anti-inflammatory molecule. Adenosine is

synthesized from ATP through the activities of two surface enzymes ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), or CD39, and ecto-5'-nucleosidease, or CD73. Both enzymes are required for adenosine generation from ATP because CD39 converts ATP to 5'AMP and CD73 converts 5'AMP to adenosine (Sakaguchi et al, 2009). nTregs express both of these enzymes on their surface and an *in vivo* study in mice concluded that adenosine contributed to the anti-inflammatory role in Tregs (Deaglio et al, 2007; Borsellino et al, 2007).

nTregs can also use cell-to-cell contact to mediate suppression. Tregs are known to suppress target cells, mainly antigen presenting cells (APCs) through cell-mediated contact suppression (Sakaguchi et al, 2009). Tregs can use a variety of mechanisms to cause effector T-cell and APC apoptosis. One possible mechanism is through galectin-1 (Garin, et al, 2006). Galectin-1 is a protein that binds to glycoproteins, which are important integral membrane proteins, causing cell cycle arrest in the target cell. This galectin-1 protein allows nTregs to use cell-to-cell contact to cause apoptosis in APC and effector T-cells. nTregs are also the only lymphocyte to express cytotoxic T-lymphocyte-associated protein (CTLA)-4 (van Loosdregt et al, 2013). CTLA-4 closely resembles the T-cell costimulatory molecule CD28, which is used for T-cell activation on dendritic cells (DCs). CTLA-4 has a high binding affinity and will bind to DCs and inhibit CD28 from binding, essentially taking up the space needed to activate naïve T-cells (Ostman et al, 2006).

INDUCED REGULATORY T-CELLS (iTregs)

Although nTregs are arguably the best-studied suppressive cells, other types of Treg exist. Induced regulatory T-cells (iTregs) are similar to nTregs because they also suppress immune reactions, but they are also different from nTregs in a number of ways. A defining distinction between nTreg an iTreg is that iTregs develop in the periphery, and mostly in the gut-associated lymphoid tissue of the intestine (Izcue et al, 2006). There are many uncertainties when it comes to distinguishing nTregs from iTregs, as there are no surface markers that can be used to effectively discriminate the two and there is no evidence as to whether they have different biological functions.

There are two common iTreg subsets: T helper 3 cells (Th3) and type 1 regulatory T-cells (Tr1). Th3 cells are identified by their production of transforming growth factor-beta (TGF-β). TGF-β helps to induce naïve T-cells into Th3 cells, and suppresses the proliferation of Th1 and Th2 cells (i.e., effector T helper cells) (Lan et al, 2005). Tr1 cells, on the other hand, are found mainly within the intestinal mucosa and are characterized by their secretion of IL-10 (Belkaid, 2007). IL-10 down-regulates the expression of pro-inflammatory cytokines and proteins; it also suppresses inflammatory mechanisms on different leukocytes (Collison et al, 2009). Tr1 cells mediate suppression using cytokine-dependent pathways and not cell-to-cell contact (Lan et al, 2005).

FOXP3

Although nTreg and iTreg are different in many ways, both are characterized by the expression of the protein forkhead box P3 (FoxP3) during their active suppression phases. FoxP3 is a transcription factor that is the key marker of Tregs. It is a member of the forkhead and winged helix family of transcriptional regulators and is essential in the development and function of Tregs. This was shown through elegant experiments where the FoxP3 gene was transferred into naïve T-cells (Hori et al, 2003). The transfer of the FoxP3 gene alone converted the naïve T-cells to Treg-like cells. These Treg-like cells were similar to nTregs in that they began to upregulate the expression of surface molecules that are found on nTregs, were very responsive to TCR stimulation, and inhibited cytokine production. FoxP3+ Tregs can, under certain conditions, such as inflammation or lymphopenia, downregulate the expression of FoxP3, lose their suppressive functions and act like effector (non-regulatory) T-cells (Hori et al, 2003). FoxP3 expression tends to be more stable in nTreg than iTreg, making iTreg more likely to "switch over" to effector T-cells (Huang et al, 2014).

The precise control mechanisms of FoxP3 on other genes have not yet been identified, but it is assumed that they exert control via DNA binding interactions during transcription. Mutations of the FoxP3 gene lead to X-linked autoimmune diseases in humans, which are characterized by severe diarrhea,

diabetes and ulcerative colitis and can be lethal (Fleskens & van Boxtel, 2013). Patients suffering from autoimmune disease show significantly lower levels of $FoxP3^+$ cells in peripheral blood mononuclear cells (lymphocytes, monocytes, and macrophages) (Lan et al, 2005). Because of its essential role and expression in all Treg types, FoxP3 is often used to estimate the number of Tregs in a tissue.

CHAPTER 2: BODY

INTRODUCTION

The thirteen-lined ground squirrel, *Ictidomys tridecemlineatus,* is a mammal that hibernates 5-8 months each year to avoid harsh winter conditions. During hibernation, thirteen-lined ground squirrels cope with periods of scarce food supply and increased energy demands by entering into a metabolically-depressed state known as torpor. Torpor is a state of decreased physiological activity that reduces body temperature, heart rate, respiratory rate, and overall metabolic rate to extremely low levels (Carey et al, 2003). Torpor is interrupted by periods known as interbout arousals, which restore metabolism close to euthermic levels.

During hibernation, the immune system changes drastically. In torpor, the number of circulating leukocytes is dramatically reduced. This leukopenia has an effect on granulocytes (neutrophils, eosinophils, and basophils), but mainly affects lymphocytes, and monocytes (Boyer & Barnes, 1999; Bouma et al, 2011). During interbout arousals, the numbers of neutrophils and monocytes increases to euthermic levels, while the numbers of lymphocytes increases to only about 50% of euthermic numbers. This profound leukopenia affects the ability of torpid animals to mount an immune response. In studies done on small hibernating mammals [European hamster (*Cricetus cricetus*), European

hedgehog (*Erinaceus europeaus*), European ground squirrel (*Spermophilus citellus*), arctic ground squirrel (*Urocitellus parryii*), and thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*)], leukopenia was found to inhibit torpid animals from mounting an immune response (Luis & Hudson, 2006; Inkovaara et al 1973; Bouma et al, 2013).

Beyond circulating (systemic) immune populations, the gastrointestinal (GI) tract of all mammals is home to a large, gut-specific immune population. Contrary to what is seen in circulation, the number of GI immune cells increases during hibernation. Looking more specifically at the GI tract, the number of Peyer's patches (PP) is unaffected, but the number of leukocytes per PP increases and the number of intraepithelial lymphocytes (IEL) and lamina propria leukocytes (LPL) increases during hibernation (Kurtz & Carey, 2007). IEL reside within the epithelium and are an innate-type lymphocyte that acts as the first line of defense against invading microbes. LPL are found within the lamina propria underlying the epithelium and are mostly comprised of T and B lymphocytes that defend against specific antigens. In most animals, a dramatic increase in IEL and LPL numbers, such as that seen in hibernating ground squirrels, would be a sign of inflammation and disease. The small intestine of hibernators, however, does not show any other signs of inflammation (thickness, redness, bleeding) and the animals show no signs of disease. One reasonable explanation for this is that the immune cells in the intestine of hibernators are a suppressive, rather than an inflammatory, type.

The major immunosuppressive cell type is the regulatory T-cell (Treg). The main function of Tregs is to suppress inflammation and prevent autoimmune disease. Tregs work by preventing T-cell activation and proliferation and by deterring antigen-presenting cells (APC) and inhibiting neutrophil effects (Sakaguchi et al, 2009). There are two types of Tregs, natural Tregs (nTregs) and induced Tregs (iTregs). nTregs develop in the thymus and iTregs are induced in the periphery, mainly at the mucosal interface of the intestine. nTregs mainly secrete IL-2, while iTregs mainly secrete IL-10 or TGF-β (Lan et al, 2005; Belkaid, 2007; Kurtz et al, 2011). The key marker of both Treg types is forkhead box P3 (FoxP3). FoxP3 is a member of the forkhead and winged helix family of transcriptional regulators. FoxP3 expression has been used in other mammals to estimate the number of Tregs in a tissue. Tregs and FoxP3 have yet to been studied in any hibernating species. I hypothesized that the increased numbers of immune cells found within the small intestine of hibernators include a large percentage of $FoxP3^+$ Tregs. In this study, I examined FoxP3 expression as an estimate of Tregs in several organs of the GI tract (ileum and colon). I also quantified the number of $FoxP3^+$ cells in the small intestine during the active, early hibernation and late hibernation seasons.

MATERIALS AND METHODS

Animals*.* Thirteen-lined ground squirrels were bred in the UWO animal facility or captured in various parts of Wisconsin in the summers of 2012-2013. The squirrels were housed in individually at 22-24°C with a light cycle roughly corresponding to the natural photoperiod and were fed a limited diet (Iams Proactive Health plus sunflower seeds) to prevent overeating and given ad lib water. In September-October, squirrels were transferred to the hibernaculum and maintained at 4°C. The room was dark except for brief periods of low red lighting once per day to check activity state. Water and food were removed after squirrels entered hibernation. The University of Wisconsin Oshkosh Institutional Animal Care and Use Committee approved all animal procedures (Protocol # 0026-000260-2-21-13).

Tissue Collection/Isolation of IEL and LPL. Thirteen-lined ground squirrels were euthanized by decapitation. Summer squirrels were euthanized in July-August when T_b=37-39°C. Hibernating squirrels were euthanized between late September and February when T_b=5-7°C**.** Torpid animals were euthanized at two points during the hibernation season, early hibernation (≤ 3 mo since initiation of first torpor bout) and late hibernation (> 3 mo since initiation of first torpor bout). The entire small intestine was removed and placed in cold calcium- and magnesium-free Hanks balanced salt solution (CMF-HBSS). The intestine was opened longitudinally, rinsed to remove luminal

contents, and Peyer's Patches were excised. To collect IEL, the remaining tissue was cut into 1-cm pieces and incubated while stirring in CMF-HBSS containing 0.1 M ethylenediamine tetraacetate (EDTA) (37°C, 3 x 20 min). The supernatants were collected and combined as the IEL fraction. The remaining intestinal pieces were incubated in RPMI-1640 containing 5% fetal bovine serum (FBS), 30 U/ml collagenase type I, 11 U/ml collagenase type III, and 20 U/ml collagenase type IV (37°C, 3 x 45 min). The supernatants were collected and combined as the LPL fraction. IEL and LPL fractions were filtered through glass wool columns and centrifuged (4°C, 600 x *g*, 10 min). The resulting pellets were resuspended in 40% Percoll, layered over 75% Percoll and centrifuged (4°C, 600 x *g,* 20 min, no brake). The lymphocytes were collected from the 40%/75% interface, washed with RPMI-1640, counted, and prepared for flow cytometry.

Flow Cytometry. Methylene blue was used to stain cells for viable (unstained) and nonviable (stained) cells. Cells were then enumerated in the counting chamber and after counting, isolated mononuclear cells were separated into tubes and resuspended in 1% bovine serum albumin (BSA) in 0.1M PBS solution. A rat Fc block was added to the cells to block the Fc receptors and prevent non-specific binding (10 min. in dark, 4°C). Monoclonal antibodies conjugated to either phycoerythrin (PE) or fluoroscein (FITC) were added to each tube, and the cells were incubated in the dark at 4°C for 20 min. The cells

were then washed twice with 1% BSA/PBS and fixed in FoxP3 permeabilization/fixation buffer (eBioscience) for 30 min - 18 hr (according to manufacturer's instructions) at 4°C in the dark. After incubation, the cells were washed with FoxP3 permeabilization wash. Monoclonal antibodies to FoxP3 were then added and the cells were mixed gently and incubated in the dark at 4°C for 20 min. The cells were washed twice with FoxP3 permeabilization wash and resuspended in BSA/PBS. Data was collected on a BD FACS Calibur (Becton Dickenson, San Jose, CA) and analyzed using WinMDI 2.9 software (The Scripps Institue, La Jolla, CA). Lymphocytes were gated by forward- and side-scatter characteristics and 50,000 gated events were collected per sample.

Quantitative RT-PCR. Total RNA was isolated from ileum, stomach, and colon using Total RNA Tissue Kit (IBI, Peosta, IA as per manufacturer's instructions). Briefly, the tissue samples were homogenized in detergents and chaotropic salt to lyse cells. The homogenate was combined with 70% EtOH and run through a glass fiber matrix. Contaminants were removed using wash buffer and purified RNA was eluted with RNase-free water. Purified RNA was quantified and 1 μg was used for the synthesis of cDNA using Moloney Murine Leukemia Virus (Promega, Madison, WI) using oligo (dT) primers to target eukaryotic mRNA. This cDNA was then used as the template for qRT-PCR with specific primers designed to amplify thirteen-lined ground squirrel mRNA sequences. cDNA samples were subjected to 40 cycles of amplification in an Applied Biosystems Real Time PCR Machine (Life Technologies, Grand Island,

NY) following manufacturers' directions for the qRT-PCR. Quantification of relative mRNA expression was determined by the comparative cycle threshold (ΔΔCT) method. The housekeeping gene succinate dehydrogenase complex, subunit A (SDHA) was examined under identical conditions as an internal control to demonstrate the equivalence of the template. At the end, a melting curve analysis was performed to confirm the specificity of amplification.

Table 1: Primers

	FORWARD 5'-3'	**REVERSE 5'-3'**
FOXP3	TATGCACCAGCTCTCCACAG	GGGCCTTGAGAGAGAAGACC
SDHA	GTGGTCTGGAACACGGATCT	TCATCAATCCGCACCTTGTA

Statistics. Statistical tests were done using Minitab v.16. After normality testing, group comparisons were analyzed by one-way ANOVA. If ANOVA was significant, Tukey's post-hoc test was used to determine significant difference between pairs of means. Quantitative data are expressed as mean ± SD P values ≤ 0.05 were considered statistically significant. qPCR was relativized by subtracting the CT value of FoxP3 genes by the average of the housekeeping gene (SDHA), then averaging that value.

RESULTS

The gut has a higher percentage of FoxP3$^+$ LPL during early hibernation. Gut mononuclear cells were isolated and changes in specific cell types were assessed using flow cytometry. The percentage of FoxP3$^+$ IEL was significantly higher in summer squirrels compared to early season, but not late season hibernators (Figure 1). The percentage of CD3$^+$FoxP3$^+$ IEL was higher in summer compared to both hibernation states (Figure 2). The percentage of FoxP3$^+$ LPL was significantly higher in early hibernation than in late hibernation and summer squirrels (Figure 3). CD3$^+$FoxP3$^+$ LPL, however did not differ between any of the states studied (Figure 4). A significant population of CD3^{lo}FoxP3$^+$ or CD3$^+$FoxP3$^+$ LPL were present during hibernation compared to summer (Figure 5). Although the exact nature of these cells is unknown they are likely Tregs with low CD3 expression. These data indicate changes in leukocyte populations in the gastrointestinal tract of hibernating ground squirrels. Overall FoxP3 protein expression increases during hibernation compared to summer, while CD3$^+$FoxP3$^+$ expression is more variable.

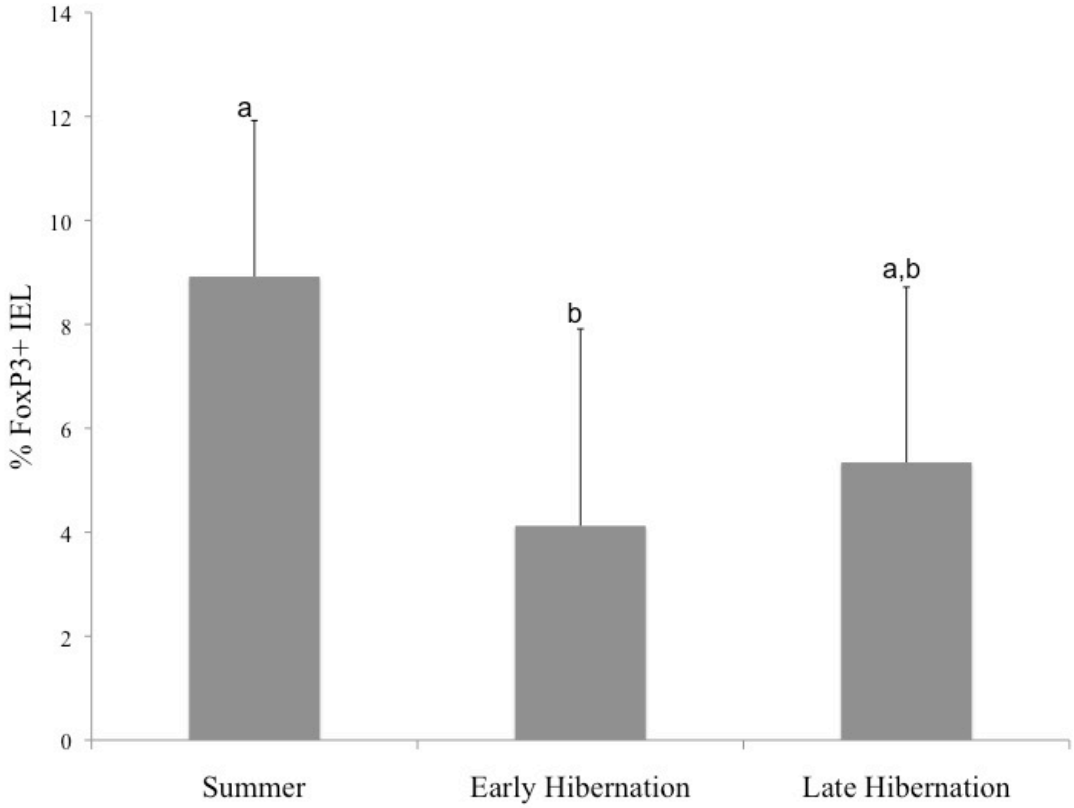

Figure 1: Percentage of **FoxP3⁺ IEL** in small intestine was significantly higher in the summer compared to early hibernation but not late hibernation animals. Summer n=11, early hibernation n=9, late hibernation n=9; P=0.01; groups with the same letter are not significantly different. Bars represent mean ± SD.

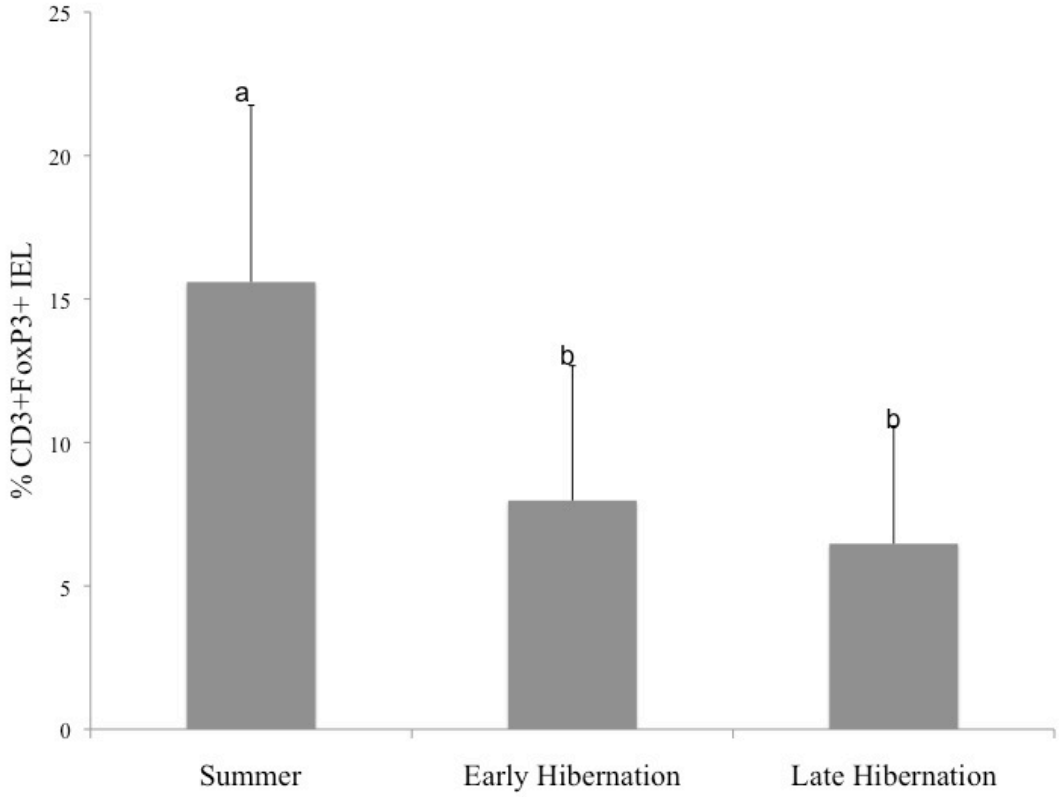

Figure 2: Percentage of **CD3⁺FoxP3⁺ IEL** in small intestine was significantly higher in the summer compared to hibernating states. Summer n=12, early hibernation n=10, late hibernation n=9; P=0.001; groups with the same letter are not significantly different. Bars represent mean ± SD.

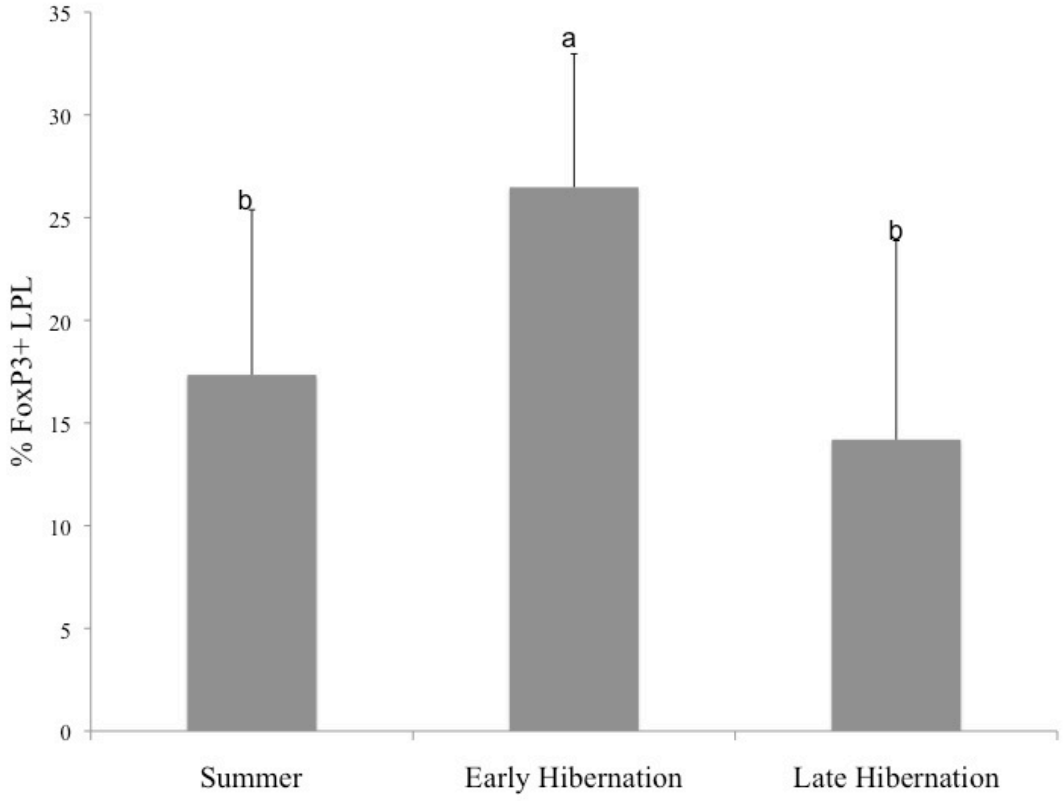

Figure 3: Percentage of **FoxP3⁺ LPL** in small intestine was significantly higher in early hibernation compared to late hibernation and summer. Summer n=14, early hibernation n=10, late hibernation n=9; P=0.006; groups with the same letter are not significantly different. Bars represent mean ± SD.

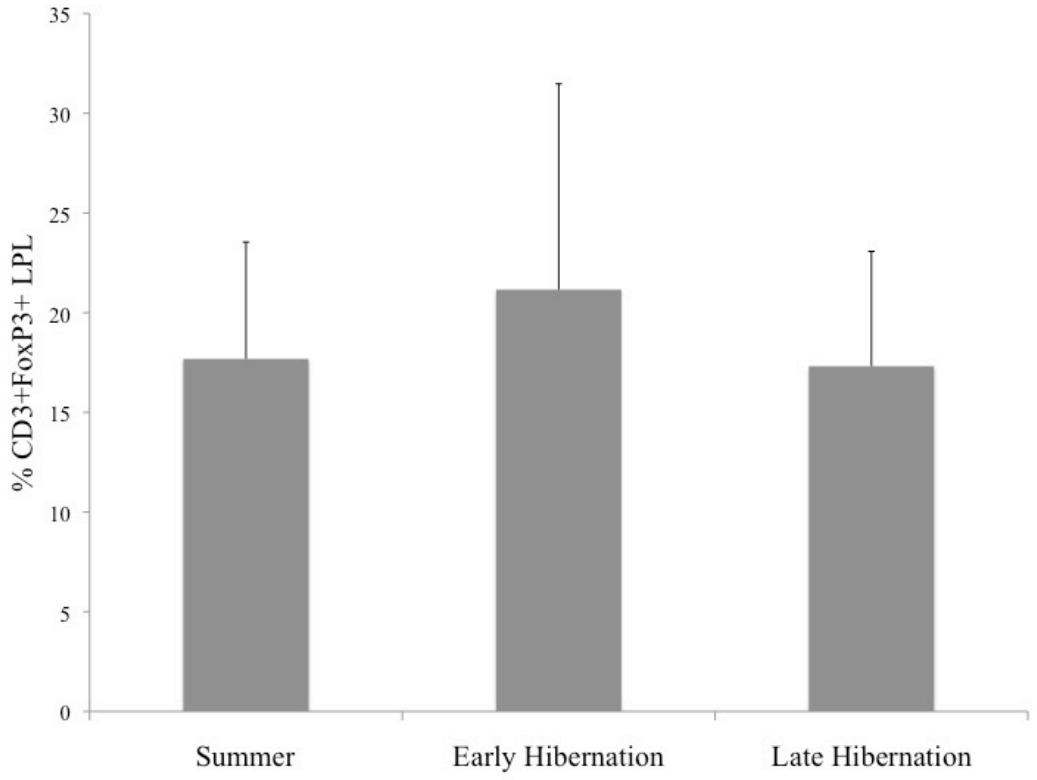

Figure 4: Percentage of **CD3⁺FoxP3⁺ LPL** in small intestine was not significantly different among any states examined. Summer n=14, early hibernation n=9, late hibernation n=9; P=0.462; Bars represent mean ± SD.

The gut has a higher percentage of $CD3^{lo}FoxP3^{+}$ or $CD3^{-}FoxP3^{+}$cells found in LPL during hibernation compared to summer. Gut mononuclear cells were isolated and studied to find changes in specific cell types using flow cytometry. The percentage of $CD3^{lo}FoxP3^{+}$ or $CD3^{-}FoxP3^{+}$ cells were examined in both LPL and IEL. The IEL cells showed no difference, whereas, there was a significant population of $CD3^{lo}FoxP3^{+}$ or $CD3^{-}FoxP3^{+}$ cells during hibernation compared to summer (Figure 5).

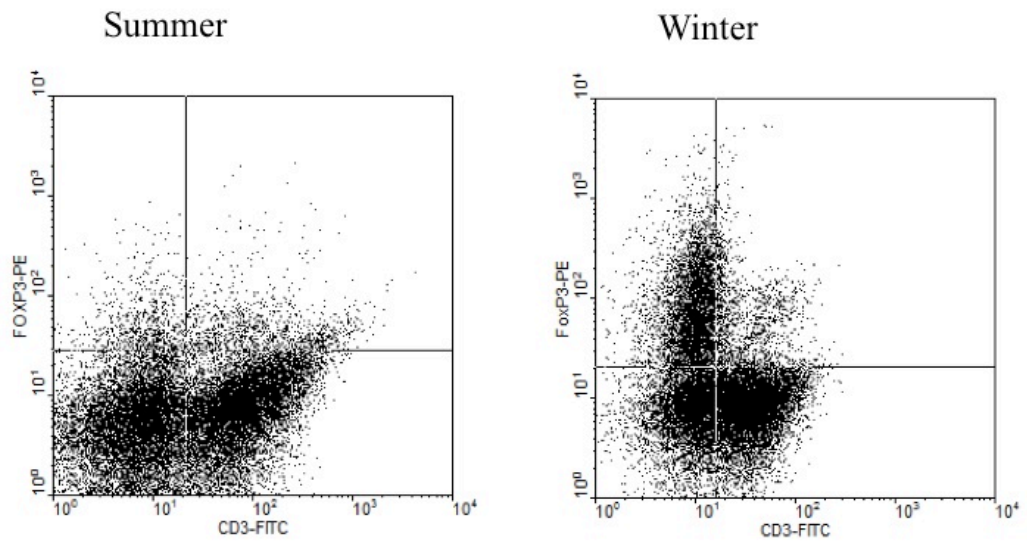

Figure 5: Significant populations of $CD3^{lo}FoxP3^{+}$ or $CD3^{-}FoxP3^{+}$ cells were found in LPL of hibernators. These $CD3^{lo}FoxP3^{+}$ or $CD3^{-}FoxP3^{+}$ cells are believed to be iTregs, although they could be a population of $FoxP3^{+}$ B cells. Further analysis is needed to determine the exact nature and phenotype of these cells. Cells were first gated by forward and side scatter before quadrant analysis.

FoxP3 expression increases in the colon of late season hibernators. cDNA from the colon of summer and early and late hibernating thirteen-lined ground squirrels was assayed by qPCR for expression of FoxP3 and compared with a housekeeping gene (SDHA). Although the expression of FoxP3 was not different between summer and either hibernating state, late season hibernators had significantly more FoxP3 transcript that early season hibernators (Figure 6).

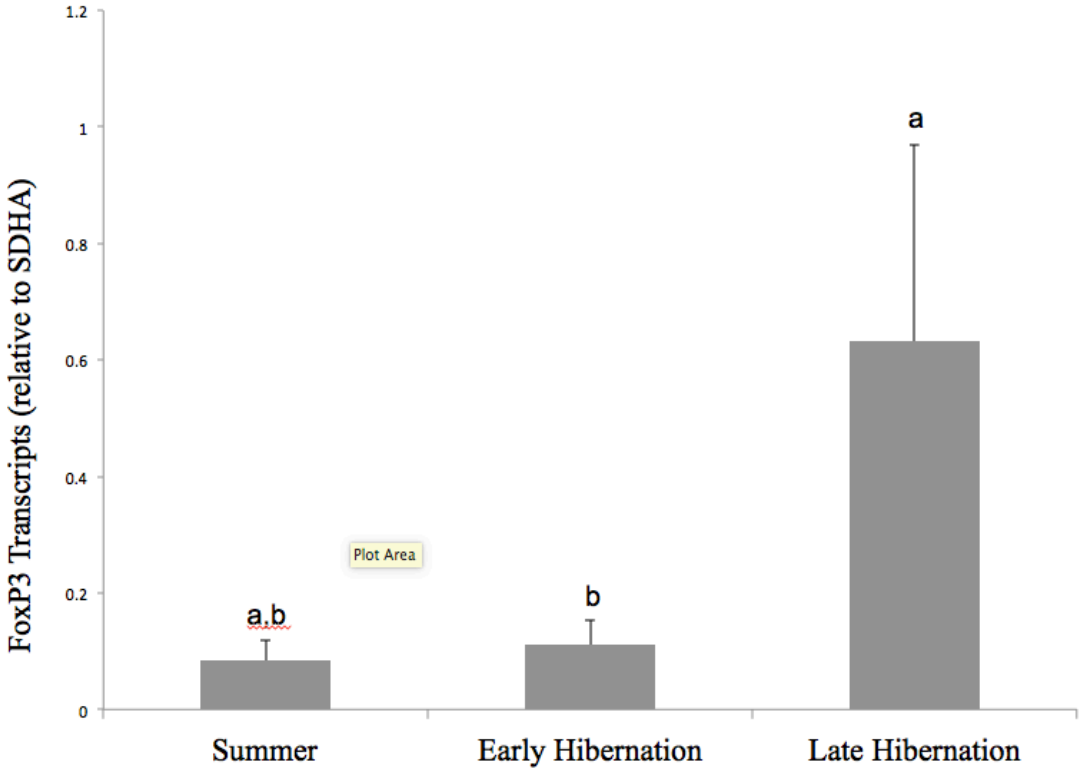

Figure 6: Expression of **FoxP3 transcript in the colon** was significantly higher in late hibernation compared to early hibernation but summer was not significantly different from either early or late hibernation. Summer n=5, early hibernation n=8, late hibernation n=7; P=0.069; groups with the same letter are not significantly different. Bars represent mean ± SD.

FoxP3 transcripts did not change in ileum. cDNA from the ileum of summer and early and late hibernating thirteen-lined ground squirrels was assayed by qPCR for expression of FoxP3 and compared with a housekeeping gene (SDHA). The expression of FoxP3 RNA in the ileum was not significantly different among the three activity states (Figure 7), although in the early hibernation state, there appears to be a trend toward significantly greater expression of FoxP3.

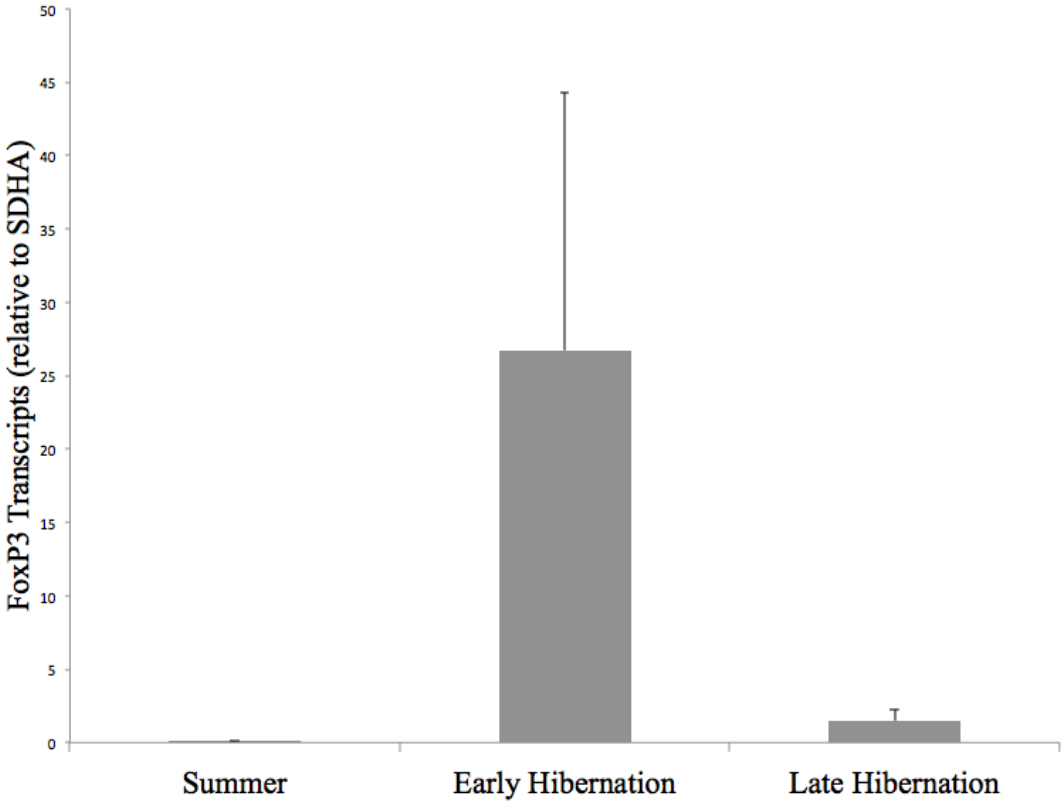

Figure 7**:** Expression of **FoxP3 transcript in the ileum** was not significantly different among any states examined. Summer n=6, early hibernation n=5, late hibernation n=8; P=0.549; Bars represent mean ± SD

DISCUSSION

In the current study, we describe natural shifts in immune parameters of the GI tract as ground squirrels transition from summer to hibernating phenotypes. Characterization of small intestine mononuclear cells by flow cytometry revealed a higher percentage of LPL cells expressing FoxP3 during torpor. qPCR of ileum, however, showed no change in FoxP3 transcripts, but there was a trend toward a significant increase in late hibernators. FoxP3 transcripts were significantly higher in the colon of late hibernators compared to both early hibernators and summer squirrels. My hypothesis that the increased numbers of immune cells found within the small intestine of hibernators contains a larger percentage of $FoxP3^+$ Tregs is supported by these data. The state of FoxP3 expression at the protein and transcript levels is higher during hibernation compared to summer.

In thirteen-lined ground squirrels, the number of IEL and LPL cells is greater in hibernators compared with summer (Kurtz & Carey, 2007). These cells are predominantly T-cells. In a study done in mice, the lamina propria of the small intestine contains large number of $FoxP3^+$ Tregs (Zhou et al, 2008). We found that within the LPL population the percentage of $FoxP3^+$ cells increased in hibernators compared to summer squirrels. Such a dramatic increase in immune cells generally indicates inflammation and disease, however, since there are no other signs of inflammation or disease. This suggests a suppressive state, where the animal can fight off any invaders that could penetrate the intestine and yet avoid costly inflammation

during hibernation. This is consistent with current data that say the GI system immune cells generally respond in suppressive manner, rather than inflammatory.

In mice, Tregs are present at higher frequencies in the gut lamina propria, but more so in the colon than in other organs (Hall et al, 2008). In thirteen-lined ground squirrels, we found that the number of FoxP3 transcripts was highest during late season hibernators compared to early season hibernators, but summer was not significantly different from each season. This suggests that more suppressive cells are present in the colon during hibernation that then dissipate during the summer and start returning again as hibernation begins. One possible explanation for the increase in suppressive cells during hibernation is to the continued presence of intestinal bacteria and increased leakiness of intestinal barriers (Carey et al, 2012a). Bacterial growth rates slow during hibernation due to low body temperature, but replication is still occurring (Luis & Hudson, 2006). Also, since the intestinal barrier is more leaky, it allows the bacteria to penetrate these openings. To prevent inflammation and possible damage to host tissues the thirteen-lined ground squirrel increases anti-inflammatory or suppressive immune cells within the colon during hibernation.

During hibernation, thirteen-lined ground squirrels lack enteral nutrition, which is similar to the clinical condition of total parenteral nutrition (TPN) in humans. TPN is used to treat many conditions ranging from short-term use in patients with gastrointestinal dysfunction to long-term use in patients with short bowel syndrome (Ohta et al, 2003). Patients on TPN are known to have damaging effects on intestinal absorption and barrier function along with a decrease in intestinal immune

cell numbers, specifically IEL and LPL (Li et al, 1995; Peterson et al, 1996; Yang et al, 2002). During hibernation the intestinal mucosa of thirteen-lined ground squirrels experiences atrophy, but in contrast to TPN, intestinal function is maintained and there is an increase in IEL and LPL numbers (Carey & Martin, 1996; Carey & Sills, 1992) The percentage of IEL decreases during TPN, but was not affected during hibernation while the percentage of LPL increases during hibernation and reduces in TPN (10). Although both hibernation and TPN lack enteral nutrition, hibernation appears to be well maintained in respect to epithelial integrity whereas TPN leads to intestinal degradation and an impaired immune population. This lack of enteral nutrition seen with TPN and during torpor might indicate why there was no significant change in the expression of FoxP3 RNA in the ileum. While hibernating, thirteen-lined ground squirrels are in an energy-conservative state and the resources they have are required to maintain an immune population. These results suggest that the removal of protein-energy within the ileum don't allow FoxP3 expression to increase during torpor and therefore show no significant difference from summer to hibernation season.

There are some discrepancies between qPCR and flow cytometry in our analysis of FoxP3 expression in LPL. This is mostly likely due to differences in the molecule detected -- flow cytometry examines FoxP3 at the protein level whereas qPCR examines it at the RNA level. Another potential reason for the differences between my qPCR and flow cytometry results is the measurement of living versus dead cells. qPCR quantifies the total number of transcripts whereas

flow cytometry measures the number of living cells that express FoxP3. This means that some cells could have undergone apoptosis and were therefore have been excluded from the cytometric counts. The overall FoxP3 expression is higher in the late hibernation season at both the protein and RNA levels.

In conclusion, this study found general increases in FoxP3+ for LPL cells during hibernation compared to summer. Hibernating animals show some variability based on season. CD3+FoxP3+ T-cells showed not significant differences between summer and hibernating months, but a significant population of CD3loFoxP3+ or CD3-FoxP3+ cells were found in hibernators. These data suggest that there is a seasonal shift within the intestinal immune phenotype of hibernators. qPCR results of the colon revealed no significant difference between summer and either hibernating state for FoxP3 expression, although late season hibernators had significantly more FoxP3 transcript than early season hibernators. Within the ileum, the expression of FoxP3 RNA did not vary significantly between summer and hibernating seasons. These data indicate changes in leukocyte populations in the gastrointestinal tract of hibernating ground squirrels. Identification of seasonal changes in intestinal Treg populations will lead to future studies of how 13 lined ground squirrels control immune activity during hibernation and could help identify the overactive components of the GI tract immune system in patients on total parenteral nutrition.

CHAPTER 3: CONCLUSION

During hibernation, the increase in intestinal lymphocytes (IEL & LPL) is associated with increased expression of FoxP3. In a study done in mice, the lamina propria of the small intestine contains a large number of FoxP3 Tregs (Zhou, 2008). In this study, the percentage of FoxP3 cells within the LPL population increase in hibernators compared to summer squirrels. This indicates that the cells are suppressive not inflammatory. Within the small intestine, FoxP3 RNA expression was increased in the colon during torpor. In a study done on mice, the gut lamina propria, especially the colon, had higher frequencies of Tregs (Hall, 2008 Immunity). This correlates with our data found in thirteen-lined ground squirrels. We found the highest FoxP3 expression in the colons of late season hibernators, with a decrease during the summer and an increase again as hibernation starts. This suggests that more suppression is needed in the colon during hibernation. In ileum, early season hibernators had the greatest FoxP3 expression, while summer and late season were significantly lower. This suggests a tissue-specific difference in the immune environment that favors the development of Tregs at different seasons in different tissues. Although there are some discrepancies FoxP3 expression is higher during the late hibernation season for both qPCR and flow cytometry for LPL cells. One possible explanation is that qPCR examines expression of mRNA whereas flow cytometry looks at proteins. Another reason for the discrepancies is qPCR quantifies the total number of transcripts,

whereas, flow cytometry measures the number of living cells that express FoxP3. This means that some cells could have undergone apoptosis and were therefore have been excluded from the cytometric counts. Together these data suggest that the increased numbers of immune cells found within the small intestine of hibernators show a suppressive phenotype due to a larger percentage of $FoxP3^{+}$ Tregs. This suppressive phenotype could be essential to the maintenance of the intestinal during the long winter fast. As the intestine becomes leakier, there is more opportunity for commensal bacterial products to infiltrate the mucosa. This could lead to detrimental inflammation and damage to the tissue. An increase in FoxP3+ Treg would curb any inflammation that may develop, thereby protecting the tissue from inflammation-induced damage. Further studies are necessary to determine how Treg populations in hibernators allow control of the immune activity during hibernation and could help identify the overactive components of the GI tract immune system in patients on total parenteral nutrition.

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