Low body temperature governs the decline of circulating lymphocytes during hibernation through Sphingosine-1-phosphate

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In deep torpor, major physiological and immunological changes occur. One of these alterations constitutes of an almost complete depletion of circulating lymphocytes, which is rapidly reversed upon arousal. Here we show that torpor induced the storage of lymphocytes in secondary lymphoid organs, in response to a temperature dependent drop in plasma levels of sphingosine-1-phosphate (S1p). Regulation of lymphocyte numbers was mediated through type 1 S1p-receptor (S1p1), as administration of a specific antagonist (W146) during torpor (Syrian hamster at ± 8°C) precluded restoration of lymphocyte numbers upon subsequent arousal. Furthermore, S1p release from erythrocytes via ATP-binding cassette (ABC)-transporters was significantly inhibited at low body temperature (4°C), but restored upon rewarming. Reversible lymphopenia was also observed during daily torpor (Djungarian hamster at ± 25°C), during forced hypothermia in anesthetized (summer active) hamsters (at ± 9°C) and in a non-hibernator (rat at ± 19°C). Our results demonstrate that lymphopenia during hibernation in small mammals is driven by body temperature, via altered plasma S1p levels. Since S1p is recognized as an important bioactive lipid involved in regulating the several other physiological processes as well, S1p may represent an important factor regulating additional physiological processes in hibernation, as well as mediating effects of therapeutic hypothermia in patients.

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**Introduction**

Hibernation is an energy conserving behavior consisting of periods of significantly inhibited metabolism (‘torpor’) that results in a largely reduced heart and ventilation rate (Milsom et al., 1999; Heldmaier et al., 2004; Hampton et al., 2010) and body temperature. Torpor bouts are interspersed by arousal periods with a duration of 8-24 h, in which metabolism increases and body temperature rapidly returns to euthermia (Hut et al., 2002; Heldmaier et al., 2004). Hibernating mammals display major changes in their physiology, which (amongst others) lead to an increased resistance to ischemia/reperfusion (Lindell et al., 2005; Kurtz et al., 2006), and a reduced immune function (Prendergast et al., 2002). Remarkably, despite the repetitive cycles of cooling and rewarming, hibernating animals do not show gross signs of organ damage (Zancanaro et al., 1999). In humans, therapeutic hypothermia is frequently used in cardiac arrest and major surgery of brain and heart to limit neuronal injury (Arrich et al., 2009). However, hypothermia as employed during cardiac surgery is associated with increased renal injury post-operatively (Kourliouros et al., 2010). Therefore, unraveling the mechanisms underlying the changes in physiology of hibernating mammals might be of substantial clinical relevance. Inflammatory responses induced by hypothermia are implicated in organ injury following therapeutic hypothermia (Moore, Jr. et al., 1988; van der Woude et al., 2004; Zhang et al., 2010). Several specific changes occur in the immune system of hibernating animals, such as lower complement levels, phagocytotic capacity, cytokine production, lymphocyte proliferation, antibody production (reviewed in Bouma et al., 2010) and a profound but readily reversible depletion of circulating leukocytes during torpor (Bouma et al., 2010c). Thereby, hibernation affects the function of both the innate and adaptive immune system (Bouma et al., 2010a). A reduced innate immune function is demonstrated by the fact that injection of LPS during torpor does not induce a febrile response (Prendergast et al., 2002). Suppression of the adaptive immune system throughout hibernation is demonstrated by the absence of rejection of skin allografts in hibernating ground squirrels until after cessation of their hibernation in spring (Shivatcheva, 1988). The underlying mechanisms of these specific changes that occur during hibernation are still unresolved. Apart from its applicability in preservation of organ function, understanding the immune function in hibernators is of growing importance as large populations of hibernating bats are currently threatened by the White Nose Syndrome (WNS). This condition has a mortality rate of 75-100 % and is caused by a psychrophilic (cold-loving) fungus which thrives on bats in torpor (Zimmerman, 2009; Blehert et al., 2009; Barlow et al., 2009; Buchen, 2010; Puechmaille et al., 2010) and is thought related to the suppressed immune function during hibernation (Wibbelt et al., 2010; Bouma et al., 2010a).

To examine mechanisms of immunological alterations in hibernation, changes in the number of circulating leukocytes were examined in different stages of hibernation in hamster species that undergo either deep, multi-day torpor bouts or shallow daily torpor. Effects were compared to those found in hamsters cooled under anesthesia. Specifically, regulation of lymphocyte numbers was studied because of their importance in adaptive immunity and immunological memory. After confirmation of lymphocyte storage in secondary lymphoid tissue during torpor, we further examined the role of sphingosine-1-phosphate (S1p), a bioactive lipid known to regulate lymphocytes egress from lymph nodes (Mandala et al., 2002; Matloubian et al., 2004; Pappu et al., 2007).
Materials and Methods

Hibernation
To induce hibernation in Syrian hamsters (*Mesocricetus auratus*), the light:dark-cycle (L:D-cycle) was shortened to 8 h:16 h for ± 10 weeks followed by continuous dim light (< 5 Lux) at an ambient temperature of 5°C. Movement detectors connected to a computer allowed to determine the animals' hibernation pattern. In the Djungarian hamsters (*Phodopus sungorus*), hibernation was induced by shortening L:D-cycle to 8 h:16 h for ± 14 weeks at an ambient temperature of 21± 1°C. Daily torpor was determined by observation at the start of the light phase (usual torpor phase) and a single body temperature measurement at the time of decapitation. All experiments were approved by the Institutional Animal Ethical Committees of the University Medical Center Groningen and University of Aberdeen.

Forced Hypothermia
Summer euthermic Syrian hamsters and Wistar rats housed at a light:dark-cycle (L:D-cycle) of 12 h:12 h were anesthetized by injecting 200 mg/kg ketamine and 1.5 mg/kg diazepam intraperitoneally. Spontaneous breathing rats were cooled, while spontaneous breathing hamsters and ventilated rats were cooled and rewarmed. A catheter was inserted into the jugular vein for blood sampling, rectal temperature was measured continuously and heart rate (ECG) was monitored on the anesthesia monitor Cardiocap S/5 (Datex Ohmeda). Animals were cooled by applying (ice-cold) water on their fur and rewarmed using a water-based heating mattress; both at a rate of ± 1°C of body temperature per 3 minutes.

Splenectomies
Splenectomies are performed on summer euthermic and torpid Syrian hamsters. Immediately after induction of anesthesia (2-2.5 % isofluorane/O₂), a blood sample was drawn by cardiac punction and flunixin-meglumin (Finadyne; Schering-Plough) was given subcutaneously (4 mg/kg) as analgesia. Summer euthermic animals that underwent splenectomy recovered in a warm room (L:D-cycle 8 h:16 h). After induction of hibernation, animals were euthanized during their third torpor bout, which was 60.3 ± 8.1 days following splenectomy. Torpid animals, having their third torpor bout, were kept < 10°C during surgery using ice-packs and recovered in the climate-controlled room, followed by euthanization upon reaching euthermia.

Blood sample analysis
After euthanization by pentobarbital intraperitoneally, 250 µl of blood collected via cardiac puncture into EDTA-coated cups (minicollect EDTA-K3; Greiner Bio-One) was analyzed on the Sysmex XE-2100, an automated hematology analyzer (Briggs et al., 2000; Ruzicka et al., 2001). Differential leukocyte counts were validated manually using Wright-Giemsa stained blood smears. The remainder of the blood was collected in a polypropylene tube mixed 1:10 v/v with a solution containing Prostaglandin E1 (94 nmol/l) (Sigma Aldrich), Na₂CO₃ (0.63 mmol/l) (Sigma Aldrich), EDTA (90 mM) (Titriplex; Sigma Aldrich), and theophyllin (10 mM) (Sigma Aldrich) to minimize platelet activation. Samples were centrifuged (30 minutes, 17,000 g, 4°C), snap-frozen in liquid nitrogen and stored at -80°C.
Labeling of lymphocytes

In order to obtain lymphocytes for autologous transfusion, Syrian hamsters were splenectomized as described above. A cannula was placed into the jugular vein connected to a subcutaneous access port (Soloport, Instech Solomon) implanted between the scapulas for infusion of cells during torpor. The spleen was cut into small pieces and washed on a cell strainer (70 µm; Greiner Bio-One) with sterile saline to obtain a suspension of splenocytes. Cells were spun down (10 minutes, 800 g, 4°C) and resuspended onto a layer of Lympholyte Mammal (Cedarlane Laboratories). Lymphocytes were then separated from other cells by centrifuging (20 minutes, 800 g, 4°C). The lymphocyte fraction was washed, centrifuged (10 minutes, 800 g, 4°C) and resuspended in saline supplemented with 10 % DMSO followed by storage in liquid nitrogen. When animals showed torpidity (around 10 weeks following splenectomy), lymphocytes were thawed and purity was assessed by analyzing a Wright-Giemsa stained smear, while viability was measured with a manual cell count after addition of Trypan blue (Sigma Aldrich). Cells were fluorescently labeled by incubation in 25 µM CFDA-SE in saline (15 minutes, 37°C) (Invitrogen). After infusion of the labeled lymphocytes during torpor, an arousal was induced by gently handling the animals. The animals were euthanized 2 days after entrance into the subsequent torpor bout.

Fluorescent microscopy

Frozen samples were embedded in Tissue Tek (Sakura), sectioned into 200 µm thick slices and counter-stained using TPO-3-TO (Invitrogen). Images were taken at 200x magnification (Leica SP2 AOBS) and processed using Imaris 6.4.

Quantitative real-time PCR (qRT-PCR)

RNA isolation was performed according to the manufacturer’s instructions (RNA Isolation Kit, Bioké). RNA concentration was determined spectrophotometrically at 260 nm (NanoDrop ND-1000, NanoDrop Technologies), while purity was checked on 1 % agarose gel. One µg of RNA was mixed with 4 µl RT buffer, 0.2 µl dNTP, 0.5 µl Rnasin, 1 µl Reverse Transcriptase, 1 µl Random Hexamers (Promega) and H2O in 20 µl. cDNA was produced on a C1000 Thermal Cycler (Biorad Laboratories). Oligonucleotide primers (Biolegio) sequences are shown in table 6.1. Amplified products CFX 384 Real-Time System (Biorad Laboratories) were checked by obtaining melting curves and verification on 1 % agarose gels.

Liquid chromatography-electrospray tandem mass spectrometry

Sphingolipids were extracted and analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) on a PE-Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ionspray source as described previously (Sullards and Merrill, Jr., 2001; Bielawski et al., 2006). HPLC separation was performed as described previously (Sullards et al., 2003), with the following changes: an Alltima C-18 column (2.1x150 mm, 5 micron; Grace Davison Discovery Sciences, USA) was used at a flow rate of 200 µl/min. N2 was used as the nebulizing gas and drying gas for the turbo ionspray source. The ion spray needle was held at 5,500 V; the orifice temperature was set to 500°C. N2 was used to collisionally induce dissociations in Q2. Multiple reaction monitoring scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant sphingolipid molecular species. MRM transitions were optimized for each individual component (C-17SoP: 366.2/250.4; C-17SaP: 368.2/270.4; C-18SoP: 380.2/264.4; C-18SaP: 382.2/284.4; C-17So: 286.2/238.1; C-17Sa: 288.2/240.1; C-18So: 300.2/252.3; C18Sa: 302.2/254.2). Quantitation was achieved by spiking the samples before extraction with
Sphingosine-1-phosphate (S1P) signaling plays a crucial role in the decline of circulating lymphocytes during hibernation. This is due to the low body temperature, which governs the expression of S1P receptors. Key S1P metabolites include sphingosine (d17:1), sphinganine (d17:0), sphingosine-1-phosphate (d17:1), and sphinganine-1-phosphate (d17:0) (Avanti Polar Lipids, Inc., USA).

**Blood rewarming ex vivo**

Washed erythrocytes derived from torpid Syrian hamsters (25 µl, 4°C) were pipetted into small polypropylene tubes containing cell culture medium (DMEM/F12; Invitrogen) supplemented with 40% probumin (Millipore). An inhibitor for ABC-A1 transporters (glyburide, 1 mM; Sigma Aldrich), ABC-C1 transporters (MK571, 50 µM; Cayman Chemicals) or both were added to the samples. Samples were then incubated at 37°C for 30 minutes, while another sample was left at 4°C for 30 minutes. After incubation, samples were centrifuged (30 minutes, 17,000 g, 4°C), while a negative control sample was centrifuged prior to incubation at 37 or 4°C. Supernatant was snap-frozen in liquid nitrogen and stored at -80°C.

**Statistical analysis and data presentation**

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by Student’s T-test or One-Way ANOVA with post-hoc LSD (SPSS 16.0 for Windows) with \( p < 0.05 \) considered significantly different. Correlations were calculated using Pearson’s correlations. Sigmaplot 11.0 was used to produce graphs shown in this manuscript.

**Table 6.1. Oligonucleotides designed for real time-PCR**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Orientation</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>CD3e</td>
<td>Forward</td>
<td>AAGGCCAAGGCCAAGCCTGTGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGCTCATAGTCTGGGTTGGGA</td>
</tr>
<tr>
<td>CD20</td>
<td>Forward</td>
<td>GCATCTCTCCTGGATGCTGATCTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCCAGCTGACAGCAGAAACACATT</td>
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Oligonucleotides were developed using Primer Designer 4.0 for Windows, based on regions of homology in the sequences of rat (Rattus Norvegicus) and mouse (Mus Musculus) that were determined using Nucleotide search and BLAST (NCBI Entrez).
Results

Figure 6.1: Average body temperature during torpor entry (A) and arousal (B) in hibernating Syrian hamsters. Graphs demonstrate the time-dependent changes in mean body temperature from 12 torpor-arousal cycles in animals equipped with temperature loggers in the peritoneum.

Lowered body temperature induces clearance of circulating lymphocytes

After entrance into deep torpor, the body temperature of the Syrian hamster (*Mesocricetus auratus*) decreases to 7.7 ± 0.8°C during the first 24 hours (Figure 6.1A). During torpor, the total number of circulating white blood cells decreases by 95%, from 1.57 ± 0.22 to 0.07 ± 0.01 (x10^6/ml). The number of circulating lymphocytes decreases even more dramatically: to about 4% of those of summer euthermic animals (Figures 6.2A, B). Lymphocyte numbers are rapidly restored upon arousal (Figure 6.2C), when body temperature increases within 2 hours to reach euthermia (Figure 6.1B). The blood lymphocyte count correlates significantly with the body temperature both during entrance into torpor (Pearson Rho^2 = 0.41; p < 0.01; figure 6.2B) and during arousal following torpor (Pearson Rho^2 = 0.71; p < 0.01; figure 6.2C).

The number of circulating erythrocytes does not change throughout the torpor-arousal cycles (Figure 6.3). In an animal showing daily torpor behaviour, the Djungarian hamster (*Phodopus sungorus*), the average body temperature during torpor is 25.2 ± 1.3°C. During these torpor bouts, the number of circulating leukocytes was reduced by about 50% while the number of circulating lymphocytes decreased by ± 30% (Figure 6.2D). To examine the role of body temperature in the decrease of circulating lymphocytes, forced hypothermia was induced in anaesthetized (summer active) hamsters that were not in hibernation to reach a body temperature of 9.1 ± 0.8°C. Forced hypothermia decreases the number of circulating leukocytes and lymphocytes (Figure 6.2B), which is fully reversed upon rewarming (Figure 6.2C). The number of circulating lymphocytes correlated significantly with body temperature during cooling (Pearson Rho^2 = 0.67; p < 0.01; figure 6.2B) as well as during rewarming following forced hypothermia (Pearson Rho^2 = 0.29; p < 0.01; figure 6.2C). The number of circulating lymphocytes in summer euthermic animals that underwent forced hypothermia is, at the start of the experiment, higher than in hibernating animals before entering torpor (Figure 6.2B; p < 0.01). The number of circulating lymphocytes in euthermic (summer active) animals is significantly higher compared to aroused (winter euthermic) animals (Figure 6.4; p < 0.05). Moreover, forced hypothermia of rats (*Rattus norvegicus*; a non-hibernating animal) to reach a body temperature of 19.2 ± 0.7°C did not affect the number of circulating erythrocytes (Figure 6.5A), but resulted in a significant decrease in the number of circulating lymphocytes as well (Figure 6.5B; p < 0.01), which was restored upon rewarming (Figure 6.5C; p < 0.01).
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Figure 6.2: Depletion of circulating lymphocytes during torpor is temperature-dependent and unaffected by splenectomy. Normal blood lymphocytes count of summer euthermic Syrian Hamster (A); body temperature dependent decrease of blood lymphocytes upon entrance into torpor and to a similar extent in forced hypothermia in non-hibernating animals (B); blood lymphocyte counts are restored rapidly during rewarming from torpor and from forced hypothermia (C); circulating lymphocyte number is reduced during daily torpor in the Djungarian hamster (D); splenectomy before hibernation does not inhibit induction of lymphopenia in torpor (E); splenectomy during torpor does not preclude restoration of blood lymphocyte count during the subsequent arousal (F). Bars represent mean ± SEM of \( n = 4 - 8 \) animals per group. Groups were compared using a Student's T-test or a One-Way ANOVA and post-hoc LSD. */** indicates significant difference at \( p < 0.05/0.01 \).

Figure 6.3: Hibernation phase does not affect the number of circulating erythrocytes. Bars represent mean ± SEM of 4 - 6 animals per group. Data was analyzed using a One-Way ANOVA with post-hoc LSD.

Figure 6.4: The number of circulating lymphocytes is reduced in aroused Syrian hamsters as compared to summer euthermic Syrian hamsters. Bars represent mean ± SEM of 11 animals per group. Groups were compared using a Two-Tailed independent samples Student's T-test. *

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indicates significant difference at $p < 0.05$.

Figure 6.5: Forced hypothermia of anesthetized rats induces lymphopenia, without affecting the number of circulating erythrocytes. Forced hypothermia of anesthetized rats does not affect the number of circulating erythrocytes (A); body temperature dependent decrease of circulating lymphocytes in forced hypothermia in anesthetized spontaneous breathing rats (B); body temperature dependent decrease of circulating lymphocytes followed by restoration upon rewarming (30 minutes after reaching 37°C) in anesthetized intubated rats (C). Bars represent mean ± SEM of 5 - 7 animals per group. Groups were compared using a One-Way ANOVA and post-hoc LSD. */** indicates significant difference at $p < 0.05/0.01$.

Table 6.2: Expression levels of CD3ε and CD20 in the spleen during torpor and arousal

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Torpor</th>
<th>Arousal</th>
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<tr>
<td>CD3ε (marker for T-lymphocytes)</td>
<td>1.00 ± 0.16</td>
<td>0.87 ± 0.10</td>
</tr>
<tr>
<td>CD20 (marker for B-lymphocytes)</td>
<td>1.00 ± 0.24</td>
<td>0.87 ± 0.24</td>
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Shown in the table is fold mRNA expression ± SEM of arousal animals ($n = 5$) as compared to torpor animals ($n = 5$). Expression of CDs was normalized to GAPDH as housekeeping gene. No significant differences were found.

Lymphopenia is due to retention of cells in peripheral lymphoid organs, but not the spleen

To establish the role of the spleen in the regulation of lymphocyte numbers during hibernation, we measured the expression of markers for lymphocytes and performed splenectomies before and during torpor. The mRNA expression of CD3ε (T-lymphocyte marker) and CD20 (B-lymphocyte marker) is unaffected by torpor (Tables 6.1 and 6.2). Although this does not directly demonstrate numbers of cells present in the spleen, we do not have any indications that mRNA expression of these specific markers changes during torpor. Hence, similar expression levels of these lymphocyte markers during torpor and arousal strongly argues against massive cellular migration into the spleen during torpor or substantial apoptosis of these cells. Surgical removal of the spleen (splenectomy) preceding the hibernation season does not influence the induction of lymphopenia during torpor (Figure 6.2E). Conversely, splenectomy during torpor does not preclude restoration of lymphocyte numbers during arousal (Figure 6.2F). To detect retention sites of lymphocytes during torpor other than the spleen, fluorescently labeled autologous lymphocytes (from surgically removed spleens) were injected intracardially into splenectomized torpid hamsters. During the subsequent torpor bout, lymphocytes are found in cervical lymph nodes (Figure 6.6A) and to a lesser extent also in gut-associated lymphoid tissue (GALT;
low body temperature governs the decline of circulating lymphocytes during hibernation through Sphingosine-1-phosphate (figure 6.6B). Importantly, liver, lung and kidney do not contain significant numbers of fluorescently labeled lymphocytes (Figures 6.6C-F).

Figure 6.6: Torpor induces storage of lymphocytes in peripheral lymphoid tissues. Panels show representative fluorescent microscopic images of CFSE-labeled lymphocytes (green) counter-stained with TO-TPO3 (red) from torpid animals. Animals were splenectomized and lymphocytes were isolated and labeled with CFDA-SE, which were injected intracardially into the same animal during torpor. Tissue was harvested during the subsequent torpor bout. Figure A shows fluorescently labeled lymphocytes in cervical lymph node B cell follicles (white arrows), and T cell zones (black arrows); figure B shows fluorescently labeled lymphocytes in lamina propria (white arrows) and Peyer’s patch (black arrows) of small intestine; figures C, D and E show lung, liver and kidney tissue, respectively.
Sphingosine-1-phosphate regulates lymphocyte dynamics

Plasma levels of S1p decrease by ± 50-60 % both during deep torpor and daily torpor, in Syrian and Djungarian hamster, respectively (Figures 6.7A, B). During arousal, plasma S1p levels rapidly raise to normal (euthermic) values in both species. Involvement of the S1p system in hibernation associated lymphopenia was further examined by intracardial injection of a specific type-1 S1p receptor (S1p1) antagonist (W146, 5 mg/kg). No significant difference was found in the number of lymphocytes between torpid animals and aroused animals that were treated with W146, while the number of circulating lymphocytes following intracardial injection of W146 in the subsequent arousal period was significantly lower compared to vehicle-treated animals ($p < 0.01$; figure 6.7C). In contrast, W146 does not affect the restoration of the numbers of circulating neutrophils (Figures 6.7D). Upon arousal, the intracellular level of S1p decreases in erythrocytes (Figure 6.8A) without a significant change in the intracellular level of sphingosine (Figure 6.8B). Plasma S1p and intracellular S1p levels of erythrocytes correlate negatively in torpor and arousal periods (Pearson Rho$^2 = -0.77$; $p < 0.001$; figure 6.8C). To establish the influence of body temperature on the release of S1p from erythrocytes, washed erythrocytes derived from torpid animals were rewarmed to 37°C. While erythrocytes maintained at 4°C do not release S1p into the medium, ex vivo rearming induces substantial S1p release. Release of S1p from rewarmed erythrocytes is significantly reduced by inhibitors of ABC-A1 (glyburide) and ABC-C1 (MK571) transporters and even more efficient when both inhibitors are combined (Figure 6.8D).
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**Figure 6.8:** The S1p level in erythrocytes negatively correlates with the S1p plasma level and is released upon rewarming through ABC-transporters. Complementary levels of S1p (A) and Sphingosine (B) in erythrocytes during different periods of hibernation; significant correlation between the level of S1p in erythrocytes and plasma of animals in torpor (closed circles) and arousal (open circles) (C); temperature governs S1p release from erythrocytes isolated from torpid animals (D). Erythrocytes were obtained from torpid animals and washed with PBS (pre); continued incubation at 4°C did not induce release of S1p from erythrocytes (30 minutes, 4°C, grey bars); *ex vivo* rewarming (30 minutes, 37°C, hatched bars) induces release of S1p that is blocked by ABC-transporter inhibitors MK571 (50 µM) and Glyburide (1 mM). Bars represent mean ± SEM of 4 - 8 animals per group. Data was analyzed using a One-Way ANOVA with post-hoc LSD. */** means significantly different at \( p < 0.05/0.01. \)

**Discussion**

Our data imply that body temperature is the driving force of lymphopenia during hibernation as shown by (1) the strong correlation between body temperature and blood lymphocyte count, (2) its occurrence during both deep and daily torpor and (3) the effect of forced cooling of (summer active) hamsters that were not in hibernation on the number of circulating lymphocytes. At the start of the experiment however, the number of circulating lymphocytes was significantly higher in animals that underwent forced hypothermia than in hibernating animals that were about to enter torpor. Since no differences were found between summer euthermic animals that did and did not undergo forced hypothermia, we speculate that this phenomenon is due to temperature-independent, seasonal changes affecting the number of circulating lymphocytes during euthermia. During daily torpor in the Djungarian hamster, the number of circulating lymphocytes also decreased, in spite of shorter torpor bout duration with a substantial higher body temperature than during deep torpor (Heldmaier et al., 2004). Although the decrease in the number of circulating
lymphocytes during daily torpor was smaller than observed during deep torpor, the fitted curve (Figure 6.2B) shows the blood lymphocyte count is ± 40 % lower at average body temperature of ± 25°C during either natural torpor or forced hypothermia in the Syrian hamster, which is about the same as observed during daily torpor in the Djungarian hamster. Temperature-dependency of this process is further supported by the fact that hypothermia in rats (non-hibernators) leads to the induction of lymphopenia as well. Since rewarming of severely hypothermic rats interacted with their breathing, we cooled and rewarmed a second group of ventilated rats to demonstrate the reversibility of lymphopenia induced by hypothermia in rats. In addition, forced hypothermia of anesthetized rats demonstrates that hypothermia-induced lymphopenia is not conserved to hibernators, but also occurs in non-hibernating animals.

*Lymphopenia during hibernation is due to storage of cells in peripheral lymphoid organs*

The notion that the number of splenic lymphocytes is stable throughout hibernation and that the lymphopenia during torpor bouts is rapidly reversed upon rewarming, strongly favors a storage-and-release mechanism over an apoptosis-and-replenishment mechanism to explain the dynamics of the lymphocytes during hibernation. Further, splenectomies preceding the hibernation season demonstrated that the spleen is not necessary for the induction of lymphopenia. Conversely, splenectomy during torpor established that recirculating lymphocytes can be obtained from a source other than spleen. Although we cannot rule out the possibility that (some) lymphocytes are retained in the spleen during torpor, it is certainly not the main organ involved. Indeed, injected fluorescently labeled lymphocytes are found in cervical lymph nodes during the subsequent torpor bout and to a lesser extent also in gut-associated lymphoid tissue (GALT). Inkovaara et al. previously showed that numbers of leukocytes increase in lung (mainly neutrophils) and gut (mainly lymphocytes) during torpor, compared to summer euthermic and arousal in hibernating hedgehogs (Erinaceus europaeus) (Inkovaara and Suomalainen, 1973). The number of lymphocytes among intraepithelial lymphocytes (IEL) and lamina propria leukocytes (LPL) increases about 3-fold while the number of lymphocytes in Peyer’s patches increases only slightly as compared to summer euthermia in the thirteen-lined ground squirrel (Ictidomys tridecemlineatus) (Kurtz and Carey, 2007). Since T-lymphocytes in the blood are mainly TCRαβ⁺, influx and retention of circulating T-lymphocytes into GALT would result in an altered ratio of TCRαβ⁺:TRCγδ⁺. Logically, the authors speculate that the unaffected ratio of TCRαβ⁺:TRCγδ⁺ in GALT reflect absence of influx and thus, that the increased lymphocyte count in GALT is due to local expansion of cells (Kurtz and Carey, 2007). However, our results suggest that there is some influx of circulating lymphocytes into GALT during torpor. Influx of circulating lymphocytes might not alter the ratio of TCRαβ⁺:TRCγδ⁺ as long as the number of cells that migrate into GALT remains relatively small compared to the number of cells already present in GALT. Therefore, the increased number of lymphocytes in GALT might well be due to a combination of influx and local expansion of cells. Taken together, we show that secondary lymphoid tissue is the main site of retention of lymphocytes during torpor.
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Figure 6.9: Proposed model of Sphingosine-1-phosphate release from erythrocytes governing lymphocyte dynamics during hibernation or hypothermia. Sphingosine is taken up by erythrocytes and phosphorylated intracellularly by sphingosine-kinase (Sphk) to S1p, which is transported into the plasma by ATP dependent ABC transporters. During torpor, in response to lower temperature, release of S1p from erythrocytes is inhibited, which lowers S1p plasma levels, thereby affecting the S1p gradient from blood to lymph node (indicated by solid line). In turn, egress of lymphocytes from lymphoid organs is inhibited resulting in profound lymphopenia. In addition, the low body temperature during torpor may affect ATP-dependent phosphorylation of sphingosine (indicated by dotted line).

**S1p stimulates lymphocyte egress upon arousal**

In our study, we demonstrate direct effects of S1p on the restoration of the number of circulating lymphocytes. Since lymphocytes continuously recirculate via the blood between various lymphoid organs, the number of circulating lymphocytes depends on the balance between influx from the blood into peripheral lymphoid organs (homing) and egress out of these organs (as mediated by S1p). Logically, a decreased S1p plasma level as occurs during torpor leads to a reduced egress from peripheral lymphoid organs and consequently to a lymphopenic state. Our data demonstrate that the retention of lymphocytes in peripheral lymphoid organs is regulated by plasma S1p levels acting via S1p1. Although the effect of S1p on lymphocyte egress from peripheral lymphoid organs has previously been demonstrated in knock-out models or by using synthetic agonists and antagonists (Mandala et al., 2002; Matloubian et al., 2004; Pappu et al., 2007; Gonzalez-Cabrera et al., 2008), our study is the first to demonstrate the importance of S1p in regulating lymphocyte numbers in the absence of pharmacological interventions or genetic changes. Lymphocytes egress from peripheral lymphoid organs is normally favored when the S1p concentration is low in lymphoid tissue interstitium and high at exit sites, i.e. blood (Pappu et al., 2007).
Although the extent of lymphopenia is larger during deep torpor, the relative decrease in S1p is larger during daily torpor. Other factors, such as time, blood flow velocity, but also expression level of S1p-receptors which might be different between species, influence the egress of lymphocytes. Therefore, the levels cannot be compared directly between species. However, both during deep and daily torpor the number of circulating lymphocytes correlated significantly with the S1p plasma level. Involvement of the S1p system is substantiated by (1) the rapid change in plasma S1p levels, (2) the correlation between plasma S1p level and blood lymphocyte count and (3) the observation that blockade of S1p1 during arousal completely blocks the restoration of the number of circulating lymphocytes. Thus, the blockade of lymphocyte egress by antagonism of the S1p1 implicates that the increase in plasma S1p levels is a causative mechanism in the restoration of circulating lymphocyte numbers during arousal. As blockade of S1p1 did not affect restoration of numbers of circulating neutrophils and monocytes or changed erythrocyte counts, a non-specific action of blockade of S1p1 seems highly unlikely. Together, these data imply a major role of S1p acting via S1p1 in regulating lymphocyte numbers in peripheral blood during hibernation.

**Erythrocytes regulate the plasma level S1p by transport through ABC-A1/C1-transporters**

Our data suggest S1p release from erythrocytes constitutes an important regulatory mechanism in S1p plasma levels. This proposal is consistent with previous observations in Sphk-deficient mice that the plasma level of S1p is mainly derived from erythrocytes (Pappu et al., 2007). Indeed, erythrocytes derived from torpid animals release S1p upon rewarming ex vivo, thus demonstrating that body temperature is the primary factor that can stimulate release. Furthermore, the strong negative correlation between plasma S1p levels and intracellular S1p content of erythrocytes suggests a prominent role for erythrocytes in regulating the plasma level of S1p. The fact that release of S1p can be reduced by inhibitors of ABC-A1 (glyburide) and ABC-C1 (MK571) transporters and is even more efficient when both inhibitors are combined, demonstrates the role for ABC-transporters in regulating S1p release from erythrocytes upon rewarming. Combination of both inhibitors demonstrates a role for both ABC-A1 and ABC-C1 transporters in the release of S1p from erythrocytes. Incomplete inhibition however, might suggest involvement of other (yet unknown) transporters in the release of S1p from erythrocytes as well. Taken together, in the absence of potential regulating factors from plasma, an increase in body temperature is sufficient to induce a rapid and substantial release of S1p from erythrocytes of torpid animals.

**Potential benefits and drawbacks of an immune-suppressed state**

Our study identifies the reduction of body temperature, resulting from metabolic suppression in torpor, as the major driving force in modulation of lymphocyte egress from lymphoid tissue that constitutes a versatile system of a rapidly reversible reduction of immune function. The induction of lymphopenia by low body temperature is widely conserved, as it seems to be a common response of mammals (shared by hibernating hamsters, hypothermic hamsters and hypothermic, non-hibernating rats). Although this seems a common response of mammals rather than an adaptation specific for hibernators, it may be beneficial during hibernation. The induction of an immune response during arousal increases the time before animals go back into torpor (Prendergast et al., 2002). Since periodic arousals account for 80-90 % of the energy utilized throughout hibernation, an
immune response might increase the energetic costs of hibernation (Kayser, 1965). Since in general no massive death of hibernating animals occurs and most microbes do not proliferate well at low temperatures, we speculate that the energetic benefits of a reduced immune function outweigh the infection risk. However, some pathogens, such as the psychrophilic (cold-loving) fungus Geomyces destructans that causes White Nose Syndrome in bats, grow well at low temperatures (Zimmerman, 2009; Blehert et al., 2009; Buchen, 2010; Bouma et al., 2010a; Wibbelt et al., 2010). Hence, immune-suppression during torpor may be detrimental. Unfortunately, reports on leukocyte dynamics during hibernation in bats are lacking. Thus, whether immune-suppression might be involved in the etiology of WNS remains to be elucidated.

**Clinical implications**

In this study we show that release of S1p from erythrocytes is significantly reduced by low body temperature. S1p may serve a key role during the induction of torpor by regulating additional aspects of the immune system as well as other physiological changes that are not primarily related to immune function. Not only does S1p regulate the function of other types of leukocytes (Rivera et al., 2008), it is also involved in other biological processes (Hannun and Obeid, 2008) and implicated in governing protection against ischemia/reperfusion-induced injury in brain, heart, liver and kidney (Hofmann et al., 2009; Bajwa et al., 2010; Park et al., 2010; Zhou et al., 2010). Therapeutic hypothermia is employed in patients with clinical conditions such as cardiac arrest, (brain) trauma and cardiac or brain surgery due to its neuroprotective properties in periods of low oxygen supply by reducing cerebral metabolism (Arrich et al., 2009). However, hypothermia is associated with increased renal injury post-operatively (Kourliouros et al., 2010). Given that the plasma level of S1p in humans is also mainly regulated by transport of S1p from erythrocytes (Kock et al., 2007; Kim et al., 2009) and the S1p-system exerts protective effects following ischemia/reperfusion in different organs (Hofmann et al., 2009; Bajwa et al., 2010; Park et al., 2010; Zhou et al., 2010), our results may be of direct consequence in understanding the benefits and detriments of therapeutic hypothermia.

**Conclusion**

Our study identifies the reduction of body temperature, resulting from metabolic suppression in torpor, as the major driving force in modulation of lymphocyte egress from lymphoid tissue because of decreased S1p plasma levels caused by inhibition of its release from erythrocytes (as summarized in figure 6.9). Understanding the mechanisms of specific physiological alterations involved in the induction of torpor increases our knowledge of natural hibernation with relevance to therapeutic hypothermia as well as pharmacologically induced suspended-animation.