Supporting Information

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SI Materials and Methods

Blood Sample Analysis. After animals were killed by i.p. pentobarbital, 250 μL of blood collected via cardiac puncture into EDTA-coated cups (minicollect EDTA-K3; Greiner) was analyzed on the Sysmex XE-2100, an automated hematology analyzer (1, 2). 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 (vol/vol) with a solution containing prostaglandin E1 (94 nmol/L) (Sigma-Aldrich), Na_2CO_3 (0.63 mmol/L) (Sigma-Aldrich), EDTA (90 mM) (Titriplex; Sigma-Aldrich), and theophylline (10 mM) (Sigma-Aldrich) to minimize platelet activation. Samples were centrifuged for 30 min at 17,000 × *g* at 4 °C, snap-frozen in liquid nitrogen, and stored at −80 °C.

Labeling of Lymphocytes. To obtain lymphocytes for autologous transfusion, Syrian hamsters were splenectomized as described in *Materials and Methods*. A cannula was placed into the jugular vein connected to an s.c. 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 a70-μm cell strainer (Greiner) with sterile saline to obtain a suspension of splenocytes. Cells were spun down for 10 min at 800 × *g* at 4 °C and were resuspended onto a layer of Lympholyte Mammal (Cedarlane Laboratories). Lymphocytes then were separated from other cells by centrifuging for 20 min at 800 × *g* at 4 °C. The lymphocyte fraction was washed, centrifuged for 10 min at 800 × *g* at 4 °C, and resuspended in saline supplemented with 10% DMSO followed by storage in liquid nitrogen. When animals showed torpidity (around 10 wk after splenectomy), lymphocytes were thawed; purity was assessed by analyzing a Wright-Giemsa–stained smear, and viability was measured with a manual cell count after addition of Trypan blue (Sigma-Aldrich). Cells were fluorescently labeled by incubation in 25 μM carboxyfluorescein diacetate succinimidyl ester in saline (Invitrogen) for 15 min at 37 °C. After the labeled lymphocytes were infused during torpor, arousal was induced by gently handling the animals. The animals were killed 2 d 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 TOTO-3 iodide (Invitrogen). Images were taken at 200× magnification (Leica SP2 AOBS) and processed using Imaris 6.4.

Real-Time PCR. RNA isolation was performed using the RNA Isolation Kit (Bioké) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm (NanoDrop ND-1000; NanoDrop Technologies), and purity was checked on 1% agarose gel. One microgram 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 H_2O in 20 μL. cDNA was produced on a C1000 Thermal Cycler (Bio-Rad Laboratories). Oligonucleotide primer sequences (Biolegio) are shown in Table S1. Amplified products (CFX 384 Real-Time System; Bio-Rad 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 on a PE-Sciex API 3000 triple-quadrupole mass spectrometer equipped with a turbo ion-spray source as described previously (3, 4). HPLC separation was performed as described previously (5), with the following changes: An Alltima C-18 column (2.1 × 150 mm, 5 μm; Grace Davison Discovery Sciences) was used at a flow rate of 200 μL/min. N2 was used as the nebulizing gas and drying gas for the turbo ion-spray source. The ion-spray needle was held at 5,500 V; the orifice temperature was set to 500 °C. N2 was used to induce dissociations collisionally 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. Multiple reaction-monitoring 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 (d17:1), sphinganine (d17:0), sphingosine-1-phosphate (S1P) (d17:1), and sphinganine-1-phosphate (d17:0) (Avanti Polar Lipids, Inc.).

Blood Rewarming ex Vivo. Washed erythrocytes (25 μL) derived from torpid Syrian hamsters (4 °C) were pipetted into small polypropylene tubes (Eppendorf) containing DMEM/F12 cell-culture medium (Invitrogen) supplemented with 40% Probumin (Millipore). An inhibitor for the ATP-binding cassette (ABC)-A1 transporters (glyburide, 1 mM; Sigma-Aldrich), the ABC-C1 transporter (MK571, 50 μM; Cayman Chemicals), or both was added to the samples. Samples then were incubated at 37 °C for 30 min. Another sample was left at 4 °C for 30 min. After incubation, samples were centrifuged for 30 min at 17,000 × *g* at 4 °C. A negative control sample was centrifuged before incubation at 37 or 4 °C. Supernatant was snap-frozen in liquid nitrogen and stored at −80 °C.

1. Briggs C, Harrison P, Grant D, Staves J, MacHin SJ (2000) New quantitative parameters on a recently introduced automated blood cell counter–the XE 2100. *Clin Lab Haematol* 22:345–350.
2. Ruzicka K, Veitl M, Thalhammer-Scherrer R, Schwarzinger I (2001) The new hematology analyzer Sysmex XE-2100: performance evaluation of a novel white blood cell differential technology. *Arch Pathol Lab Med* 125:391–396.
3. Bielawski J, Szulc ZM, Hannun YA, Bielawska A (2006) Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Methods* 39:82–91.
4. Sullards MC, Merrill AH, Jr. (2001) Analysis of sphingosine 1-phosphate, ceramides, and other bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Sci. STKE.* 2001, 11.
5. Sullards MC, Wang E, Peng Q, Merrill AH, Jr. (2003) Metabolomic profiling of sphingolipids in human glioma cell lines by liquid chromatography tandem mass spectrometry. *Cell Mol Biol* 49:789–797.

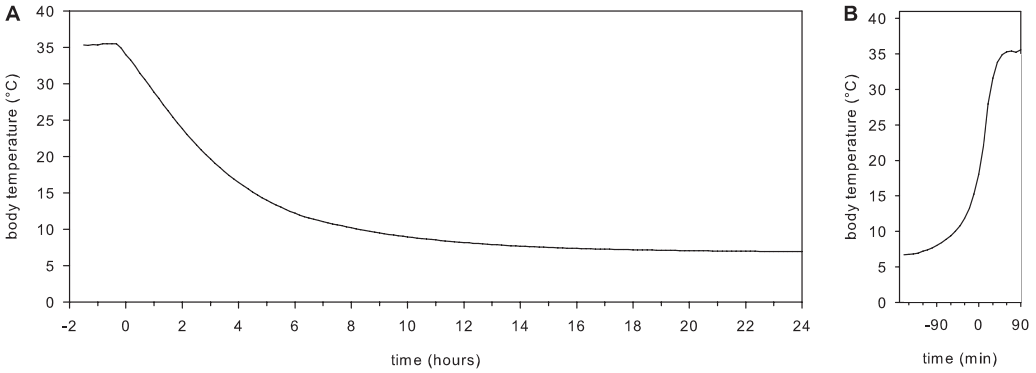

Fig. S1. Average body temperature during (*A*) torpor entry and (*B*) arousal in hibernating Syrian hamsters. Graphs demonstrate the time-dependent changes in mean body temperature from 12 torpor–arousal cycles measured by temperature loggers in the animals' peritoneum.

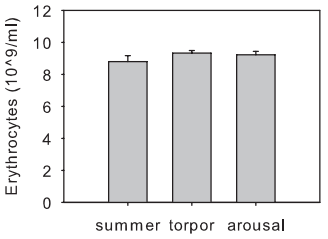

Fig. S2. Hibernation phase does not affect the number of circulating erythrocytes. Bars represent mean ± SEM of four to six animals per group. Data were analyzed using one-way ANOVA with post hoc least significant difference.

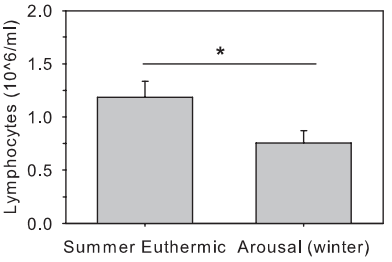

Fig. S3. The number of circulating lymphocytes is reduced significantly in aroused (winter-euthermic) Syrian hamsters compared with summer-euthermic Syrian hamsters. Bars represent mean ± SEM of 11 animals per group. Groups were compared using two-tailed independent samples student's *t*-test. $*P < 0.05$.

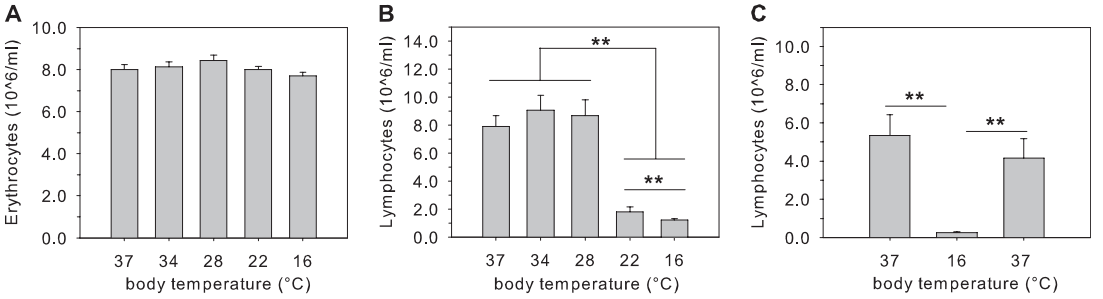

Fig. S4. Forced hypothermia of anesthetized rats induces lymphopenia without affecting the number of circulating erythrocytes. (*A*) Forced hypothermia of anesthetized rats does not affect the number of circulating erythrocytes. (*B*) Body temperature-dependent decrease in circulating lymphocytes in forced hypothermia in anesthetized spontaneously breathing rats. (*C*) Body temperature-dependent decrease in circulating lymphocytes followed by restoration upon rewarming (30 min after reaching 37 °C) in anesthetized intubated rats. Bars represent mean ± SEM of five to seven animals per group. Groups were compared using one-way ANOVA and post hoc least significant difference. $**P < 0.01$.

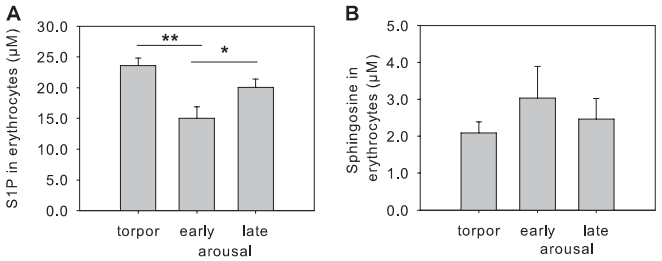

Fig. S5. Complementary levels of (*A*) S1P and (*B*) sphingosine in erythrocytes during different periods of hibernation. Bars represent mean ± SEM of four to six animals per group. Data were analyzed using one-way ANOVA with post hoc least significant difference. */** indicates significant difference at $*P < 0.05$; $**P < 0.01$.

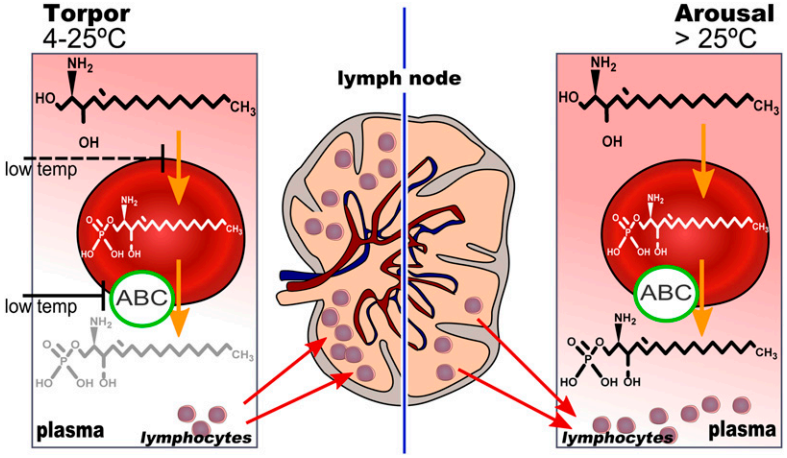

Fig. S6. Proposed model of S1P release from erythrocytes governing lymphocyte dynamics during hibernation or hypothermia. Sphingosine is taken up by erythrocytes and is phosphorylated intracellularly by sphingosine kinase to S1P, which is transported into the plasma by ATP-dependent ABC transporters. During torpor, in response to lower temperature, release of S1P from erythrocytes is inhibited. The inhibition lowers S1P plasma levels, 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).

Table S1. Oligonucleotides designed for real time PCR

Transcript	Orientation	Sequences
CD3ε	Forward	AAGGCCAAGGCCAAGCCTGTGAC
	Reverse	GGCTCATAGTCTGGGTTGGGA
CD20	Forward	GCATTCTGTCGGTGATGCTGATCTC
	Reverse	CTCCAGCTGACAGCAGAACAACATT

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).

Table S2. Expression levels of CD3ε and CD20 in the spleen during torpor and arousal

Transcript	Torpor	Arousal
CD3ε (marker for T lymphocytes)	1.00 ± 0.16	0.87 ± 0.10
CD20 (marker for B lymphocytes)	1.00 ± 0.24	0.87 ± 0.24

The table shows fold mRNA expression ± SEM of aroused animals ($n = 5$) compared with torpid animals ($n = 5$). Expression of CDs was normalized to *GAPDH* as housekeeping gene. No significant differences were found.