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Hibernation is associated with depression of T-cell independent humoral immune responses in the 13-lined ground squirrel

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ABSTRACT

Mammalian hibernation consists of periods of low metabolism and body temperature (torpor), interspersed by euthermic arousal periods. The function of both the innate and adaptive immune system is suppressed during hibernation. In this study, we analyzed the humoral adaptive immune response to a T-cell independent (TI-2) and a T-cell dependent (TD) antigen. Thirteen-lined ground squirrels were immunized in summer or during hibernation with either a TI-2 or TD antigen on day 0 and day 14. Blood was drawn on day 0, 7, 14, 21 and 28. Both types of antigens induced a significant rise in antibody titer in summer animals. Much to our surprise, however, only immunization with the TD antigen, and not with the TI-2 antigen induced a humoral response in hibernators. Flow cytometric analysis of CD4 (helper T-lymphocytes), CD8 (cytotoxic T-lymphocytes) and CD45RA (B-lymphocytes) in blood, spleen and lymph nodes ruled out massive apoptosis as explanation of the absent TI humoral response during hibernation. Rather, reduced TI-2 stimulation of B-lymphocytes, possibly due to lowered serum complement during torpor, may explain the reduced antibody production in response to a TI-2 antigen. These results demonstrate that hibernation diminishes the capacity to induce a TI-2 humoral immune response, while the capacity to induce a humoral response to a TD antigen is maintained.

1. Introduction

Mammalian hibernation consists of periods of torpor that last several days to weeks and are characterized by lowered metabolism and body temperature, which typically reaches ±0–4 °C (Kenagy et al., 1989). These torpor bouts are interspersed by shorter (euthermic) periods called arousals (Carey et al., 2003; Storey, 1997; van Breukelen and Martin, 2002). The repetitive cycles of cooling and rewarming that occur during hibernation do not lead to gross signs of organ injury (Arendt et al., 2003; Fleck and Carey, 2005; Sandovici et al., 2004; Talaei et al., 2011; Zancanaro et al., 1999), despite the fact that such extreme conditions can induce apoptosis or necrosis in non-hibernating animals (Aslami and Juffermans, 2010; Boutilier, 2001; Hochachka, 1986; Storey, 2010). One of the adaptations that hibernators might use to resist tissue damage during torpor is the reversible suppression of the immune system. Immune suppression during torpor is characterized by leukopenia (Bouma et al., 2010b, 2011; Frerichs et al., 1994; Reznik et al., 1975; Spurrier and Dawe, 1973; Suomalainen and Rosokivi, 1973), a reduced complement level (Maniero, 2002) and absence of an innate immune response to injection of lipopolysaccharide (LPS) (Prendergast et al., 2002). In addition, specific suppression of the adaptive immune system is illustrated by extremely delayed rejection of skin allografts (Shivatcheva, 1988) and a reduced production of antibodies during torpor and arousal (Burton and Reichman, 1999; Sidky et al., 1972) (reviewed in (Bouma et al., 2010a)). Thus, hibernation reduces the capacity of the innate, cellular and humoral immune function. We showed previously that low body temperature reduces the number of circulating lymphocytes during torpor due to a lowered sphingosine-1-phosphate plasma level (Bouma et al., 2011). Further, we demonstrated that neutropenia during torpor is due to margination of cells secondary to low body temperature (unpublished data, submitted). To date, the mechanisms that underlie the reduced humoral immune function during hibernation have not been clarified.

A reduced humoral immune function in hibernation was first demonstrated by the absence of antibody production of spleen cells from torpid Syrian hamsters (*Mesocricetus auratus*) in response to *in vitro* incubation at 37 °C with sheep red blood cells (SRBCs), in contrast to spleen cells derived from euthermic hamsters (Sidky and Auerbach, 1968). This finding is in line with

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observations *in vivo*. Formation of plaque forming cells in the spleen following intraperitoneal (i.p.) injection of SRBCs in hibernating 13-lined ground squirrels (*Ictidomys tridecemlineatus*) is delayed for the duration of torpor (up to 40 days) (Jaroslow and Serrell, 1972). Further, secondary antigen challenge using hen egg-white lysozyme (HEL) leads to lower antibody titers in hibernating Turkish hamsters (*Mesocricetus brandti*) as compared to euthermic animals (Burton and Reichman, 1999). A humoral immune response induced by a T-cell dependent (TD) antigen such as ovalbumin or HEL depends on proper B-lymphocyte function and the presence of an adequate co-stimulatory signals provided by CD4+ T-lymphocytes through T-lymphocytes by CD40L-CD40 binding and cytokine production (Abbas and Lichtman, 2003; Noelle et al., 1992). In the case of a T-cell independent type 2 (TI-2) antigen, such as the carbohydrate ficoll, stimulation of B-lymphocytes occurs primarily by the antigen itself, potentially augmented by complement through the complement receptor 2 (CR2, CD21) that is expressed on B-lymphocytes as part of the B-cell coreceptor complex (Abbas and Lichtman, 2003; Dempsey et al., 1996; Frank and Atkinson, 2001). In the work presented here we assessed the capacity to induce a humoral immune response to both a TD antigen and a TI-2 antigen during hibernation. Summer active and winter hibernating 13-lined ground squirrels were primarily and secondarily immunized with these two types of antigen. We determined the effect on the hibernation pattern and the induction of a humoral immune response by measuring the antibody titer following injection. This experiment allowed us to differentiate between the capacity to mount a humoral immune response to a TD antigen and a TI-2 antigen during hibernation.

2. Materials and methods

2.1. Animals

Adult and juvenile (born spring of the same year) 13-lined ground squirrels of both sexes were obtained by capture in the Madison area. After capture, ivermectin (0.02 mg/kg, delivered orally) was given to all squirrels as a de-worming agent. Animals were housed individually in plastic cages with paper nesting material and free access to food (rat chow and sunflower seeds) and water. Summer animals ($n = 16$) were injected with antigens in July and euthanized at the end of the protocol on day 28 (see "Immunization Protocol" below). Animals allocated to the hibernating groups were moved to a dark room maintained at 4 °C in mid-late September of the same year. Food and water were removed once a squirrel began to show regular torpor cycles, on average within 2 weeks of entering the cold room. Hibernation patterns were monitored by placing saw dust on the animals and daily inspections for signs of (previous) movement. Hibernating animals were immunized after their third torpor bout in the fully aroused state, which was induced by moving them from the cold room into the warm room. Animals were transported back into the cold room after immunization, to allow the animals to go into torpor again. At the end of the protocol (see "Immunization protocol" below), animals were euthanized by isoflurane anesthesia (3% in O_2) followed by decapitation upon arousal. Blood (100 μl) was collected in EDTA-coated tubes for flow cytometric analysis. The remainder was collected in heparin-coated tubes and was separated by centrifugation (10 min, 800*g*) and plasma was snap-frozen in liquid nitrogen and stored at −80 °C. The spleen was removed and divided into two parts: one half was snap-frozen in liquid nitrogen and stored at −80 °C for quantitative PCR (qPCR) analysis. The other half of the spleen as well as the cervical lymph nodes were used for isolation of lymphocytes for flow cytometric analysis.

2.2. Immunization protocol

Animals were immunized at day 0 and again at day 14 by i.p. injection of either 50 μg of 4-Hydroxy-3-nitrophenylacetic-Amino-EthylCarboxyMethyl-FICOLL (NP-AECM-FICOLL; Biosearch Technologies, Novato, USA) in 300 μl phosphate buffered saline (PBS; Sigma–Aldrich, Zwijndrecht, The Netherlands) to stimulate a TI-2 humoral immune response ($n = 16$) (adapted from (Shih et al., 2002)) or with 100 μg NP-ovalbumin (Biosearch Technologies, Novato, USA) adsorbed to 2 mg alum (Thermo Scientific, Rockford, USA) in 300 μl PBS to induce a TD humoral immune response ($n = 15$) (adapted from (Randolph et al., 1999; Vora et al., 2009)). Immunization was performed by i.p. injection as this is relatively easy to perform and therefore, minimizes injection errors. Blood (~50 μl) was drawn into a heparin-coated glass capillary tube after punctuation of the pedal vein by a 27G needle under brief anesthesia (isoflurane 0.5–5% in O_2) at days 0 (before immunization), 7, 14 and 21. The final blood sample was drawn at day 28 upon euthanization of the animals. Blood samples were centrifuged immediately upon collection (10 min, 800*g*), separated into plasma and pellet and snap-frozen in liquid nitrogen followed by storage at −80 °C. Succeeding each blood draw an equal volume of saline (0.9% NaCl) was injected i.p. to prevent dehydration of the animals.

2.3. Flow cytometry

Directly after euthanization of the animals, lymphocytes were isolated from lymph nodes and spleen by cutting these organs into small pieces and washing with 0.9% NaCl on a 100 μm cell strainer (BD Biosciences, San Jose, California). Next, following centrifugation (10 min, 800*g*), cells were resuspended in 3 ml flow cytometry buffer, which consisted of PBS supplemented with 2% v/v heat-inactivated newborn calf serum (NCS; Sigma–Aldrich, Zwijndrecht, The Netherlands) and 0.1% w/v sodium azide. This cell suspension was then pipetted on top of 3 ml Lympholyte solution (Cedarlane Labs, Ontario, Canada) in a 15 ml tube and centrifuged (20 min, 800*g*). Lymphocytes from blood were isolated by incubating 100 μl whole blood with 500 μl erythrocyte lysis buffer (8.26 g NH_4Cl, 1 g $KHCO_3$ and 0.037 g EDTA in 1 L H_2O) for 15 min on ice, followed by the addition of 1 ml PBS and centrifugation (10 min, 800*g*). After centrifugation of isolated lymphocytes derived from blood, cervical lymph nodes or spleen, the pelleted cells were resuspended in 100 μl flow cytometry buffer supplemented with the following antibodies at the appropriate dilution: APC-conjugated mouse anti-rat CD4 (BioLegend, San Diego, USA), FITC-conjugated mouse anti-rat CD8 (1:25, BD Biosciences, San Jose, California) and PE – conjugated mouse anti-rat CD45RA (BD Biosciences, San Jose, California). Next, cells were incubated for 1 h on ice in the dark, followed by washing using an equal volume of flow cytometry buffer, centrifugation (10 min, 800*g*) and resuspension in 200 μl flow cytometry buffer prior to analysis on a FACS-Calibur (BD Biosciences, San Jose, California).

2.4. Elisa

NP-specific immunoglobulins were determined by coating Immunolon 4HBX ELISA plates (Thermo Scientific, Rockford, USA) with 100 μl PBS containing 1 μg NP-KLH (Biosearch Technologies, Novato, USA) at 4 °C overnight. Plates were then washed with PBS supplemented with 0.05% v/v Tween-20 and blocked with 3% Probumin (Millipore, Amsterdam, The Netherlands) in PBS for 2 h at room temperature (RT). After washing, plates were incubated with 50 μl of plasma diluted 1:5000 in blocking buffer (2 h, RT), washed and incubated with 100 μl (1:1000) of either polyclonal goat anti-rat IgG:HRP (Thermo Scientific, Rockford, USA) or polyclonal goat anti-rat IgM:HRP (AbD Serotec, Kidlington, UK) (1 h,

RT). After washing the plates, 50 μl TMB (1-step-Ultra TMB, Thermo Scientific, Rockford, USA) was added. After 15 min incubation in the dark, 50 μl of stop solution (2 M H_2SO_4) was added. Absorbance was read at 450 nm on a spectrophotometer. The total titer of immunoglobulins was measured to determine specificity of the protocol by incubating the plates with 100 μl of anti-rat IgG or IgM (1:1000), rather than with NP-KLH. Titers are expressed as relative OD-value of [NP-specific IgX]:[total IgX] and normalized to the values obtained at baseline, where IgX is either IgM or IgG. All reagents were allowed to warm to room temperature before use and plates were sealed during the incubation and blocking periods.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 20 for Windows. Survival curve analysis was performed using a Log-Rank test. Antibody titers were compared over time using a paired *T*-test with the animals' own baseline as reference, while differences between groups were calculated using a one-Way ANOVA and post hoc least-significant differences in the case of three groups or an independent samples Student's *T*-test in the case of two groups. Antibody titers measured at baseline and up to 4 weeks after immunization were compared between seasons using a General Linear Model for repeated measures. In all cases, $p < 0.05$ was considered significantly different. Data are presented as means ± standard error of the mean (S.E.M.).

3. Results

3.1. Immunization with a T-cell dependent antigen disturbs torpor and leads to increased mortality as compared to a T-cell independent antigen

Primary immunization with ovalbumin precluded torpor behavior in 2 out of 7 animals after 1 week and in all animals after 2 weeks. In contrast, immunization of hibernating animals with ficoll, either primary or secondary, did not disturb their hibernating behavior (Fig. 1A). Further, the mortality of both summer active and hibernating animals that were immunized with ovalbumin, was significantly increased as compared to animals that were immunized with ficoll (Log Rank $X^2 = 10.1$, df = 3, $p < 0.05$; Fig. 1A). In contrast, immunization of either summer active or hibernating animals with ficoll did not lead to increased mortality. No difference could be found in the body weight of ficoll- and ovalbumin-immunized animals, either in summer or during hibernation ($p > 0.05$, Fig. 1B). The body mass of hibernating animals upon euthanization however, was significantly lower as compared to summer active animals ($p < 0.05$, Fig. 1B). Except a lower body temperature upon euthanization of hibernating animals immunized with ficoll ($p < 0.05$), no major differences in body temperature were found, since all animals were euthanized upon summer euthermia or interbout arousal (Fig. 1C).

3.2. The humoral response to a TI-2 antigen, but not to a TD antigen, is disturbed in hibernating animals

In order to determine whether NP-ovalbumin and NP-ficoll induce a humoral response in summer and hibernating animals, we measured by ELISA the titers of NP-specific IgM antibodies (NP-IgM) and NP-specific IgG antibodies (NP-IgG) produced in response to immunization. Immunization with NP-ovalbumin induces significant production of NP-IgM in summer animals in the 2nd week after secondary immunization ($p < 0.05$), while the NP-IgM titers increase in the 1st week following primary immunization in hibernating animals ($p < 0.05$; Fig. 2A). Further, NP-ovalbumin leads to higher NP-IgG titers at 2 weeks following primary immunization both in summer active and in hibernating animals ($p < 0.05$; Fig. 2B). No differences were detected in the titers of NP-IgG (GLM repeated measures: $F(1,0.14)$, $p = 0.72$) or NP-IgM (GLM repeated measures: $F(1,0.77)$, $p = 0.42$) between animals immunized in the summer and during hibernation that were immunized with ovalbumin (Fig. 2A and B). In contrast to immunization with a TD antigen (NP-ovalbumin), immunization with a TI-2 antigen (NP-ficoll) induces only a humoral immune response in summer animals, but not in hibernating animals. The NP-IgM titer increases in summer active animals at the 1st week following primary immunization only ($p < 0.05$), but did not rise in hibernating animals (Fig. 2C). The titer of NP-IgG in summer active animals increases 1 week following primary immunization as compared to baseline ($p < 0.05$). No significant rise in NP-IgG titers could be detected in hibernating animals (Fig. 2D). Although the NP-IgM titers were not different between summer active and hibernating animals (GLM repeated measures: $F(1,2.40)$, $p = 0.15$), the NP-IgG titers was increased in summer active animals only (GLM repeated measures: $F(1,6.36)$, $p = 0.01$) following immunization with NP-ficoll (Fig. 2C and D). Thus, significant augmented antibody titers are found in response to a TD-antigen both summer active and hibernating animals, while only summer active animals produce antibodies after immunization with a TI-2 antigen.

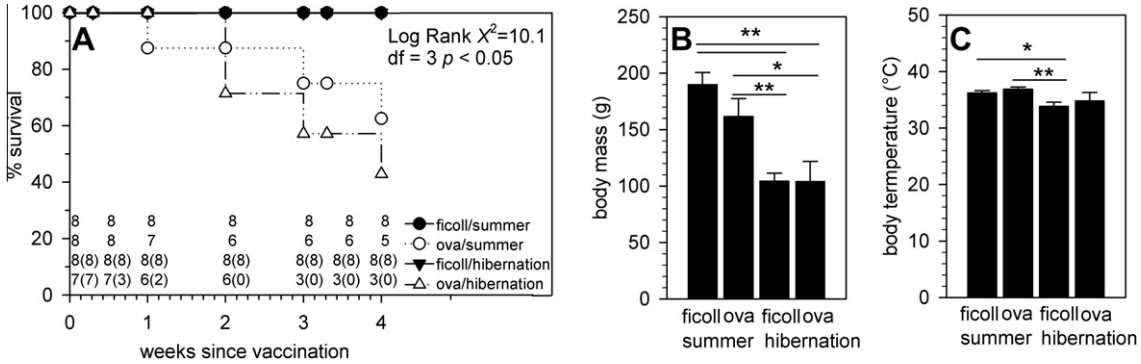

Fig. 1. Survival curves, body mass and temperature of summer active and hibernating ground squirrels following immunization. Mortality is significantly higher in summer and hibernating animals that were injected with ovalbumin compared to animals immunized with ficoll (Log Rank test $X^2 = 10.1$, df = 3, $p < 0.05$); the number of animals showing torpidity during the last 24 h is shown in brackets, as can be seen ovalbumin substantially disturbed hibernation (A); body mass of hibernators is significantly lower than in summer animals ($p < 0.05$) (B); at sacrification, T_b of hibernating animals immunized with ficoll was lower compared to summer animals ($p < 0.05$), but was not significantly different from ovalbumin immunized hibernating animals (C). Bars represent mean ± S.E.M.; */**indicates significant different at $p < 0.05/0.01$.

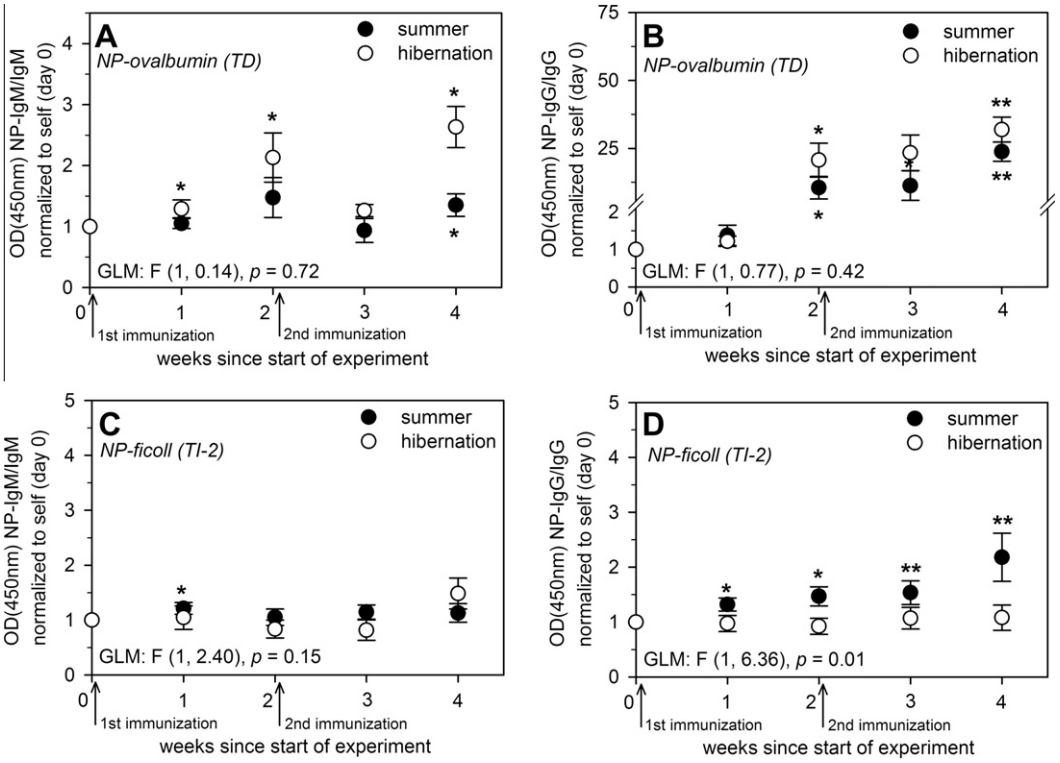

Fig. 2. Measurement of antigen-specific immunoglobulins reveals the absence of a humoral response to a T-cell independent antigen during hibernation. Immunization with ovalbumin induces a T-cell dependent humoral response with a significantly increased NP-specific IgM titer both in summer and hibernating animals ($p < 0.05$) (A); following immunization with ovalbumin, the NP-specific IgG titer increases significantly both in summer ($n = 5–8$) and hibernating animals ($n = 3–7$; $p < 0.01$) (B); in contrast, immunization with ficoll leads to a transient rise in NP-specific IgM titers in summer animals ($n = 8$; $p < 0.05$) but not in hibernating animals ($n = 8$) (C); the NP-specific IgG titers increase following immunization with ficoll in summer ($p < 0.01$), but not during hibernation (GLM repeated measures $p = 0.01$) (D). Dots represent mean ± S.E.M. of the ratio [NP-specific-Ig]:[total Ig] as normalized to the baseline measurement obtained from the same animal on day 0 of the experiment; GLM: general linear model for repeated measures; */**indicates significant different at $p < 0.05/0.01$.

3.3. Number of CD4+ T-lymphocytes, CD8+ T-lymphocytes and B-cells are not affected by immunization with NP-ficoll or NP-ovalbumin, but are affected by hibernation

To reveal additional alterations in the adaptive immune system during hibernation, we performed flow cytometry analysis of circulating lymphocytes in blood and lymphocytes in peripheral lymphoid organs (cervical lymph nodes and spleen). The numbers of lymphocytes are expressed either as absolute count per ml of blood or relative to the total number of lymphocytes (i.e. CD4+, CD8+ and CD45RA+) that were counted in the lymphoid organ. The numbers of circulating CD4+, CD8+ or CD45RA+ (a marker for B-lymphocytes) lymphocytes are not affected by immunization with either NP-ovalbumin or NP-ficoll in summer active animals (Fig. 3A) or hibernating animals (Fig. 3B). However, the number of circulating CD4+, CD8+ and CD45RA+ lymphocytes are significantly increased in hibernating animals as compared to summer animals (pooled groups from TI and TD immunization) ($p < 0.05$; Fig. 3C). In the spleen, again no differences were found between the relative number of lymphocytes between animals immunized with NP-ovalbumin and NP-ficoll in summer (Fig. 4A) or during hibernation (Fig. 4B). In pooled groups, the relative number of CD4+ lymphocytes remains stable during hibernation, while the number of CD8+ lymphocytes is increased ($p < 0.01$) and the number of CD45RA+ lymphocytes is lower as compared to summer active animals ($p < 0.01$; Fig. 4C). Thus, although the numbers of lymphocytes are not affected by immunization with NP-ovalbumin or NP-ficoll, pooling of the groups revealed significant differences in the number of circulating lymphocytes and the relative numbers of lymphocytes in lymph nodes and spleen between summer active and hibernating animals.

4. Discussion

Our study shows that the capacity to induce a humoral immune response against a TI-2 antigen (NP-ficoll) is impaired during hibernation, while the ability to mount a response to a TD antigen (NP-ovalbumin) remains normal. Immunization with NP-ficoll did not disturb torpor behavior or induce mortality. Further, no production of specific antibodies was found in hibernating animals, while both NP-IgM and NP-IgG titers increased after immunization with NP-ficoll in summer active animals. In contrast to NP-ficoll, immunization of hibernating animals with NP-ovalbumin was associated with a disturbance of the animals' hibernation pattern and increased mortality. Further, both NP-IgM and NP-IgG significantly increased after immunization with NP-ovalbumin, both in summer and during hibernation. The numbers T- and B-lymphocytes in blood, cervical lymph nodes and spleen is not affected by immunization with either NP-ficoll or NP-ovalbumin. However, analysis of pooled groups reveals significantly increased number of both T- and B-lymphocytes (i.e. CD4+, CD8+ and CD45RA+) in blood during hibernation as compared to summer. In cervical lymph nodes and spleen, the relative number of CD45RA+ B-lymphocytes is reduced in (immune-challenged) hibernating animals. The relative number of CD8+ T-lymphocytes on the other hand, is decreased in cervical lymph nodes, but increased in spleen during hibernation in immune-challenged animals. The relative

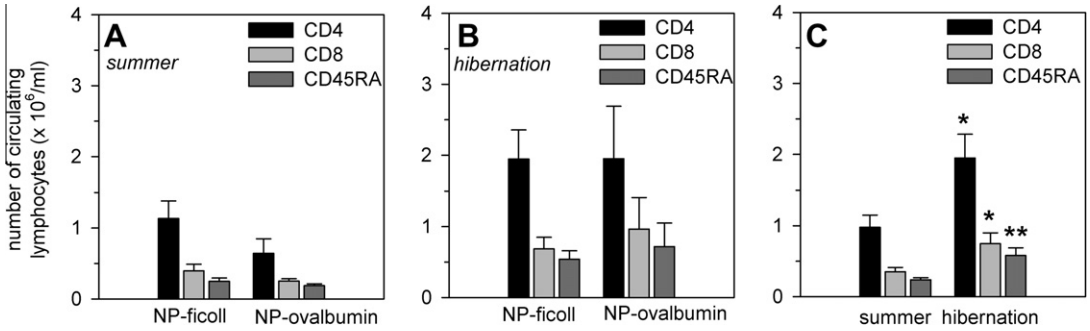

Fig. 3. Flow cytometric analysis of circulating lymphocytes demonstrates significant differences between summer and hibernating animals, but not between ficoll and ovalbumin immunized animals. No differences are observed in the number of circulating CD4+, CD8+ or CD45RA+ cells following immunization with ficoll ($n = 8$) or ovalbumin ($n = 5$) in summer animals (A); no differences are observed in the number of circulating CD4+, CD8+ or CD45RA+ cells following immunization with ficoll ($n = 7$) or ovalbumin ($n = 3$) in hibernating animals (B); the number of circulating lymphocytes is significantly higher in hibernating animals ($n = 9$) during interbout arousals compared with summer animals ($n = 13$), due to increased numbers of CD4+ ($p < 0.05$), CD8+ ($p < 0.05$) and CD45RA+ cells ($p < 0.01$) (C). Bars represent mean ± S.E.M.; */**indicates significant different at $p < 0.05/0.01$.

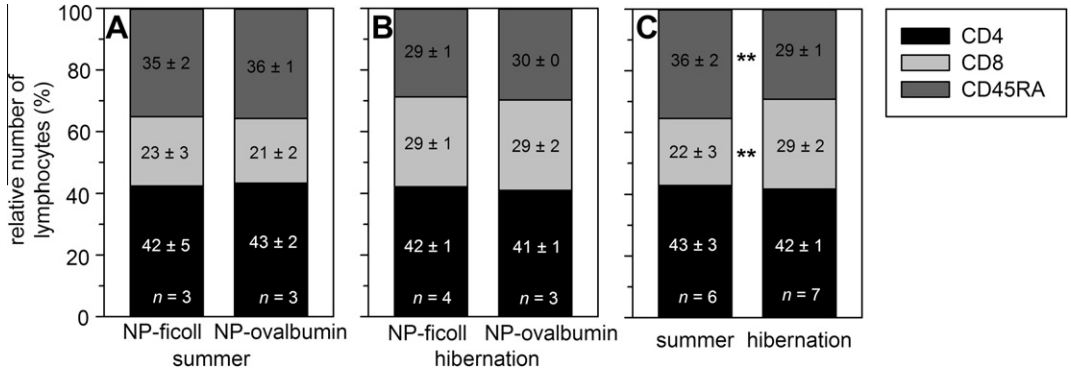

Fig. 4. Flow cytometric analysis of lymph nodes and spleen demonstrates significant differences between summer and hibernating animals. No differences are found in the relative number of CD4+, CD8+ or CD45RA+ cells between NP-ficoll and NP-ovalbumin immunized animals in the spleen in summer ($n = 5–8$) or during hibernation ($n = 3–8$) (A and B); the number of CD8+ is greater ($p < 0.01$) and CD45RA+ is lower ($p < 0.001$) in the spleen during hibernation, while the number of CD4+ cell remains unaffected (C). Bars represent mean relative number of cells (% of lymphocytes, defined as the sum of CD4, CD8 and CD45RA); **indicates significant different at $p < 0.01$.

number of CD4+ T-lymphocytes is higher in cervical lymph nodes during hibernation, while no significant differences were found in spleen. Thus, although differences in numbers of lymphocytes exist between summer and hibernating animals, major subtypes of lymphocytes are present in significant numbers in hibernating animals. Concluding, humoral responses to TD antigens are not reduced during hibernation, while hibernating animals do not respond to a TI-2 antigen.

Previous studies report a delayed formation of antibody-forming cells in response to i.p. injection of SRBC's (a mixed TD and TI-2 antigen) in 13-lined ground squirrels. This immune response is highly dependent on the amount of time spent in torpor, as the authors conclude that the response mainly took place during interbout arousals (Jaroslow and Serrell, 1972). In line with this finding, immunization of Turkish hamsters (*M. brandti*) by s.c. injection of HEL (a TD antigen) leads to a minor humoral response during hibernation, but induces substantial production of IgG when animals are moved to a warm room, which precludes torpor behavior (Burton and Reichman, 1999). In contrast to these studies, immunization with the TD antigen in our experiments disturbed torpor behavior. Based on previous data, we speculate that disturbance of hibernation pattern leads to a prolonged euthermic interval, which allows for the induction of a humoral immune response that is not significantly different from summer animals. Not only torpor, but also intermittent forced hypothermia of summer squirrels leads to a delayed formation of antibodies (Andjus et al., 1964). Together, these data suggest that low body temperature during torpor precludes the induction of a humoral immune response to a TD antigen. Different effects of the TD antigen during hibernation between previous studies and our current work might be due to differences in dosage, type of antigen or administration route, since all animals were housed in the same climate chamber during the experiments and experimental procedures were standardized as described in the methods. Taken together, although antibody titers might not rise until animals reach euthermia, humoral immune responses to TD can take place during hibernation (i.e. torpor and arousal), while TI-2 induced humoral responses are suppressed.

The observed effects of hibernation on the humoral immune function could be due to the low body temperature during torpor or might be due to temperature-independent effects of hibernation. In order to exclude potential (acute) effects of body temperature on the absorption and distribution of the antigen, antibody titers and numbers of (circulating) lymphocytes, we performed all injections and blood draws during interbout arousals. However, this experimental design does not completely rule out a role for temperature-dependent mechanisms. In addition, temperature-independent factors might be involved in the suppressed humoral immune function. The existence of temperature-independent factors that affect the humoral immune system is suggested by the observation that spleen cell suspensions derived from torpid Syrian hamsters (*M. auratus*) cultured *in vitro* at 37 °C produce less antibodies in response to SRBC's, as compared to spleen cell suspension derived from winter or summer euthermic animals (Sidky and Auerbach, 1968). Sidky et al. (1972) attempted to differentiate

effects mediated by hypothermia and hibernation itself (i.e. seasonally-induced differences) by keeping Richardson's ground squirrels (*Urocitellus richardsonii*) at an ambient temperature of ±22–24 °C, which prevents the animals from entering periods of deep torpor. Injection of SRBC's into these non-hibernating animals revealed reduced antibody titers and antibody producing cells in the spleen as compared to summer animals, in line with observations in hibernating squirrels (Sidky et al., 1972). This suggests that humoral immune function is, at least in part, affected by (yet unknown) temperature-independent factors during hibernation (i.e. seasonal variation).

Differences in the route of B-lymphocyte activation by TD and TI-2 antigens might reveal more information about potential temperature-independent factors that lead to a reduced response to TI-2 antigens during hibernation. In the case of TI-2 immune response, stimulation of B-lymphocytes occurs by the antigen itself, which binds components of the complement pathway to augment the stimulation of B-lymphocytes. Further, the complement system appears to be involved in distribution of TI-2 antigens, as it mediates follicular localization of ficoll in the spleen, which is important for the induction of a memory response (van den Eertwegh et al., 1992). During torpor the level of serum complement of golden-mantled ground squirrels (*Callospermophillus lateralis*) is reduced and then restored to levels observed in summer animals upon arousal (Maniero, 2002). The importance of complement is illustrated by the observation that complement deprivation of mice diminishes the capacity to induced a response to immunization with ficoll (van den Eertwegh et al., 1992). Not only does complement seem to augment stimulation of B-lymphocytes by TI-2 antigens, signaling through the complement receptor CR2 also plays an important role in inducing the isotype switch from IgM to IgG (Ochsenbein et al., 1999). Hence, the reduced capacity to induce a humoral immune response with production of both IgM and IgG antibodies to a TI-2 antigen might be explained by the reduced amount of complement during torpor. In addition to complement-mediated mechanisms, marginal zone (MZ) macrophages can recognize blood-borne microbes through pathogen pattern receptors (PPR's) and provide additional stimulation of B-lymphocytes (Kraal and Mebius, 2006; Zandvoort and Timens, 2002). In the response to TI-2 antigens however, MZ macrophages do not seem to play an important role, as experimental elimination of these cells does not affect the response against ficoll (Kraal et al., 1989). Further, changes in cytokine-mediated signaling might lead to a reduced capacity to mount a humoral immune response during hibernation. Cytokines that are involved in the induction of an immune response to TI-2 antigens are type 1 interferon (IFN) (Swanson et al., 2010), interleukin-1 (IL-1), IL-2, IL-4, IL-5 (Vos et al., 2000) and IL-12 (Buchanan et al., 1998). These cytokines might either enhance, suppress or have both enhancing and suppressive effects on the induction of a humoral response. Thereby, cytokine mediated signaling can stimulate polarization of the immune response towards a type-1 or type 2 response (Vos et al., 2000). Unfortunately, despite their important signaling role in the immune system, data on cytokine levels during hibernation are scarce. Of the cytokines mentioned above, production of IL-4 is upregulated in the small intestine during hibernation (Kurtz and Carey, 2007). In addition, hibernating ground squirrels produce less IFN in response to tilorone hydrochloride, LPS and poly rI:rC as compared to summer active animals (Kandefer-Szerszen et al., 1994). Thus, the reduced humoral immune response to TI-2 antigens during hibernation might be explained, at least in part, by the reduced amount of complement and changes in the cytokine environment during torpor.

The results presented in this work might help to explain the pathogenesis of White Nose Syndrome (WNS) in bats. This infectious dieases is associated with the deaths of millions of bats. WNS is caused by the psychrophilic fungus *Geomyces destructans*, which has an optimal growth temperature close to the bats' body temperature during torpor (Barlow et al., 2009; Blehert et al., 2009; Buchen, 2010; Lorch et al., 2011; Puechmaille et al., 2010; Zimmerman, 2009). Despite substantial growth of the fungus on muzzle and wings, no signs of inflammation can be seen in deceased hibernating bats, in contrast to deceased bats in spring (Meteyer et al., 2009). Growth of the fungus is likely permitted to occur during torpor when the immune function is reduced (Bouma et al., 2010a). Both the innate, cellular and humoral immune system play a role in the immune response against a fungus, which carries TD- and TI-antigens (Hamad, 2012). Whereas low burden fungal (airway) infection in mice only induces activation of Th2-lymphocytes, high burden fungal infection activates Th2-lymphocytes and B-lymphocytes, leading to the production of circulating antibodies (Porter et al., 2011). In the current work, we demonstrate that in contrast to a TI-2 antigen, immunization with a TD antigen during hibernation induces a potent immune response with disturbance of hibernation patterns, antibody production and increased mortality. Therefore, we speculate that upon reaching a certain amount of fungal load in WNS-affected bats, the fungus might induce a TD immune response with disturbance of torpor behavior. This is supported by the observation that the frequency of arousals in affected bats is associated with the severity of the disease and predicts early mortality (Reeder et al., 2012). Hence, disturbance of torpor by an immune response might increase energy consumption and potentially cause mortality.

5. Conclusion

Mammalian hibernation is associated with reduced function of the innate, cellular and humoral adaptive immune systems. We demonstrate that a TI-2 antigen does not induce a humoral response during torpor, while a TD antigen can induce a humoral immune response with disturbance of torpor behavior, increased mortality and antibody production. We speculate that suppressed TI-2 humoral immune function is secondary to the reduced concentration of complement during torpor, which plays an important role in the induction of TI-2 humoral immune responses. Further understanding of the mechanisms that lead to a diminished humoral immune function during hibernation may aid in the understanding of the pathogenesis of WNS and potentially lead to the discovery of novel pharmacological therapies for immunological disorders.

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