Kumulative Habilitationsschrift

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Einfluss zirkadianer Rhythmik auf die Migration und Funktion von Leukozyten

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Inhalt

1. Vorwort

Die Publikationen, die von mir als Erst- und Letztautor generiert wurden, bilden den Schwerpunkt dieser kumulativen Habilitationsschrift. Die diesen Publikationen zugrunde liegenden Experimente wurden von mir selbstständig konzipiert oder gleichwertig mitkonzipiert und von mir selbst oder meinen ehemaligen und aktuellen Doktoranden und wissenschaftlichen Mitarbeitern unter meiner unmittelbaren Anleitung und Mitarbeit durchgeführt. Die Abbildungen stammen aus den angegebenen Publikationen und wurden von mir ins Deutsche übertragen.

Ich danke ganz besonders Herrn Prof. Dr. Ulrich Pohl für die Aufnahme meiner Emmy-Noether-Arbeitsgruppe an das Walter-Brendel-Zentrum für Experimentelle Medizin und seiner essentiellen Unterstützung insbesondere bei Platz- und initialen Ausstattungsfragen, die es mir erlaubten, unsere experimentellen Studien adäquat durchzuführen und zu publizieren. Weiterer, wichtiger Dank gilt den uns unterstützenden Fördergesellschaften, der Deutschen Forschungsgemeinschaft (DFG) und dem Europäischen Research Council (ERC).

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2. Zusammenfassung

Inhaltsangabe

Die Rekrutierung von Leukozyten in Organe spielt eine entscheidende Rolle bei Entzündungen, aber auch unter homöostatischen Bedingungen. In dieser Habilitationsschrift wurde untersucht, inwieweit zirkadiane und neuronale Prozesse die Migration von Leukozyten ins Gewebe beeinflussen. Die Daten zeigen, dass zentral initiierte, systemische Programme über adrenerge Nerven des sympathischen Nervensystems (SNS) lokal die Infiltration von myeloiden Zellen ins Gewebe beeinflussen. Dies führt zur zirkadianen Oszillation in der Rekrutierung von Leukozyten aus dem Blut ins Knochenmark. Die meisten Zellen dringen zu Beginn der täglichen Aktivität ins Gewebe ein, d.h. zu Beginn der Nacht in der Maus, dem hier untersuchten Modellorganismus. Auf diesen Studien aufbauend wurde untersucht, ob migratorische Oszillationen auch in der Emigration von Lymphozyten aus dem Blut existierten. In der Tat gibt es eine starke Oszillation in der Migrationsbewegung von T- und B-Zellen aus dem Blut in den Lymphknoten, sowie aus dem Lymphknoten in die efferente Lymphe. Diese rhythmische Migration hat erheblichen Einfluss auf die adaptive Immunantwort, da Immunisierungsreaktionen auch nach mehreren Wochen eine Tageszeitabhängigkeit in ihrer Stärke aufweisen. Die Ergebnisse dieser Habilitationsschrift liefern somit neue Erkenntnisse in die Mechanismen der systemischen Regulation des Migrationsverhalten sowohl von myeloiden als auch lymphoiden Zellen durch sympathische Nerven und zirkadiane Rhythmen, mit dem Potential für chronotherapeutisch-basierte Interventionen von Entzündungs- und Immunisierungsreaktionen.

Einleitung

Das Konzept der zirkadianen Rhythmik wurde in den späten 1950er Jahren eingeführt, um autonome Rhythmen zu beschreiben, die unter konstanten Bedingungen auftreten - beispielsweise konstanter Dunkelheit oder Temperatur (Halberg, 1959). Sie sind somit nicht direkt von externen Faktoren abhängig, sondern werden von diesen synchronisiert. Zirkadiane Rhythmen zeigen eine Periodenlänge von etwa 24 Stunden (von lateinisch circa diem = etwa einen Tag) und spielen eine entscheidende Rolle bei der optimalen Anpassung von Organismen an ihre Umwelt (Golombek and Rosenstein, 2010). Licht ist

der Hauptsynchronisationsfaktor, auch Zeitgeber genannt, der auf den Organismus wirkt. Licht wird im Auge über lichtempfindliche retinale Ganglienzellen verarbeitet (Lucas et al., 1999). Dies sind nicht-bilderzeugende Zellen, die aber dennoch Photonen detektieren können und diesen Reiz über den retinohypothalamischen Trakt zum suprachiasmatischen Nukleus (SCN) transferieren (**Abb. 1**). Der SCN sitzt über dem *Chiasma opticum* im vorderen Hypothalamus und besteht aus etwa 20.000 hochvernetzten Neuronen. Der SCN ist die zentrale Komponente eines komplexen, schwingungsfähigen Systems, das zirkadiane Rhythmen vermittelt. Er ist somit der grundlegende Zeitmesser der zirkadianen Uhr in Wirbeltieren (Klein et al., 1991).

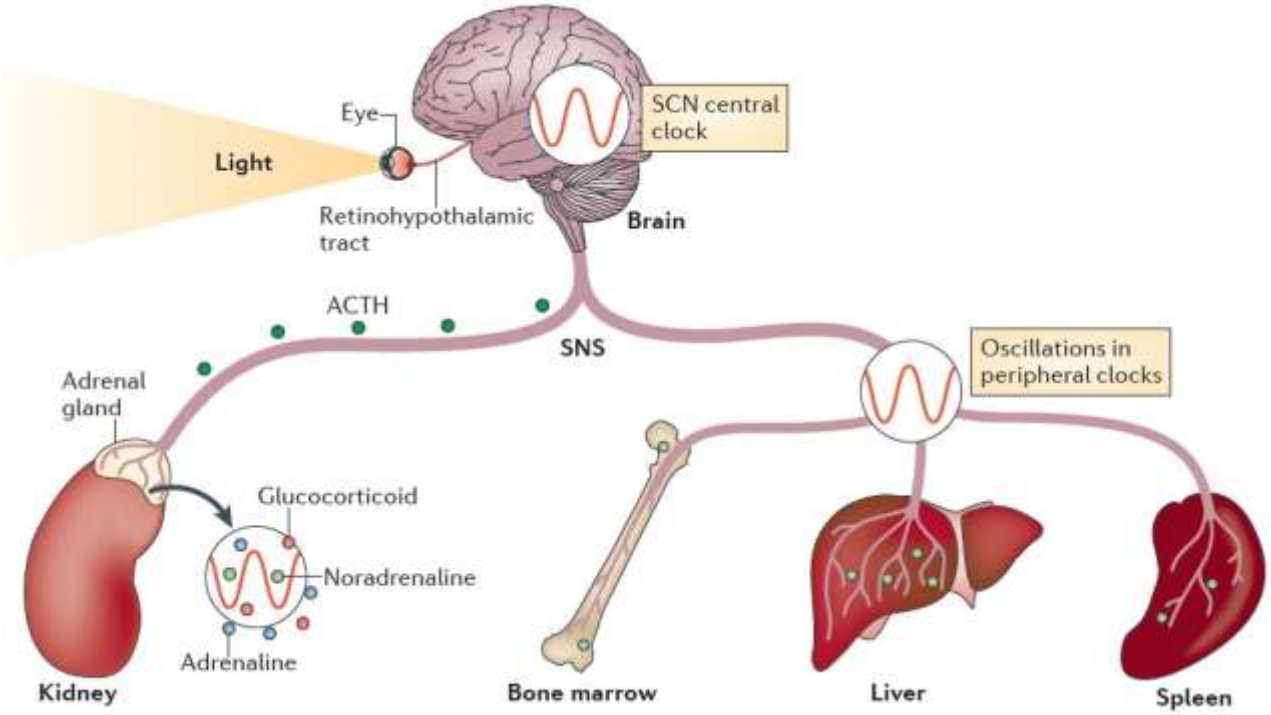

Abb. 1. ***Synchronisation des Körpers durch Licht.***
Licht ist der Hauptsynchronisationsfaktor der Umgebung für den Körper. Durch den retinohypothalamischen Trakt gelangt der Reiz ins Gehirn zum vorderen Hypothalamus, wo er Neuronen des suprachiasmatischen Nukleus synchronisiert. Der SCN agiert als Uhr des Körpers. Von hier werden Gewebe, die Licht nicht direkt wahrnehmen können mit der Umgebung synchronisiert. Das geschieht über humorale Faktoren wie das Adrenocorticotropische Hormon, Glukokortikoide, Adrenalin und Noradrenalin. Signale können aber auch durch die direkte Innervierung eines Gewebes durch das sympathische Nervensystem übertragen werden. Das Resultat ist die Gleichschaltung der Uhren in peripheren Geweben mit der Uhr im SCN und somit eine Synchronisation mit der täglichen ~24h Hell-Dunkel Oszillation der Umgebung. Entnommen aus (Scheiermann et al., 2013).

Die zirkadiane Uhr

Die meisten physiologischen Funktionen in Organismen weisen zirkadiane Rhythmen auf, die durch molekulare Oszillatoren gesteuert werden.

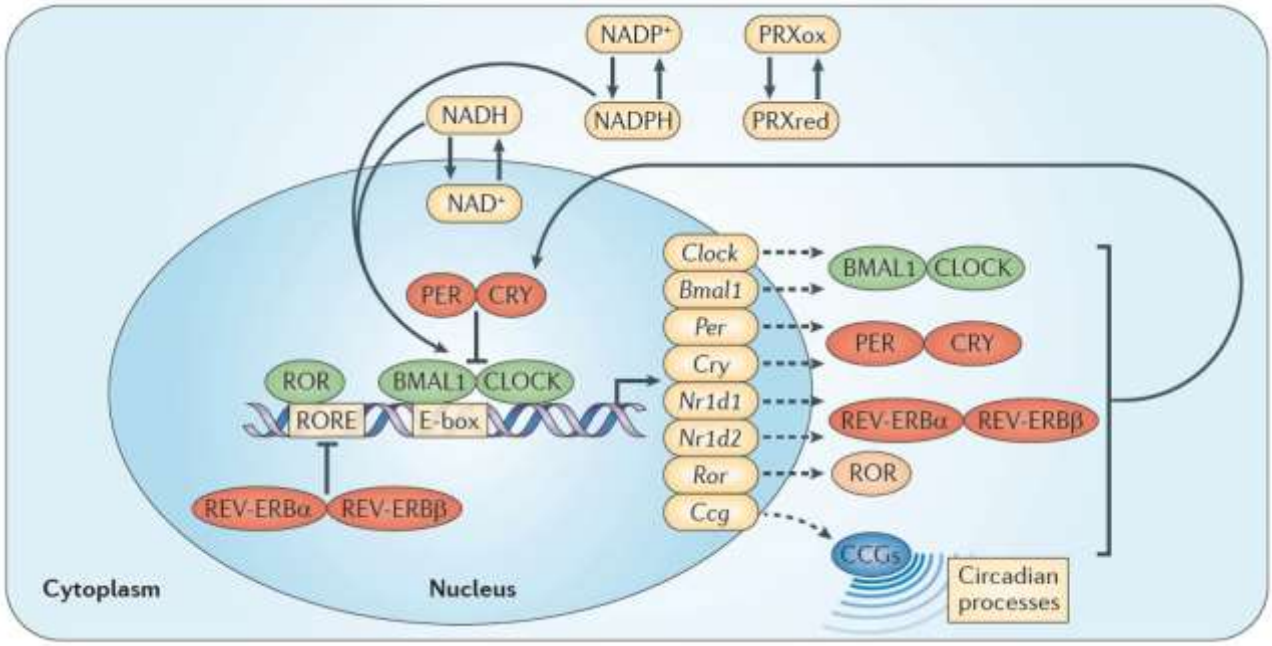

Abb. 2. ***Die molekularen Komponenten der zirkadianen Uhr.***
Transkription von *Bmal1* und *Clock* führt zu Anreicherung der Proteine im Zytoplasma der Zelle. Sobald hier die Konzentration deren Dimerisierung begünstigt, kann das Heterodimer in den Zellkern eindringen. Als Transkriptionsfaktor bindet BMAL1/CLOCK E-Box DNA Elemente im Promoter von anderen zirkadianen Faktoren (wie *Per*, *Cry*, *Ror*, und *Nr1d1* (Rev-Erba) und *Nr1d2* (Rev-Erbb) und induziert deren Transkription. Diese wiederum können dann die Transkription von *Bmal1* und *Clock* sowohl positiv (ROR) als auch negativ (PER, CRY, REV-ERB-α, REV-ERB-β) beeinflussen. Entnommen aus (Scheiermann et al., 2013).

Lichtinput im SCN führt zur Synchronisation in der Expression von positiven (BMAL1, CLOCK, NPAS2) und negativen Transkriptionsfaktoren (CRY1, CRY 2, PER1, PER 2, REV-ERB-α, REV-ERB-β) (Levi and Schibler, 2007) (**Abb. 2**). Diese Transkriptionsfaktoren sind die molekularen Komponenten der zirkadianen Uhr, die sehr robust autoregulatorische Transkriptions-Translations-Rückkopplungsschleifen vermitteln können. Licht induziert die Transkription von *Bmal1* (auch *Arntl* genannt) und *Clock*, die Heterodimerisierung der BMAL1 und CLOCK Proteine und deren Bindung an die Promotoren von negativen Transkriptionsfaktoren, was eine autoregulatorische Rückkopplungsschleife induziert, die von einem neuen Licht-Zyklus zurückgesetzt wird. Zudem binden BMAL1/CLOCK an Promotoren von Uhr-induzierbaren Genen, die selber nicht Teil der Uhr sind, aber zirkadiane Prozesse ausüben. Neben der Oszillation im SCN

des Gehirns, gibt es auch in peripheren Organen Oszillationen, so z. B. in Leukozyten (Boivin et al., 2003; Du et al., 2005; Yamazaki et al., 2000) und Endothelzellen (Maemura et al., 2000), die auch autonom vom SCN oszillieren können (Nagoshi et al., 2004).

Synchronisation von peripheren Geweben

Der Mechanismus und die Routen für die Vermittlung der zirkadianen Rhythmik vom Gehirn in nicht-neurale, periphere Gewebe sind unklar. Vom SCN werden verschiedene Signale ausgesandt, die dann periphere Gewebe mit dem SCN synchron setzen (**Abb. 1**). Humorale Faktoren, wie Glukokortikoide und Melatonin sind seit langem in der Regulation der peripheren Rhythmen bekannt. Glukokortikoide umfassen eine Klasse von Hormonen, die in der Nebennierenrinde produziert werden, und die homöostatische Funktionen regulieren. Die primären Glukokortikoide bei Mensch und Maus sind Cortisol, bzw. auch das Kortikosteron, deren Plasmaspiegel starken Tagesschwankungen unterliegen, mit Höchstwerten zu Beginn der Wachphase (frühe Morgenstunden im Menschen und früher Abend in der nachtaktiven Maus) (Haus et al., 1983). Melatonin wird in der Zirbeldrüse in der Nacht synthetisiert und ist das wichtigste Signal für die Vermittlung von Dunkelheit (Korf, 1994). Deswegen ist der Zeitpunkt der Melatoninausschüttung beim Menschen und der Maus gleich, nämlich während der Nacht. Auch bei anderen humoralen Faktoren, wie dem Zytokinspiegel im Plasma wurde gezeigt, dass er Tagesschwankungen aufweist. So weisen die pro-inflammatorischen Zytokine TNF-α, IL-1β und IFN-γ einen Höchstwert in den frühen Morgenstunden beim Menschen auf, während bei Mäusen dies in der Nacht stattfindet (Kovshik et al., 2007).

Es gibt außerdem Hinweise darauf, dass das sympathische Nervensystem ein wichtiger Faktor in der Vermittlung der zirkadianen Oszillationen in der Peripherie ist. Im Gegensatz zu humoralen Faktoren kann das sympathische Nervensystem sowohl lokal über die Freisetzung von Noradrenalin aus Nervenenden Gewebe innervieren als auch systemisch über Adrenalin und Noradrenalin-Sekretion aus dem Nebennierenmark (**Abb. 1**). Insbesondere lokale noradrenerge Signale, die durch den β3-adrenergen Rezeptor auf Stromazellen des Knochenmarks vermittelt werden, führen zu Herabregulation des Chemokins CXCL12 (Mendez-Ferrer et al., 2008). Dies führt zur rhythmischen Freisetzung von hämatopoietischen Stamm- und Vorläuferzellen aus dem Knochenmark in die Blutzirkulation.

Rhythmen im Immunsystem

Molekulare Interaktionen zwischen zirkadianen Rhythmen und den Bestandteilen des Immunsystems haben sich in den letzten Jahren herauskristallisiert (Arjona et al., 2012; Curtis et al., 2014; Druzd et al., 2014; Haus and Smolensky, 1999; Labrecque and Cermakian, 2015; Lange et al., 2010; Scheiermann et al., 2013). In Bezug auf die Migration von Leukozyten gibt es erste Indizien dafür, dass Leukozyteninteraktionen mit dem vaskulären Endothel vermehrt nachts stattfinden können (House et al., 1997). Zudem ist die Anzahl der Lymphozyten im Thymus zu diesem Zeitpunkt erhöht (Litvinenko et al., 2005). Es wurden auch zirkadiane Schwankungen in der Funktion und Zahl von Immunzellen im Blut beobachtet, der Sensibilität von Lymphozyten auf Mitogene, sowie der Phagozytoseaktivität von Neutrophilen (Haus et al., 1983; Hriscu, 2005). In dieser Habilitationsschrift wurden Oszillationen in der Migrationsbewegung von Leukozyten der angeborenen als auch der adaptiven Immunantwort sowohl unter physiologischen als auch pathologischen Bedingungen untersucht.

Rekrutierung von Leukozyten ins Gewebe - die Leukozytenadhäsionskaskade

Zelladhäsionsmoleküle auf der Oberfläche von vaskulären Endothelzellen stellen eine Schnittstelle zwischen Geweben und Blut dar, wobei sie Interaktionen zwischen Leukozyten und dem Endothel vermitteln. Sie dienen somit als potente Modulatoren für die Einwanderung von Leukozyten. Interaktionen zwischen Leukozyten und Endothel laufen in einem mehrstufigen Prozess ab, der sogenannten Leukozytenadhäsionskaskade, die beschreibt, wie Leukozyten aus dem Gefäßlumen in das darunter liegende Gewebe gelangen. Ursprünglich waren drei Schritte in diesem Prozess beschrieben worden (Butcher, 1991; Springer, 1994), welche dann im Detail weiter definiert wurden (Ley et al., 2007; Wagner and Frenette, 2008). Frei im Blutstrom zirkulierende Leukozyten müssen zuerst ihre Geschwindigkeit verlangsamen, um mit dem Endothel in Kontakt treten zu können. Leukozyten, die aus dem Mittelstrom an die Seite gelangen, können über Adhäsionsmoleküle mit Liganden auf dem Endothelium interagieren, was zu einem Rollen der Zellen entlang des Endothels führt, und zu einer enormen Reduzierung in ihrer Geschwindigkeit. Dies wird durch das sogenannte ‚Tethering' von Leukozyten am Endothel initiiert, gefolgt vom Rollen von Leukozyten entlang der Endothelzellen der Gefäßwand. Rollende Leukozyten können nun weitere Interaktionen eingehen, was zu einer Aktivierung des Leukozyten führt. Dies geschieht über Chemokine, die auf dem

Endothel präsentiert werden und von Chemokinrezeptoren auf dem Leukozyten erkannt werden. Die Signaltransduktion, die durch diese Ligation ausgelöst wird, führt zu einer Konformationsänderung in Integrinen, die aus einer niederaffinen Konformation in eine hochaffine wechseln. Dies führt zur Adhäsion der langsamer rollenden Zelle. Adhärente Zellen sind in der Lage, an Endothelzellen entlang zu kriechen, und können nun zwischen Endothelzellen (parazellulär) oder durch den Endothelzellenkörper (transzellulär) in dem Prozess der transendothelialen Migration (auch Diapedese genannt) in das darunter liegende Gewebe eindringen. Direkt unterhalb des Endothels durchbrechen Leukozyten die Basalmembran als letzte Barriere und wandern daraufhin in das Gewebe ein. Die anfänglichen Interaktionen von Leukozyten mit dem Endothel und das anschließende Rollen entlang der vaskulären Gefäßwand werden fast ausschließlich durch die Mitglieder der Selektin-Familie vermittelt, nämlich P- und E-selektin (von Blutplättchen und / oder Endothelzellen exprimiert), und L-selektin auf Leukozyten (Vestweber and Blanks, 1999). Während Selektine überwiegend das Rollen von Leukozyten vermitteln, wird die Adhäsion von Leukozyten durch Interaktionen zwischen Leukozyten-Integrinen und Molekülen der Immunoglobulin-(Ig)-Superfamilie des Endothels erreicht. Die Hauptmoleküle in diesem Schritt der Leukozytenadhäsionskaskade sind Leukozyten β2-Integrine, nämlich leukocyte function-associated antigen (LFA)-1 und Makrophagenantigen (Mac)-1, sowie das α4-Integrin Very Late Antigen (VLA)-4. Auf dem Endothel sind die Bindungspartner die Ig-Familienmitglieder intercellular cell adhesion molecule (ICAM)-1 und vascular cell adhesion molecule (VCAM)-1 (Luster et al., 2005), aber auch ICAM-2 und MadCAM-1 sind in den vaskulären Betten unterschiedlicher Organe von Bedeutung. Der letzte Schritt der Leukozyten-Transmigration ist der Prozess der endothelialen Diapedese, die vorzugsweise entlang von Endothelzellverbindungen auftritt (parazelluläre Transmigration). Die endothelialen Zelladhäsionsmoleküle, die diesen Schritt vermitteln, sind überwiegend Mitglieder der Ig-Superfamilie, nämlich Platelet endothelial cell adhesion molecule (PECAM-1), junctional adhesion molecule (JAM) -A, -B und -C, und ICAM-2, sowie CD99 (Ley et al., 2007).

Zirkadiane Rhythmik in der Migration von Zellen der angeborenen Immunantwort

Ein Großteil der Literatur hat sich mit der Rekrutierung von Leukozyten in entzündlichen Szenarien befasst, aufgrund der Tatsache, dass hier eine Regulation und eine selektive Intervention von großer klinischer Bedeutung sein können. Im Gegensatz dazu, ist die

Regulation der Migration von Leukozyten ins und aus dem Blut unter homöostatischen Bedingungen nicht ebenso detailliert erforscht. Eine wichtige Studie in diesem Gebiet konnte zeigen, dass hämatopoietische Stamm- und Vorläuferzellen nicht konstant aus dem Knochenmark ins Blut mobilisiert werden, sondern dass dieser Prozess rhythmisch abläuft (Mendez-Ferrer et al., 2008) und abhängig ist von lokaler Innervierung des Knochenmarks durch sympathische Nerven (Katayama et al., 2006).

Das bedeutet, dass Oszillationen in hämatopoietischen Zellen im Blut zum Teil durch eine rhythmische, zirkadiane Mobilisierung von Zellen aus dem Knochenmark zustande kommen. Wie es sich aber mit der Rekrutierung von Zellen in Gewebe verhält, und ob die Adhäsionskaskade auch zirkadianer Rhythmik unterliegt, ist nicht bekannt. Im ersten Teil dieser Habilitationsschrift wird gezeigt, dass die Migration von myeloiden Zellen zu Geweben von der Tageszeit abhängig ist. Diese Studie wurde 2012 in der Fachzeitschrift ‚*Immunity*' publiziert (Scheiermann et al., 2012).

Zunächst isolierten wir Knochenmarkszellen zu einem Tageszeitpunkt, markierten diese mit dem fluoreszenten Farbstoff CFSE und injizierten diese Zellen intravenös in Rezipiententiere, die in unterschiedlichen Lichtrhythmen gehalten wurden. Dies ist mittels Kammern möglich, die eine zum Tierhaus verschobene Lichtrhythmik aufweisen. Eine Stunde nach Injektion konnten wir feststellen, dass die markierten Zellen, die sich im Knochenmark befanden und somit gerade eingedrungen waren, deutlich zahlreicher zu Beginn der aktiven Phase in der Maus auftraten als während des Tages (**Abb. 3A**). Spezifisch kam es zu einem Höchstwert 13h, nachdem das Licht (der Zeitgeber) eingeschaltet wurde (Zeitgeber time (ZT) = 13). In einer rhythmischen 12h hell:12h dunkel Umgebung ist dies gleichbedeutend mit 1h, nachdem das Licht ausgeschaltet wurde. Der Tiefstwert trat zum Zeitpunkt ZT1 auf, also 1h nach Einschalten des Lichts (**Abb. 3A**). Mittels Durchflusszytometrie konnten wir auch die genaue Zusammensetzung der Zellpopulationen feststellen und konnten zeigen, dass alle untersuchten Zellen um ZT13 die höchste Kapazität in der Migration aufwiesen. Dies galt speziell für lineage negative, Sca-1 positive, c-Kit positive (sogenannte LSK Zellen), unter denen sich auch hämatopoietische Stammzellen befinden, sowie für Vorläuferzellen (colony-forming units in culture (CFU-C) und ausdifferenzierte Neutrophile (Gr1+ Mac1+) (**Abb. 3B-D**).

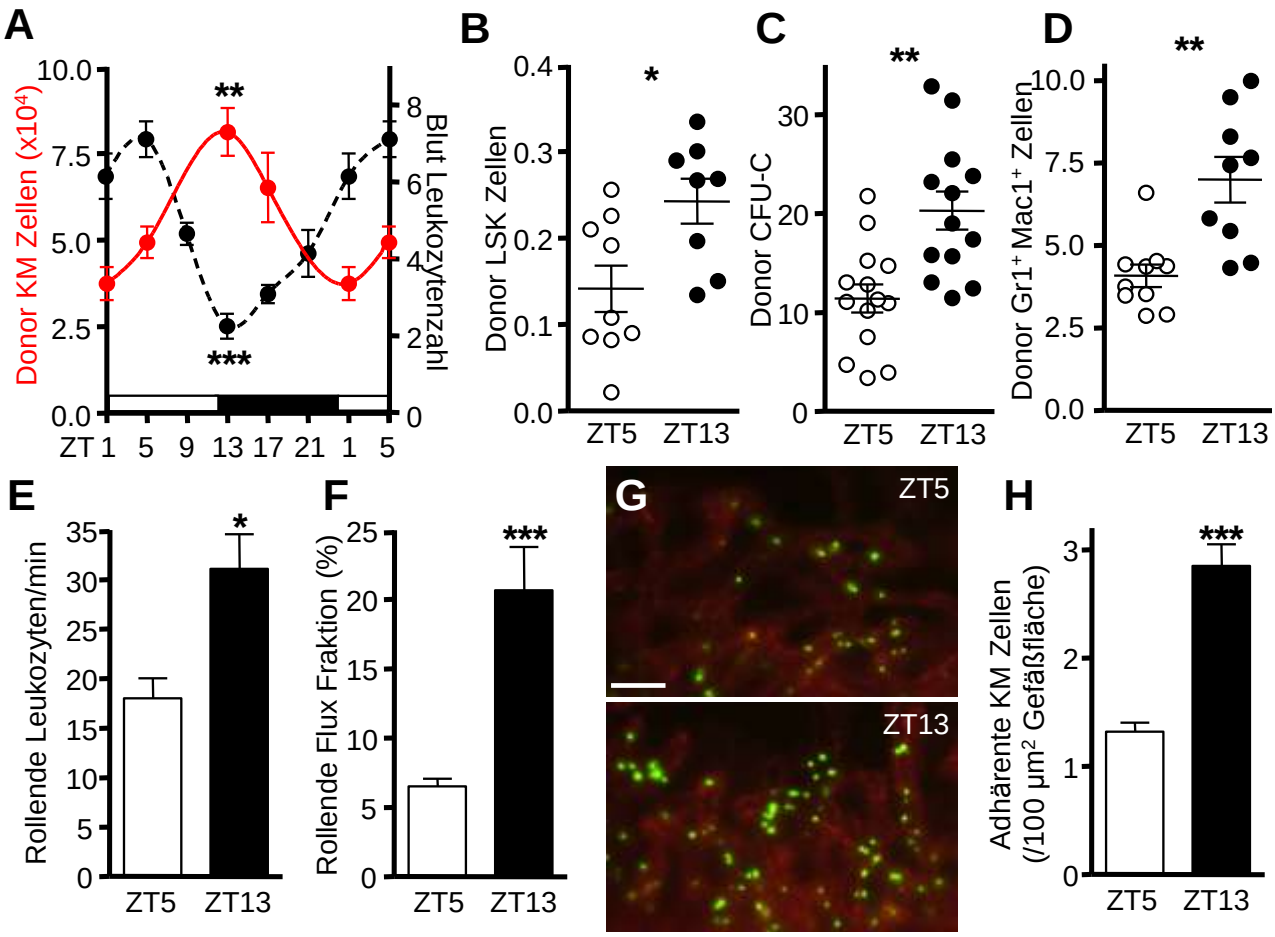

Abb. 3. ***Rhythmische Leukozytenmigration ins Knochenmark*.**
(A) Oszillationen von Blutleukozytenzahlen (schwarze Linie) und Migration von Leukozyten ins Knochenmark (rote Linie). (B-D) LSK Zellen, Vorläuferzellen (CFU-C) und Neutrophile migrieren stärker abends ins Knochenmark. (E-H) Intravitalmikroskopische Analysen und Aufnahmen der Leukozytenmigration ins Knochenmark des Schädeldachs. Entnommen in abgewandelter Form aus (Scheiermann et al., 2012).

Um zu zeigen, welche Schritte in der Adhäsionskaskade speziell betroffen waren (Rollen, Adhäsion, Transmigration) analysierten wir in der Maus die Leukozytenadhäsion im Knochenmark in vivo. Dies ist im Knochenmark des Schädels möglich, das sich direkt unterhalb des Schädeldachs befindet und mittels Fluoreszenz-Intravitalmikroskopie visualisierbar ist. Nach Injektion des Fluoreszenzfarbstoffs Rhodamin 6G konnten Knochenmarkssinusoide, in denen die Rekrutierung hauptsächlich stattfindet, untersucht werden. Wir konnten nun die Anzahl der rollenden und adhärenten Zellen quantifizieren und zeigen, dass beide Prozesse rhythmisch ablaufen, das heißt, mehr rollende Zellen und adhärente Zellen wurden zum ZT13 Zeitpunkt observiert (**Abb. 3E-H**). Das ist besonders dadurch interessant, dass zu diesem Zeitpunkt deutlich weniger Zellen im Blut vorhanden sind, was die Fraktion, der rollenden Zellen dramatisch erhöht (**Abb. 3F**). Das

bedeutet, dass eine größere Anzahl an Zellen im Blut nicht gleichbedeutend ist mit einer erhöhten Migration dieser Zellen ins Gewebe. Diese Daten zeigen, dass ein rhythmisches Emigrieren von Leukozyten aus dem Blut existiert.

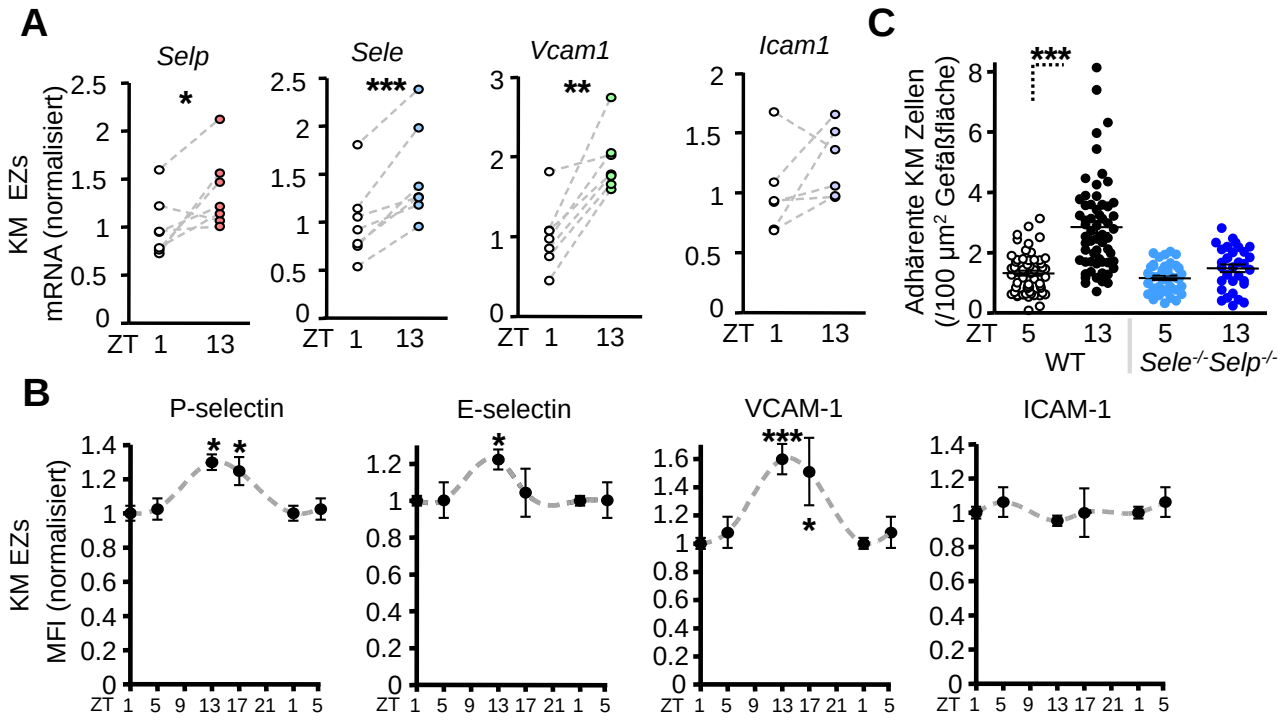

Abb. 4. ***Rhythmische Expression von Adhäsionsmolekülen auf Endothelzellen des Knochenmarks.***
Knochenmarksendothelzellen exprimieren mehr P-Selektin, E-Selektin und VCAM-1 mRNA (A) und Protein (B) zu Beginn der Nacht. (C) P- und E-selektin doppeldefiziente Mäuse zeigen keine Oszillation in der Adhäsion von Leukozyten. Entnommen in abgewandelter Form aus (Scheiermann et al., 2012).

Um zu untersuchen, ob E- und P-selektin die Rhythmik vermittelten, untersuchten wir die Expression dieser Moleküle in sortierten Endothelzellen des Knochenmarks mittels quantitativer PCR (Q-PCR) und Durchflusszytometrie. Sowohl Q-PCR (**Abb. 4A**) als auch FACS Analyse (**Abb. 4B**) zeigten, dass E- und P-selektin und auch VCAM-1, die sogenannten Homing-Rezeptoren im Knochenmark (Mazo et al., 1998), aber nicht ICAM-1, über den Tagesverlauf oszillierten. Dies war von Relevanz, denn in P-und E-selektin defizienten (*Sele*$^{-/-}$*Selp*$^{-/-}$) Mäusen, konnten keine Oszillationen in der Leukozytenrekrutierung detektiert werden (**Abb. 4C**). Dies weist darauf hin, dass eine lokale Oszillation dieser Moleküle auf dem Endothel für die rhythmische Rekrutierung von Leukozyten im Knochenmark verantwortlich ist.

Wie synchronisieren Umgebungsreize die zirkadiane Oszillation im Knochenmark? Eine Möglichkeit ist der humorale Weg über Adrenocorticotropes Hormon (ACTH) oder das sympathische Nervensystem zur Nebenniere, wo es dann zur zirkadianen Ausschüttung von Glukokortikoiden, Adrenalin und Noradrenalin kommt (**Abb. 1**). Ein anderer Weg ist die direkte Synchronisation durch die lokale Innervierung über das SNS. Um den Einfluss letzterer Möglichkeit zu prüfen, denervierten wir mittels eines chirurgischen Eingriffs das Ganglion cervicale superius. Dieser sympathische Nervplexus ist für die unilaterale Innervierung einer Seite des Kopfes zuständig und sitzt hinter der Bifurkation der Arteria carotis. Mäusen wurde einseitig das Ganglion cervicale superius entfernt, wobei auf der Gegenseite ein Schein-Eingriff durchgeführt wurde. Auf der ipsilateralen, denervierten Seite zeigten Tiere ein Horner-Syndrom, ein leichtes Hängen des Augenliedes, was auf einen stark verminderten adrenergen Tonus hinweist (Scheiermann et al., 2012; Walton and Buono, 2003).

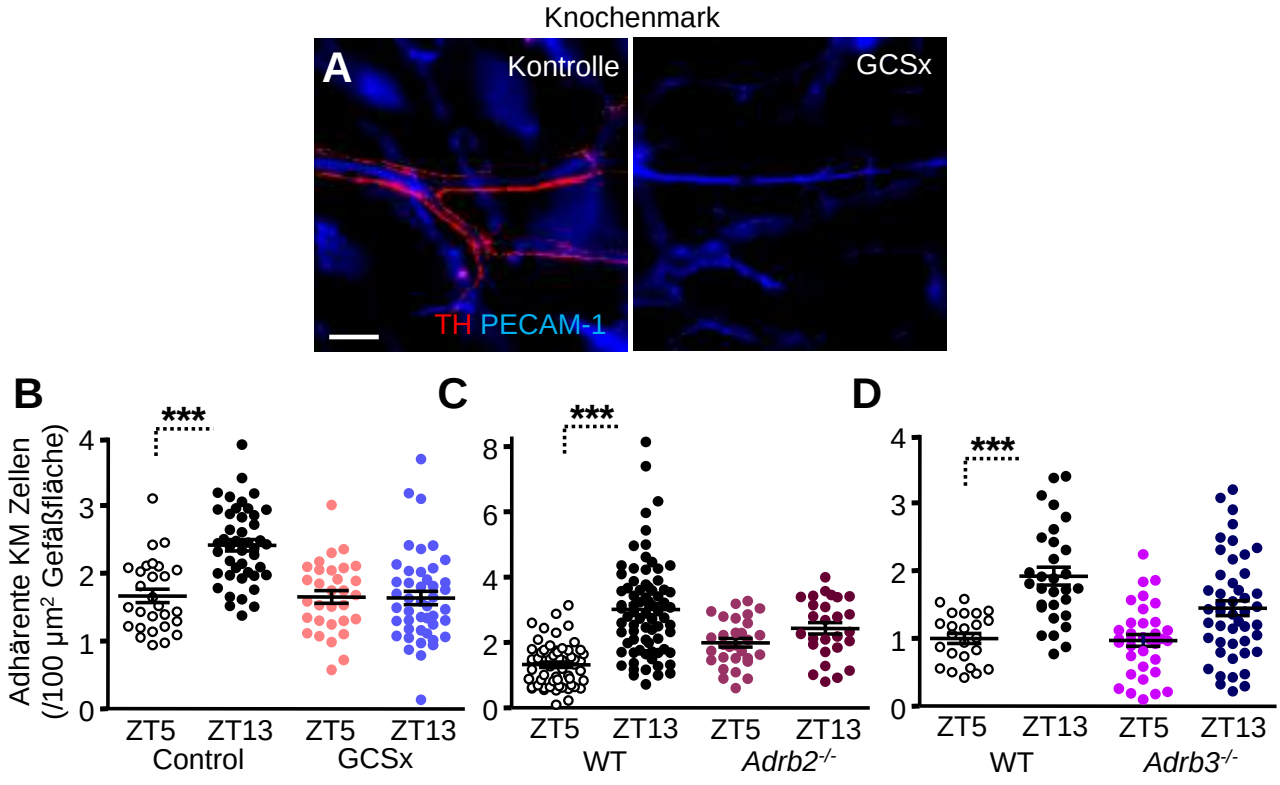

Abb. 5. ***Rhythmik ist abhängig von lokaler sympathischer Innervierung.***
(A) Chirurgische Denervierung des Ganglion cervicale superius (GCSx) führt zur sympathischen Denervierung des ipsilateralen Knochenmarks des Schädeldachs. (B-D) GCSx sowie Mäuse, die defizient in β2- oder β3 adrenergen Rezeptoren sind, weisen keine Oszillation in der Adhäsion von Leukozyten im Knochenmark auf. Entnommen in abgewandelter Form aus (Scheiermann et al., 2012).

In der Tat zeigten Immunofluoreszenzanalysen von Tyrosinhydroxylase-positiven Nerven eine verminderte sympathische Innervierung des Knochenmarks (**Abb. 5A**). Mäuse wurden nun intravenös mit fluoreszenzmarkierten Knochenmarkszellen injiziert, und eine Stunde später wurde mittels Intravital-Mikroskopie das Knochenmark des Schädeldachs untersucht. Während auf der kontralateralen, nerv-intakten Seite eine rhythmische Rekrutierung von Zellen mit hohen Werten abends zu sehen war, gab es in der denervierten Seite keinerlei Oszillationen (**Abb. 5B**). Zudem wiesen auch Mäuse, die defizient in β2 oder β3-adrenergen Rezeptoren waren, keine Oszillationen in der Rekrutierung auf (**Abb. 5C-D**). Diese Daten weisen darauf hin, dass eine direkte, lokale Innervierung durch das sympathische Nervensystem über β2- und β3-adrenerge Rezeptoren für die rhythmische Immigration von Leukozyten ins Knochenmark von essentieller Bedeutung ist.

Um die Relevanz dieser Oszillationen in einem Krankheitsmodell zu testen, induzierten wir in Mäusen einen starken systemischen inflammatorischen Reiz durch Gabe von Lipopolysacchariden (LPS) und untersuchten betroffene Gewebe. Interessanterweise wiesen Leberendothelzellen sowohl unter homöostatischen als auch inflammatorischen Bedingungen eine starke zirkadiane Rhythmik in der Expression von ICAM-1 und VCAM-1 auf (**Abb. 6A-B**). Auch eine zirkadiane Infiltration von Leukozyten ins Leber-Gewebe war unter homöostatischen und inflammatorischen Bedingungen zu sehen, mit vermehrter Infiltration abends (**Abb. 6C-D**). *Icam1*-defiziente Tiere wiesen hingegen diese Oszillationen nicht mehr auf (**Abb. 6D**). LPS führt bei hoher Dosis zum Tod. Interessanterweise ist diese Letalität rhythmisch: Gabe von LPS zu Beginn der Nachtphase wies die höchste Letalität auf, im Gegensatz zu Tagesbeginn, also zu der Zeit, wo auch die ICAM-1 Expression am höchsten war (**Abb. 6E**). Des Weiteren waren *Icam1*-defiziente Tiere vor dem Tod durch LPS geschützt. Nur bei äußerst hohen Dosen, die bei allen WT Tieren zum Tod führten (nicht gezeigt), starben auch *Icam1*$^{-/-}$ Mäuse, allerdings ohne hierbei eine zirkadiane Rhythmik zu zeigen (**Abb. 6F**). ICAM-1 ist demnach sowohl für die LPS-vermittelte Letalität von entscheidender Bedeutung als auch für deren Rhythmik.

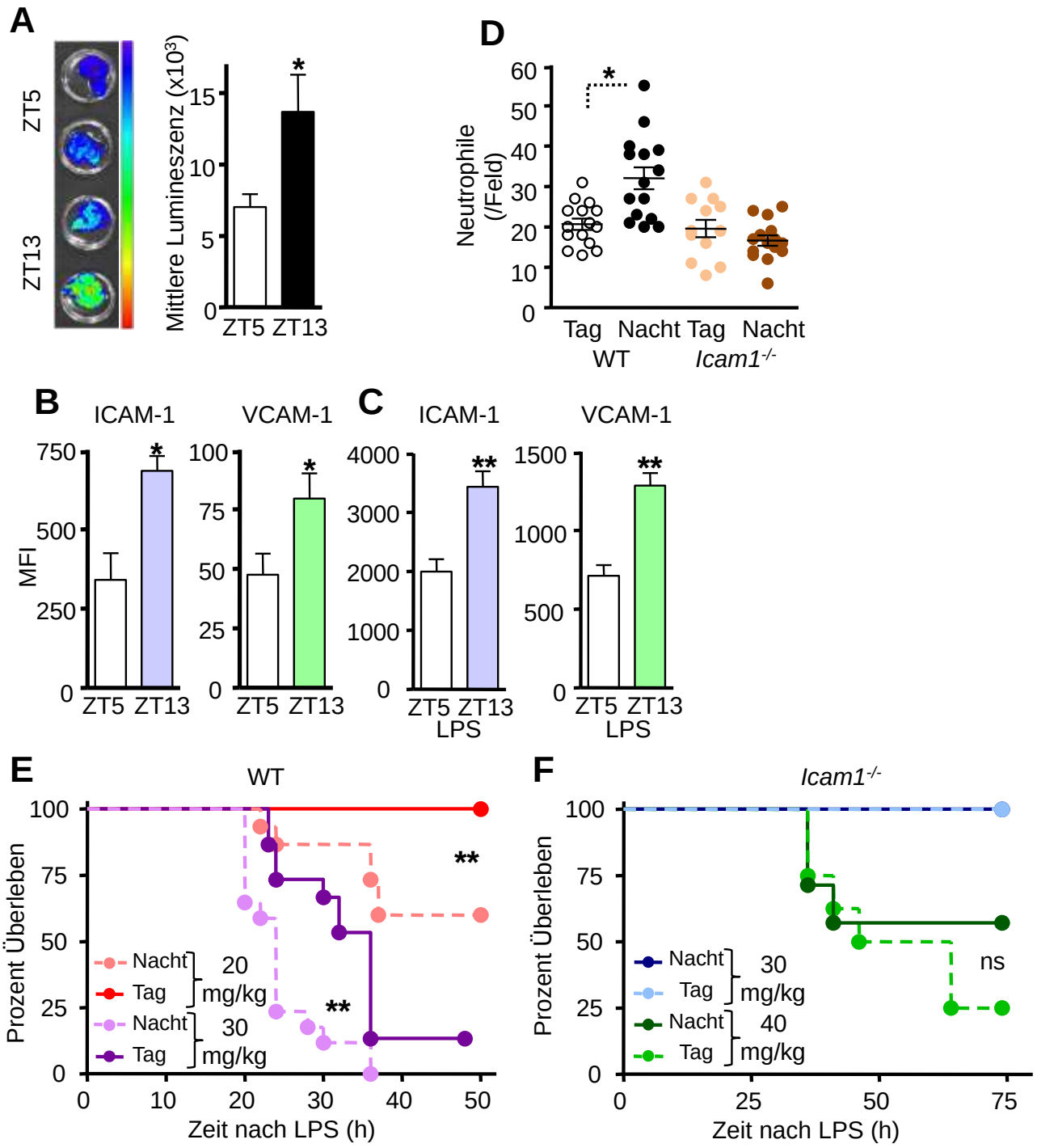

Abb. 6. ***Die Reaktion auf LPS ist stark tageszeitabhängig*.**
(A) Bioluminezent-markierte Leukozyten migrieren stärker abends in die Leber. (B) Leberendothelzellen weisen Abends höhere Mengen an ICAM-1 und VCAM-1 auf, sowohl unter homöostatischen (B) als auch LPS-inflammatorischen (C) Bedingungen. (E-F) Wildtyp Mäuse weisen starke Oszillationen auf in der Letalität von LPS auf (E); ICAM-1-defiziente Tiere tun dies nicht (F). Entnommen in abgewandelter Form aus (Scheiermann et al., 2012).

Zirkadiane Rhythmik in der Migration von Zellen der adaptiven Immunantwort

Lymphozyten zirkulieren durch Lymphknoten auf der Suche nach Antigen, was als ein kontinuierlicher Prozess angesehen wird. Im zweiten Teil dieser Habilitationsschrift wird gezeigt, dass die Lymphozytenmigration durch Lymphknoten und Lymphe allerdings rhythmisch abläuft. Diese Studie wurde 2017 in der Fachzeitschrift ‚*Immunity*' publiziert (Druzd et al., 2017).

Um in den Lymphknoten zu gelangen, gehen Lymphozyten umfangreiche Wechselwirkungen mit hochendothelialen Venolen (HEV) ein (Butcher, 1991; Forster et al., 2008; Ley et al., 2007; Muller, 2011; Springer, 1994; Vestweber and Blanks, 1999; von Andrian and Mempel, 2003; Wagner and Frenette, 2008). Lymphozyten binden zunächst an das Molekül Peripheral Nodal addressin (PNAd) auf HEVs mittels L-selektin. Lymphozyten rollen entlang der HEVs und werden über Wechselwirkungen der Chemokinrezeptoren CCR7 und CXCR4 mit ihren jeweiligen Liganden CCL21 und CXCL12 aktiviert. Aktivierte Leukozyten verwenden das Integrin LFA-1, um an ICAM-1 zu binden, und wandern schließlich in das Lymphknoten Parenchym aus. Im Lymphknoten interagieren Lymphozyten mit dendritischen Zellen, um Antigen zu scannen (Gasteiger et al., 2016). Danach wandern sie über efferente Lymphgefäße ab. Für den Prozess der Emigration ist die Expression des Sphingosin-1-phosphat-Rezeptor-1 (S1P1, kodiert durch *S1pr1*) auf Lymphozyten kritisch, der das Phospholipid Sphingosin-1-phosphat (S1P) erkennt (Matloubian et al., 2004). S1P ist in höherer Konzentration in Blut und Lymphe vorhanden, und es ist niedrig im Gewebe, was zu einem Konzentrationsgradienten führt (Cyster and Schwab, 2012). Die Antagonisierung der Funktion von S1P1 mit dem Medikament FTY720 (fingolimod) wird zur Behandlung von Patienten mit multipler Sklerose benutzt, um autoreaktive T-Zellen am Verlassen des Lymphknoten und der Infiltration ins zentrale Nervensystem zu hindern (Massberg and von Andrian, 2006). Es war unklar, inwieweit Rhythmen in der adaptiven Immunantwort und der Regulation der Lymphknotenzellularität eine Rolle spielen (Arjona and Sarkar, 2005; Esquifino et al., 1996; Fortier et al., 2011; Hemmers and Rudensky, 2015). Zellen des adaptiven Immunsystems wie T- und B-Zellen sowie dendritische Zellen, besitzen die molekularen Komponenten der zirkadianen Uhr (Bollinger et al., 2011; Hemmers and Rudensky, 2015; Silver et al., 2012). Im Gegensatz zu Monozyten des angeborenen Immunsystems

(Nguyen et al., 2013) ist aber die funktionelle Relevanz dieser zellintrinsische Oszillationen für Lymphozyten unklar (Hemmers and Rudensky, 2015; Yu et al., 2013).

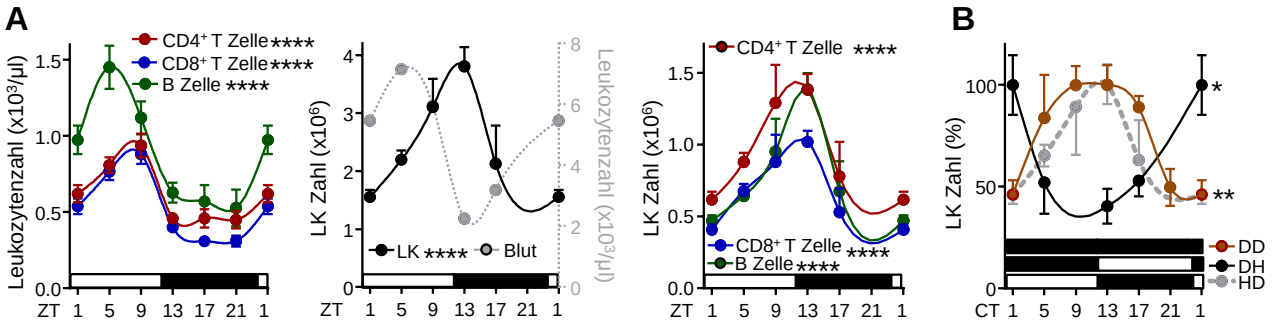

Abb. 7. *Zirkadiane Oszillationen von Lymphozytenzahlen im Lymphknoten*.
(A) CD4 und CD8 T Zellen sowie B Zellen weisen diurnale Rhythmen in der Menge ihrer Zellen im Blut (links) und Lymphknoten (LK) auf (Mitte und rechts). (B) Im Hell-Dunkel Rhythmus (grau, HD) weist die Anzahl an Zellen im Lymphknoten einen Höchstwert zu Beginn der Dunkelphase auf. Dieser Rhythmus lässt sich durch Änderung des Lichtzyklusses invertieren (Dunkel-Hell (DH), schwarz) und ist aber immer noch präsent in konstanter Dunkelheit (rot, Dunkel-Dunkel (DD)), Zeichen eines tatsächlichen zirkadianen Rhythmus. Entnommen in abgewandelter Form aus (Druzd et al., 2017).

In unseren initialen Untersuchungen konnten wir zeigen, dass Lymphozyten in Blut und Lymphknoten starke Oszillationen aufweisen, wobei diese um etwa 8h verschoben sind: Während im Blut zwischen ZT5 und ZT9 ein Höchstwert erreicht wurde, war die höchste Zahl im Lymphknoten gegen ZT13 zu sehen (**Abb. 7A**). Oszillationen waren immer noch präsent, wenn Tiere in konstanter Dunkelheit gehalten wurden (**Abb. 7B**). Zudem glichen sie sich nach einigen Tagen einer veränderten Umgebungsrhythmik an, wenn der Lichtrhythmus um 12h verändert wurde. Beides sind wichtige Kennzeichen für die Existenz eines tatsächlichen zirkadianen Rhythmus.

Um zu bestimmen, ob Lymphozyten rhythmisch in Lymphknoten gelangen, verwendeten wir adoptive Transfertechniken mit fluoreszenz-markierten Lymphozyten. Die Infiltration von Lymphozyten-Subpopulationen erreichte zu Beginn der Nacht (ZT13) einen Höhepunkt und blieb während des Tages niedrig (**Abb. 8A**). Um festzulegen, ob diese Oszillationen von Lymphozyten-intrinsischen und / oder Signalen der Umgebung bestimmt wurden, transferierten wir Zellen bei ZT5 (‚Tag') oder ZT13 (‚Nacht') in Empfängertiere, entweder zu ZT5 oder ZT13. Während ‚Tag' (Zellen) in ‚Tag' (Empfänger) Transfers die niedrigste Rekrutierungskapazität besaßen und ‚Nacht' in ‚Nacht' die höchste, wurden bei den Chimären gemischte Beiträge erkennbar (**Abb. 8B**). Expression

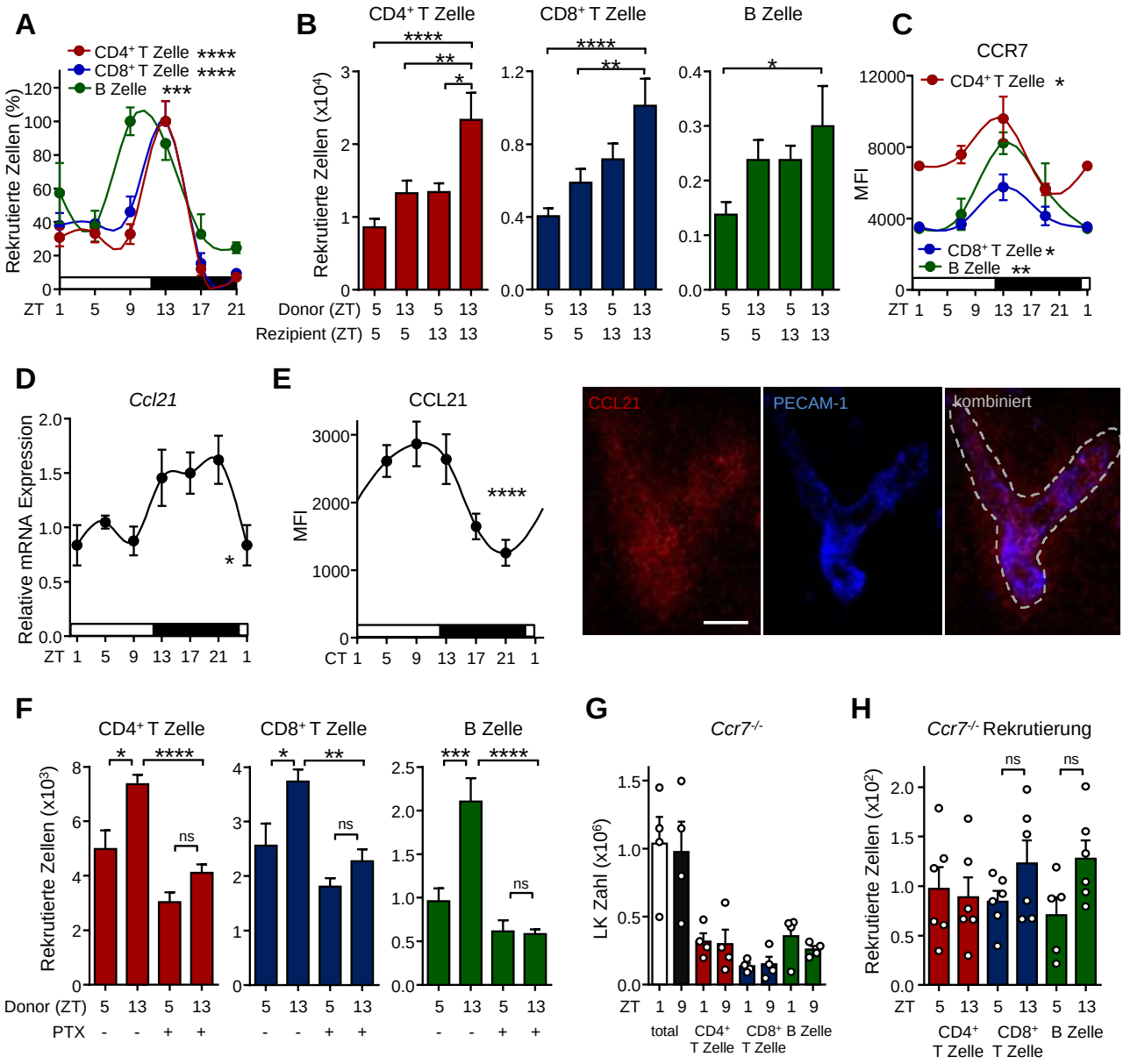

Abb. 8. ***Rhythmik in der Rekrutierung von Lymphozyten in Lymphknoten*****.**
(A) Rekrutierung von Lymphozyten in Lymphknoten über 24h. (B) Rekrutierung von Lymphozyten in Lymphknoten an zwei Zeitpunkten, wobei die Zeitpunkte von Donor- und Rezipiententieren permutiert wurden. (C) Expression von CCR7 auf Lymphozyten über 24h. (D) Q-PCR Analyse des *Ccl21* Gens in Lymphknoten. (E) Proteinexpressionsanalyse von CCL21 in HEV. (F) Rekrutierungsexperimente von Lymphozyten in Lymphknoten, nach Vorbehandlung der injizierten Zellen mit Pertussis Toxin (PTX). (G) Anzahl von Zellen in Lymphknoten von *Ccr7*-defizienten Tieren. (H) Rekrutierungsexperimente von *Ccr7*-defizienten Lymphozyten in Lymphknoten.

des Chemokinrezeptors CCR7 zeigte eine deutliche Rhythmik mit einem Höchstwert bei ZT13 (**Abb. 8C**). Zusätzlich zeigten HEVs Oszillationen in der Expression des Chemokins CCL21, einem Liganden für CCR7, mit einem höheren Niveau zu Beginn der Nacht (**Abb.**

8D-E). Oszillationen in Chemokin-Rezeptoren waren kritisch für die rhythmische Rekrutierung, da eine titrierte, kurze Vorbehandlung von adoptiv transferierten Zellen mit Pertussis Toxin (PTX) (Lo et al., 2005), einem Inhibitor der Chemokin-Rezeptor-Funktion, die Rhythmik vollständig aufhob (**Abb. 8F**). *Ccr7*-defiziente Tiere zeigten keine tageszeitbedingten Schwankungen in der Zahl von Leukozyten im Lymphknoten (**Abb. 8G**). Zudem wiesen Ccr7-defiziente Lymphozyten auch keine Rhythmik in der Infiltration des Lymphknotens auf (**Abb. 8H**). Dies zeigte, dass die Lymphozyten-Rekrutierung in Lymphknoten von Rhythmen in Leukozyten und der Mikroumgebung bestimmt wird, zusammen mit der Expression der CCR7-CCL21 Rezeptor-Liganden-Achse.

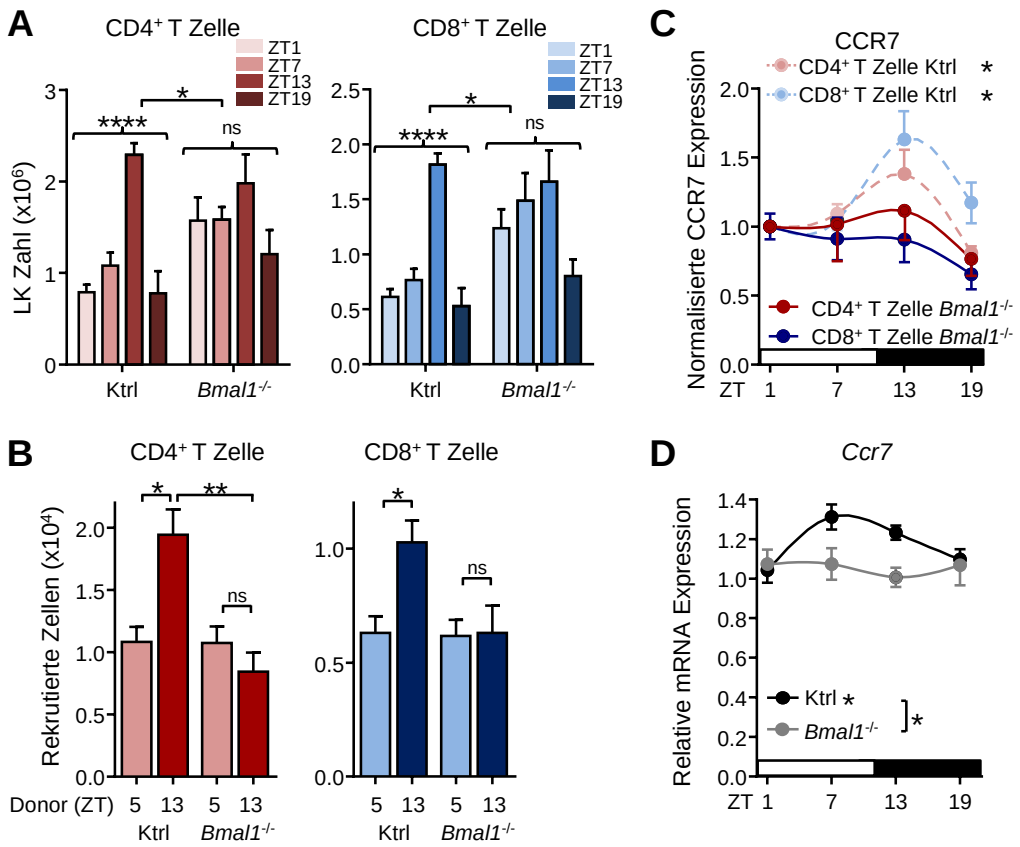

Abb. 9. ***T-Zell-intrinsische Oszillationen regulieren die Migration von Lymphozyten.*** (A) CD4 und CD8 T-Zellzahlen in Lymphknoten von Kontrolltieren (Ktrl) und T-zellspezifischen *Bmal1*-defizienten Tieren über 24h. (B) Rekrutierung von Ktrl oder *Bmal1*-defizienten T Zellen in Wildtyp-Rezipienten an ZT5 und ZT13. Durchflusszytometrie (C) und Q-PCR (D) von T Zellen für den Chemokinrezeptor CCR7. Entnommen in abgewandelter Form aus (Druzd et al., 2017).

Wir generierten als nächstes Mäuse, in denen das zirkadiane Gen *Bmal1* (**Abb. 2**) in T-Zellen ausgeschaltet wurde (T-Zelle *Bmal1*$^{-/-}$). Bemerkenswerterweise resultierte der

Verlust von Lymphozyten-BMAL1 in der Aufhebung des Rhythmus in T-Zellzahlen im Lymphknoten (**Abb. 9A-B**). Darüber hinaus fehlte die rhythmische Expression von CCR7 bei *Bmal1*-defizienten CD4-T-Zellen, was wir durch Q-PCR und Durchflusszytometrie zeigen konnten (**Abb. 9C-D**). Dies ist ein Indiz für die Regulierung des Moleküls auf Transkriptionsebene durch die innere Uhr. Zusammengenommen bieten diese Daten Hinweise für eine Rolle von zellautonomen Uhren in der Lymphozytenmigration.

Da unsere Daten darauf schließen ließen, dass, zusätzlich zu einer rhythmischen Rekrutierungs-Komponente, ein rhythmischer Efflux von Lymphozyten aus dem Lymphknoten Oszillationen in der Anzahl von Lymphozyten im Lymphknoten verursachen könnte, quantifizierten wir Lymphozytenzahlen in Lymphflüssigkeit. Zu diesem Zweck etablierten wir eine neue Methode der Kanülierung von mesenterischen efferenten Lymphgefäßen. Interessanterweise observierten wir prominente Rhythmen in Zellzahlen in der Lymphe, mit einem Höchstwert bei ZT9 und einem Tiefstwert bei ZT21 (**Abb. 10A**). Diese Rhythmen waren – wie schon zuvor im Lymphknoten beobachtet (**Abb. 7B**) – auch in konstanter Dunkelheit zu sehen, ein wichtiges Zeichen eines tatsächlichen zirkadianen Rhythmus (**Abb. 10B**). Um zu überprüfen, ob diese Oszillationen in der Lymphzellularität wirklich auf rhythmischen Efflux zurückzuführen waren und nicht Folge einer rhythmischen Rekrutierung, transferierten wir Lymphozyten zu verschiedenen Zeiten des Tages, blockierten die Rekrutierung nachfolgender Zellen in den Lymphknoten und quantifizierten ihre Migration durch Lymphknoten und Lymphe (Mandl et al., 2012). Ein höheres Lymphknoten Rückhaltevermögen wurde erkennbar für Zellen, die zum Zeitpunkt ZT13 injiziert wurden im Vergleich zu ZT5 (**Abb. 10C-D**). Die T-Zell-spezifische *Bmal1*-Deletion hob auch die Oszillationen in der Lymphe auf, was auf eine Bedeutung für zellautonome Uhren auch im Efflux von Lymphozyten hinwies. Bemerkenswerterweise zeigten auch adoptiv transferierte *Bmal1*-defiziente CD4 T-Zellen keine Tageszeit-abhängige Auswirkungen in ihren Lymphknoten Halbwertszeiten (**Abb. 10E-F**). Dies bewies die Bedeutung von T-Zell-Uhren für das rhythmische Verhalten dieser Zellen. Lymphozyten-Uhren und der tageszeitabhängige Eintritt der Zellen in Lymphknoten hat demnach funktionelle Konsequenzen für den Lymphknoten Transit und den Austritt in die Lymphe.

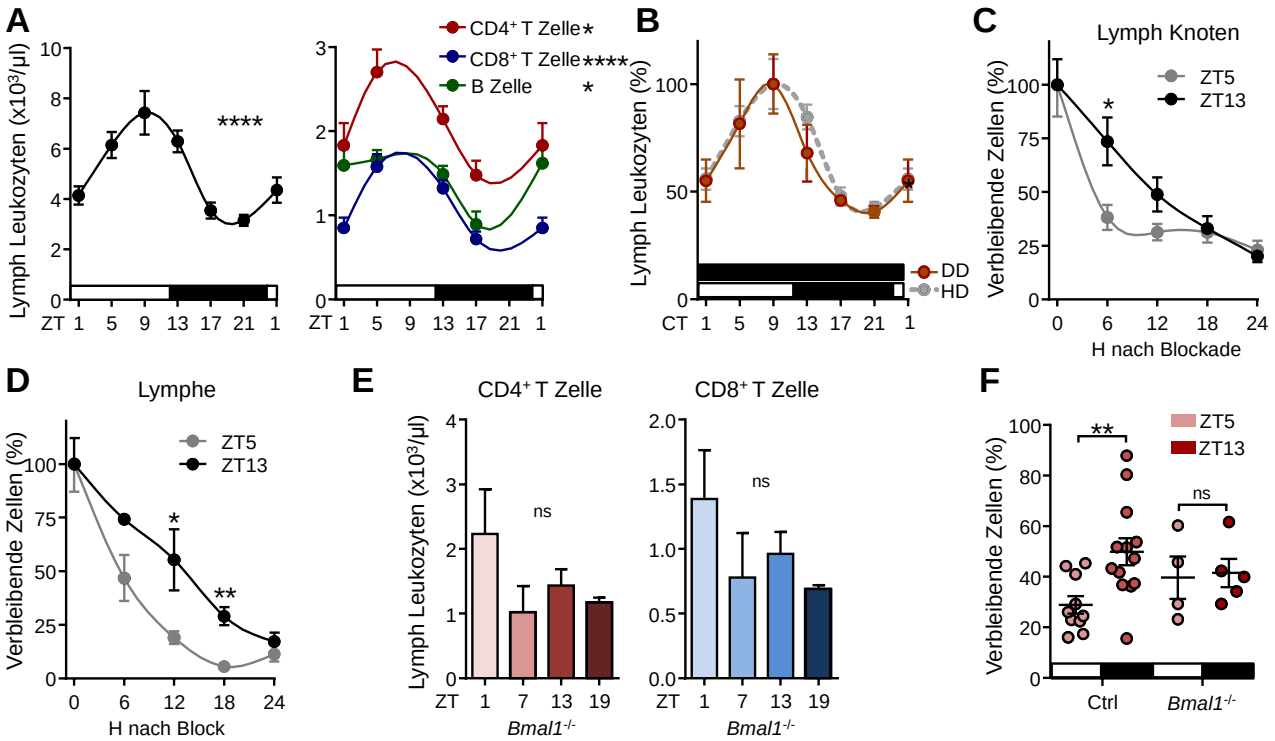

Abb. 10. *Zirkadiane Oszillationen von Lymphozytenzahlen in der Lymphe*.
(A) Anzahl von Zellen in Lymphe über 24h. (B) Anzahl von Zellen in Lymphe über 24h in Hell-Dunkel (HD) und Dunkel-Dunkel (DD) Bedingungen. (C) Prozentsatz verbleibender Zellen in Lymphknoten zu zwei Tageszeitpunkten. (D) Prozentsatz verbleibender Zellen in Lymphe zu zwei Tageszeitpunkten. (E) Anzahl von CD4- und CD8- *Bmal1*-defizienten T Zellen in Lymphknoten über 24h. (F) Prozentsatz verbleibender CD4 Kontroll- und *Bmal1*-defizienten T Zellen in Lymphknoten zu zwei Tageszeitpunkten.

Wir untersuchten nun, ob die Expression des S1P1-Rezeptors tageszeitabhängigen Schwingungen unterliegt. In der Tat zeigte *S1pr1* robuste Oszillationen mit einem Höchstwert bei ZT5, der mit hohen Lymphozytenzahlen in der Lymphe einherging (**Abb. 11A**). Interessanterweise wiesen *Bmal1*-defiziente T-Zellen diese Oszillation nicht auf, was für eine Kontrolle des Gens durch die zirkadiane Uhr spricht (**Abb. 11B**). Um die Rolle von S1P1 in dem zeitabhängigen Efflux genetisch zu bestätigen, generierten wir T-Zell-spezifische Mäuse, die heterozygot für *S1pr1* waren, um nicht vollständig den Efflux von Lymphozyten zu blockieren (Matloubian et al., 2004), sondern zu titrieren, da Verlust eines Allels von *S1pr1* zu Haploinsuffizienz führt (Lo et al., 2005). In *S1pr1* heterozygoten Mäusen wurden keine Oszillationen in Lymphknoten bzw. veränderte Oszillationen in der Lymphe. beobachtet (**Abb. 11C-D**). Zudem wurden keine Oszillationen im S1P Spiegel in efferenter Lymphe beobachtet (**Abb. 11E**), so dass Rhythmen im Rezeptor

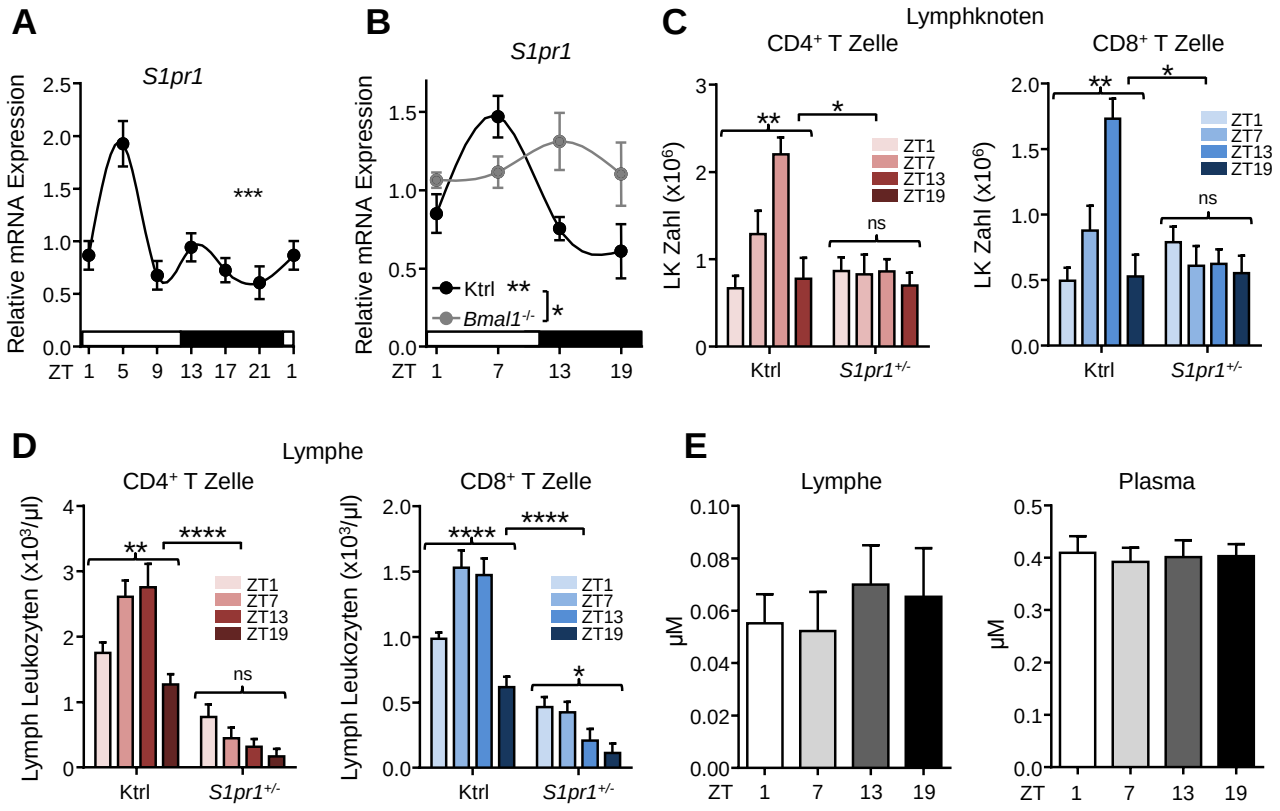

Abb. 11. *Oszillation in S1pr1 reguliert die rhythmische Emigration von Lymphozyten*.
(A) Expression von *S1pr1* in Lymphknoten über 24h. (B) Expression von *S1pr1* in CD4 Ktrl und *Bmal1*-defizienten T Zellen über 24h. Anzahl an T Zellen über 24h in (C) Lymphknoten und (D) Lymphe. (E) Massenspektrometrische Analyse von S1P in Lymphe und Serum. Entnommen in abgewandelter Form aus (Druzd et al., 2017).

(*S1pr1*) und nicht im Liganden (S1P) der Hauptantrieb für die rhythmische Lymphozyten Emigration zu sein scheint. Zusammen zeigen diese Daten eine wichtige Rolle für S1P1 in der zirkadianen Emigration von Lymphozyten aus Lymphknoten in efferente Lymphe.

Wir stellten nun die Hypothese auf, dass oszillierende Lymphozytenzahlen im Lymphknoten funktionelle Konsequenzen für adaptive Immunantworten bedeuten könnten. Tatsächlich variierte der Aktivierungsstatus von Lymphozyten im Verlauf des Tages, was über eine erhöhte CD69-Expression festgestellt werden konnte (**Abb. 12A**). Da dendritische Zellen kritisch für die Aktivierung von Lymphozyten und die Erzeugung adaptiver Immunreaktionen sind, untersuchten wir als nächstes, ob auch die Zahl dieser Zellen oszillatorischen Schwankungen unterläge. In der Tat zeigte die Zellularität von migratorischen dendritischen Zellen eine ausgeprägte Rhythmik, die in Phase mit Lymphozyten auftrat (**Abb. 12B**).

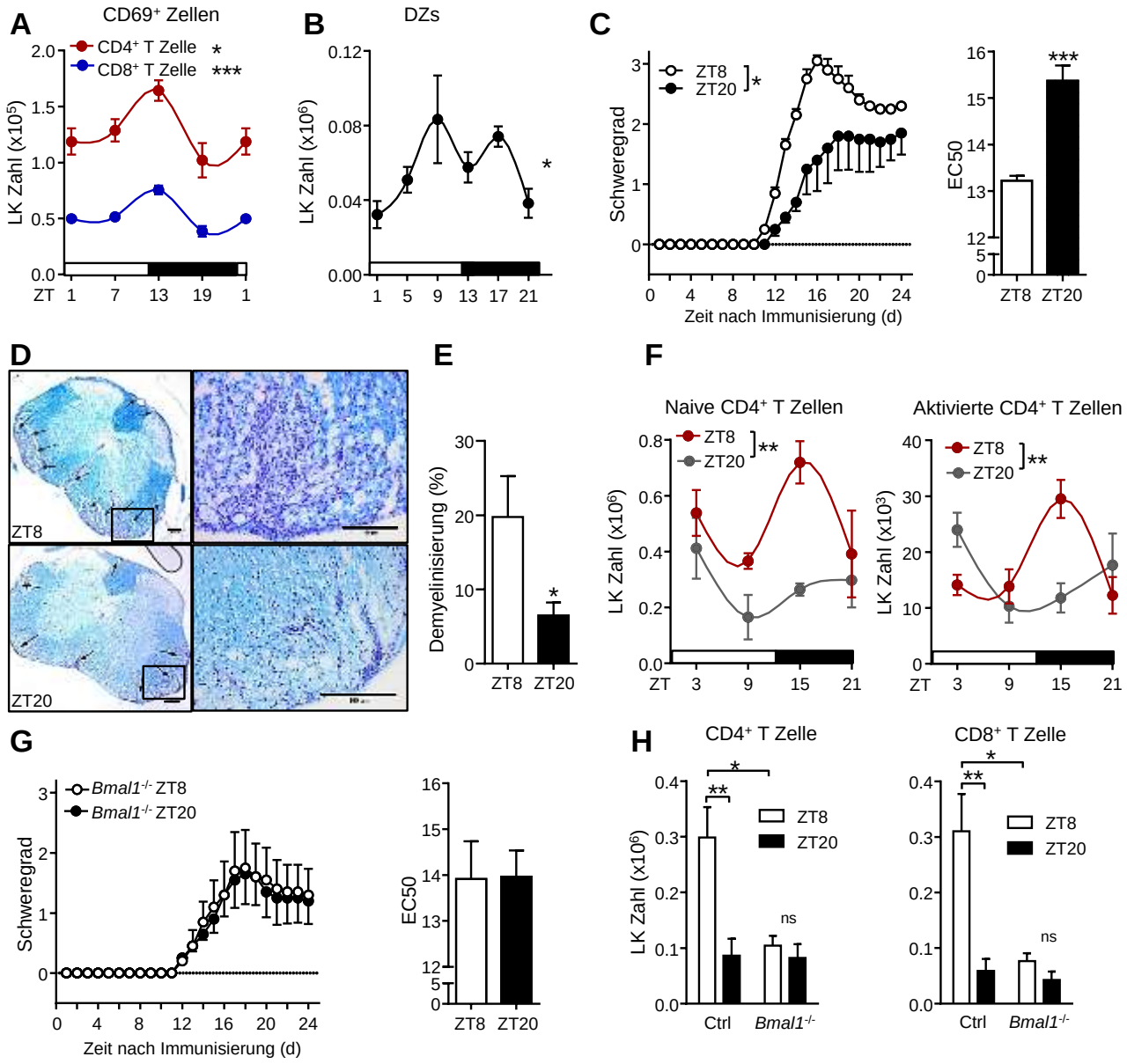

Abb. 12. ***Die Immunisierungsreaktion in Experimenteller Enzephalomyelitis (EAE) ist tageszeitabhängig.***
(A) Anzahl an CD69$^+$ T Zellen im Lymphknoten über 24h. (B) Anzahl an migratorischen Dendritischen Zellen (DZ) im Lymphknoten über 24h. (C) Klinische Schweregrade in Mäusen nach Immunisierung zur Induktion von EAE zum Zeitpunkt ZT8 und ZT20. (D-E) Bilder und Quantifizierung der Demyelinisierung in der Wirbelsäule in EAE. (F) Anzahl von T Zellen in Lymphknoten zwei Tage nach EAE Induktion. (G) Klinische Schweregrade in T-Zell-defizienten *Bmal1*-Mäusen nach Immunisierung zur Induktion von EAE zum Zeitpunkt ZT8 und ZT20. (H) Anzahl von T Zellen in Lymphknoten zwei Tage nach EAE Induktion zum Zeitpunkt ZT15. Entnommen in abgewandelter Form aus (Druzd et al., 2017).

Wir untersuchten nun die pathophysiologische Bedeutung der zirkadianen Lymphknotenzahlen bei der experimentellen Enzephalomyelitis (EAE), einem Tiermodell der multiplen Sklerose. Interessanterweise zeigten Mäuse, die nachmittags (ZT8) induziert

wurden, deutlich stärkere Krankheitssymptome als Tiere, die in der Nacht induziert wurden (ZT20) (**Abb. 12C**). Dieser Unterschied war mit einer höheren Immunzellinfiltration und Demyelinisierung im Rückenmark assoziiert (**Abb. 12D-E**). Zwei Tage nach der Induktion von EAE wurde in Lymphknoten von ZT8-induzierten Tieren eine Zunahme von naiven und aktivierten CD4 und CD8 T-Zellen nachgewiesen, während in ZT20-immunisierten Tieren T-Zellzahlen relativ niedrig blieben (**Abb. 12F**). Somit scheinen Oszillationen in der Zahl der CD4-T-Zellen in Lymphknoten bei der ersten Begegnung mit dem Antigen für den Schweregrad der Krankheit verantwortlich zu sein. Um zu untersuchen, ob T-Zell-autonome Uhren diese Antwort regulierten, verwendeten wir T-Zell-defiziente *Bmal1* Tiere. In diesen Mäusen traten keine tageszeitabhängigen Unterschiede auf (**Abb. 12G**). Bei ZT15 am Tag 2 nach der Immunisierung unterschieden sich die T-Zellzahlen in Lymphknoten zwischen Tag- und Nacht-immunisierten Kontrolltieren, aber nicht in T-Zell-defizienten *Bmal1* Tieren. Somit bestimmt die Tageszeit die Stärke der adaptiven Immunantwort (**Abb. 12H**).

Im Vergleich zur unmittelbaren Antwort des angeborenen Immunsystems, die unter zirkadianer Kontrolle ist (Gibbs et al., 2014; Keller et al., 2009; Nguyen et al., 2013; Scheiermann et al., 2012), ist es bemerkenswert, dass auch die Reaktion des adaptiven Immunsystem von der Tageszeit abhängt, da letztere Reaktionen sehr viel länger brauchen. Somit scheint es, dass die ersten Stunden in der Erzeugung der adaptiven Immunantwort und die Anzahl der vorhandenen Zellen in Lymphknoten kritisch sind für die Stärke der Immunantwort. Dies kann nicht durch den nächsten zirkadianen Zyklus kompensiert werden. Kritische Faktoren des Immunsystems oszillieren in Mäusen und Menschen mit entgegengesetzten Phasen entsprechend ihrer invertierten Ruhe- und Aktivitätszeiten. Leukozytenzahlen in der Zirkulation sind hoch während der jeweiligen Ruhephasen, die beim Menschen im Laufe der Nacht, in Mäusen während des Tages auftreten. Diese Daten legen nahe, dass adaptive Immunreaktionen auch beim Menschen unter zirkadianer Kontrolle stehen könnten. Da in unserem EAE-Modell, die Immunisierung zu Beginn der Aktivitätsphase eine höhere Immunantwort in Mäusen auslöste, sollten beim Menschen die frühen Morgenstunden eine höhere adaptive Immunreaktionsfähigkeit generieren. Tatsächlich scheint dies der Fall zu sein: Studien mit Patienten haben gezeigt, dass die Verabreichung von Hepatitis A (Phillips et al., 2008) und Grippe-Impfstoffen (Long et al., 2016) am Morgen höhere Antikörpertiter induzierten im Vergleich zu anderen

Zeiten. Zusammengenommen bieten unsere Daten somit Mechanismen für eine tageszeitabhängige Reaktivität in der Migration und der Funktionalität von myeloiden und lymphoiden Leukozyten. Darauf aufbauende weitere Untersuchungen sollten eine Optimierung von Immuntherapien und Impfprogrammen zum Ziel haben.

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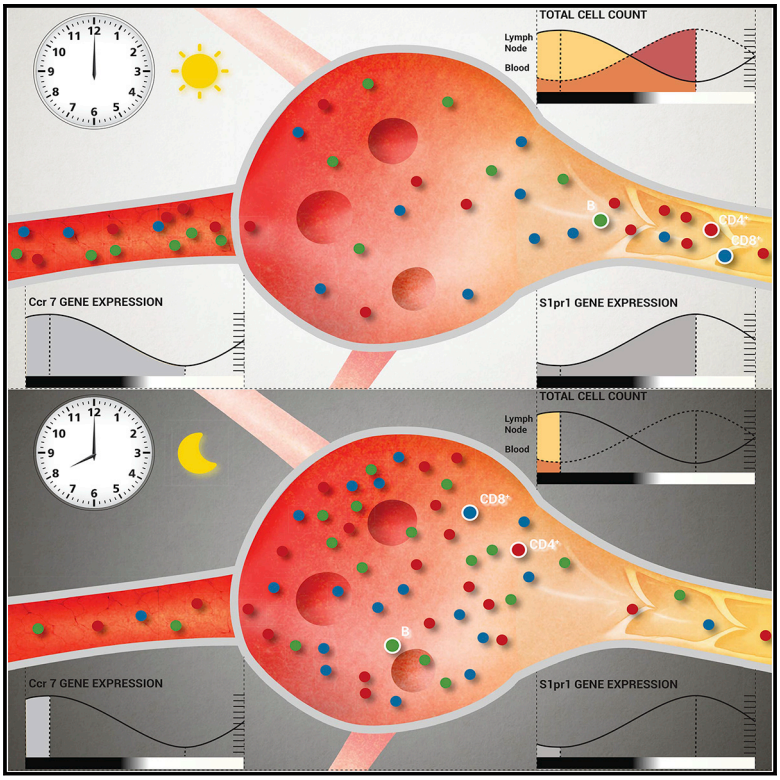
Article

Immunity

Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses

Graphical Abstract



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In Brief

Lymphocyte trafficking through lymph nodes and lymph is an important immune surveillance mechanism of the body. Druzd et al. (2017) demonstrate that this trafficking occurs in a circadian manner and that adaptive immune responses are also time-of-day dependent and are ablated when circadian clock function is lost in T cells.

Highlights

- Lymphocyte numbers in lymph nodes and lymph oscillate over the course of the day
- Rhythmic *Ccr7* and *S1pr1* expression drives rhythmic lymphocyte homing and egress
- Adaptive immune responses to immunization and pathogens are time-of-day dependent
- Loss of circadian clocks in lymphocytes ablates rhythmic adaptive immune responses

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Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses

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SUMMARY

Lymphocytes circulate through lymph nodes (LN) in search for antigen in what is believed to be a continuous process. Here, we show that lymphocyte migration through lymph nodes and lymph occurred in a non-continuous, circadian manner. Lymphocyte homing to lymph nodes peaked at night onset, with cells leaving the tissue during the day. This resulted in strong oscillations in lymphocyte cellularity in lymph nodes and efferent lymphatic fluid. Using lineage-specific genetic ablation of circadian clock function, we demonstrated this to be dependent on rhythmic expression of promigratory factors on lymphocytes. Dendritic cell numbers peaked in phase with lymphocytes, with diurnal oscillations being present in disease severity after immunization to induce experimental autoimmune encephalomyelitis (EAE). These rhythms were abolished by genetic disruption of T cell clocks, demonstrating a circadian regulation of lymphocyte migration through lymph nodes with time-of-day of immunization being critical for adaptive immune responses weeks later.

INTRODUCTION

Lymphocytes survey antigen by circulating through blood, lymph nodes (LNs) and lymph and shape specific immune responses in LNs. To enter LNs, lymphocytes must undergo extensive interactions with high endothelial cell venules (HEVs) (Butcher, 1991; Förster et al., 2008; Ley et al., 2007; Muller, 2011; Springer, 1994; Vestweber and Blanks, 1999; von Andrian and Mempel, 2003; Wagner and Frenette, 2008). Lymphocytes initially tether on peripheral nodal addressin (PNAd) expressed on HEVs using L-selectin (CD62L) as a ligand. Lymphocytes roll along the vascular endothelium and become activated via interactions of the chemokine receptors CCR7 and CXCR4 with their respective ligands CCL21 and CXCL12. Activated leukocytes use the integrin LFA-1 (CD11a) to bind to ICAM-1 to promote adhesion and finally emigrate into the LN parenchyma.

After LN entry, lymphocytes interact with dendritic cells in order to scan presented antigen (Gasteiger et al., 2016), and finally emigrate into efferent lymphatic vessels. For this egress, expression of the sphingosine-1-phosphate-receptor 1 (S1P1, encoded by *S1pr1*) on lymphocytes is critical, recognizing the chemoattractant phospholipid sphingosine-1-phosphate (S1P) (Matloubian et al., 2004). S1P concentrations are high in blood and lymph but low in tissues, thus providing a gradient that guides lymphocytes out of the LN and into efferent lymph (Cyster and Schwab, 2012). This mechanism is therapeutically exploited for treating multiple sclerosis patients by antagonizing S1P1 function with the drug FTY720 (fingolimod) to keep autoreactive T cells from exiting LNs and entering the central nervous system (Massberg and von Andrian, 2006).

Lymphocyte trafficking into LNs is believed to occur in a continuous fashion, and not to be influenced by time-of-day variables. Moreover, it is generally unclear whether circadian rhythms regulate overall cellularity in these tissues (Arjona and Sarkar, 2005; Esquifino et al., 1996; Fortier et al., 2011; Hemmers and Rudensky, 2015).

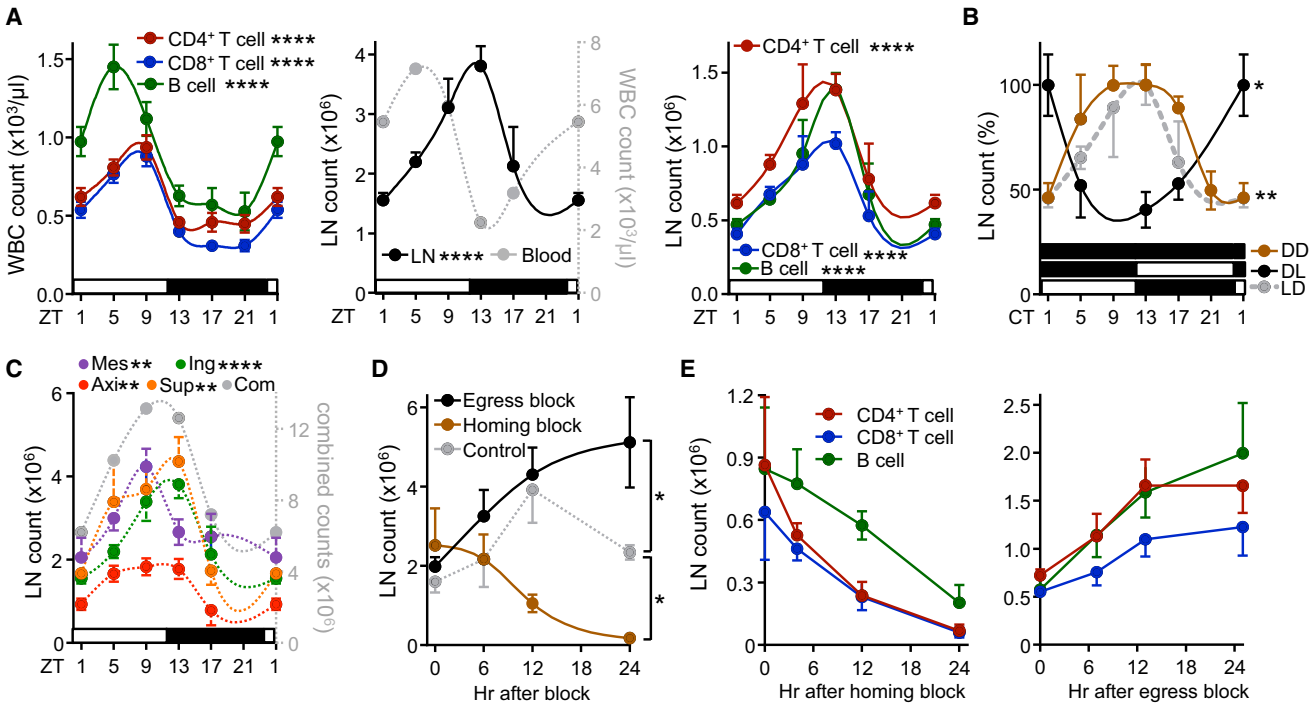

Figure 1. Lymphocyte Numbers Exhibit Circadian Oscillations in Lymph Nodes

(A) Lymphocyte oscillations in blood (left panel) and inguinal lymph node (middle and right panels) over 24 hr. Zeitgeber time (ZT, time after light onset) 1 is double-plotted to facilitate viewing; n = 4–49 mice, one-way ANOVA, WBC: white blood cells.

(B) Lymph node oscillations under light-dark (LD), dark-dark (DD) and inverted, dark-light (DL) conditions, normalized to peak times; CT, circadian time in constant darkness conditions; n = 3–15 mice, one-way ANOVA.

(C) Oscillations across multiple lymph nodes, axi: axillary, sup: superficial cervical, ing: inguinal, mes: mesenteric, com: combined counts; n = 3–19 mice, one-way ANOVA, counts are plotted per single lymph node.

(D) Lymph node counts after treatment with FTY720 (egress block) or integrin-blocking antibodies (homing block); n = 3–5 mice, one-way ANOVA with Tukey's multiple comparisons test.

(E) Lymphocyte subpopulations after homing block (left) and egress block (right); n = 3 mice. *p < 0.05, **p < 0.01, ****p < 0.0001. All data are represented as mean ± SEM. See also Figure S1.

Circadian rhythms are important drivers for most physiological processes as they align the body with rhythmically occurring daily changes in the environment (Dibner et al., 2010). They normally rely on an intricate interplay of cell-intrinsic clock genes driving circadian responses (Mohawk et al., 2012). Daily oscillations of lymphocyte counts in blood have been described (Arjona et al., 2012; Curtis et al., 2014; Haus and Smolensky, 1999; Labrecque and Cermakian, 2015; Scheiermann et al., 2013) and cells of the adaptive immune system such as T and B cells, as well as dendritic cells, possess the components of the molecular clock machinery (Bollinger et al., 2011; Hemmers and Rudensky, 2015; Silver et al., 2012a). In contrast to monocytes of the innate immune system (Nguyen et al., 2013), however, the functional relevance of these cell-intrinsic oscillations for lymphocytes is unclear (Hemmers and Rudensky, 2015). Stimulated by previous findings, which described periodic oscillations in innate immune cell function (Gibbs et al., 2014; Nguyen et al., 2013; Scheiermann et al., 2012) and T helper-17 (Th17) cell differentiation (Yu et al., 2013), we postulated that the migration of lymphocytes through murine LNs might be regulated in a circadian manner with direct relevance for the mounting of adaptive immune responses.

RESULTS

Lymphocyte Numbers Exhibit Circadian Oscillations in Lymph Nodes

In contrast to circulating blood lymphocyte numbers, which peak during the day in mice around *Zeitgeber* time (ZT) 5 (i.e., 5 hr after light onset) (Figure 1A), numbers for $CD4^+$ and $CD8^+$ T cells as well as B cells showed delayed oscillations (by ~8 hr) in inguinal lymph nodes (iLNs), with highest counts occurring at the beginning of the dark phase (ZT13, i.e., 1 hr after lights off) (Figure 1A). These rhythms were consistently observed for naive and central memory T cells, demonstrating a broad phenomenon also affecting T lymphocyte subpopulations (Figures S1A–S1C). Oscillations were not only observed in the rhythmic environment represented by 12 hr light:12 hr dark conditions (LD) but were sustained in constant darkness (dark:dark, DD), indicating their bona fide endogenous circadian nature (Figure 1B). Light exposure was an important entrainment factor, since rhythms were inverted when the light regime was reversed (DL) (Figure 1B). Rhythms were furthermore detected across various types of LNs (Figure 1C and Figures S1D–S1F), indicating a relevant phenomenon across the LN compartment. To investigate the

underlying mechanisms driving these oscillations, we focused on the cellular LN input and output pathways by blocking lymphocyte homing or egress, both critical determinants of LN cellularity (Lo et al., 2005). Blocking homing with anti-integrin antibodies dramatically decreased LN cellularity over 24 hr while blocking lymphocyte egress with FTY720 increased LN cellularity over the same time frame, confirming the temporally highly dynamic cellular nature of this tissue (Figures 1D and 1E). Both treatments ablated rhythmicity, indicating that lymphocyte homing and egress—but not intranodal proliferation (Figures S1G and S1H)—were the central determinants of circadian oscillatory cellularity. These data demonstrate a striking circadian oscillation in lymph node cellularity, peaking at night onset.

Lymphocyte Homing Is Dependent on Oscillations in Lymphocytes and Microenvironment

We next used adoptive transfer techniques to determine whether lymphocyte homing to the LN was occurring in a rhythmic manner. LN infiltration of lymphocyte subpopulations peaked around night onset and remained low during the day (Figure 2A). To define whether oscillations were determined by lymphocyte-intrinsic and/or microenvironmental signals, we adoptively transferred cells harvested at ZT5 (“day”) or ZT13 (“night”) into LD-entrained recipients at either ZT5 or ZT13. While “day” (cells) into “day” (recipient) transfers exhibited the lowest homing capacity and “night” into “night” transfers the highest, a mixed contribution of both lymphocyte and microenvironment timing was observed in the “day” into “night” and “night” into “day” chimeras (Figure 2B). A screen for oscillations of promigratory factors on T and B cells revealed that expression of the chemokine receptor CCR7 exhibited rhythmicity peaking at ZT13 (Figure 2C) while the adhesion molecules CXCR4, CD11a, and L-selectin showed either no oscillations or not for all lymphocyte subpopulations (Figures S2A and S2B). In addition, expression analyses of whole lymph node mRNA and extracellular protein on HEVs revealed oscillatory amounts of the chemokine CCL21, a ligand for CCR7—but not CXCL12 (not shown)—being high around night onset (Figures 2D and 2E). HEVs also exhibited rhythmic expression of ICAM-1 but not of PNAd (Figures S2C and S2D). Oscillations in lymphocyte chemokine receptors were critical for rhythmic homing because a titrated, short pretreatment of adoptively transferred cells with pertussis toxin (PTX) (Lo et al., 2005), an inhibitor of chemokine receptor signaling, ablated rhythmicity (Figure 2F). To investigate the involvement of CCR7 in this process, we analyzed total lymph node cellularity of CCR7-deficient mice, as well as the rhythmic homing capacity of isolated CCR7-deficient cells. *Ccr7*$^{-/-}$ mice exhibited no oscillations in lymph node cell counts while also exhibiting the expected lower overall numbers (Förster et al., 1999) (Figure 2G). In addition, *Ccr7*$^{-/-}$ cells failed to show rhythmic lymph node homing (Figure 2H). These data demonstrated that lymphocyte recruitment to LNs is determined by rhythms in leukocytes and the microenvironment, along with in-phase expression of the CCR7-CCL21 receptor-ligand axis.

Circadian Clocks Control Cellular Oscillations in Lymph Nodes

LNs exhibit oscillations of clock genes (Figure 3A), prompting us to investigate the role of lymphocyte clocks in their migratory behavior. We generated mice in which the core clock gene *Bmal1* (also known as *Arntl*) was deleted in T cells (*Bmal1*$^{flox/flox}$x*Cd4-cre*) or B cells (*Bmal1*$^{flox/flox}$x*Cd19-cre*) (Figure 3B and Figures S2E and S2F). Remarkably, loss of lymphocyte BMAL1 ablated the overall rhythmicity of T and B cell numbers in lymph nodes (Figure 3C and Figure S2G). In addition, rhythmic homing of *Bmal1*$^{flox/flox}$x*Cd4-cre* T cells into WT recipients was ablated (Figure 3D). In agreement with these findings, rhythmic expression of CCR7 surface protein (Figure 3E) and mRNA (Figure 3F) was absent in BMAL1-deficient CD4$^+$ T cells, indicating the regulation of the molecule at the transcriptional level by the circadian clock. Together, these data provide evidence for a functional role of cell-autonomous clocks in lymphocyte migration.

Lymphatic Egress from Lymph Nodes Is under Circadian Control

Because our data indicated that, in addition to a rhythmic homing component, lymphocyte egress might counterbalance LN oscillations (Figures 1D and 1E), we quantified lymphocyte numbers in lymph fluid by cannulating efferent lymphatic vessels. Prominent rhythms in cellular counts were detected, peaking at ZT9 and exhibiting a low at ZT21 (Figure 4A). These oscillations were observed for different lymphocyte populations (Figure 4A and Figures S2H and S2I) and were bona fide circadian in nature as they persisted in constant darkness (Figure 4B). Rhythms were not due to higher lymph volume or flow rates at different times of the day (Figure S2J). To verify whether oscillations in lymph cellularity were truly attributable to rhythmic egress and not secondary to rhythmic input into LNs (Figure 2A), we transferred lymphocytes at different times of the day, blocked subsequent LN entry and quantified their transit through the LN into lymph over time (Mandl et al., 2012). A higher LN retention capacity of cells injected at ZT13 was observed compared to ZT5 and a less rapid accumulation of cells in lymph (Figures 4C and 4D and Figure S3), demonstrating lymphocyte egress to be highly rhythmic. This effect resulted in longer LN half-lives of cells injected at ZT13 (CD4: 12 hr, CD8: 12 hr, B cells: 16 hr) compared to ZT5 (CD4: 9 hr, CD8: 9 hr, B cells: 13.5 hr) (Figures 4C and 4D and Figure S3). T- and B cell-specific BMAL1-deletion ablated oscillations in lymph, indicating the importance of cell-autonomous clocks also for lymphocyte egress (Figure 4E and data not shown). Of importance, adoptively transferred BMAL1-deficient CD4$^+$ T cells exhibited no time-of-day variations in their LN half-life (Figure 4F), demonstrating the relevance of T cell clocks in their rhythmic trafficking behavior.

Using a mathematical approach, we assessed whether oscillatory LN counts could be modeled with only either homing or egress to be rhythmic or whether both components needed to oscillate. Although oscillations were also observed when only one component was rhythmic, the best fit was achieved when both homing and egress were assumed to oscillate, thus supporting our experimental data (Figure 4G and Figure S4). In summary, lymphocyte clocks and the time-of-day entry of cells into LNs have functional consequences for LN transit and egress into lymph.

Rhythmic Lymphocyte Egress Depends on Oscillatory *S1pr1* Expression

S1P-receptors are critical in regulating lymph node egress (Cyster and Schwab, 2012). We therefore investigated whether

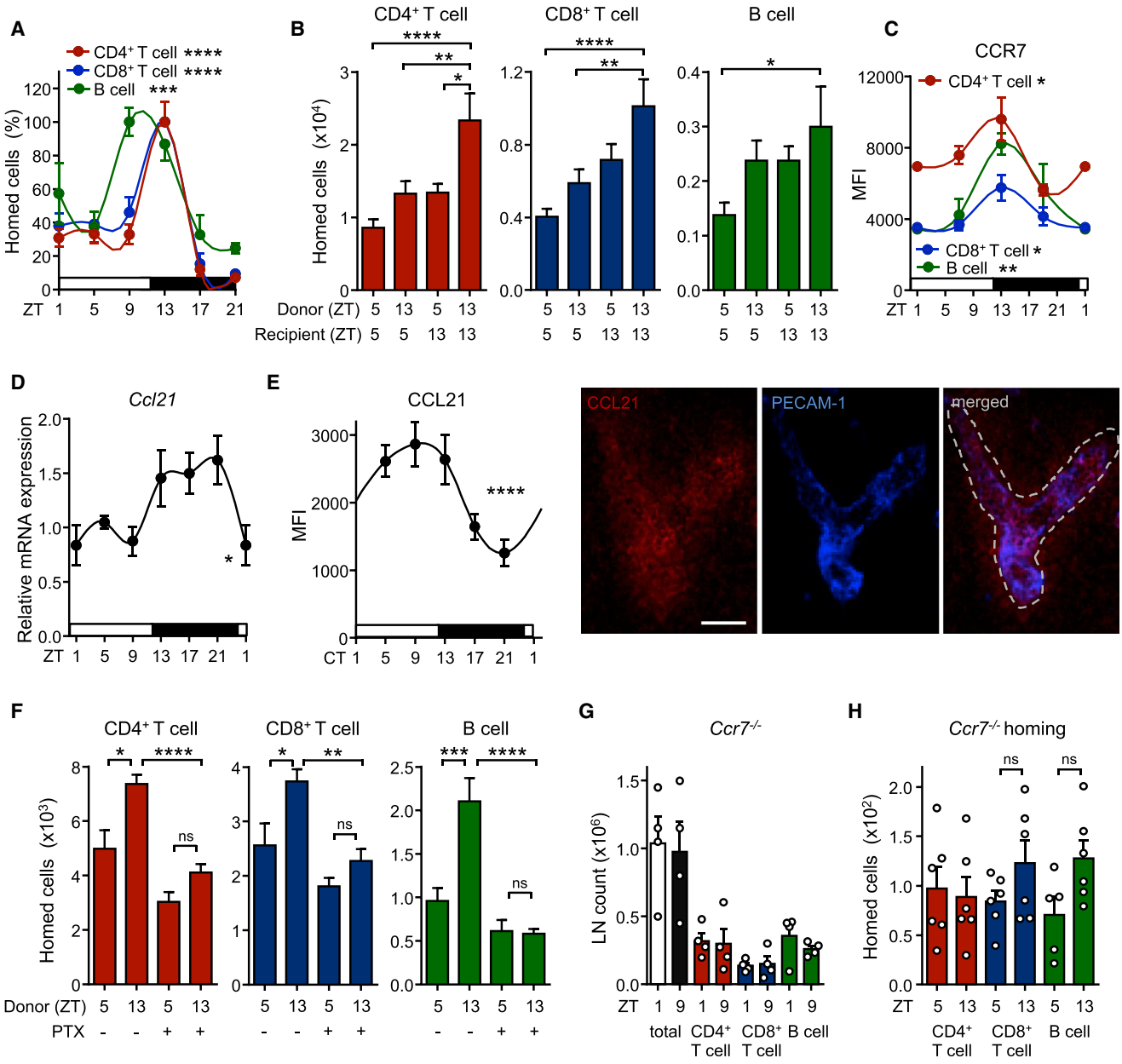

Figure 2. Rhythmic Lymphocyte Homing Is Dependent on Oscillations in Both Lymphocytes and Microenvironment

(A) Lymph node homing of lymphocyte populations over the course of the day, normalized to peak times; n = 3–17 mice, one-way ANOVA.

(B) Adoptive transfer of lymphocyte populations using donor and recipient mice kept at ZT5 or ZT13; n = 6–17 mice, one-way ANOVA with Tukey's multiple comparisons test.

(C) Oscillations of CCR7 surface expression on lymphocyte subpopulations in LN; n = 3–5 mice, one-way ANOVA.

(D) Q-PCR analysis of LN *Ccl21* amounts over 24 hr; n = 3–5 mice, one-way ANOVA.

(E) Quantification and images of expression of CCL21 on HEV over 24 hr in constant darkness (CT, circadian time: the corresponding light and dark phase are indicated); n = 3–18 mice, one-way ANOVA. Scale bar represents 50 μm.

(F) LN homing of lymphocytes harvested at ZT5 or ZT13 and treated with or without pertussis toxin (PTX); n = 5–11 mice, one-way ANOVA with Tukey's multiple comparisons test.

(G) Lack of oscillations in LN cellularity of *Ccr7*$^{-/-}$ mice; n = 4 mice, unpaired Student's t test.

(H) Lack of rhythmic LN homing of *Ccr7*$^{-/-}$ cells into WT hosts; n = 5–6 mice, unpaired Student's t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are represented as mean ± SEM. See also Figure S2.

expression of S1P-receptor family members exhibited oscillations using quantitative PCR (Q-PCR). All S1P receptors exhibited robust diurnal oscillations peaking between ZT1 and ZT9 (Figure 5A and Figure S5A), which coincided with high lymphocyte egress. In addition, FTY720, as well as the S1P1-specific functional antagonist SEW2871 strongly down-modulated lymphatic

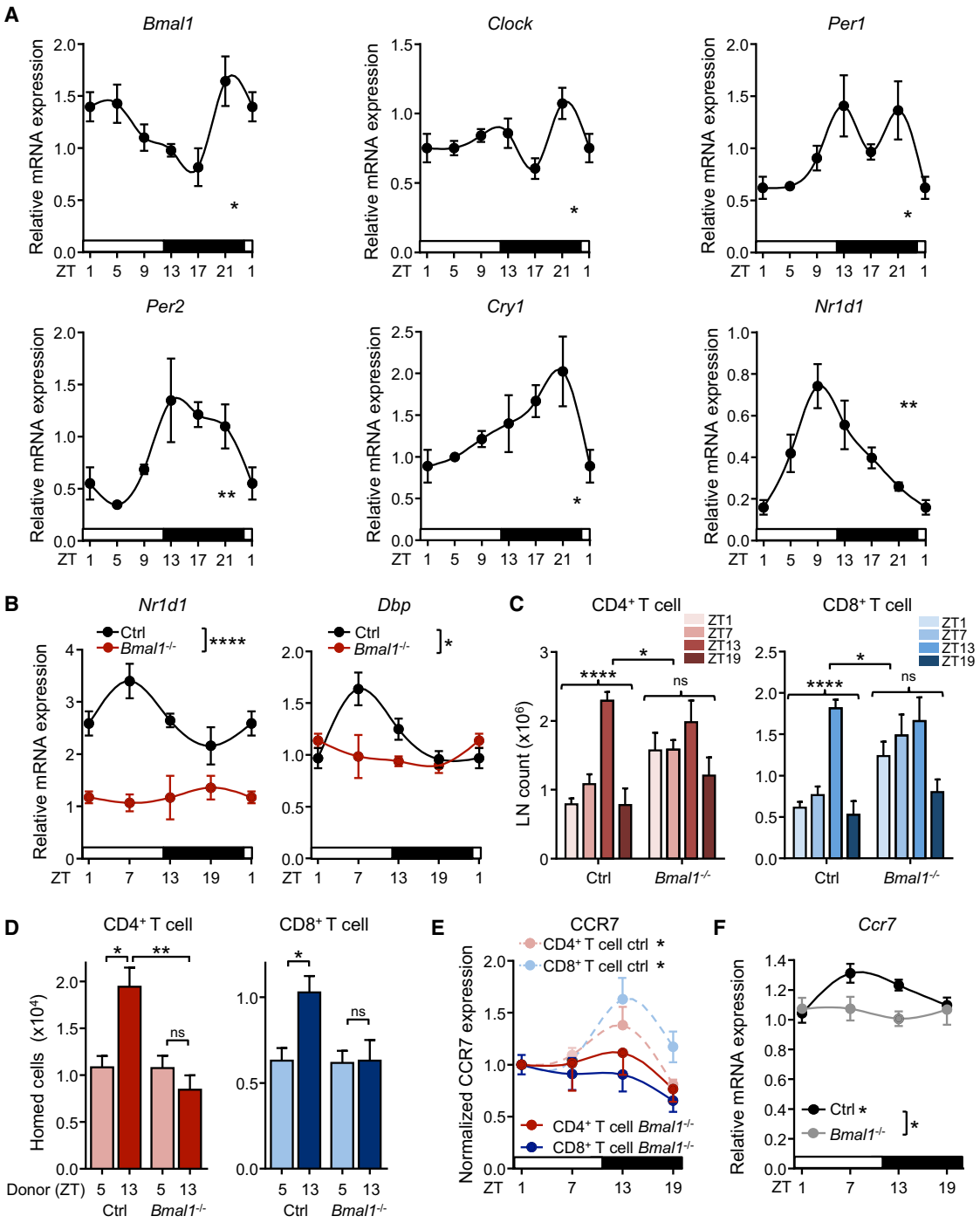

Figure 3. Oscillations of Circadian Clock Genes in Lymph Nodes Control Cellularity

(A) Q-PCR analysis of circadian clock genes in LN over 24 hr; n = 3–5 mice, one-way ANOVA.

(B) Circadian clock gene mRNA profiles in sorted CD4$^+$ T cells from *Bmal1*$^{flox/flox}$x*Cd4-cre* and control animals; n = 3–10 mice, two-way ANOVA.

(C) Lymph node CD4 and CD8 T cell counts in control and T cell specific *Bmal1*$^{-/-}$ mice, n = 3–9 mice, one-way and two-way ANOVA.

(D) LN homing of lymphocytes harvested from control or T cell-specific *Bmal1*$^{-/-}$ mice at ZT5 or ZT13 into WT hosts; n = 10–34 mice, one-way ANOVA with Tukey's multiple comparisons test.

(E) CCR7 surface expression on T lymphocyte subpopulations in LN of control and T cell-specific *Bmal1*$^{-/-}$ mice; n = 3–5 mice, one-way ANOVA.

(F) Q-PCR analysis of CD4$^+$ T cell *Ccr7* over 24 hr in control and T cell-specific *Bmal1*$^{-/-}$ mice; n = 4–8 mice, one-way and two-way ANOVA. *p < 0.05, **p < 0.01, ****p < 0.0001. All data are represented as mean ± SEM. See also Figure S2.

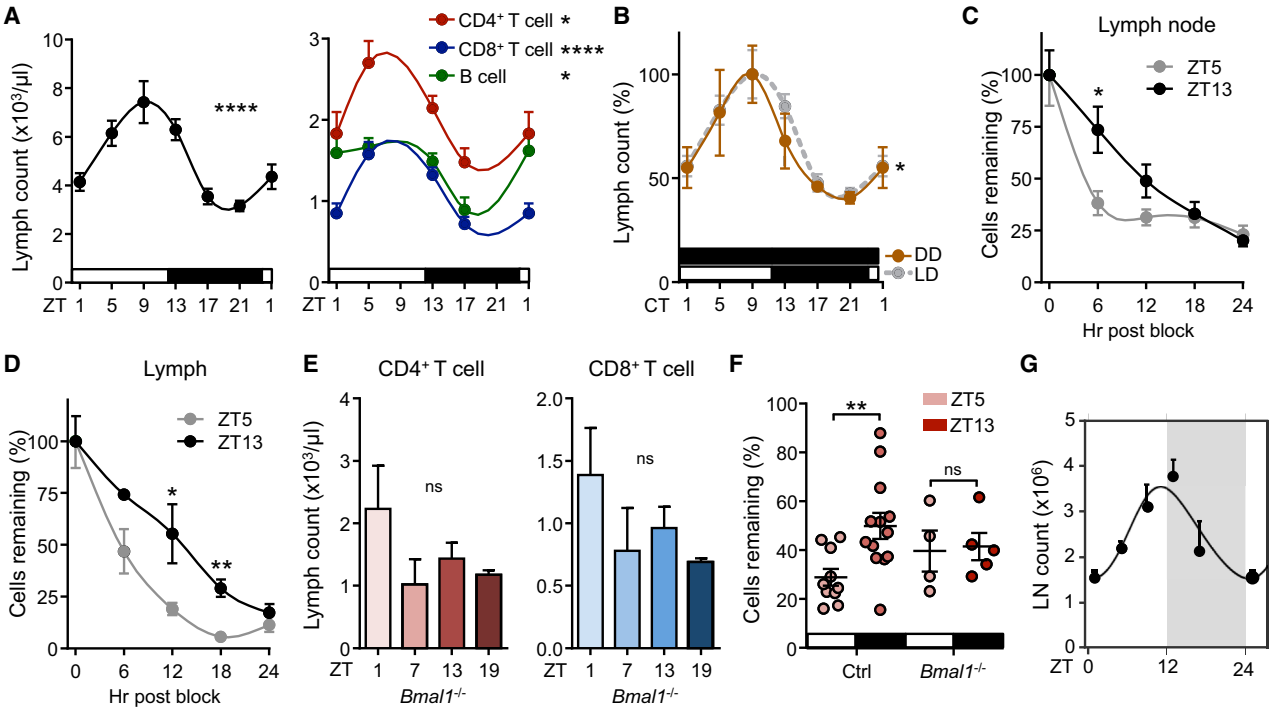

Figure 4. Circadian Oscillations in Lymphocyte Egress from LNs

(A) Oscillations of total leukocyte (left panel) and lymphocyte (right panel) counts in efferent lymph over 24 hr; n = 6–33 mice, one-way ANOVA.

(B) Lymph leukocyte count oscillations under light-dark (LD) and dark-dark (DD) conditions; n = 3–37 mice, one-way ANOVA.

(C and D) Remaining cellular numbers (in %) in lymph node (C) and lymph (D) over 24 hr after block of leukocyte homing. Lymph node: n = 3–10 mice; lymph: n = 3–6 mice, unpaired Student's t test.

(E) Lymph CD4$^+$ and CD8$^+$ T cell counts in T cell-specific *Bmal1*$^{-/-}$ mice; n = 3–8 mice, one-way ANOVA.

(F) Remaining cells (in %) in lymph nodes of adoptively transferred control and BMAL1-deficient CD4$^+$ T cells 12 hr after transfer; n = 4–13, unpaired Student's t test.

(G) Mathematical model of leukocyte homing and egress. The model is expressed as a line based on the indicated data points. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. All data are represented as mean ± SEM. See also Figures S2–S4.

egress in a time- and concentration-dependent manner (Figures 5B and 5C and Figure S5B). The observation that FTY720-treated animals exhibited reduced but still rhythmic lymph cellularity indicated a daytime-sensitive role for S1P1 in mediating lymphocyte exit. Rhythmic expression of *S1pr1* was ablated in BMAL1-deficient CD4$^+$ T cells, pointing toward a regulation of the gene by the circadian clock (Figure 5D). To investigate this in more detail, we performed an in vitro assay, in which the promoter region of *S1pr1* was cloned in front of the luciferase (*luc*) reporter gene. Luciferase activity in HEK293 cells transfected with the *S1pr1-luc* reporter was decreased after co-transfection of increasing amounts of *Bmal1* and *Clock* expression plasmids (Figure 5E). This demonstrated that expression of *S1pr1* is regulated by BMAL1 and CLOCK.

To confirm the role of S1P1 in the time-of-day-dependent egress genetically, we generated T cell-specific mice that were heterozygous for *S1pr1* in order not to completely block lymphocyte egress (Matloubian et al., 2004) but to titrate S1P1 amounts, as loss of one allele had been demonstrated to result in haploinsufficiency (Lo et al., 2005). *S1pr1*$^{+/flox}$*xCd4-cre* mice exhibited no more oscillations in LN counts and altered lymph rhythmicity, demonstrating the importance of S1P1 in the proper timing of lymphocyte egress (Figures 5F and 5G and Figure S5C). Importantly, no diurnal oscillations were observed in amounts of S1P in efferent lymph (Figure 5H) or in S1P synthesizing or degrading enzymes in lymph node (Figure S5D), suggesting that oscillatory expression of the receptor (S1P1) and not its ligand (S1P) was the driver for rhythmic lymphocyte egress. Together, these data demonstrate a critical role for S1P1 in mediating circadian lymphocyte egress from lymph nodes into efferent lymph.

Relevance of Circadian Oscillations in Lymph Node Cellularity

We hypothesized that oscillatory lymphocyte counts in LNs might have functional consequences in a potential time-of-day dependence of adaptive immune responses. We therefore tested whether the activation status of lymphocytes in LNs varied over the course of the day. More activated T cells were present in LNs at night onset as assessed by CD69 staining, coinciding with higher overall lymphocyte counts at this time (Figure 6A). Because dendritic cells (DCs) are key antigen-presenting cells critical in the activation of lymphocytes and the generation of adaptive immune responses (Girard et al., 2012), we

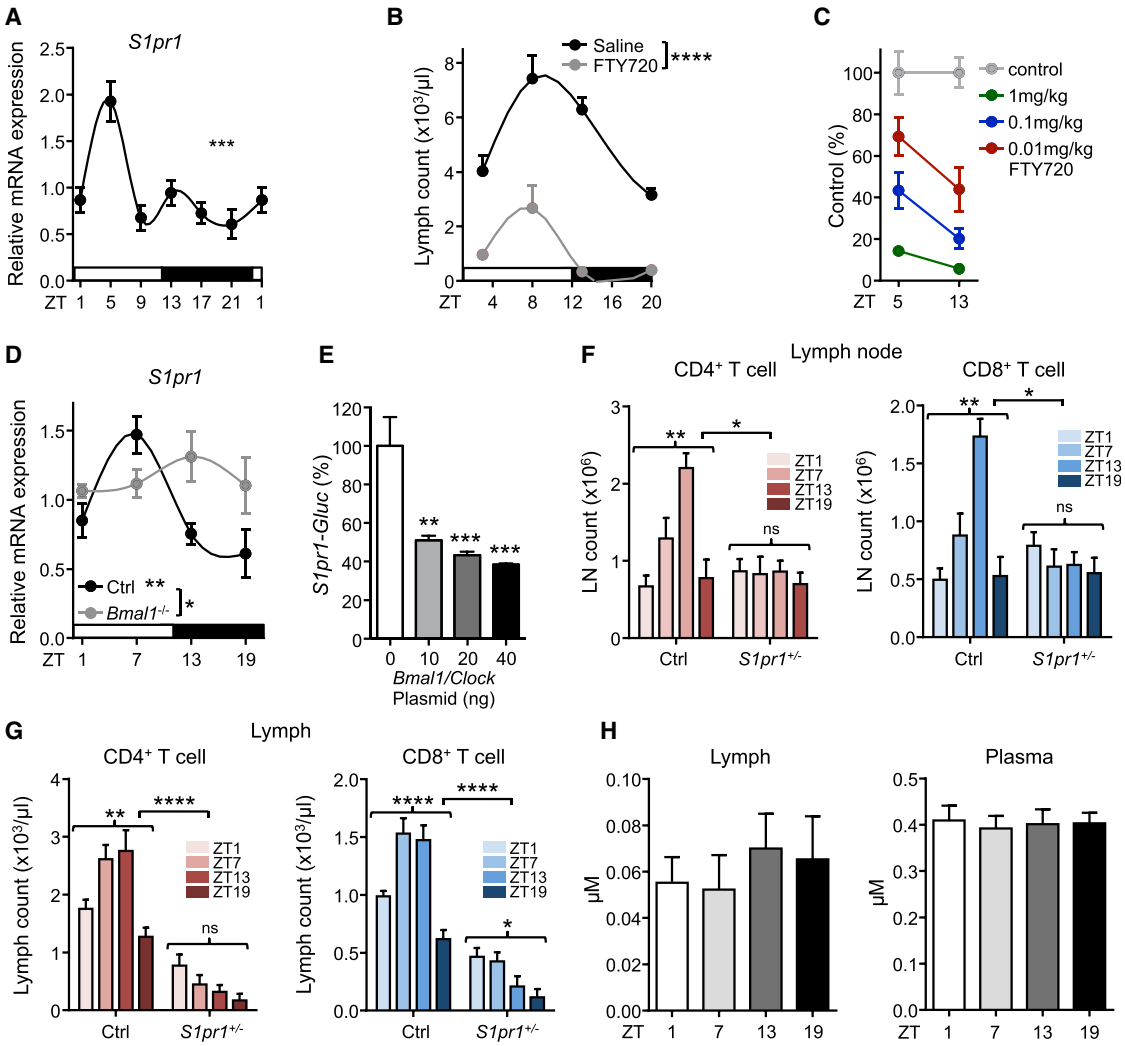

Figure 5. Rhythmic Lymphocyte Egress Depends on Oscillatory S1pr1 Expression

(A) Q-PCR analysis of LN *S1pr1* over 24h. n = 3–5 mice, one-way ANOVA.

(B) Lymph counts after blockade of S1P-receptor function using FTY720 at the indicated times; n = 3–33 mice, two-way ANOVA.

(C) FTY720 titration and respective lymph counts at two time points. n = 3–5 mice.

(D) Q-PCR analysis of CD4$^+$ T cell *S1pr1* over 24 hr in control and T cell-specific *Bmal1*$^{-/-}$ mice; n = 4–9 mice, one-way and two-way ANOVA.

(E) Normalized activity (in %) of Gaussia Luciferase driven by murine *S1pr1* promoter (*S1pr1*-GLuc) after co-transfection with various doses of *Clock* and *Bmal1* plasmids in HEK293 cells (n = 6). Data shown are pooled from two independent experiments, one-way ANOVA with Tukey's multiple comparisons test.

(F) LN CD4$^+$ and CD8$^+$ T cell counts in control and T cell-specific *S1pr1* heterozygous mice; n = 3–6 mice, one-way and two-way ANOVA.

(G) Lymph CD4$^+$ and CD8$^+$ T cell counts in control and T cell-specific *S1pr1* heterozygous mice; n = 3–12 mice, one-way and two-way ANOVA.

(H) Mass spectrometric analysis of sphingosine-1-phosphate (S1P) in lymph and blood plasma; n = 9–11 mice. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are represented as mean ± SEM. See also Figure S5.

next investigated whether these cells also exhibited oscillatory LN counts. Migratory DC cellularity showed strong oscillations peaking in phase with lymphocytes (Figure 6B and Figure S6A). These data point to the existence of a concerted circadian migration pattern of antigen-bearing (DCs) and antigen-recognizing (T cells) cells in LNs.

Recent evidence indicates that the immune system can respond to challenges in a rhythmic fashion (Fortier et al., 2011; Gibbs et al., 2014; Nguyen et al., 2013; Scheiermann et al., 2012; Silver et al., 2012b). We therefore investigated the pathophysiological significance of circadian oscillatory LN counts in the autoimmunity model of EAE. Mice immunized during the late light phase (ZT8, when cell counts are high in LNs, Figures 1A and 1C) showed a dramatically accelerated disease progression 2 weeks later, with higher clinical scores compared to late night-immunized animals (ZT20, when LN counts trough) (Figure 6C). Differences in disease scores were associated with higher immune cell infiltration and demyelination in the spinal cord at the peak of the disease (Figures 6D and 6E). We detected elevated interleukin-2 (*Il2*) mRNA amounts (Figure 6F) and a higher number of IL-17 producing as well as very-late antigen (VLA)-4 integrin positive CD4$^+$

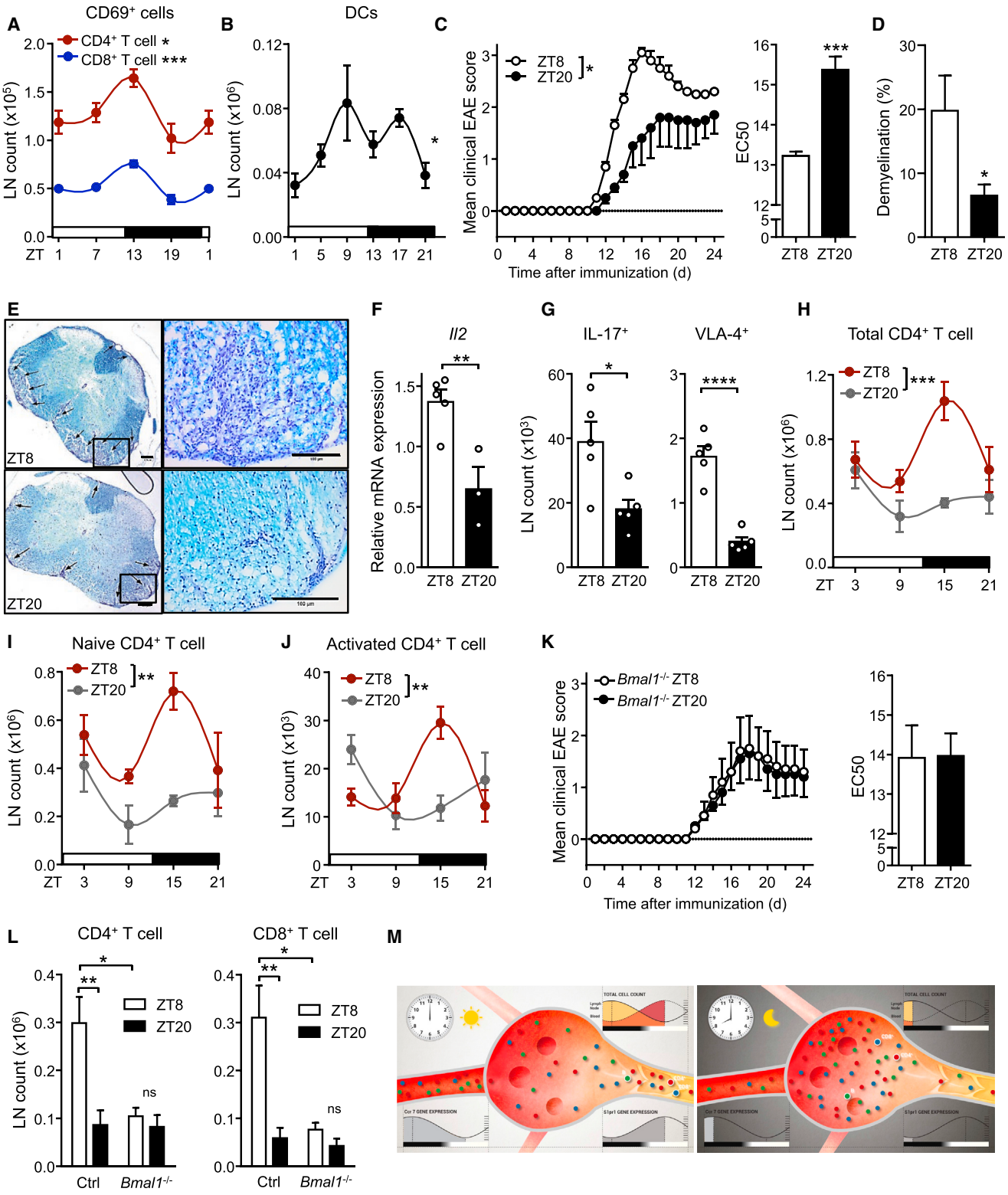

Figure 6. T Cell Clock Function Regulates Disease Severity in EAE

(A) Oscillations of CD69$^+$ T cell numbers in lymph node; n = 3–5 mice, one-way ANOVA.

(B) Oscillations of migratory dendritic cells (DCs) in lymph node; n = 6–12 mice, one-way ANOVA.

(C) EAE disease scores of mice immunized at ZT8 or ZT20. Disease score EC_{50} comparisons show accelerated symptom progression in ZT8-immunized mice; n = 5 mice, two-way ANOVA (left panel) and unpaired Student's t test (right panel).

(legend continued on next page)

T cells in LNs of ZT8-immunized mice, subtypes that have been shown to be critical for the induction of EAE (Kawakami et al., 2012) (Figure 6G). This indicated that circadian regulation of immunization occurred at a very early phase of the process when T cells are activated in draining lymph nodes (Figure S6B). Two days after induction of EAE, an increase of both naive and activated $CD4^+$ and $CD8^+$ T cells was detected in draining lymph nodes of ZT8-immunized animals, while in ZT20-immunized animals T cell numbers remained relatively low (Figures 6H–6J and Figure S6C). Thus, oscillations in the numbers of $CD4^+$ T cells in lymph nodes during initial encounter with antigen appear to be pivotal for the severity of EAE. To investigate whether T cell autonomous clocks regulate this response, we genetically deleted T cell circadian clock function. Although in control animals disease development depended on the time of immunization, in T cell specific *Bmal1*$^{-/-}$ mice it did not (Figure 6K). Two days after immunization, total and T cell counts in draining lymph nodes were different at ZT15 between day- and night-immunized control, but not in T cell specific *Bmal1*$^{-/-}$ mice (Figure 6L and Figure S6D). Hence, T cell clocks determine time-of-day function and, after challenge, development of autoimmune sequelae. We finally investigated whether adaptive immune responses to pathogens exhibited similar circadian rhythmicity. Mice were infected with the gastric bacterial pathogen *Helicobacter pylori* at three different time points during the day, and lymph node counts were quantified 3 weeks later. Also in this chronic infection model, LN counts showed strong circadian responsiveness to the initial infection with highest numbers present at ZT7, analogous to the EAE immunization experiments (Figure S6E). In addition, acute viral infection with influenza A virus led to stronger pulmonary infiltration of $CD8^+IFN\text{-}\gamma^+$ T cells when animals were infected at ZT8 compared to ZT20, 8 days post infection (Figure S6F). Together, these data strongly indicate that immunization reactions and the adaptive immune responses to various pathogens follow a circadian rhythm (Figure 6M and Movie S1).

DISCUSSION

We have described here the mechanisms that govern a circadian rhythmicity in the capacity of lymphocytes to enter and exit lymph nodes, which depend on cell-autonomous, clock-gene-controlled expression of promigratory factors. Lymphocytes entered LNs most prominently at the onset of the night phase and egressed from the tissue during the day. This resulted in oscillatory cell counts in lymph nodes and lymph and time-of-day differences in the adaptive immune response weeks after immunization. In addition, DCs were found to be present in LN in highest numbers around night onset, peaking in phase with the lymphocyte populations. Our data reveal that T cell-autonomous circadian oscillations are critical in regulating adaptive immunity.

It is surprising to note that lymph nodes exhibit circadian differences in their cellularity, given that they represent such a central tissue of the immune system and, accordingly, have thus been intensely studied. Since we observed oscillations in all investigated lymph nodes, the phenomenon appears to be broad and robust and not restricted to specific body locations. It is noteworthy that other lymphoid organs such as the thymus (data not shown) and the bone marrow do not exhibit overt circadian oscillations in absolute numbers. At least the latter, however, still displays circadian activity in cellular trafficking as hematopoietic stem and progenitor cells (HSPCs) are mobilized into blood (Lucas et al., 2008; Méndez-Ferrer et al., 2008) and recruited back into the bone marrow (Scheiermann et al., 2012) at different times. The fraction of mobilized and homed cells might be small compared with the overall numbers, though, which might explain why overt oscillations of total cells in the BM are not observed. In contrast to the BM, lymph node total cellularity is highly dynamic over 24 hr, as seen when homing or egress is blocked. Still, homing of leukocytes to lymph nodes and bone marrow occurs predominantly at night, while egress (or mobilization in the case of the bone marrow) occurs predominantly during the day. Thus, rhythmic egress of lymphocytes via efferent lymph is a major mechanism underlying the oscillatory leukocyte numbers in blood. Whether other egress routes for lymphocytes, from the thymus or the spleen, occur in a circadian manner is currently unclear.

Our data point to a critical role of cell-intrinsic clock-dependent mechanisms in the regulation of T and B lymphocyte trafficking. While global BMAL1 deficiency results in a diverse array of phenotypes (Bunger et al., 2000), such as altered B cell numbers (Sun et al., 2006), few studies have focused on cell-type specific deletion of BMAL1 in the immune system. Lineage-specific ablation of BMAL1 in myeloid cells results in a pro-inflammatory state (Nguyen et al., 2013), yet a similar approach targeting lymphocytes yielded no obvious phenotype (Hemmers and Rudensky, 2015). The latter finding might be due to mice with clock-deficient lymphocytes exhibiting phenotypes only at specific times, so that only when tested over multiple time points across the day can alterations be detected.

(D) Quantification of demyelination in lumbar spinal cord sections; n = 5 mice, unpaired Student's t test.
(E) Luxol Fast Blue staining of lumbar spinal cord sections of mice immunized at ZT8 (top) or ZT20 (bottom) at the peak of the disease showing demyelinated areas (arrows), scale bar represents 200 μm (left images), 100 μm (right images).
(F) LN IL-2 mRNA after EAE induction; n = 3–5 mice, unpaired Student's t test.
(G) LN counts of $IL\text{-}17^+$ and $VLA\text{-}4^+$ $CD4^+$ T cells after EAE induction; n = 5 mice, unpaired Student's t test.
(H–J) Diurnal profiles of inguinal lymph node counts of total $CD4^+$ T cells (H), naive $CD4^+$ T cells (I), and activated $CD4^+$ T cells (J) on day 2 after EAE induction; n = 4 mice, two-way ANOVA with Bonferroni's post hoc test.
(K) EAE disease scores and EC_{50} values in T cell-specific *Bmal1*$^{-/-}$ mice immunized at ZT8 or ZT20; n = 5 mice.
(L) Inguinal lymph node counts of $CD4^+$ T cells, and $CD8^+$ T cells in T cell-specific *Bmal1*$^{-/-}$ mice at ZT15 on day 2 after EAE induction; n = 4–5 mice, unpaired Student's t test.
(M) Schematic diagram of circadian lymphocyte migration through lymph nodes. At night onset, increased homing due to higher CCR7 amounts leads to enhanced lymphocyte counts in the lymph node. During the day, higher *S1pr1* expression induces the egression of lymphocytes into efferent lymph. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. All data are represented as mean ± SEM. See also Figure S6 and Movie S1.

Our data showing that lack of BMAL1 in T cells ablated oscillations in EAE disease scores indicates an important role for T cell clocks in the adaptive immune response. This is in line with a previous observation that T cells harvested from *Clock* mutant mice exhibited altered proliferation responses at specific times (Fortier et al., 2011).

We demonstrated the critical regulators of lymphocyte trafficking, *Ccr7* and *S1pr1*, to be under circadian control. *Ccr7* and *S1pr1* oscillations showed opposite phases, being high in the evening for the former and high during the day for the latter. This agrees with the additional role of CCR7 as a retention factor for T cells in lymph nodes that can antagonize S1P1 function (Pham et al., 2008). In addition, more T cells expressed the activation marker CD69 at night onset, a negative regulator of S1P1 (Shiow et al., 2006). These inverted oscillations of retention and egress factors appeared to be responsible for keeping lymphocytes retained in the node at night and promoting daytime egress. In addition to these lymphocyte intrinsic signals, the microenvironmental fluctuation of CCL21 indicated an additional rhythmic component that was non cell-autonomous. Whether direct autonomic innervation governs these oscillations—as has been shown for the bone marrow and skeletal muscle (Méndez-Ferrer et al., 2008; Scheiermann et al., 2012)—and/or humoral factors as has been shown for the lung (Gibbs et al., 2014), is outside the scope of this manuscript and should be investigated in the future.

Lymph nodes act as the body's immunological sieve, capturing lymph-borne antigens and antigen-presenting cells (APCs) and bringing these components in close contact with lymphocytes. Our data show that not only T cells migrate to the lymph node in a circadian manner but that also dendritic cells, the major APCs, are present more prominently around night onset, peaking at the same time as lymphocytes. Both cell types need to interact to produce a functional adaptive immune response (Gasteiger et al., 2016). It therefore appears likely that having them present at the same time in the confined environment of the lymph node enhances the likelihood of antigen encounter by the very few specific T cell clones, as opposed to cells entering and exiting at random times. This process of more effective cellular interactions appears to be further helped by the fact that during the night fewer cells exit the tissue than during the day, thus giving APC-T cell pairs more time to interact functionally. Whether also antigen encounter with APCs at peripheral surfaces is occurring in a rhythmic manner, which could ultimately be driving a circadian migratory DC process, should be the subject of future investigations.

Compared to the immediate response of the innate immune system, which is under circadian control (Gibbs et al., 2014; Keller et al., 2009; Nguyen et al., 2013; Scheiermann et al., 2012), it is remarkable that also the adaptive immune system appears to be temporally regulated, because these responses take much longer to be mounted. How can the time-of-day dependent differences encountered during the initial stimulus be preserved to exhibit circadian gating of immune responses weeks later? It appears that the initial hours in the generation of the adaptive immune response and the number of cells present in the lymph node are critical in regulating the strength of the response (Moon et al., 2007), which cannot be compensated for by the next circadian cycle.

In steady state, critical factors of the immune system oscillate in mice and humans with opposite phases according to their inverted rest-activity times (Arjona et al., 2012; Curtis et al., 2014; Labrecque and Cermakian, 2015; Scheiermann et al., 2013). Circulating leukocyte counts are high during the respective resting phases, which occur during the day in mice and at night in humans. In addition, also under stimulated conditions timing matters, as administration of granulocyte colony-stimulating factor (G-CSF) to patients in order to mobilize hematopoietic stem cells from bone marrow into blood yields higher numbers at different times than in mice (Lucas et al., 2008). These data suggest that also adaptive immune responses in humans could be under circadian control. Since in our EAE model, immunization close to the onset of the activity phase produced a higher immune response in mice, in humans the early morning hours should produce a higher adaptive immune reactivity. Indeed, this appears to be the case as recent studies have unveiled that administration of hepatitis A (Phillips et al., 2008) and flu vaccines (Long et al., 2016) in the morning yielded highest antibody titers compared to other times. Together, our data provide mechanistic insights for a time-of-day difference in lymphocyte trafficking and adaptive immune responses, thus warranting further investigations into time-of-day optimization of immune therapies and vaccination programs.

EXPERIMENTAL PROCEDURES

Animals

Cd4cre, *Lckcre*, *Cd19cre*, *Bmal1*$^{flox/flox}$, and *S1pr1*$^{flox/flox}$ mice were obtained from Jackson Laboratories and crossbred to target T cells and B cells, respectively. *Ccr7*$^{-/-}$ mice were a gift from Reinhold Förster. 7- to 8-week-old wild-type C57BL/6 mice were purchased from Charles River and Janvier. Mice were housed under a 12 hr:12 hr light-dark cycle with food and water ad libitum. To induce changes in light regime, we placed mice in a light cycler (Park Bioservices) with a 12 hr-inverted light cycle for a minimum of 2 weeks to completely establish an inverse light cycle or kept in constant darkness. All animal experimental procedures were carried out in accordance with the German Law of Animal Welfare and approved by the Regierung of Oberbayern or the ethics committee of the Schleswig-Holstein State Ministry of Energy, Agriculture, Environment and Rural Areas.

Flow Cytometry

Blood was collected into EDTA-coated capillary tubes (Microvete 300). Counts were obtained using an IDEXX ProCyte DX cell counter or a Hemavet Hematology Analyzer 950FS (Drew Scientific). Erythrocytes were lysed by incubation in 0.8% NH_4Cl. Lymph was collected as described below. Spleens, lymph nodes, or thymi were harvested from animals and processed through a cell strainer (40–70 μM pore size) and resuspended in PBS. For quantification of dendritic cells, lymph nodes were first chopped into small pieces and incubated in collagenase IV (1 mg/ml, C5138, Sigma) and DNase I (0.2 mg/ml, Roche) for 30 min at 37°C with gentle agitation. Following digestion, cells were passed through a 40 μm strainer and resuspended in PBS supplemented with 2% fetal bovine serum (GIBCO). A 20 min blocking step using anti-CD16/32 antibody (2.4G2; BD Biosciences) was performed on ice prior to fluorescent staining. Single-cell suspensions were stained with fluorescence-conjugated antibodies and analyzed by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter) or a FACSCanto II flow cytometer (BD Biosciences). Prior to staining of intracellular cytokines, cells were restimulated for 5 hr with PMA and ionomycine (cell stimulation cocktail with protein transport inhibitors, eBioscience). Data were analyzed with FlowJo software (Tree Star).

Adoptive Transfer Studies

Lymphocytes from spleen and peripheral lymph nodes (ratio 80:20) were labeled with 1.5 μM of carboxyfluorescein succinimidyl ester (CFSE,

Thermo Fisher Scientific) or 2.5 μM CellTracker Red CMTPX (Thermo Fisher Scientific) in PBS containing 0.2% BSA and 2 mM EDTA for 20 min at 37°C and washed 4 times. 20 × 10^6 cells were then injected i.v. into recipient mice. In some experiments, lymph node entry was blocked two and 12 hr later by intraperitoneal (i.p.) administration of 100 μg anti-α_L (CD11a, clone M17/4; BioXCell) and anti-α_4 (CD49d, clone PS/2; BioXCell) integrin antibodies in PBS. In some experiments, cells were pre-incubated with a 200 ng/ml pertussis toxin (PTX) pulse of 10 min at 37°C and washed 4 times before injection together with control-treated cells (Lo et al., 2005). The subsets of adoptively transferred cells in the recipient mice were analyzed via flow cytometry. Dyes were switched to avoid dye-specific effects.

S1pr1 Promoter Luciferase Reporter Assay

The *S1pr1*-Gaussia Luciferase (*S1pr1*-GLuc) GLuc-ON promoter clone containing a ~1 kb fragment upstream of the transcription start site and a ~0.3 kb fragment of the exon 1 of murine *S1pr1* in pEZX-PG04 vector (also containing a constitutively expressed secreted alkaline phosphatase (SEAP) secondary reporter as an internal control) was obtained from GeneCopoeia. HEK293AAV cells (Cellbiolabs) were plated onto 96-well plates and maintained in DMEM with 2 mM stable glutamine supplemented with 10% fetal bovine serum (FBS) and 10,000 U penicillin/streptomycin at 37°C with 5% CO2. Upon 80%–90% confluence, cells were transfected with the following expression plasmids: 20 ng *S1pr1-luc* with various combinations of the following clock gene constructs: *HA-Clock*, *HA-Bmal1*, and *pcDNA3.1* (mock transfection) as indicated per well using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Medium was changed once on the next day and was collected for measurement 48 hr after transfection. The secreted GLuc and SEAP activities were measured with the Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia) according to the manufacturer's protocol using the Berthold TriStar LB 941 plate reader (Berthold Technologies). Normalized promoter activity was calculated as the ratio of GLuc to SEAP activities.

Lymph Cannulation

40 min prior to cannulation, 200 μl olive oil was administered intragastrally to identify mesenteric lymph vessels that are located just upstream of the thoracic duct. Mice were anesthetized (100 mg/kg ketamine, 20 mg/kg xylazine, 1% acepromazin i.p.), vessels were cannulated and lymph was drawn via a fine bore polythene tubing (Smiths Medical) that had previously been flushed with PBS containing EDTA. In some experiments, mice were injected i.p. with 1 mg/kg (or less) of FTY720 or 10 mg/kg of SEW2871 1 or 2 hours, respectively, before cannulation. Cell numbers were determined using an IDEXX ProCyte DX cell counter.

Immunofluorescence Microscopy and Quantitative Imaging Analysis

Harvested lymph nodes were embedded in OCT (TissueTec), frozen and sectioned with a thickness of 10 μm on a cryostat (Leica). Sections were fixed in methanol or left unfixed, incubated with fluorescently coupled antibodies and imaged on an Axio Examiner.D1 microscope (Zeiss) equipped with LEDs of 405 nm, 488 nm, 561 nm, and 642 nm excitation wavelengths or equipped with a 4-color laser stack and a confocal spinning-disk head (Intelligent Imaging Innovations). For investigations of protein expression in HEVs, all quantifications were performed using mask analysis (Zeiss software) based on PECAM-1 expression and quantifying expression of other fluorescent channels within the mask containing PECAM+-pixels delineating HEV vascular structures as previously detailed (Scheiermann et al., 2012).

EAE Induction

For EAE induction the MOG35-55-CFA Emulsion PTX Kit (EK-2110, Hooke Labs) was used according to the manufacturer's protocol. Mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine i.p.) and bilaterally s.c. injected with 200 μg myelin oligodendrocyte glycoprotein (MOG) 35–55 in 200 μL Complete Freund's Adjuvant (CFA) at the indicated time points, followed by two i.p. injections of 200 ng pertussis toxin (PTX) in phosphate buffered saline (PBS), immediately after immunization and 24 hr later. From day 8 on, paralysis was assessed using the clinical scoring system: 0, no obvious signs of disease; 0.5, distal paralysis of the tail; 1, complete tail paralysis; 1.5, mild paresis of one or both hind legs; 2, severe paresis of both hind legs; 2.5, complete paralysis of one hind leg; 3, complete paralysis of both hind legs; 3.5, complete paralysis of hind legs and paresis of one front leg. Animals with scores of 2 and up were provided access to food and water on the bottom of the cage.

T and B Cell Isolation

$CD4^+$ T cells for Q-PCR analysis were purified from lymph nodes of C57BL/6 WT, × *S1pr1*$^{+/flox}$x*Cd4-cre*, *Bmal1*$^{flox/flox}$x*Cd4-cre* or littermate control mice using the EasySep Mouse $CD4^+$ T cell enrichment kit (StemCell Technologies). Purity was confirmed via flow cytometry and was generally > 96%. $B220^+$ B cells for western blot analysis were purified from spleens of *Bmal1*$^{flox/flox}$x*Cd19-cre* or littermate control mice using the EasySep Mouse B cell enrichment kit (StemCell Technologies). The Pan T Cell Isolation Kit (Miltenyi Biotec) was used to purify total T cells from mouse splenocytes. Splenocytes were labeled with a mixture of biotin-conjugated monoclonal antibodies directed against cells other than T cells and followed by conjugate binding with anti-biotin microbeads. Magnetically labeled non-T cells were depleted with autoMACS (Miltenyi Biotec) and the negative fraction was collected, yielding an average 90% purity of ($CD3^+$) T cells.

Antibodies, Western Blotting, Mass Spectrometry Analyses, Quantitative Real-Time PCR, Histology, T Cell Proliferation Analyses, *Helicobacter Pylori* Infection and Influenza A Virus Infection

See Supplemental Experimental Procedures.

Statistical Analyses

All data are represented as mean ± SEM. Comparisons between two samples were performed using the paired and unpaired Student's t tests or Mann-Whitney test. One-way ANOVA analyses followed by Tukey's multiple comparison test, two-way ANOVA analyses followed by Bonferroni's post hoc test were used for multiple group comparisons. Statistical analyses were performed with GraphPad Prism 6 software. Statistical significance was assessed as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.12.011.

AUTHOR CONTRIBUTIONS

D.D. and O.M. designed and performed experiments, analyzed results, and wrote the manuscript; L.I., U.H., R.H., W.H., A.H.T., A.L., M.E., C.-S.C., K.K., A.d.J., S.M.H., N.K., O.U., L.Y., and L.E.S. performed experiments and provided valuable inputs on the manuscript; C. Schmal and H.H. performed modeling analyses; B.K. provided access to mass spectrometers; W.S. and H.O. designed and supervised experiments, discussed data, and wrote the manuscript; C. Scheiermann conceived and supervised the study, designed and performed experiments, discussed data, and wrote the manuscript.

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Article

Adrenergic Nerves Govern Circadian Leukocyte Recruitment to Tissues

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SUMMARY

The multistep sequence leading to leukocyte migration is thought to be locally regulated at the inflammatory site. Here, we show that broad systemic programs involving long-range signals from the sympathetic nervous system (SNS) delivered by adrenergic nerves regulate rhythmic recruitment of leukocytes in tissues. Constitutive leukocyte adhesion and migration in murine bone marrow (BM) and skeletal-muscle microvasculature fluctuated with circadian peak values at night. Migratory oscillations, altered by experimental jet lag, were implemented by perivascular SNS fibers acting on β-adrenoreceptors expressed on nonhematopoietic cells and leading to tissue-specific, differential circadian oscillations in the expression of endothelial cell adhesion molecules and chemokines. We showed that these rhythms have physiological consequences through alteration of hematopoietic cell recruitment and overall survival in models of septic shock, sickle cell vaso-occlusion, and BM transplantation. These data provide unique insights in the leukocyte adhesion cascade and the potential for time-based therapeutics for transplantation and inflammatory diseases.

INTRODUCTION

Leukocyte recruitment is critical for combating pathogens in the periphery as well as for bone marrow (BM) repopulation after transplantation. Much progress has been made over the past two decades in our understanding of the major molecular mechanisms involved in leukocyte recruitment in response to an inflammatory challenge. Leukocytes initially tether and roll on endothelial cell P- and E-selectins, allowing signals from chemokines and endothelial receptors to activate leukocyte integrins for binding to intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These high-affinity interactions lead to leukocyte arrest on endothelial cells and, subsequently, diapedesis toward an inflammatory site or for engraftment in the BM (Butcher, 1991; Ley et al., 2007; Muller, 2011; Springer, 1994; Vestweber and Blanks, 1999; Wagner and Frenette, 2008). This sequential multistep process is regulated by signals in situ from adhesion receptors and by soluble factors (e.g., cytokines and chemoattractants), thereby enabling endothelial cells to serve as gatekeepers at the interface of blood and tissues.

Although leukocyte migration in inflammatory scenarios has been studied intensely, the regulation of leukocyte trafficking under homeostasis is less understood. Steady-state migration of hematopoietic stem cells (HSCs) and lymphocytes in lymphoid and nonlymphoid tissues has been described as part of normal immunosurveillance that maximizes encounters with potential pathogens (Massberg et al., 2007; Sigmundsdottir and Butcher, 2008; von Andrian and Mackay, 2000). It has been assumed that similar surveillance mechanisms exist for myeloid cells whose migration to tissues exposed to the external environment (e.g., skin and gut) keeps pathogens at bay. Constitutive, low-level expression of endothelial adhesion molecules probably regulates myeloid cell trafficking, because mice lacking major adhesion pathways are susceptible to spontaneous bacterial infections (Bullard et al., 1996; Forlow et al., 2002; Frenette et al., 1996). Given that leukocytes play key roles in regenerative processes, one would predict that the organism also possesses broad "housekeeping" programs that maintain the integrity of all tissues, irrespective of probabilities of infection.

Circadian rhythms regulate several vital biological processes through internal molecular clocks (Dibner et al., 2010; Green et al., 2008). Blood leukocyte numbers have long been known to exhibit circadian oscillations (Haus and Smolensky, 1999), and more recent studies have revealed that the release of HSCs and progenitor cells (HSPCs) from the BM follows similar rhythms (Lucas et al., 2008; Méndez-Ferrer et al., 2008). Interestingly, specific circadian times have been linked with the onset of acute diseases, notably in the cardiovascular system (Muller et al., 1985; Willich et al., 1987). Emerging data, in turn, indicate that chronic perturbations of circadian rhythms promote vascular diseases (Anea et al., 2009; Brown et al., 2009). Although the mechanisms are still undefined, numerous studies have demonstrated strong associations between high leukocyte counts and various ischemic vascular diseases (Coller, 2005; Margolis et al., 2005). Here, we tested the hypothesis that circadian-controlled neural signals influence leukocyte behavior and the inflammatory response. We show that leukocyte recruitment to tissues under homeostasis was not a continuous process but

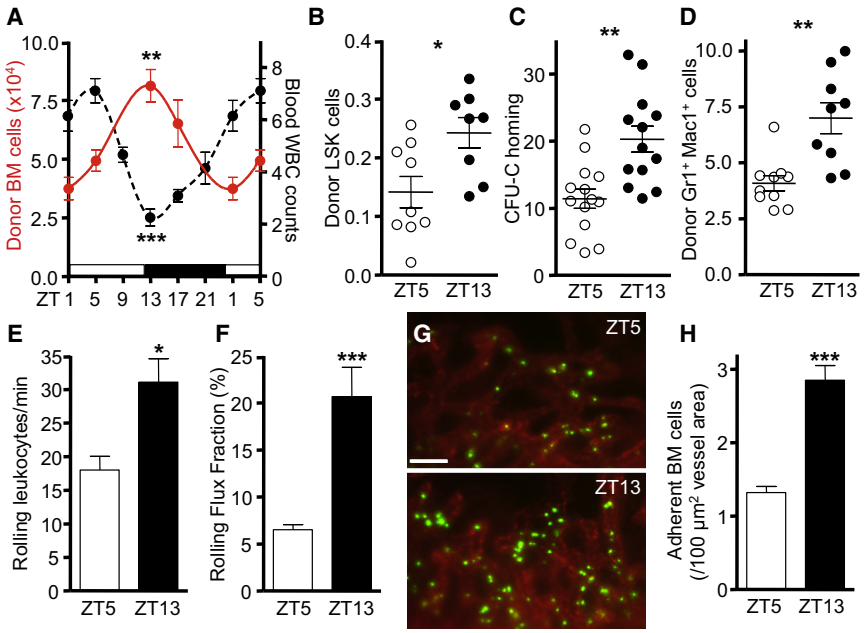

Figure 1. Circadian Oscillations of Hematopoietic Cell Recruitment to the Bone Marrow

(A) Time course of homed adoptively transferred BM cells (red) and corresponding WBC numbers in blood (× 10^3/µl, black). Light and dark cycles are indicated by white and black bars, respectively. n = 4–15.

(B) Quantification of homing of donor LSK cells (× 10^3) 3 hr after 5 × 10^6 BM cells were transferred to recipients. n = 8–9.

(C and D) Circadian oscillations in homing of CFU-C (in %) (C) and neutrophils (× 10^4) (D). n = 9–14.

(E and F) Quantifications of absolute numbers of rolling leukocytes in BM sinusoids (E) and the rolling flux fraction (F). n = 33–43 vessels from 8–9 mice per group.

(G and H) In vivo images (G) and quantification (H) of fluorescently labeled adherent BM cells (green) after adoptive transfer. BM sinusoids were identified with R6G (red). n = 60 areas from 7 mice per group.

*p < 0.05, **p < 0.01, ***p < 0.001. The scale bar represents 50 µm. See also Figure S1 and Table S1.

rather exhibited circadian oscillations, and that these rhythms, orchestrated by the molecular clock via adrenergic nerves, can impact the outcome of disease.

RESULTS

Hematopoietic Cell Recruitment in the BM Operates under Circadian Control

Total circulating leukocyte counts oscillate in murine blood, peaking ~5 hr after the onset of light (zeitgeber time, ZT5) and exhibiting a trough at ZT13 (Figure 1A), confirming previous reports (Haus and Smolensky, 1999). These rhythms are observed for HSCs (Méndez-Ferrer et al., 2008) as well as all major leukocyte subsets (Figure S1A available online), but not for erythrocytes or platelets (data not shown).

To test whether the recruitment of leukocytes from the blood to tissues also exhibited circadian preferences, we initially investigated hematopoietic cell recruitment to the BM. When we assessed the recruitment potential of adoptively transferred BM cells throughout the day, we observed significant circadian oscillations in the homing of total hematopoietic cells to the BM, whose pattern ran in antiphase with that of blood, exhibiting a peak at ZT13 and a trough in the daytime (ZT5) (Figure 1A). Circadian recruitment was observed for Lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells (Figure 1B), colony-forming units in culture (CFU-C, Figure 1C), and neutrophils (Figure 1D).

To investigate the sequence of events in vivo, we visualized the interactions between circulating leukocytes and BM sinusoidal endothelium using multichannel fluorescence intravital microscopy (MFIM) of the calvarial BM as previously described (Chiang et al., 2007; Mazo et al., 1998). The number of endogenous rolling leukocytes in BM sinusoids was increased at ZT13 compared to ZT5 (Figures 1E and 1F), as was the number of adherent cells after adoptive transfer (Figures 1G and 1H and Figures S1B and S1C). These findings suggested that the circadian time has a significant impact on the rhythmic recruitment of different hematopoietic cell populations to the BM.

Rhythmic Leukocyte Recruitment to Skeletal Muscle

We next investigated whether circadian oscillations also dictated leukocyte recruitment to peripheral tissues using the cremaster muscle as a model to study leukocyte-endothelial cell interactions in real time. Initial investigations by whole-mount immunofluorescence staining of unstimulated muscle tissues for the pan-leukocyte marker CD45 and myeloid antigens F4/80 or Gr-1 revealed numerous extravascular CD45$^+$ cells alongside postcapillary venules identified by expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1), vascular-endothelial cadherin (VE-Cadherin), and ephrin-receptor B4 (EphB4) (Figures 2A and S2A). Interestingly, extravascular leukocyte numbers exhibited circadian fluctuations, with CD45$^+$F480$^+$ macrophages representing the predominant leukocyte population in muscle (81% of CD45$^+$ cells versus 2% for neutrophils, data not shown), peaking at ZT13 along postcapillary venules, where leukocyte infiltration mainly occurs (Figures 2B, 2C, S2B, and S2C), but not along arterioles or capillaries (Figure S2D). Staining for Ki67 revealed very few positive leukocytes with no detectable circadian rhythm (data not shown), suggesting that the observed leukocyte increase at night was not due to proliferation.

We next quantified leukocyte recruitment in real time in the exteriorized cremaster muscle through MFIM after injection of very low doses of fluorescently conjugated antibodies for identification of leukocyte populations in vivo (Figure 2D) (Chiang et al., 2007). Under surgically induced trauma conditions, the numbers of adherent neutrophils and monocytes significantly increased at night, whereas lymphocyte numbers remained unchanged (Figures 2E–2G), and overall leukocyte rolling was not affected (data not shown). No circadian differences in hemodynamic parameters such as blood flow or wall shear rates were observed

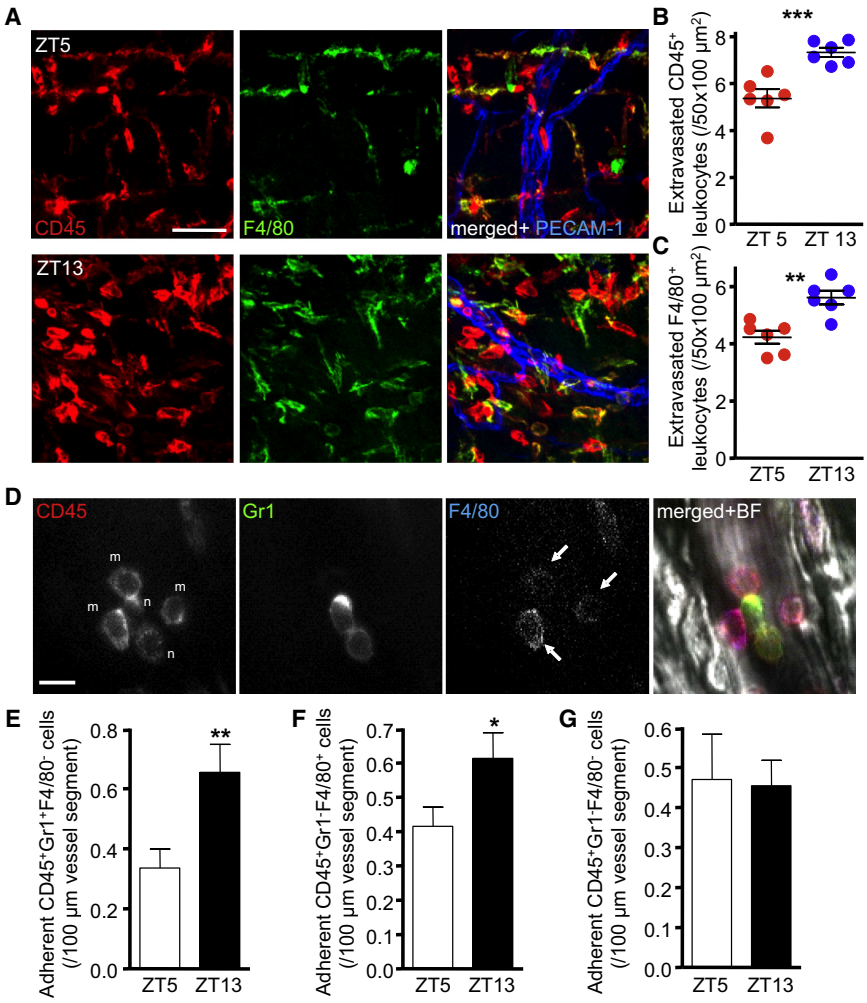

Figure 2. Circadian Oscillations of Leukocyte Recruitment to Skeletal Muscle

(A–C) Ex vivo images (A) and quantifications (B and C) of extravasated CD45$^+$ and F4/80$^+$ leukocytes situated around postcapillary venules as analyzed by whole-mount immunofluorescence staining of cremaster-muscle tissues. n = 6.

(D–G) Adherent leukocyte subsets as determined by multichannel fluorescence intravital microscopy. In vivo images showing antibody-stained adherent leukocytes, (D); adherent neutrophils, (E) and n in (D); monocytes, (F) and arrows, m in (D); and lymphocytes, (G). n = 142–148 vessels quantified from 7 mice per group.

*p < 0.05, **p < 0.01, ***p < 0.001. Scale bars represent 50 µm (A) and 10 µm (D). See also Figure S2 and Table S1.

at either time point in BM, nor in skeletal muscle (Table S1). Thus, even when an inflammatory response is triggered, the experimental time can influence the recruitment of myeloid cells in trauma-stimulated tissues. Together, these data argue for analogous mechanisms orchestrating circadian recruitment to BM and skeletal muscle across a wide range of hematopoietic lineages and differentiation stages.

Oscillations of Adhesion Molecules and Chemokines Mediate Circadian Leukocyte Recruitment

The circadian differences in leukocyte rolling and/or adhesion in the BM and skeletal muscle suggest substantial diurnal changes in the ability of endothelial cells to recruit leukocytes. We used transgenic mice expressing green fluorescent protein (GFP) under the Tie-2 promoter (Tie2-*Gfp*) to isolate endothelial cells (Motoike et al., 2000). Whole-mount staining of cremaster-muscle and BM tissues from Tie2-*Gfp* mice, as well as flow cytometry of collagenase IV-digested tissues using antibodies directed against PECAM-1 and VE-Cadherin, confirmed endothelial GFP expression (Figures S3A–S3D). We therefore sorted the CD45$^-$VE-Cadherin$^+$PECAM-1$^+$GFP$^+$ cell subset for quantitative PCR (qPCR) analyses (Figures S3C and S3D). Interestingly, we observed significant fluctuations in *Icam1* expression in sorted cremasteric endothelial cells; peak and trough values overlapped with those of leukocyte recruitment (Figure 3A). By contrast, and consistent with the circadian differences observed in adhesion, but not rolling, in the muscle (Figures 2E and 2F and data not shown), there was no difference in the expression of endothelial selectins (*Sele* and *Selp*), *Vcam1*, *Icam2*, or *Cd44* (Figures 3A and S3E). Immunofluorescence staining of frozen tissue sections corroborated these data at the protein level (Figures 3B and S3F). We confirmed circadian fluctuations of ICAM-1 on the endothelial cell surface in vivo by quantifying the numbers of intravenously (i.v.) injected adherent green or red fluorescent microspheres coated with anti-ICAM-1 or isotype control antibodies using MFIM (Figure 3C, left bars). Oscillations were also seen in hematopoietic *Icam1*$^{-/-}$ radiation chimeras, excluding the potential contribution from ICAM-1-bearing blood leukocytes (Figure 3C, right bars). We also investigated the circadian expression levels of chemokines known to be involved in myeloid cell trafficking in cremasteric endothelial cells via qPCR. We detected a clear circadian rhythm for *Ccl2* (Figure 3D), but not for other chemokines tested, including *Ccl3*, *Ccl5*, *Cxcl1*, *Cxcl2*, and *Cx3cl1* (Figure S3G). In contrast to the muscle, BM endothelial cells exhibited circadian differences in P-selectin, E-selectin, and VCAM-1, but not in ICAM-1 at either the RNA or the protein level (Figures 3E and S3H).

To investigate the functional relevance of ICAM-1 and CCL2 fluctuations in skeletal muscle, we examined the numbers of extravasated leukocytes in *Icam1*$^{-/-}$ mice and in mice deficient in the CCL2 receptor (*Ccr2*$^{-/-}$) via whole-mount ex vivo immunofluorescence. Consistent with our expression results, no circadian rhythm was apparent, demonstrating the critical requirement of these molecules in this activity (Figure 3F).

To assess the functional relevance of fluctuations in adhesion-molecule expression in BM, we examined the role of P- and E-selectins. MFIM of BM microvessels of *Sele*$^{-/-}$*Selp*$^{-/-}$ mice

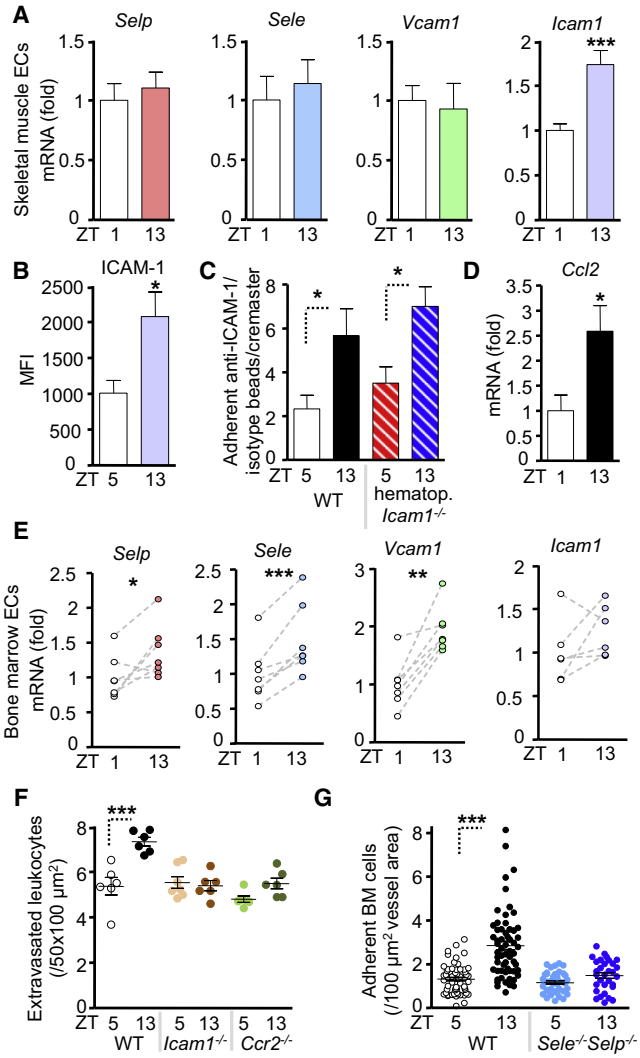

Figure 3. Oscillations of Promigratory Factors Mediate Circadian Leukocyte Recruitment

(A) qPCR analysis of sorted cremasteric endothelial cells (ECs) for P-selectin (*Selp*), E-selectin (*Sele*), *Vcam1*, and *Icam1*. n = 12–16.

(B) Quantification of ICAM-1 protein expression in muscle by confocal immunofluorescence imaging of frozen sections. n = 9. MFI, mean fluorescence intensity.

(C) MFIM quantification of specific (anti-ICAM-1-coated) versus nonspecific (immunoglobulin G-coated) fluorescent microsphere adhesion in the cremasteric microvasculature in WT and hematopoietic *Icam1*$^{-/-}$→WT BM chimeras. n = 5–6.

(D) qPCR analysis of sorted cremasteric endothelial cells for *Ccl2*. n = 6.

(E) qPCR of sorted BM endothelial cells (ECs). n = 6–7.

(F) Numbers of extravasated CD45^{+} leukocytes as analyzed by whole-mount immunofluorescence staining of cremaster-muscle tissues in WT control, *Icam1*$^{-/-}$, and *Ccr2*$^{-/-}$ animals. n = 6–7.

(G) Quantification of adherent fluorescently labeled cells to BM sinusoids in WT and *Selp*$^{-/-}$*Sele*$^{-/-}$ mice after adoptive transfer. n = 31–75 areas from 4–8 mice per group.

*p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S3.

revealed that, in these mice, circadian oscillations in leukocyte recruitment were ablated (Figure 3G). In stark contrast, and in agreement with the expression data, circadian leukocyte recruitment to the BM was independent of ICAM-1, because *Icam1*$^{-/-}$ mice exhibited no alterations in circadian oscillations in the BM relative to wild-type (WT) animals (Figure S3I).

Taken together, these data demonstrate that tissue-specific expression of key promigratory factors fluctuates within the endothelial cells of these tissues. Of importance, the differences in their molecular signatures correlate with the oscillations observed in their respective functions, i.e., adhesion in the cremaster muscle (ICAM-1, CCL2) and both rolling and adhesion in the BM (endothelial selectins, VCAM-1).

Requirements of Local Adrenergic Nerves

Most prior studies have focused on the relevance of humoral factors in synchronizing circadian rhythms (Dimitrov et al., 2009; Haus, 2007). We have recently described a key role for local innervation in the circadian release of HSCs from the BM (Méndez-Ferrer et al., 2008). To assess the function of local innervation in hematopoietic cell recruitment to tissues, we adopted two surgical approaches to denervate the cremaster muscle and BM. For the cremaster muscle, we denervated mice unilaterally through microsection of the genitofemoral nerve (GFNx) (Lucio et al., 2001) that innervates this tissue (Zempoalteca et al., 2002). For the BM, we sympathectomized mice through unilateral surgical ablation of the superior cervical ganglion (SCGx) (Alito et al., 1987), which resulted in ptosis on the denervated side (Figure S4A), as seen in Horner's syndrome (Walton and Buono, 2003). Denervation was confirmed via whole-mount immunofluorescence staining for the sympathetic-nerve marker tyrosine hydroxylase (TH) (Zhou et al., 1995), which revealed marked reductions in the number of TH^{+} nerve fibers in the neurectomized sides of the tissues compared to the sham-operated contralateral sides (Figures 4A and 4B). Thus, these approaches allowed us to investigate the role of local innervation in nerve-intact and denervated tissues of the same mouse.

Whereas brightfield intravital microscopy (BIM) studies of the cremasteric microvasculature revealed no circadian oscillations in leukocyte rolling (Figure 4C), leukocyte adhesion (Figure 4D) and extravasation (Figure 4E) were significantly elevated at night in contralateral muscle tissues, with no oscillations observed in the denervated side. In addition, GFNx completely abolished the nightly increase in ICAM-1 expression (Figure S4B), suggesting that the nighttime surge in ICAM-1 is controlled locally by adrenergic sympathetic-nerve fibers. Adoptively transferred BM cells in SCGx animals exhibited circadian fluctuations in the numbers of adherent cells in the nerve-intact side, whereas the denervated side did not show any oscillations (Figure 4F). No circadian differences were observed in hemodynamic parameters, tissue weight, or vascular density in neurectomized animals (Figures S4C–S4E and Table S2). Together, these studies demonstrate the importance of local innervation for circadian oscillations in hematopoietic cell recruitment to both skeletal muscle and BM.

Signals from the SNS are transmitted from the brain to peripheral tissues by norepinephrine through adrenoreceptors (Elenkov et al., 2000). To examine which adrenergic receptors were important for the circadian regulation of hematopoietic cell trafficking, we adoptively transferred fluorescently labeled WT BM cells into β2- or β3-adrenoreceptor (*Adrb2* or *Adrb3*)-deficient recipients. We found that circadian oscillations in cell

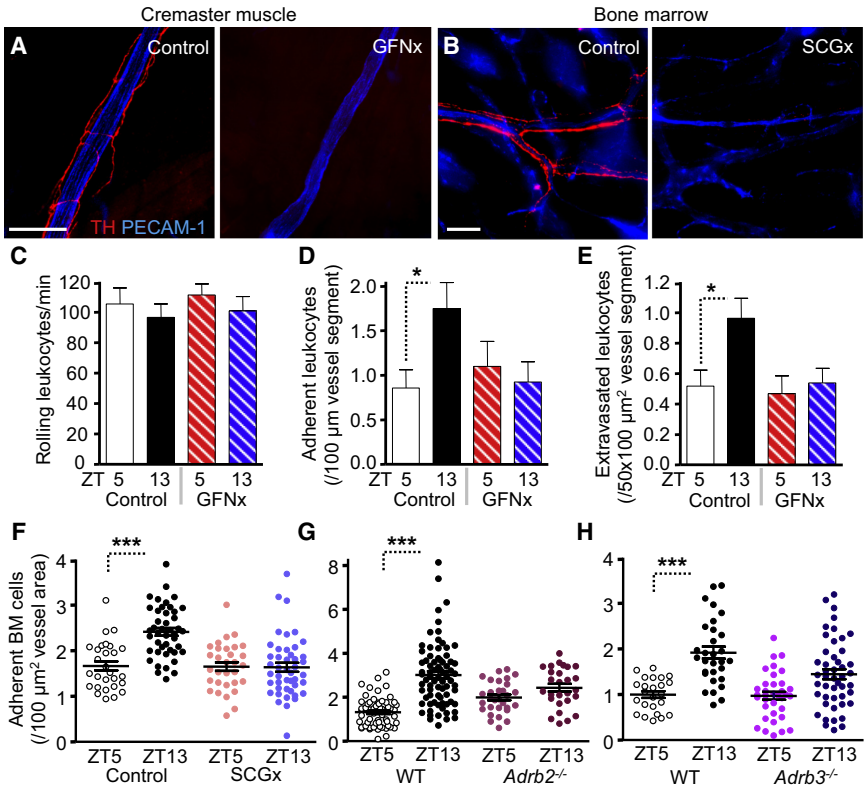

Figure 4. Requirements of Local Adrenergic Nerves

(A–F) Unilateral surgical denervation of the genitofemoral nerve (GFNx) or the superior cervical ganglion (SCGx).

(A and B) Images of TH^+ nerve fibers (red) and associated vessels (PECAM-1, blue) in cremaster muscle (A) or calvarium (B) 4 weeks after GFNx or SCGx of the contralateral control and operated sides.

(C–E) BIM microscopy of exteriorized cremaster-muscle tissues quantifying rolling (C), adhesion (D), and transmigration (E) after GFNx. n = 28–40 vessels from 3–4 mice per group.

(F–H) Quantification of adherent fluorescently labeled cells to BM sinusoids after SCGx (F) or in *Adrb2*$^{-/-}$ (G) or *Adrb3*$^{-/-}$ (H) mice after adoptive transfer. n = 28–45 vessels from 5–6 mice per group.

*p < 0.05, ***p < 0.001. Scale bars represent 100 µm. See also Figure S4 and Table S2.

recruitment to the BM were significantly reduced in both recipient strains (Figures 4G and 4H). In addition, circadian differences in the expression of BM endothelial cell adhesion molecules were ablated in these mice (Figures S4F and S4G). Similarly, no circadian oscillations were observed in the numbers of extravascular leukocytes in the cremaster muscle of *Adrb2*$^{-/-}$ or *Adrb3*$^{-/-}$ animals at steady state (Figures S4H and S4I), and BM chimeras for both strains revealed the critical role of non-hematopoietic β2- and β3-adrenoreceptors in circadian leukocyte recruitment to this tissue (Figures S4J–S4L). Furthermore, the nighttime surge in ICAM-1 expression was averted when mice were treated with the β3-adrenoreceptor-specific antagonist SR59230A (Figure S4M). These data clearly indicate that the endothelial oscillations in adhesion-molecule expression require local delivery of adrenaline and signaling through β-adrenoreceptors.

Biological Relevance of Circadian BM Recruitment

We next examined whether the circadian regulation of hematopoietic cell recruitment to the BM could be exploited in the setting of BM transplantation. The injection of lethally irradiated mice with limiting numbers of BM cells (25,000 cells) led to marked differences in survival. Whereas most animals transplanted in the morning succumbed to death due to hematopoietic failure (Figure S5A), all animals survived the procedure when transplanted at ZT13 (Figures 5A and 5B). To test whether mimicking an enhanced adrenergic tone at night could emulate this phenotype, we treated mice with the pan-β-adrenoreceptor agonist isoproterenol, which significantly upregulated expression of P- and E-selectins and VCAM-1, but not ICAM-1, in irradiated recipients (Figure 5C). After isoproterenol treatment, the number of recruited donor BM cells and progenitor cells was significantly increased compared to that of the control (Figures 5D and 5E), whereas this effect was abrogated in *Selp*$^{-/-}$*Sele*$^{-/-}$ or anti-VCAM-1-treated animals (Figures 5E and 5F). Furthermore, survival was markedly improved in the isoproterenol-treated animals when limiting numbers of BM cells were injected for transplantation (Figure S5B). To determine whether this effect was dependent on Adrβ2 or Adrβ3, we treated mice with specific agonists (clenbuterol or BRL37344, respectively). We found that only BRL37344 exhibited a similar effect to that of isoproterenol (Figures 5G and S5A and data not shown), indicating that β3-adrenoreceptor signaling is sufficient to promote hematopoietic recruitment to the BM. BRL37344 treatment also enhanced the homing of long-term repopulating HSCs (LT-HSCs) (Figure 5H). Because we performed BM transplantation 24 hr after treatment with isoproterenol or BRL37344, the enhanced recruitment was probably due to an effect on the stroma rather than on the infused BM cells. We found no differences in CXCL12 levels between groups at the time of BM transplantation (Figures S5C and S5D). These data suggested that transplantation performed at night or after pharmacological treatment with β3 agonists could potentially reduce the number of HSPCs needed for successful long-term engraftment.

Circadian Rhythms in Leukocyte Recruitment Are Entrained by Photic Cues

Circadian rhythms are entrained by light in that photic input is interpreted by suprachiasmatic nuclei, the fundamental timekeeper of the mammalian clock (Klein et al., 1991; Ralph et al., 1990). We thus tested whether alterations in photic input by induction of an experimental jet lag would be sufficient to modify rhythms in leukocyte recruitment. Advancing the light regime by 12 hr after the light phase (Figure 6A) completely abolished the circadian rhythms in leukocyte recruitment to skeletal

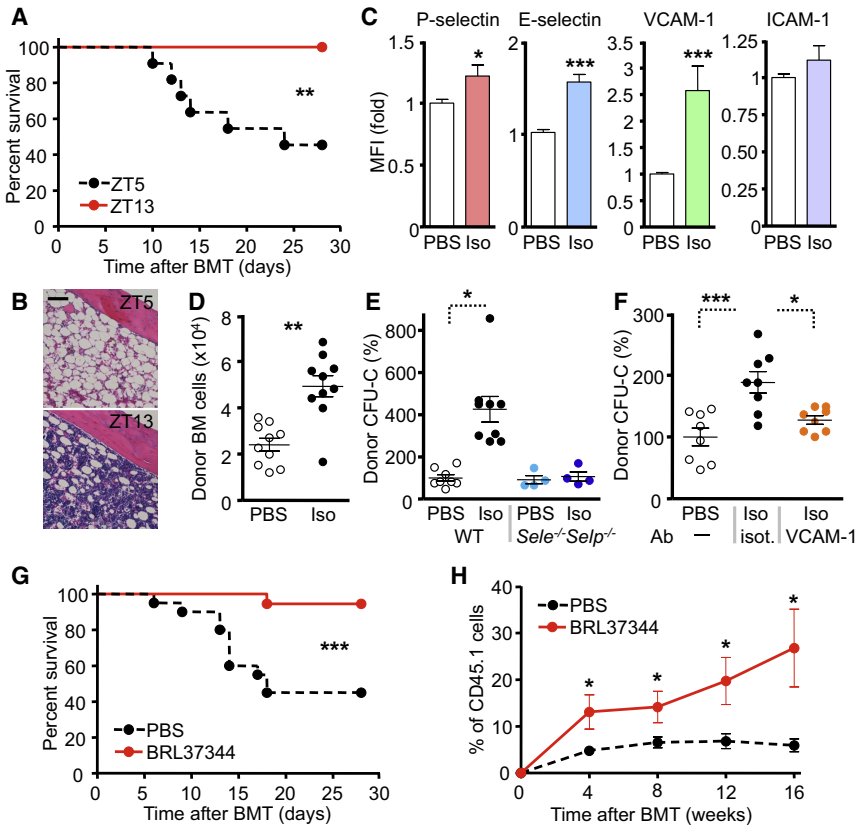

Figure 5. β-Adrenergic Stimulation Enhances Hematopoietic Progenitor Cell Reconstitution after Transplantation

(A) Survival curves after circadian BM transplantation (BMT) with limiting numbers of BM cells (2.5×10^4) into lethally irradiated recipients. n = 10.

(B) Representative micrographs of hematoxylin and eosin (H&E)-stained sections 30 days after circadian BMT.

(C) Flow-cytometric analysis of endothelial cell adhesion molecule expression after PBS or isoproterenol (iso) treatment in recipients. n = 5. MFI, mean fluorescence intensity.

(D) Quantification of donor BM cells that homed to the BM of recipients treated with PBS or iso. n = 10.

(E and F) Quantification of donor CFU-C that homed to the BM of recipients treated with PBS or iso in WT or *Selp*$^{-/-}$*Sele*$^{-/-}$ mice (E) or after treatment with an isotype (isot.) or anti-VCAM-1 blocking antibody (F). n = 4–9.

(G) Survival curves after transplantation with limiting numbers of BM cells (2.5×10^4) into lethally irradiated recipients pretreated with PBS or BRL37344. n = 18–20.

(H) Effect of BRL37344 on homing of LT-HSCs. n = 6.

*p < 0.05, **p < 0.01, ***p < 0.001. The scale bar represents 100 μm. See also Figure S5.

muscle (Figure 6B) and the oscillations in endothelial ICAM-1 expression (Figure 6C). Experimental jet lag also eliminated the increased homing to the BM at night (Figure 6D). To assess further the role of the molecular clock in the circadian fluctuations of leukocytes, we evaluated the role of BMAL-1, a key transcription factor required to entrain circadian oscillations (Levi and Schibler, 2007). We found that circadian oscillations of leukocyte numbers in both blood and tissues were completely abolished in *Bmal1*$^{-/-}$ animals kept in constant darkness for three weeks, in contrast to the normal oscillations observed in heterozygous and WT littermates (Figures 6E–6G). These results underscore the importance of light as an environmental cue required for synchronizing the clock machinery that entrains rhythms in leukocyte recruitment to tissues.

Circadian Time Influences Leukocyte Recruitment in Inflammation

To assess whether circadian leukocyte recruitment could alter the inflammatory response, we injected mice with tumor necrosis factor alpha (TNF-α) and analyzed cremaster-muscle tissues 8 hr later, either at ZT5 or ZT13. Whereas leukocyte rhythms in blood were significantly blunted after stimulation (Figure 6I, left bars), neutrophil infiltration showed a dramatic increase compared to PBS-treated animals (Figures 6H, left panel and 6J, left bars). Circadian rhythms in leukocyte recruitment remained intact in inflammation, with ZT13 showing significantly higher neutrophil infiltration than ZT5 (Figures 6H, left and 6J, left). In addition, ICAM-1 expression in endothelial cells was significantly increased after TNF-α stimulation and exhibited circadian oscillations (Figure 6K, left bars). We also observed induction of endothelial cell adhesion molecule expression (P- and E-selectin, VCAM-1, and *Cd44)* as well as induction of chemokines (*Ccl2*, *Ccl5*, *Cxcl1*, and *Cxcl2)* after TNF-α stimulation (Figures S6A–S6H), but no rhythm was detectable. *Icam2*, *Ccl3*, and *Cx3cl1* levels were not altered after inflammation and did not exhibit oscillations (Figures S6I–S6K).

When we induced jet lag and treated mice with TNF-α using the same protocol, circadian rhythms in white blood cell (WBC) counts were inhibited (Figure 6I, right bars) and neutrophil infiltration was almost completely abolished (Figures 6H, right panel and 6J, right bars), whereas the expression of endothelial cell adhesion molecules was dramatically reduced compared to controls kept under a normal light regime (Figure 6K, right bars and Figures S6A–S6C). These experiments further illustrate the significance of circadian time and light in leukocyte recruitment under both steady-state and inflammatory conditions.

Inflammatory Leukocyte Recruitment Oscillates in Sickle Cell Disease

We next aimed to evaluate the relevance of circadian leukocyte recruitment in inflammation in models of sickle cell disease (SCD) and septic shock, pathologies in which neutrophil recruitment has been shown to play a critical role (Frenette and Atweh, 2007; Hewett et al., 1992; Thomas et al., 1992). SCD mice challenged with a TNF-α-induced model of vaso-occlusion (Figure S7A) exhibited significantly increased leukocyte recruitment to the cremaster muscle when the experiment was performed at night compared to during the daytime (Figures S7B and S7C). In addition, adherent leukocytes showed marked elevations in Mac-1-integrin activation at night (Figure S7D), a key molecule

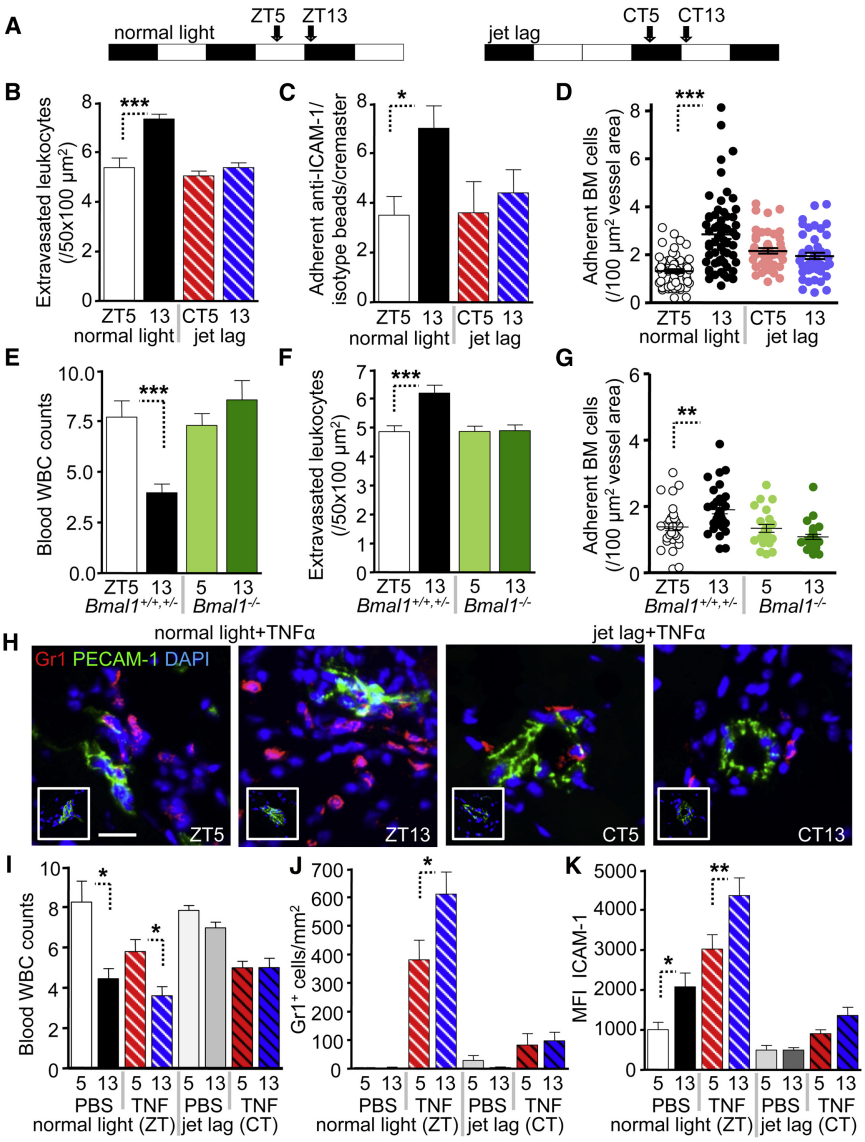

Figure 6. Circadian Rhythms in Leukocyte Recruitment Are Entrained by Photic Cues

(A) Light regime under normal and jet-lag conditions as entrained using a light cycler.

(B) Quantification of extravasated CD45$^+$ leukocytes in the cremaster muscle as analyzed by whole-mount immunofluorescence staining in mice under normal-light and jet-lag conditions. n = 3–6.

(C) Quantification of specific versus nonspecific sphere adhesion in hematopoietic *Icam1*$^{-/-}$→WT BM chimeras under normal-light and jet-lag conditions. n = 5–6.

(D) Quantification of adherent fluorescently labeled cells to BM sinusoids in mice under normal-light and jet-lag conditions. n = 46–61 areas quantified from 4–5 mice per group.

(E–G) Circadian oscillations in *Bmal1*$^{-/-}$ mice and control littermates after 3 weeks of constant darkness.

(E) Blood leukocyte counts (× 10^3/µl). n = 4–8.

(F) Numbers of extravasated CD45$^+$ leukocytes as analyzed by whole-mount immunofluorescence staining of cremaster-muscle tissues. n = 24–30 vessels from 4 mice per group.

(G) Numbers of adherent fluorescently labeled cells to BM sinusoids after adoptive transfer. n = 22–31 areas quantified from 3 mice per group.

(H–J) Representative images (PBS in insets) (H), blood leukocyte counts (× 10^3/µl) (I), and quantification of TNFα-induced neutrophil infiltration in sections of the cremaster muscle (J) under normal-light and jet-lag conditions. n = 3–9.

(K) Quantification of ICAM-1 protein expression in frozen sections harvested from the same mice. MFI, mean fluorescence intensity.

CT, circadian time. *p < 0.05, **p < 0.01, ***p < 0.001. The scale bar represents 50 µm. See also Figure S6.

mediating heterotypic interactions between activated adherent leukocytes and circulating red blood cells (RBCs) (Hidalgo et al., 2009). This translated to a ~2-fold increase in WBC-RBC interactions (Figure S7E) and a significant decrease of the mean venular-blood-flow rates (Figure S7F). Ultimately, the overall survival of SCD mice was significantly reduced at night. Lethality was due to disseminated intravascular coagulation, as evidenced by microthrombi in the lungs as well as scattered foci of liver necrosis (Figures S7G–S7I and data not shown). Importantly, we did not observe circadian differences in TNF-receptor expression at the investigated time points (Figure S7J). These data indicate that circadian leukocyte recruitment can exacerbate sickle cell vaso-occlusion and compromise survival.

Circadian Time Governs Leukocyte Recruitment and Survival in Septic Shock

To gain further insight into the relevance of circadian leukocyte recruitment to vital organs, we performed adoptive transfer studies using BM harvested from Vav1-*Cre;Rosa26*-lsl-*Luc* mice expressing luciferase selectively in hematopoietic cells. Consistent with our studies in muscle and BM, we observed strong recruitment of adoptively transferred cells to the liver that had a circadian rhythm peaking at night (Figure 7A). These rhythms coincided with oscillations in endothelial ICAM-1 and VCAM-1 expression (Figure 7B), whereas expression of endothelial selectins was unchanged (data not shown). To test further the relevance of these fluctuations, we challenged mice with a lethal dose of endotoxin (lipopolysaccharide [LPS]) in the morning or at night. Consistent with our results with TNF-α, both adhesion-molecule expression (Figure 7C) and leukocyte recruitment exhibited strong oscillations, with neutrophil infiltration into liver peaking when LPS was administered at night (Figures 7D–7F). In contrast, diurnal variations in neutrophil recruitment to this tissue were ablated in *Icam1*$^{-/-}$ animals (Figures 7D–7F). Similarly to TNF-receptor expression, we also did not observe circadian rhythms of TLR4 (the principal receptor for LPS) at these time points (data not shown). In line with prior studies (Feigin et al., 1969; Halberg et al., 1960), survival was greatly reduced when mice were

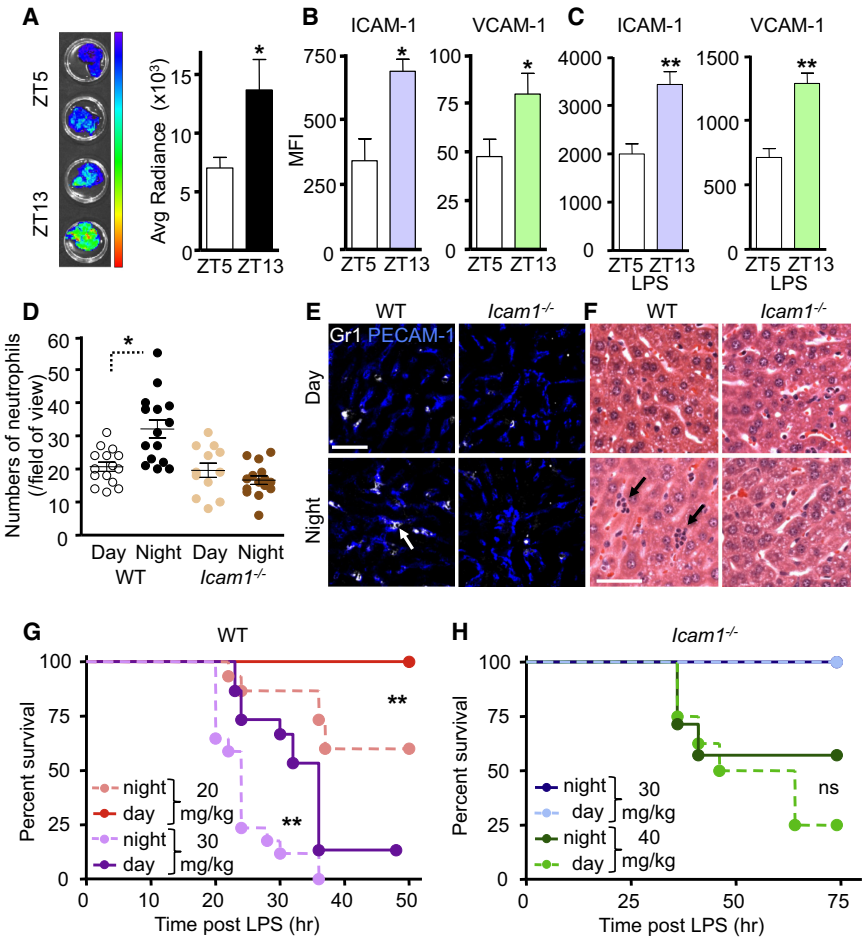

Figure 7. Circadian Time Influences Leukocyte Recruitment in Septic Shock

(A) Bioluminescence imaging and quantifications of harvested liver after adoptive transfer of *Vav-Cre:luciferase*-expressing BM cells to recipients at ZT5 or ZT13. n = 7.

(B and C) Quantification of endothelial cell adhesion molecule expression levels in liver at steady state (B) and after 12 hr stimulation with LPS (C) by immunofluorescence confocal imaging of frozen tissue sections. n = 4–7. MFI, mean fluorescence intensity.

(D) Quantification of LPS-induced neutrophil infiltration in frozen liver sections of WT or *Icam1*$^{-/-}$ animals. n = 6.

(E and F) Representative images of neutrophil infiltration in liver sections as assessed by immunofluorescence confocal imaging (E) and H&E staining (F) showing neutrophils clusters (arrows).

(G and H) Survival curves of WT (G) and *Icam1*$^{-/-}$ (H) animals after LPS-induced septic shock. n = 7–17.

*p < 0.05, **p < 0.01. Scale bars represent 50 μm. See also Figure S7.

challenged with LPS in the night compared to the morning (Figure 7G). Mortality was highly dependent on leukocyte recruitment, because *Icam1*$^{-/-}$ animals were significantly more resistant to LPS-induced lethality (Xu et al., 1994) (Figure 7H). Additionally, the impaired survival of night-challenged animals was abrogated in *Icam1*$^{-/-}$ mice, indicating that oscillating ICAM-1 levels are critical for the observed diurnal rhythm (Figure 7H). *Icam*$^{+/-}$ animals exhibited an intermediate phenotype of survival compared to WT and *Icam1*$^{-/-}$ mice, indicating the functional importance of the differences in ICAM-1 expression observed in this model (Figure S7K). Taken together, these results demonstrate that circadian rhythms in leukocyte recruitment can directly impact the outcome of inflammatory diseases.

DISCUSSION

Here, we show that the emigration of leukocytes to tissues is regulated by signals from the autonomic nervous system and that the peak recruitment occurs at night in rodents, during a period of activity. Circadian hematopoietic cell recruitment is synchronized by the molecular clock via sympathetic nerves, which induce through β-adrenoreceptors oscillations in endothelial cell expression of ICAM-1 and CCL2 in skeletal muscle and endothelial selectins, VCAM-1, and CXCL12 (Méndez-Ferrer et al., 2008) in the BM. In addition, our data suggest that rhythmic leukocyte recruitment is not restricted to these tissues but occurs in many vital organs. The difference in the molecular signature of circadian expression of endothelial cell adhesion molecules and chemokines in tissues is probably responsible for the diurnal preference in hematopoietic cell populations recruited to various organs.

Changes in the light cycle, a major zeitgeber for circadian rhythms (Golombek and Rosenstein, 2010), were sufficient to ablate oscillations in hematopoietic cell recruitment. This finding, along with the involvement of BMAL-1, strongly indicates genuine circadian rhythms and suggests their central orchestration by the molecular clock (Dibner et al., 2010; Green et al., 2008). Additionally, the surprising anti-inflammatory effect seen after acute jet lag suggests a potent and broad interference of light in the normally rhythmically generated inflammatory response. It is possible that chronic jet lag may perturb circadian immunosurveillance mechanisms and contribute to the higher incidence of cancer in shift workers (Filipski et al., 2004). How jet lag exerts these broad changes in the inflammatory response should be the subject of future investigations.

Most prior studies of the regulation of circadian rhythms have focused on the involvement of humoral factors, notably of glucocorticoid (GC) levels, which peak at night in mice and reach trough values during the day (Dickmeis, 2009). Given that GCs exhibit anti-inflammatory properties, we would expect that GC hormones might antagonize the rhythms described herein, which depend on local innervation. It is possible, however, that neural and hormonal pathways complement each other to fine-tune the physiological and inflammatory responses (Elenkov et al., 2000). Thus, higher levels of GC at night in mice may in fact keep the nighttime surge in leukocyte infiltration in check.

Although catecholamine treatment of donor progenitor cells in vitro enhanced their engraftment ability through the β2-adrenoreceptor (Spiegel et al., 2007), we have excluded a role for adrenergic receptors on transplantable hematopoietic cells in our studies using radiation chimeras and adoptive-transfer experiments. Therefore, endothelial cells in BM and skeletal muscle, which can express both the β2- and β3-adrenoreceptors (Steinle et al., 2003), probably represent a logical cellular target, given that they also express the adhesion molecules necessary to mediate hematopoietic cell-endothelial cell interactions. Our results indicate that BM transplantation performed after stimulation with β3-adrenergic agonists in the recipient may improve clinical outcomes through enhanced engraftment efficiency. Given that adrenergic stimulation can also induce the mobilization of HSPCs (Méndez-Ferrer et al., 2008), how the cells targeted by adrenergic signals interpret these signals at different times remains unclear. The fact that the same adrenergic receptors can lead to distinct adhesion-molecule expression in different vascular beds suggests tissue-specific signaling responses that remain undefined. The present findings are consistent with previous studies in which circadian oscillations of BM CXCL12 with trough expression levels of the chemokine occurring in the morning were associated with the peak of HSC egress (Méndez-Ferrer et al., 2008). It is probable that the nightly increase of CXCL12 in the BM cooperates with endothelial upregulation of adhesion molecules for maximal hematopoietic cell homing in the evening. In the BM, β3-adrenoreceptor expression is enriched in rare Nestin^{+} perivascular mesenchymal stem cells, which express high amounts of CXCL12 and are targeted by the SNS in the stem cell niche (Méndez-Ferrer et al., 2010). How microvascular pericytes collaborate with endothelial cells to entrain rhythms in extramedullary tissues will be the subject of future studies in the laboratory.

The rhythmic pattern of leukocyte recruitment may have evolved for the benefit of having readily available tissue phagocytes for enhancement of the response to pathogens during periods of activity, when injuries and encounters with microorganisms are more likely to occur. Although macrophages represent the major leukocyte subset oscillating under homeostasis, we show that neutrophil recruitment after trauma or in the setting of inflammatory diseases is significantly influenced by endogenous rhythms of endothelial cell adhesion. It is conceivable that the diurnal clearance of senescent neutrophils by the BM and liver is mediated by these circadian mechanisms. It is possible that the HSPC pool is rejuvenated by diurnal migratory incentives. We have presented clear evidence, however, that circadian timing can influence the engraftment efficiency when HSPC counts are suboptimal, demonstrating the significance of circadian hematopoietic cell recruitment to the BM. As such, given the inverted rhythms between rodents and humans (Lucas et al., 2008), we would predict that human HSC transplantation is optimal early in the morning, rather than later in the day.

We postulate that the diurnal mechanisms of keeping neutrophil numbers in check may affect cardiovascular diseases, which are influenced by circadian time, given that their levels are known to correlate positively with the degree of severity (Coller, 2005; Muller et al., 1985). When exaggerated, these mechanisms may exert detrimental effects. Indeed, in models of TNF-α-induced vaso-occlusion in SCD mice and LPS-induced lethality, we have found that circadian rhythms modulate the robustness of the inflammatory response, with implications for survival. Whereas we recognize potential mechanistic differences between atherosclerosis and inflammatory models studied herein, these data are in line with prior studies that have shown persistent oscillations in the susceptibility of mice to bacterial infection and inflammatory leukocyte trafficking (Castanon-Cervantes et al., 2010; Feigin et al., 1969; Gibbs et al., 2012; Halberg et al., 1960; House et al., 1997; Keller et al., 2009; Liu et al., 2006; Shackelford and Feigin, 1973; Silver et al., 2012). Sensitivity to inflammatory stimuli at times of higher SNS tone probably interacts at multiple levels, encompassing local neural input as we describe herein and endogenous rhythms within tissues, as well as interactions with humoral factors and the parasympathetic nervous system.

Given the reported critical role of blood leukocytes in ischemic vascular diseases, the present results argue that circadian leukocyte adhesion or an exaggeration thereof might contribute to triggering acute vascular diseases in the morning. Our own as well as published studies have clearly shown that leukocyte recruitment promotes LPS-induced lethality (Xu et al., 1994) and SCD vaso-occlusion (Hidalgo et al., 2009; Turhan et al., 2002), which led us to evaluate experimentally the effect of circadian time in inflammatory diseases. Our results provide the proof of principle that time-dependent differences in leukocyte recruitment translate into significant changes in survival. Thus, a better understanding of the rhythms in leukocyte migration will allow the design of targeted chronotherapy that may lead to meaningful clinical impacts on disease outcome.

EXPERIMENTAL PROCEDURES

Animals

Bmal1$^{-/-}$ (gift from C.A. Bradfield), *Adrb2*^{tm1Bkk} (gift from G. Karsenty), and Berkeley SCD mice [Tg(Hu-miniLCRα1$^{G}\gamma^{A}\gamma\delta\beta^{S}$) *Hba*$^{-/-}$ *Hbb*$^{-/-}$] (gift from N. Mohandas); *Sele*$^{-/-}$*Selp*$^{-/-}$, FVB/N-*Adrb3*^{tm1Lowl}/J, *Icam1*^{tm1Jcgr}/J, *Vav1-cre*^{A2Kio}/J, and STOCK Tg(TIE2GFP)287Sato/J mice (all from Jackson Laboratories); and *ROSA26Sor*$^{tm2(ACTB-Luc)Tyj}$ and the inbred FVB/NJ and C57BL/6-CD45.1/2 congenic strains (all from the National Cancer Institute) were used in this study. See Supplemental Experimental Procedures for references and details. All mice used were males, housed on a 12 hr light/dark cycle (lights on/off at 7 a.m./7 p.m.) with food ad libitum. All experimental procedures were approved by the Animal Care and Use Committees of Albert Einstein College of Medicine and Mount Sinai School of Medicine.

Reagents

Details can be found in Supplemental Experimental Procedures.

Whole-Mount Immunofluorescence

Whole-mount immunostaining of cremaster-muscle and BM tissues was performed as previously detailed (Scheiermann et al., 2007). See Supplemental Experimental Procedures for details.

Intravital Microscopy

BIM determination of leukocyte-vessel wall interactions in murine cremasteric venules, hemodynamic characteristics, and image analyses were studied as previously detailed (Chang et al., 2008; Scheiermann et al., 2009). For MFIM, animals were prepared as previously detailed for the cremaster muscle (Chiang et al., 2007) and the BM (Mazo et al., 1998). See Supplemental Experimental Procedures for details.

Surgical Denervation Techniques

The genitofemoral nerve (Lucio et al., 2001) and the superior cervical ganglion (Alito et al., 1987; Walton and Buono, 2003) were dissected as described. See Supplemental Experimental Procedures for details.

BM Transplantation and Generation of Chimeric Mice

Transplantation procedures were carried out using congenic CD45.1/2 mice. Chimeric mice were generated by i.v. injection of 1×10^6 donor mice BM nucleated cells into lethally irradiated (12 Gy, split dose, 3 hr apart) mice as previously detailed (Katayama et al., 2006). For survival studies, the recovery of donor mature WBC in peripheral blood and survival were monitored as indicated. In some experiments, recipient mice were irradiated with 4 Gy of sublethal irradiation, and donor-recipient chimerism in mature peripheral leukocytes were analyzed via flow cytometry.

Flow Cytometry, Sorting, and qPCR

Tissues were harvested from Tie2-GFP animals, minced, and digested in type IV collagenase (*Clostridium histolyticum*, Sigma-Aldrich). Single-cell suspensions were stained with DAPI and fluorescence-conjugated antibodies and analyzed through flow cytometry using an LSRII (BD Biosciences). Data were analyzed with FlowJo (Tree Star) or FACSDiva 6.1 software. Endothelial cells were sorted into qPCR Dynabeads lysis buffer (Invitrogen) and processed with SYBR Green as previously described (Méndez-Ferrer et al., 2008). Details can be found in Supplemental Experimental Procedures.

Adoptive Transfer of Labeled BM Cells

BM cells were labeled with carboxyfluorescein succinimidyl ester (Invitrogen) per the manufacturer's instructions and injected into nonirradiated recipient mice (at ZT4 and ZT12). Cells were allowed to circulate for 1 hr. The trafficking of labeled BM cells was assessed via MFIM of the BM immediately after 10 μg of rhodamine 6G (R6G) was injected for visualization of BM sinusoids. In some experiments, BM cells were labeled with VT680 (VisEn Medical). The subsets of adoptively transferred cells in the recipient BM were analyzed via flow cytometry.

Homing Assay of HSPCs

Experiments were performed as previously described (Katayama et al., 2003). See Supplemental Experimental Procedures for details.

β-Adrenergic Agonist Treatment

Isoproterenol, or the β3-adrenergic agonist BRL37344, or the β2 adrenergic agonist clenbuterol (5 mg/kg) was injected intraperitoneally for 5 days, and an in vitro homing assay or BM transplantation was performed 24 hr after the last injection to minimize the direct effect of the drugs on the injected donor cells.

CXCL12 ELISA

Details can be found in Supplemental Experimental Procedures.

Induction of Light Regime

For inducing changes in light regime, mice were placed in a light cycler (Park Bioservices) with a 12-hr-inverted light cycle for 12 hr (to induce jet lag) or for a minimum of 2 weeks (to completely establish an inverse light cycle), or were kept for 3 weeks in complete darkness.

Histopathology

Details can be found in Supplemental Experimental Procedures.

Statistics

All data are represented as mean ± SEM. Comparisons between two samples were done using the paired and unpaired Student's t tests. One-way ANOVA analyses followed by Tukey's multiple comparison tests were used for multiple group comparisons. Statistical analyses were performed with GraphPad Prism 5. Log rank analyses were used for Kaplan-Meier survival curves. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.05.021.

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7. Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die schriftliche Habilitationsleistung selbständig verfasst und die Herkunft des verwendeten oder zitierten Materials ordnungsgemäß kenntlich gemacht habe.

München, den 08. Juni 2018
Dr. Christoph Scheiermann