Immunological aspects of hibernation as leads in the prevention of acute organ injury
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Lymphopenia in 5′-AMP induced torpor is regulated by the decrease in sphingosine-1-phosphate levels and activation of Adenosine-2b receptors

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Natural hibernation consists of torpid phases alternated with arousals. The suppression of metabolism during torpid phases substantially reduces the animal’s energy demand and causes lowering of body temperature. Induction of torpor holds substantial promise in various medical conditions, including trauma, major surgery and transplantation. Torpor in mice can be induced pharmacologically by injection of 5′-AMP. Previously, we showed that the reduction in body temperature governs lymphopenia in natural torpor via reduction in plasma sphingosine-1-phosphate (S1p). Here we show that 5′-AMP induces torpor, as evidenced by a reduction of body temperature to ~ 23°C 4 h after i.p. injection, and reduces the number of circulating lymphocytes due to retention in lymph nodes. Lymphopenia was caused by stimulation of A2b-receptors, as it was precluded by the adenosine A2b-receptor-antagonist MRS1754, without affecting body temperature. In addition, forced cooling of mice induces lymphopenia as well, which occurs independently of A2b-receptor stimulation. In addition, administration of 5′-AMP substantially decreases the migration of lymphocytes both into lymph nodes and inside lymph nodes, as demonstrated by tracing labeled lymphocytes and two-photon intravital microscopy. The S1p plasma level is reduced during 5′-AMP induced torpor, but restores upon arousal. Injection of a S1p1-antagonist prior to rewarming prevents the restoration of lymphocyte counts upon arousal. Together, these data suggest that lymphopenia during 5′-AMP induced torpor results from both a strong inhibition of lymphocyte egress via A2b-receptor stimulation and a reduction in S1p plasma level. In conclusion, 5′-AMP induces a state of pharmacological torpor in mice during which lymphopenia is primarily governed by body temperature-independent suppression of lymphocyte egress from lymph nodes.

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Introduction

Torpor is a widely conserved behavior in which metabolism—and hence energy demands—are decreased, which is presumed to allow animals to cope with periods of harsh environmental conditions with low food supply (Melvin and Andrews, 2009). During deep torpor, the body temperature might be as low as ± 0-4°C (Kenagy et al., 1989) lasting from a few days up to 35 days, depending on the species (Twente and Twente, 1965; Andjus et al., 1964; Hut et al., 2002; Carey et al., 2003a; Heldmaier et al., 2004). In animal species exhibiting daily torpor the minimum body temperature typically remains above 18°C (Heldmaier et al., 1999; Heldmaier et al., 2004; Geiser, 2004). Both types of torpor alternate with euthermic arousal periods. Specific adaptations are thought to be crucial in allowing animals to safely undergo these states of physiological extremes without signs of organ injury (Zancanaro et al., 1999; Arendt et al., 2003; Sandovici et al., 2004; Fleck and Carey, 2005; Talaei et al., 2011; Bouma et al., 2012). For instance, the superior resistance to ischemia and hypothermia of hibernating animals is suggested to play an important role in maintaining homeostasis (Frerichs et al., 1994; Storey, 2004; Lindell et al., 2005). In addition, changes in the immune system occur during torpor. These lead to the induction of a reversible immunodeficient state, which might not only conserve energy but also prevent tissue injury by limiting inflammatory responses (Bouma et al., 2010a). Upon entrance into deep torpor, number of circulating leukocytes drop by ± 90% in all hibernating mammals studied so far (Bouma et al., 2010a). Previously, we showed that lowering of the body temperature during torpor causes lymphopenia through retention of lymphocytes in secondary lymphoid organs subsequent to a reduction in the plasma level of sphingosine-1-phosphate (S1p) (Bouma et al., 2011). Lowering of metabolism while maintaining homeostasis, with depression of the immune system as occurs during torpor may be of benefit in a number of medical conditions where ischemia and/or inflammation are involved in the induction of organ injury, including trauma, cardiac arrest, organ transplantation, major cardiac and brain surgery, or in patients in the intensive care unit (ICU) (Aslami and Juffermans, 2010). Metabolic suppression in donor organs may improve preservation of transplant organs compared with the currently utilized cold and static preservation methods that are hampered by cell swelling, acidosis and the production of reactive oxygen species (ROS) upon reperfusion (Maathuis et al., 2007).

To date, the exact mechanism(s) leading to the induction of natural torpor is not fully understood, although fasting (e.g. due to scarce food supply in winter) might play an important role (Melvin and Andrews, 2009). Indeed, fasting of mice housed in constant darkness induces a state of torpor that is characterized by a drop in body temperature and numb behavior. Torpor in fasted mice is associated with an increased plasma level of 5'-AMP (Zhang et al., 2006). Interestingly, injection of 5'-AMP in mice induces a similar state of torpor (Zhang et al., 2006; Lee, 2008). Mechanisms suggested to be involved in the induction of torpor by 5'-AMP include the activation of AMP-activated protein kinase (AMPK) (Lindsley and Rutter, 2004; Lee, 2008; Melvin and Andrews, 2009), a principal cytoplasmic molecular energy sensor (Bouma et al., 2010b), and the reduction in cardiac output due to stimulation of adenosine receptors by 5'-AMP derived adenosine (Swoap et al., 2007). Thus, 5'-AMP may be considered a pharmacological tool to induce torpor. However, effects of 5'-AMP on the immune system are not known. Lowering of body temperature after injection of 5'-AMP might induce lymphopenia. Alternatively, 5'-AMP derived adenosine might activate
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Adenosine receptors expressed on high endothelial venules (HEVs) and influence lymphocyte migration into lymph nodes. The only adenosine receptor expressed on the HEV-like cell line KOP2.16 is the adenosine 2b (A2b)-receptor, which reduces lymphocyte migration upon activation by adenosine (Takedachi et al., 2008). Here we assessed whether 5′-AMP induced torpor in mice (1) affects lymphocyte recirculation and (2) whether these effects are mediated by activation of the A2b-receptor or lowered body temperature.

Materials and methods

Animals

C57BL/6 and CD45.1 congenic mice were housed under standard L:D-conditions (12:12) in the animal facilities of the University of Groningen, The Netherlands and the National Institutes of Health, Bethesda, USA. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and Harlan Netherlands B.V., while CD45.1+ B6 mice were obtained from the NIAID contract colony at Taconic Farms. Prior to the experiments, animals were fed ad libitum using standard animal lab chow. All animal experiments were approved either by the Animal Experimental Committees of the University of Groningen, The Netherlands or the National Institutes of Health, Bethesda, USA.

Torpor induction by 5′-AMP

Torpor was induced pharmacologically by injecting 7.5 mmol/kg of 5′-AMP (Sigma Aldrich) in 0.9% saline (pH 7.5) intra-peritoneally. To record body temperature during experiments, we either surgically implanted miniaturized iButtons intra-peritoneally (Maxim Direct), and allowed mice to recover for a period of 3 weeks before torpor induction, or measured the body temperature using a rectal probe (Physitemp Instruments). Mice were euthanized at different times after injection of 5′-AMP. The minimum body temperature during torpor was reached at 4-5 hours following 5′-AMP injection and full arousal with normalization of body temperature occurred by 10 hours after 5′-AMP administration. Euthermic animals served as controls and were euthanized at the time that other animals were injected with 5′-AMP. At euthanization, animals were anesthetized using 3% isoflurane/oxygen and ± 800 µl blood was drawn immediately by cardiac puncture into small EDTA-coated tubes. Automated hematological analysis was performed within 5 hours using a Sysmex XE-2100 (Briggs et al., 2000; Ruzicka et al., 2001) that was validated by manual counting of a Giemsa-stained blood smear. The remaining blood was collected for mass-spectrometry measurement of sphingosine/sphingosine-1-phosphate (S1p) in a polypropylene tube mixed 1:10 v/v with a solution that minimizes platelet activation and contained 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). These samples were centrifuged (30 minutes, 17,000 g, 4°C), snap-frozen in liquid nitrogen and stored at -80°C.

T cell lymph node homing assay

To investigate the impact of 5′-AMP on T cell homing to lymph nodes, CD45.1+ T cells were purified by negative selection (MACS, Miltenyi Biotec) and 1x10⁷ cells were adoptively transferred i.v. into congenic C57BL/6 (CD45.2+) recipients. Simultaneously, 0.9% saline or 7.5 µmol/g of 5′-AMP was injected i.p.. Two hours later, inguinal, brachial and mesenteric lymph nodes were collected from each recipient, cell suspensions made by homogenization through 70 µm cell strainers (BD Biosciences), cell numbers enumerated and cell fractions analyzed by flow cytometry. To assess whether blockade of T cell homing from blood to
lymph nodes prevents 5’-AMP-induced lymphopenia, lymph node entry was blocked in mice by i.p. administration of 100µg anti-αL-integrin (clone M17/4) and 100µg anti-α4-integrin (clone PS/2, both from BioXcell) antibodies in PBS two hours prior to 5’-AMP injection (Lo et al., 2005). Blood samples were drawn as described above 4 hours after torpor induction.

**Two-photon imaging data acquisition and analysis**

Surgical preparation of the inguinal or popliteal lymph node was performed using a protocol modified from previous reports (Miller et al., 2003; Mempel et al., 2004) after having injected 5x10^6 purified T cells labeled with 1µM CMFDA (Invitrogen) 18-24 hours prior. Mice were anesthetized with 1-1.5% isoflurane (Baxter) vaporized in a 80:20 mixture of O_2 and air during the surgery and microscopy. To inject 5’-AMP during imaging, a catheter was inserted intra-peritoneally via a 29.5GA insulin needle attached to plastic 0.01 inch wide microbore tubing (Tygon) and secured with Durapore tape (Fischer Scientific) prior to placing the mouse under the microscope. Images were acquired on an LSM 710 NLO multiphoton imaging system (Carl Zeiss Microimaging) enclosed in a custom-built environmental chamber kept at 35ºC through a 20x water immersion lens (N.A. 1.0) and fluorescent excitation provided by a Chameleon ULTRA (II) Ti:Sapphire laser (Coherent) tuned to 800nm wavelength. Imaging planes collected at 3µm steps to form z-stacks that were repeated every 30 sec to yield 4D datasets that were processed with Imaris (Biplane) and analyzed in MatLab as described previously (Egen et al., 2008) to obtain cell velocities. AfterEffects (Adobe) was used to produce video clips.

**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) was used at a flow rate of 200 µl/min. N_2 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 500ºC. N_2 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 (d17:1), sphinganine (d17:0), sphingosine-1-phosphate (d17:1), and sphinganine-1-phosphate (d17:0) (Avanti Polar Lipids).

**Flow Cytometry**

Peripheral blood mononuclear cells were isolated from blood samples with erythrocyte lysis buffer (0.83 % w/v ammoniumchloride in H_2O; incubated for 10 minutes on ice) or by spinning through a histopaque gradient according to the manufacturer’s protocol (Sigma Aldrich). Cells were stained using anti-mouse antibodies CD44 (IM7), CD3ε (145-2C11), B220 (RA3-6B2), CD19 (1D3) purchased from eBioscience. All samples were acquired on a LSR-II flow cytometer (Becton Dickinson) and data analyzed using FlowJo (Tree Star).
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**Statistical analysis**
To calculate statistical differences, SPSS 20.0 for Windows workstations was used. Data was analyzed using a One-Way ANOVA and after testing for homogeneity of variances post-hoc testing was performed using LSD or Games-Howell. In the case where less than three groups were to be compared, an independent samples Student’s T-test was used. To estimate the effect of different dosages, an ANOVA linear trend analysis was applied. In all situations, \( p < 0.05 \) was considered significantly different. Data in the manuscript are presented as means ± standard error of the mean (SEM).

**Results**

**Administration of 5’-AMP reduces the body temperature and induces lymphopenia**
As previously described (Zhang et al., 2006), injection of 5’-AMP into mice leads to the induction of a torpor-like state as characterized by numb behavior and a profound reduction of body temperature. After injection of 5’-AMP, body temperature gradually reduces from 34.5 ± 0.4°C to 23.4 ± 0.3°C at 4 hours, after which a biphasic increase towards normalization was observed (Figure 9.1A). At 4 hours after injection of 5’-AMP, the number of circulating lymphocytes decreases significantly (\( p < 0.01 \); Figure 9.1B). Flow cytometric analysis reveals that both T-lymphocytes (\( p < 0.01 \); Figure 9.1C) and B-lymphocytes (\( p < 0.05 \); Figure 9.1D) are significantly reduced. The numbers of circulating T- and B- lymphocytes restores at 10 h following 5’-AMP to values not significantly different from those measured before injection (Figure 9.1B-D). In contrast to lymphocytes, 5’-AMP induced torpor does not affect the number of circulating erythrocytes, monocytes or neutrophils (Figure 9.1E-G). Upon arousal, however, the number of neutrophils is strongly increased (Figure 9.1F), while the number of erythrocytes and monocytes remains unaffected. Hence, injection of 5’-AMP leads to a significant drop in body temperature and is associated with a reversible reduction in the number of circulating T- and B-lymphocytes.

**A2b-receptor activation induces lymphopenia independent of low body temperature**
Activation of the purinergic A2b-receptor might play a role in the observed lymphopenia by 5’-AMP in mice (Takedachi et al., 2008). To investigate this, we injected a specific A2b-receptor antagonist (12.5 mg/kg MRS1754) to block effects of 5’-AMP derived adenosine mediated through the A2b-receptor, fifteen minutes prior to injection of 5’-AMP. Pretreatment with MRS1754 did not affect the reduction of body temperature of mice induced by 5AMP, which was 24.8 ± 1.1°C at 4 hours after injection of 5’-AMP (\( p > 0.05 \) vs. 5’-AMP only). However, MRS1754 precluded the induction of lymphopenia by 5’-AMP (\( p < 0.05 \) vs. 5’-AMP only; Figures 9.1B-D). To further consolidate the role of A2b-receptors in the induction of lymphopenia, we injected a specific A2b agonist (LUF6210) in the absence of 5’-AMP and measured the number of circulating lymphocytes 4 hours after injection. Injection of the A2b agonist LUF6210 does not affect the body temperature (Figure 9.1A), which is 37.7 ± 0.1°C at 4 hours after injection and is not significantly different from the body temperature before injection. Similar to injection of 5’-AMP alone, administration of LUF6210 alone reduces the number of circulating lymphocytes (\( p < 0.01 \); Figure 9.1B), due to a reduction in the number of circulating T-lymphocytes (\( p < 0.05 \); Figure 9.1C) and B-lymphocytes (\( p < 0.01 \); Figure 9.1D). In addition, MRS1754 pretreatment also precludes the reduction of circulating lymphocytes induced by LUF6210 (Figure 9.1B-D). Neither LUF6210 alone nor MRS1754 combined with either LUF6210 or 5’-AMP, induces a significant change in the number of circulating erythrocytes, neutrophils of monocytes (Figures 9.1E-G).
Figure 9.1. 5'-AMP induced torpor is associated with a reduced number of T- and B-lymphocytes and an increased number of circulating neutrophils upon arousal. Intraperitoneal (i.p.) injection of 5'-AMP (7.5 mmol/kg) leads to the induction of torpor characterized by a reduced body temperature, which is not observed after i.p. injection of the specific A2b-receptor agonist LUF6210 (3 mg/kg) (A); the number of circulating lymphocytes is reversibly reduced by injecting 5'-AMP or LUF6210, which is precluded by pretreatment with the specific A2b-receptor antagonist MRS1754 (12.5 mg/kg) (B); the reduction in the number of circulating lymphocytes by 5'-AMP and LUF6210 is due to a reduced number of both T-lymphocytes (C) as well as B-lymphocytes (D) and can be precluded by MRS1754; the number of circulating erythrocytes is not affected by 5'-AMP, LUF6210 or the specific A2b-receptor antagonist MRS1754 (E); the number of circulating granulocytes increases upon arousal following torpor induced by 5'-AMP (F); the number of circulating monocytes is not affected by 5'-AMP, LUF6210 or MRS1754 (G). Bars represent mean ± standard error of the mean (SEM); */** = p < 0.05/0.01 (n = 6-9 animals per group).
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Figure 9.2. Effect of body temperature on 5′-AMP induced lymphopenia. Housing mice at an ambient temperature of ± 30°C precludes the drop in body temperature that is observed in mice housed at ± 22°C after injection of 5′-AMP i.p. (A); housing mice at a relatively high ambient temperature does not attenuate the decrease in the total number of circulating lymphocytes following injection of 5′-AMP (B); the number of circulating T-lymphocytes is reduced by 5′-AMP, independent of body temperature (C); the number of circulating B-lymphocytes is only reduced in mice housed at ± 22°C following injection of 5′-AMP (D); 5′-AMP does not affect the number of circulating erythrocytes (E); injecting 5′-AMP in animals housed at ± 30°C increases the number of circulating neutrophils (F); 5′-AMP does not affect the number of circulating monocytes (G). Since torpor duration (as noticed by numb behaviour) at an ambient temperature of ± 30°C lasts ± 2 h, we injected these mice with 7.5 mmol/kg i.p. at t = 0 h and with 1.9 mmol/kg (25%) at t = 2 h. Ta = ambient temperature. Bars represent mean ± standard error of the mean (SEM); */** = p < 0.05/0.01 (n = 3-9 animals per group).

5′-AMP induced lymphopenia occurs in the absence of lowered body temperature
Since data from the experiment described above suggest that lymphopenia during 5′-AMP induced torpor might be secondary to temperature-independent activation of A2b-receptors, we further elucidated the role of the body temperature on lymphopenia during 5′-AMP induced torpor in mice. Therefore, we housed the animals after injection of 5′-AMP at an ambient temperature of ± 30°C to preclude the reduction in body temperature by 5′-AMP. Numb behavior of the animals was readily observed upon 5′-AMP injection, but lasted only about 2 h at an ambient temperature of ± 30°C, possibly caused by more rapid metabolization of 5′-AMP. To allow torpor for up to 4 hours at this relatively high ambient temperature, we injected a second dose of 1.9 mmol/kg 5′-AMP i.p. (25% of the initial dose) 2 hours after injection of the initial dose. In these animals, we found that although 5′-AMP does not affect the body temperature under these conditions (Figure 9.2A), the number of circulating lymphocytes are reduced as compared to baseline (p < 0.05; Figure 9.2B). This is largely influenced by a reduced number of T-lymphocytes (p < 0.05; Figures 9.2C), since the number of B-lymphocytes is not significantly decreased (Figure 9.2D). Although the number of circulating erythrocytes and monocytes are not affected by housing animals at a relatively high ambient temperature following injection of 5′-AMP (Figures 9.2E, G), the number of
circulating neutrophils is significantly increased at 4 hours after injection \((p < 0.05; \text{Figure } 9.2F)\). Hence, the reduction in number of circulating T-lymphocytes as a consequence of injection of 5’-AMP is secondary to activation of A2b-receptors and occurs independent of body temperature.

### Restoration of lymphocyte counts upon arousal requires lymphocyte egress

Plasma S1p levels influence lymphocyte egress from lymphoid organs through S1p\(_1\) receptors expressed on lymphocytes (Pappu et al., 2007), and herewith affect the number of circulating lymphocytes. Previously, we showed that the drop in the number of circulating lymphocytes during deep torpor in hibernating hamsters is due to a lowered plasma level of S1p (Bouma et al., 2011). For this reason, we measured the plasma S1p levels after injection of 5’-AMP in mice and determined whether restoration of normal numbers of circulating lymphocytes is induced by S1p. Injection of 5’-AMP in mice significantly reduces the plasma S1p level by ± 40% at 4 hours after injection \((p < 0.05 \text{ as compared to baseline})\). S1p levels are fully normalized during arousal at 10 h \((p < 0.05 \text{ as compared to 4 hours; } \text{Figure } 9.3A)\). Injection of the A2b-receptor agonist LUF6210 alone did not affect S1p levels (Figure 9.3A). To test whether S1p might play a role in the restoration of the number of circulating lymphocytes upon arousal, we injected a S1p\(_1\) specific antagonist (W146, 10 mg/kg i.p.) at 6 hours following injection of 5’-AMP, i.e. at the initial stage of arousal. Indeed, injection of this S1p\(_1\) antagonist precludes the restoration of the total number of circulating lymphocytes (Figure 9.3B). Restoration of normal number of lymphocytes upon rewarming is mainly due to an attenuation of the increase in the number of circulating T-lymphocytes \((p < 0.01; \text{Figure } 9.3C)\), since the number of B-lymphocytes is not yet significantly increased at 10 hours as compared to 4 hours after injection of 5’-AMP (Figure 9.3D). Importantly, the S1p\(_1\) specific antagonist did not affect restoration of body temperature (Figure 9.3E) or the number of circulating erythrocytes or monocytes (Figure 9.3F,H), although it prevented the increase in the number of circulating neutrophils upon arousal (Figure 9.3G). Taken together, changes in the plasma level of S1p following injection of 5’-AMP likely affects the number of circulating lymphocytes, particularly through modulation of T-lymphocyte egress from lymphoid organs.

### Forced hypothermia leads to a concomitant decrease in the number of lymphocytes

Although activation of A2b-receptors induces lymphopenia without affecting the body temperature, this finding does not rule out that 5’-AMP induced reduction of the number of circulating lymphocytes is (partly) temperature-dependent. To assess whether a the reduction of body temperature as occurs following injection of 5’-AMP affects the number of lymphocytes, we cooled anesthetized mice using ice packs and measured the number of circulating lymphocytes 20 minutes after reaching a body temperature of ± 24°C. In line with injection of 5’-AMP, forced hypothermia leads to a similar reduction in the number of lymphocytes, which seems more prominent for T-lymphocytes than for B-lymphocytes \((p < 0.05; \text{Figures } 9.4A-C)\). Cooling did not affect the number of circulating erythrocytes, neutrophils and monocytes (Figures 9.4D-F). To assess whether A2b-receptors might be involved in cooling induced lymphopenia, we i.p. injected animals with its antagonist MRS1754 (1.25 mg/kg, \(n = 2\) and 12.5 mg/kg, \(n = 2\)) 30 minutes prior to forced hypothermia. We found no significant effects of MRS1754 on the reduction in the number of circulating lymphocytes in hypothermic animals (Figures 9.4A-C), demonstrating that A2b-receptors are not involved in lymphopenia induced by hypothermia.
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Figure 9.3. Restoration of circulating lymphocyte counts upon arousal from torpor in mice is governed by Sphingosine-1-phosphate (S1p). Injection of 5'-AMP leads to a reduced plasma level of S1p at 4 hours following injection, which is normalized upon arousal while the A2b agonist LUF6210 did not affect S1p levels (A); injection of a specific S1p1-antagonist (W146; 10 mg/kg i.p.) prevents the restoration during arousal of the total number of circulating lymphocytes (B) which is due to an increased number of circulating T-lymphocytes (C), since no significant increase in the number of B-lymphocytes was observed during arousal (D); W146 does not affect the restoration of normothermia upon arousal (E) or the number of circulating erythrocytes (F); in addition, W146 precluded the increased number of circulating neutrophils following rewarming (G), but did not affect the number of circulating monocytes (H). Bars represent mean ± standard error of the mean (SEM); */** = p < 0.05/0.01; (n = 6-9 animals per group).
Figure 9.4. Effect of forced hypothermia on the number of circulating lymphocytes. Forced hypothermia of anesthetized mice to reach a body temperature ± 24°C leads to a similar decrease in the total number of lymphocytes as observed following i.p. injection of 5'-AMP, which was not affected by injecting an A2b-receptor antagonist (MRS1754) 30 minutes prior to cooling (A); the number of circulating T-lymphocytes is reduced by 5'-AMP and forced hypothermia, the latter not being affected by MRS1754 (B); both 5'-AMP and forced hypothermia reduce the number of circulating B-lymphocytes and the latter is unaffected by MRS1754 (C); the number of circulating erythrocytes (D), neutrophils (E) and monocytes (F) was not affected by 5'-AMP or hypothermia in mice. Bars represent mean ± standard error of the mean (SEM); */** = p < 0.05/0.01 (n = 7-9 animals per group).

Figure 9.5. Although lymphopenia induced by 5'-AMP is due to retention in lymph nodes, 5'-AMP reduces the homing rate of lymphocytes. Blocking lymph node homing by administration of antibodies against α4/αL-integrins (100 µg/mouse) 2 hours before injection of 5'-AMP precludes the induction of lymphopenia at 4 hours after injection of 5'-AMP (A); neutralizing antibodies against homing receptors prevent the 5'-AMP induced decrease the number of circulating T-lymphocytes (B) or B-lymphocytes (C); injection of 5'-AMP (i.p.) reduces homing of labelled lymphocytes to inguinal (iLN), brachial (bLN) and mesenteric lymph nodes (mLN). Adoptively transferred CD45.1+ congenic T-lymphocytes were i.v. injected in recipient mice (1x10^7) at 2 hours following transfer as compared to saline controls (D). Bars represent mean ± standard error of the mean (SEM); */** = p < 0.05/0.01 (n = 4-6 animals per group).
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5'-AMP induced torpor reduces lymphocyte recirculation rates and causes the retention of lymphocytes within secondary lymphoid organs

To study whether retention of lymphocytes in secondary lymphoid organs leads to lymphopenia after injection of 5'-AMP, we blocked homing by injecting neutralizing antibodies against α4/α-L-integrins prior to the induction of torpor. α4/α-L-integrins are expressed on lymphocytes and are required for lymph node entry (Lo et al., 2005). Blocking lymphocyte homing to lymph nodes resulted in a concomitant increase in the number of circulating T- and B-lymphocytes 2 hours later, before injection of 5'-AMP. Subsequent induction of torpor did not lead to a reduction in the number of circulating T- or B-lymphocytes at 4 hours following injection of 5'-AMP (p < 0.05; Figures 9.5A-C). Hence, lymph node homing of both T- and B-lymphocytes is essential for the induction of lymphopenia by 5'-AMP. Both the augmentation of migration into lymphoid organs (‘homing’) or a decreased migration out of lymphoid organs (‘egress’) can lead to a reduction in the number of circulating lymphocytes. As described above, S1p mediated egress upon arousal after injection of 5'-AMP stimulates restoration of normal numbers of circulating lymphocytes. Likely, the reduced plasma level S1p after injection of 5'-AMP diminishes the rate of egress of lymphocytes. Alternatively, a lowered migration rate of lymphocytes inside lymph nodes impairs the migration of lymphocytes towards exit sites and hence, leads to a reduced egress rate. Therefore, we examined the migratory behavior of lymphocytes in lymph nodes by two-photon intravital microscopy to follow dye-labelled adoptively transferred T cells. Injection of 5'-AMP severely impaired the motility of lymphocytes (at 37°C), possibly leading to a reduced egress of lymphocytes in secondary lymphoid organs (as can be seen in this movie derived by 2-photon intravital microscopy: http://www.hjalmarbouma.nl/20120930.mov). In order to quantify the effects of 5'-AMP on homing of lymphocytes, we adoptively transferred CD45.1+ T-lymphocytes and measured their number in lymph nodes 2 hours following intravenous injection. We found that 5'-AMP reduces homing of CD45.1+ T-lymphocytes to inguinal, brachial and mesenteric lymph nodes (p < 0.05; Figure 9.5D). Thus, although lymphopenia upon 5'-AMP administration is blocked by the prevention of homing, administration of 5'-AMP reduces the homing rate of lymphocytes and results in lower numbers of lymphocytes in lymphoid tissue. Taken together, lymphopenia induced by 5'-AMP is due to retention of lymphocytes in lymph nodes through a decreased egress rate. Likely, the reduced motility of lymphocytes in lymph nodes and potentially also the lowered S1p plasma level inhibit lymphocyte egress after injection of 5'-AMP.

Discussion

Both temperature-dependent and temperature-independent effects lead to clearance of circulating lymphocytes following injection of 5'-AMP

As described in the introduction, hibernating animals are able to withstand physiological extreme conditions without signs of organ injury (Zancanaro et al., 1999; Arendt et al., 2003; Sandovici et al., 2004; Fleck and Carey, 2005; Talaei et al., 2011; Bouma et al., 2012). Changes in key physiological parameters occur during natural torpor, including a profound, reversible reduction in the number of circulating lymphocytes (Bouma et al., 2011). In this study, we exploited the ability to pharmacologically induce a torpor-like state in non-hibernators by injecting 5'-AMP into mice. Following injection of 5'-AMP, the number of T-lymphocytes and B-lymphocytes is reduced, without significant effects on other blood cells. 5'-AMP induced lymphopenia depends on the activation of the purinergic A2b-receptor, as co-infusion of an
A2b-specific antagonist precluded lymphopenia but did not affect the reduction in body temperature. Further, lymphopenia could also be induced by injecting an A2b-specific agonist, which in contrast to 5′-AMP did not reduce the body temperature. In addition, lowering of the body temperature to reach a similar value as induced by 5′-AMP leads to a similar decrease in the number of total, T- and B- lymphocytes, which however could not be prevented by antagonizing A2b-receptors. Together, these observations imply that the induction of lymphopenia during a 5′-AMP induced torpor in mice occurs independently of body temperature and is governed by A2b-receptors, as summarized schematically in Figure 9.6.

**Lymphopenia is not due to increased homing, but depends on decreased egress of cells**

Upon induction of torpor using 5′-AMP, the induction of lymphopenia was precluded by blocking lymphocyte homing receptors. Migration to the spleen is not prevented by blocking homing receptors. Therefore, lymphopenia during torpor induced by 5′-AMP is unlikely to be caused by retention of cells in the spleen. Rather, this finding suggests that retention of lymphocytes in peripheral lymph nodes leads to a reduced number of circulating lymphocytes, similar to the situation during natural deep torpor in the Syrian hamster (Bouma et al., 2011). Retention of lymphocytes in lymphoid organs might be due to increased homing or reduced egress of cells. Therefore, we estimated the homing rate of lymphocytes by counting the numbers of CD45.1+ T-lymphocytes in lymph nodes at 2 hours following transfer. The number of CD45.1+ T-lymphocytes in lymph nodes is not yet influenced by egress of cells at this time-point. Results from this experiment suggest that 5′-AMP impairs the homing rate of lymphocytes into lymph nodes. Inhibition of homing by 5′-AMP might be secondary to activation of A2b-receptors, as activation of A2b-receptors in HEVs restricts lymphocyte migration into lymphocytes in CD73–/– mice (Takedachi et al., 2008). Thus, lymphopenia during 5′-AMP induced torpor is not caused by an increased homing rate of lymphocytes. Consequently, induction of 5′-AMP related lymphopenia must be dependent on the inhibition of lymphocyte egress from lymph nodes back into the circulation.

**Lowered body temperature might lead to lymphopenia by a lowered S1p**

Injection of 5′-AMP might reduce lymphocyte egress through the lowered plasma level S1p or the decreased motility of lymphocytes within lymph nodes. Inhibition of lymphocyte egress from lymph nodes in mice leads to maximal lymphopenia within 2-4 hours (Mandala et al., 2002). S1p is a bioactive lipid that plays an important role in stimulating lymphocyte egress from lymph nodes, as demonstrated in other models (Mandala et al., 2002; Matloubian et al., 2004; Pappu et al., 2007; Sensken et al., 2011). Previously we have shown that in naturally hibernating hamsters, when animals enter deep torpor and the body temperature is lowered, the plasma level of S1p drops, which is likely due to a diminished release from erythrocytes (Bouma et al., 2011). In the current work, we reveal that 5′-AMP lowers the body temperature and leads to a reduced plasma level of S1p as well. Upon arousal, the plasma level S1p increases towards euthermic values. Administration of a S1p1 specific antagonist before rewarming, however, precludes the restoration of normal numbers of circulating T-lymphocytes. The number of B-lymphocytes on the other hand did not increase significantly upon rewarming and was not affected by injection of a S1p1 specific antagonist. The observation that the number of circulating B-lymphocytes is not (yet)
lymphopenia in 5’-AMP induced torpor is regulated by the decrease in sphingosine-1-phosphate levels and activation of adenosine-2b receptors

restored at 10 hours following injection of 5’-AMP, might be explained by the longer lymph node dwell time of B-lymphocytes as compared to T-lymphocytes (Tomura et al., 2008). Thus, lowering of the body temperature after injection of 5’-AMP might lead to diminished egress secondary to the lowered S1p plasma level. On the other hand, injection of an A2b-specific agonist does not affect the body temperature or the plasma level S1p. Hence, activation of A2b-receptors induces additional temperature-independent effects, which are not governed by S1p and lead to the induction of lymphopenia. In accord, we found that 5’-AMP (at 37°C) induces an immediate and almost complete reduction in lymphocyte motility within brachial lymph node. We speculate that the reduced motility of lymphocytes, precludes cells to reach the medullary and cortical lymphatic sinuses and to exit from the lymph nodes (Sinha et al., 2009). Taken together, our data imply that inhibition of lymphocyte motility conveys the reduction of circulating lymphocytes in 5’-AMP induced torpor by reducing their egress rate from secondary lymphoid organs. Restoration of normal numbers of circulating T-lymphocytes is stimulated by S1p upon arousal.

While 5’-AMP induced torpor displays profound effects on lymphocyte recirculation, it also induced substantial neutrocytosis upon arousal. This increase in number of circulating neutrophils was precluded also by blocking the S1p-receptor during arousal. In vitro experiments show that S1p stimulates Fc-γ receptor signal transduction (Florey and Haskard, 2009) and interleukin-8 (IL-8) production (Milara et al., 2009), which both promote neutrophil chemotaxis. Further, S1p lyase deficient (Sgp11−/−) mice have a higher plasma level S1p, an augmented granulopoiesis and a higher number of circulating neutrophils (Allende et al., 2011). Thus, the rise in the number of circulating neutrophils above baseline may be caused by S1p as well. Previously, we demonstrated that low body temperature inhibits the release of S1p from erythrocytes into the plasma through ATP-binding cassette (ABC) transporters, which can be stimulated by (ex vivo) rewarming of erythrocytes (Bouma et al., 2011). In conclusion, rewarming following 5’-AMP induced torpor in mice leads to an increased plasma level of S1p, which leads to an increased number of circulating neutrophils.

A2b-mediated signaling in the immune system

While we demonstrate in this study that activation of A2b-receptors affects the number of circulating lymphocytes, A2b-receptors can exert additional immunomodulatory actions upon activation. A2b-receptors can be activated by (endogenous) adenosine, while A2b-mediated signaling can be inhibited by interferon gamma (IFN-γ) (Kolachala et al., 2005). Formation of endogenous adenosine is regulated by the inducible enzymes CD73 (ecto-5’-nucleotidase; 5’-NT) and CD39 (nucleoside triphosphate diphosphohydrolase-1; ENTPD1) which are expressed on subsets of T- and B-lymphocytes, on follicular dendritic cells, on thymic medullary reticular fibroblasts, epithelial cells and afferent lymphatic vessels (Resta et al., 1998; Algars et al., 2011; Linden and Cekic, 2012). Subsequent metabolism of adenosine can be mediated through adenosine deaminase (ADA). Interestingly, ADA-/- mice exhibit increased levels of adenosine and purine metabolites, leading to severe immunodeficiency with recurrent infections (ADA-SCID) (Sauer et al., 2012). The role of adenosine in lymphocyte migration is illustrated by the reduced homing rate (Takedachi et al., 2008) and increased influx into tissues in CD73-/- mice as compared to wild-type animals following experimental cerebral ischemia (Petrovic-Djergovic et al., 2012) or following cardiac allotransplantation (Hasegawa et al., 2008a). Activation of A2b-receptors can reduce
lymphocyte homing (Takedachi et al., 2008) and decrease the barrier function of vascular endothelium (Hasegawa et al., 2008; Linden and Cekic, 2012). The importance of A2b-mediated signaling the adaptive immune system is illustrated by the fact that genetic ablation of CD73 or A2b antagonism leads to increased lymphocyte transmigration into tissues, while agonism of the A2b-receptor prolongs graft survival in a cardiac allograft model (Hasegawa et al., 2008). Taken together, (5′-)AMP can be metabolized into adenosine by ectonucleotidases, which has diverse immune suppressive properties and plays an important role in regulating lymphocyte migration. The immune suppressive effects of 5′-AMP might be of relevance to the treatment of infectious diseases, auto-immune diseases and tumor immunology (Beldi et al., 2008; Mandapathil et al., 2010; Sun et al., 2010).

Clinical implications
The potential to pharmacologically induce a torpor-like state with increased resistance to ischemia/reperfusion and hypothermia combined with immune suppression might be of major clinical relevance. One of the applications might be optimization of the application of therapeutic hypothermia, as currently employed during brain and cardiac surgery (Arrich et al., 2009). Although hypothermia during surgery is thought to be crucial in limiting neuronal injury during periods of low oxygen supply by reducing cerebral metabolism, hypothermia is associated with kidney injury post-operatively (Kourliouros et al., 2010), which represents an important risk factor for in-hospital and long-term mortality following cardiac surgery (Loef et al., 2009).

Conclusion
Injection of 5′-AMP leads to the induction of a torpor-like state in mice, which is characterized by a drop in body temperature and is associated with a substantial, transient lymphopenia due to temperature-independent activation of A2b-receptors. Inhibition of lymphocytes motility by 5′-AMP diminishes egress of cells and induces retention of lymphocytes in lymph nodes. Upon arousal, the plasma level S1p restores to euthermic levels and stimulates restoration of normal numbers of circulating lymphocytes. We speculate that the signaling pathways activated by 5′-AMP might represent an important pharmacological target to safely suppress metabolism and the immune system, leading to optimization of outcome following major surgery and hence, reduction of mortality during follow-up.
lymphopenia in 5'-AMP induced torpor is regulated by the decrease in sphingosine-1-phosphate levels and activation of adenosine-2b receptors

Figure 9.6. 5'-AMP governs lymphopenia due to retention of lymphocytes in lymph nodes. Injection of 5'-AMP in mice induces torpor and a decline body temperature. 5'-AMP reduces both homing and egress rate of lymphocytes through activation of the purinergic adenosine 2b (A2b) receptor, ultimately resulting in a decreased number of circulating lymphocytes independent of its effects on body temperature. In addition, the lowering of body temperature inhibits the release of Sphingosine-1-phosphate (S1p) from erythrocytes (Bouma et al., 2011), which is restored upon arousal, thus allowing for restoration of numbers of circulating lymphocytes.