

# Seasonal changes in the intestinal immune system of hibernating ground squirrels

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## Abstract

Hibernation is associated with a prolonged fast (5–8 mo) which has the potential to affect intestinal immunity. We examined several aspects of the intestinal immune system in summer (non-hibernating) and hibernating ground squirrels. Peyer's patches were largely unaffected by hibernation, but numbers of intraepithelial lymphocytes (IEL) and lamina propria leukocytes (LPL) were greater in hibernators compared with summer. Hibernator IEL were less mature as demonstrated by low numbers of cells expressing activation-associated markers and co-receptors. Compared with summer, the percentage of B cells was higher and percentage of T cells was lower in the hibernator LPL. Hibernation was associated with greater mucosal levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-10 and IL-4, but IL-6 and TGF- $\beta$  were unchanged. Mucosal IgA levels were greater in entrance and torpid hibernators compared with summer. The results suggest that modifications of the intestinal immune system during hibernation may help preserve gut integrity throughout the winter fast.

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## 1. Introduction

The mucosal surfaces of the body (e.g., respiratory airways, gastrointestinal tract, urogenital tract) are important for immune defense because of their direct contact with the external environment. The intestine, in particular, is exposed to large numbers of antigens from the diet and resident microbes [1]. Only a single layer of epithelial cells separates luminal antigens from the intestinal immune system. This helps explain why the immune

system at mucosal sites, like the intestine, displays generalized suppression of inflammation, rather than stimulation in response to antigenic challenge [1].

The immune system of the small intestine is composed of organized and diffuse gut-associated lymphoid tissue (GALT). Peyer's patches (PP) are GALT organized into dome-shaped clusters of lymphoid follicles overlaid by a specialized epithelium containing microfold (M) cells that transport luminal antigens to underlying antigen-presenting cells [2]. Antigens are processed and presented to B and T lymphocytes which induce specific immune responses at intestinal effector sites, such as the lamina propria (LP). Most intestinal immune cells

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are diffusely distributed throughout the LP and intraepithelial compartments. LP leukocytes (LPL) are primarily T or B lymphocytes and often include a significant number of IgA-producing plasma cells [3]. Intraepithelial lymphocytes (IEL) are distributed throughout the basolateral side of the intestinal epithelium and are predominantly T cells [4]. Because of their close proximity to the luminal environment and the epithelium, IEL are exposed to a wide variety of luminal antigens and are thought to be involved in the development of immune tolerance to orally ingested antigens [4,5] and the maintenance of epithelial integrity [6,7].

Mammalian hibernation is a seasonal adaptation to periods of low food availability characterized by extensive time spent in a hypometabolic, low body temperature ( $T_b$ ) state called torpor. Torpor bouts are periodically interrupted by rapid arousal to normothermia [8]. Throughout the hibernation season (5–8 mo), most fat-storing hibernators like ground squirrels ingest no food and rely almost entirely on lipids from hydrolysis of white adipose tissue for energy [9]. The lack of luminal nutrition during hibernation is similar in some respects to the clinical condition of total parenteral nutrition (TPN), which is associated with a decrease in intestinal immune cell numbers and detrimental effects on intestinal absorption and barrier function [10–12]. Previous studies from our laboratory have shown that although the intestinal mucosa undergoes significant atrophy during hibernation, intestinal function, including the activities of brush border enzymes and the uptake of nutrients, is maintained [13–16]. Histological studies of ground squirrel small intestine have reported involution of PP and, in contrast to TPN, an increase in IEL and LPL during hibernation [17–19]. Considering the intestinal derangements that occur concomitant with a decrease in immune cells in models of TPN, we hypothesized that the altered intestinal immune population during hibernation represents a protective phenotype that aids in the maintenance of epithelial integrity and function during the prolonged winter fast. For the purposes of this study, we consider a protective phenotype to include maintenance or enhancement of anti-bacterial immune functions and shifts in mucosal cytokine levels toward anti-inflammatory dominance. To that end, we examined the phenotypic characteristics of three populations of intestinal immune cells, levels of mucosal cytokines and production of mucosal IgA in summer and hibernating ground squirrels.

## 2. Animals, materials and methods

### 2.1. Animals

Adult 13-lined ground squirrels (*Spermophilus tridecemlineatus*) of both sexes were trapped in the vicinity of Madison, WI in summer. Squirrels were housed individually with free access to water and food (Purina rodent chow 5001, supplemented with sunflower seeds) at 22 °C with a 12:12 h light-dark cycle. Squirrels were held in these conditions for at least 1 mo before use in experiments. In September–October, squirrels were transferred to a room maintained at 4 °C. The room was dark except for brief periods (<20 min) of low lighting once per day to check activity state. Water and food were removed after squirrels began regular bouts of torpor. The University of Wisconsin Institutional Animal Care and Use Committee approved all animal procedures.

### 2.2. Tissue collection

Squirrels were killed by decapitation. Summer squirrels were killed in late June–August when  $T_b = 37–39$  °C. Activity states during hibernation (October–March; Fig. 1) were: “entrance”, entering torpor ( $T_b = 20–25$  °C); “early torpor”, <24 h in torpor ( $T_b \sim 5–7$  °C); “late torpor”,  $\geq 7$  d in torpor ( $T_b \sim 5–7$  °C); “arousal”, arousing from torpor ( $T_b = 20–25$  °C); and “interbout arousal”, active between torpor bouts (>3 h at  $T_b = 37–39$  °C). The small intestine starting approximately 3–4 cm distal of the pylorus was removed and placed in ice-cold 0.01 M phosphate-buffered saline (PBS). A 1-cm segment near the proximal end (jejunum) was fixed in 10% buffered formalin for 2–4 h and processed for histology. Mucosa was scraped from the remaining tissue. A portion (100–200 mg) of mucosa was homogenized in a buffer containing 10 mM HEPES, 0.1% Triton X-100, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, and protease inhibitor cocktail. The homogenate was centrifuged (4 °C, 3000 × g, 10 min) and the supernatant containing cytosolic proteins was collected for immunoblotting. The remainder of scraped mucosa was frozen in liquid nitrogen.

### 2.3. Isolation of Peyer's patches, IEL, and LPL

Summer, late torpor, and interbout arousal ground squirrels were killed by decapitation and

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