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Hibernation induces immune changes in the lung of 13-lined ground squirrels (*Ictidomys tridecemlineatus*)

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ABSTRACT

During hibernation, significant changes occur in the systemic and intestinal immune populations. We found that the lungs of hibernating 13-lined ground squirrels (*Ictidomys tridecemlineatus*) also undergo shifts in immune phenotype. Within the population of mononuclear cells, the percentage of T cells increases and the percentage of CD11b/c^{+} cells decreases in hibernators. E-selectin, which promotes endothelial attachment, increases during arousal from torpor. Levels of the anti-inflammatory cytokine interleukin (IL)-10 in the lung are lower during hibernation while levels of the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α remain constant. Expression of suppressor of cytokine signaling (SOCS) proteins is also decreased in torpid hibernators. Our data point to a unique immune phenotype in the lung of hibernating ground squirrels in which certain immunosuppressive proteins are downregulated while some potentially inflammatory proteins are maintained or amplified. This indicates that the lung houses an immune population that can potentially respond to antigenic challenge during hibernation.

1. Introduction

Mammalian hibernation is a physiological adaptation to periods of low ambient temperature and limited food availability. During winter, thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) spend the majority of time in a metabolically depressed state called torpor, which is characterized by drastically reduced heart rate, ventilation and body temperature (<10 °C). Torpor is not constant, however, and squirrels periodically arouse to euthermic body temperature (~37 °C) and metabolic rate in a state referred to as interbout arousal (IBA). Many physiological changes occur throughout the torpor-arousal cycles of hibernation including alterations in blood flow and upregulation of protective mechanisms (reviewed in Carey et al., 2003). The immune system undergoes significant changes during hibernation as well. During torpor, the number of circulating leukocytes drops to approximately 10% of summer levels, but is largely replenished during IBA (Bouma et al., 2011; Reznik et al., 1975). Not surprisingly, the ability to respond to systemic challenge is impaired during torpor (Bouma et al., 2013; Prendergast et al., 2002). While changes in systemic leukocyte populations are striking, organ-specific populations, derived at some point by migration from the blood, are also affected by the physiological extremes of hibernation.

In the small intestine, lymphocyte numbers in the intraepithelial and lamina propria compartments increase during the hibernation season (Fichtelius and Jaroslow, 1969; Kurtz and Carey, 2007; Shivatcheva and Hadjioloff, 1987). This is sustained through IBA and corresponds to elevated tissue levels of interferon (IFN)-γ, TNF-α, and interleukin (IL)-10 (Kurtz and Carey, 2007). IL-10, in particular, is higher in all phases of hibernation compared to summer. Despite the dramatic increase in cell numbers and cytokine production, intestinal tissue of hibernators is not inflamed. In fact, when exposed to an inflammatory stimulus (ischemia-reperfusion), the intestine of IBA hibernators is more resistant to damage and less likely to experience neutrophil infiltration than the intestine of summer squirrels or non-hibernators (rats) (Kurtz et al., 2006). In humans, an interesting phenomenon exists in patients suffering from inflammatory bowel diseases (IBD) and chronic obstructive pulmonary disease (COPD) where inflammation in the primary organ (intestine and lung, respectively) leads to inflammation in the other (i.e. pulmonary inflammation in IBD) (Keely et al., 2012). This may be due to a common embryonic origin or the similarities in cellular and molecular structure of these organs, although the exact mechanism is still under investigation (Wang et al., 2013). Given the dramatic immune modifications in the intestine during hibernation and the apparent immunological connections between the gut and the lung, we hypothesized that lung immune populations are affected by hibernation as well.

Few studies have examined the effect of hibernation on the lungs and most of these have focused on surfactant production, tissue remodeling and ventilation (McArthur and Milsom, 1991; Ormond et al., 2003; Talaei et al., 2011; Zimmer and Milsom, 2001). Little

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is known about the immunology of the hibernator lung. A study in hedgehogs reported that the number of lung leukocytes was greater during hibernation compared to summer (Inkovaara and Suomalainen, 1973). Among specific leukocyte types, the number of neutrophils increased and the numbers of eosinophils decreased during hibernation. Lymphocyte numbers were not different between summer and hibernating hedgehogs. Lungs of hibernating hamsters display only a modest rise in expression of inflammatory markers and no change in the number of leukocytes, as assessed histologically and by expression of CD45 (Talaei et al., 2012). In the current study we examined immune parameters in the lungs of summer squirrels and hibernators during torpor and arousal. We characterized lung mononuclear cells using flow cytometry and examined the expression of proteins involved in modulation of immune pathways. We hypothesized that, like the intestine, the lung would demonstrate a generally suppressive, rather than inflammatory, phenotype during hibernation.

2. Materials and methods

2.1. Animals

Male and female thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) were collected near Madison, WI during summer or bred in the University of Wisconsin Oshkosh animal facility. Squirrels were housed individually with unlimited access to water at approximately 20 °C with a light cycle roughly corresponding to the natural photoperiod. Squirrels were fed a limited diet of IAMS Proactive Health Chunks (10 g/day) to prevent overeating. In the late summer/early autumn, squirrels were transferred to a cold room (~4–5 °C) with only short periods (<10 min) of red light daily for monitoring activity state. Once squirrels entered torpor, food and water were removed. Interbout arousal (IBA) was induced by moving squirrels to a 20 °C room under low-level lighting. Arousals were induced within 5 d of a squirrel's next anticipated natural arousal. All procedures were approved by the University of Wisconsin Oshkosh Institutional Animal Care and Use Committee.

2.2. Tissue collection

Lung tissues were harvested from ground squirrels following decapitation. Summer squirrels were euthanized in July and August when T_b = 37–39 °C. Hibernating squirrels were euthanized between late September and mid-March as either torpid (T_b~5–7 °C) or IBA (T_b = 36–39 °C). Torpid squirrels were in constant torpor for at least 4 days prior to tissue collection. IBA squirrels were euthanized 2 h after reaching euthermic T_b (36–39 °C). Tissues from IBA squirrels that were used for cytokine and protein analysis (but not flow cytometry) were obtained through a collaborator. In this case, animals were briefly (<5 min) anesthetized with isoflurane prior to decapitation (in accordance with the IACUC-approved protocol at University of Wisconsin Madison). Lungs were removed, rinsed with PBS and flash frozen in liquid nitrogen.

2.3. Lung leukocyte isolation

Leukocytes were extracted using a protocol modified from (Zhang et al., 2005). Both lungs were collected from ground squirrels immediately after decapitation, placed in PBS and teased apart with forceps on ice. The remaining pieces were transferred to a flask containing 25 mL RPMI with 5% FBS and agitated for 20 min at 37 °C to remove the majority of blood (as determined by the pale color of the tissue after incubation). The pieces were further agitated in 25 mL of fresh RPMI (5% FBS) containing 15 mg type I collagenase (Worthington Biochemical, Lakewood, NJ) until the tissue was digested (1–3 h). The remaining mixture was run through a 100-μm cell strainer and centrifuged (600 × *g*, 5 min, 4 °C) to collect the cells. The cells were resuspended in 40% Percoll, layered over 70% Percoll and centrifuged (600 × *g*, 20 min, 4 °C, no brake). Mononuclear cells were collected at the 40%/70% Percoll interface, washed and counted prior to use in flow cytometry. Cell viability was confirmed to be ≥95% by trypan blue staining.

2.4. Flow cytometry

Isolated mononuclear cells (1–1.5 × 10^6) were resuspended in 1% BSA in PBS and incubated with rat Fc block (D34-485; BD Biosciences, San Diego) for 10 min at 4 °C in the dark. Cells were then incubated with fluorescently-conjugated antibodies to CD3 (eBioG4.18), CD11b/c (OX42), TCRαβ (R73), TCRγδ (V65) (eBioscience, San Diego) and CD45RA (OX-33; BioLegend, San Diego) for 20 min (4 °C in dark). After washing, cells were fixed overnight in 2% paraformaldehyde (4 °C in dark) prior to analysis using a BD FACS Calibur (Becton Dickenson, San Jose, CA). Flow cytometry data were analyzed using WinMDI 2.9 software (The Scripps Institute, La Jolla, CA).

2.5. ELISA

Frozen lung tissues (100 mg) were homogenized in 500 μl of PBS containing protease inhibitors. Rat-specific ELISAs were used to measure concentrations of TNF-α (eBioscience, San Diego) and IL-10 (BD Biosciences, San Diego) by comparison with a standard curve. Values were normalized to total protein concentration of the sample (as determined by BCA Protein Assay Kit; Thermo Scientific Pierce, Rockford, IL).

2.6. Immunoblotting

Lung tissue (100–200 mg) was homogenized in a buffer (10 mM HEPES, 0.1% Triton-X 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) containing protease inhibitors and incubated on ice for 30 min. The homogenate was centrifuged (4 °C, 5000 × *g*, 10 min) and the supernatant containing cytosolic proteins was collected for immunoblotting. Total protein concentration was determined using BCA Protein Assay Kit according to manufacturer's instructions. Proteins (55–60 μg) were separated using SDS-PAGE and relative expression of IgA (Bethyl Laboratories, Montgomery, TX), E-selectin, SOCS5 (both from Santa Cruz Biotechnology, Inc., Dallas, TX) and SOCS1 (Cell Signaling Technology, Beverly, MA) were determined using immunoblotting. A Kodak Image Station and Kodak Molecular Imaging Software 4.0 were used to capture chemiluminescent images of blots. Quantity-One 1-D analysis software (BIO-RAD, Hercules, CA) was used for densitometric analysis of protein bands which were normalized to beta-actin (Novus Biologicals, Littleton, CO). Beta-actin was shown to be consistent with protein loading by comparison with Ponceau S staining of membranes before blocking.

2.7. Statistics

After normality testing, one-way ANOVA or Kruskal–Wallis U test (CD45RA, TCRγδ and SOCS1 analysis) was used to compare means from summer, IBA and torpid squirrels. If ANOVA was significant, Fisher's LSD test was used to determine significant differences between groups. If Kruskal–Wallis U test was significant, pairwise comparisons were performed using Mann–Whitney tests with a Bonferroni correction. Graphs present data as means ± standard error.