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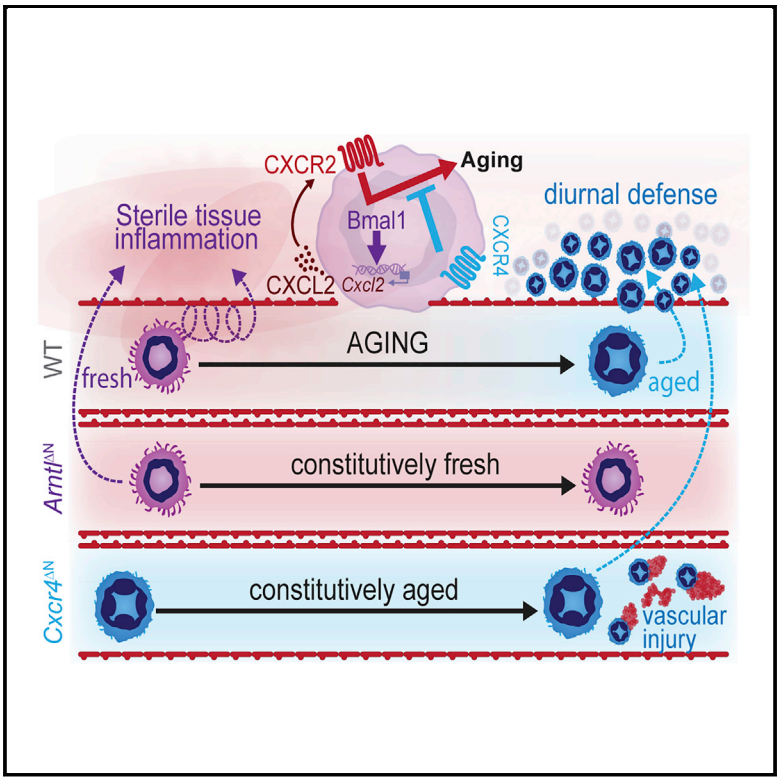
Immunity

A Neutrophil Timer Coordinates Immune Defense and Vascular Protection

Graphical Abstract



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In Brief

Neutrophils display circadian oscillations in numbers and phenotype in the circulation. Adrover and colleagues now identify the molecular regulators of neutrophil aging and show that genetic disruption of this process has major consequences in immune cell trafficking, anti-microbial defense, and vascular health.

Highlights

- Neutrophil aging is an intrinsically driven, bona fide circadian process
- Bmal1 and CXCR2 induce neutrophil aging, whereas CXCR4 antagonizes it
- Diurnal aging critically dictates how and when neutrophils migrate into tissues
- Aging favors neutrophil clearance, thereby protecting the cardiovascular system

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A Neutrophil Timer Coordinates Immune Defense and Vascular Protection

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SUMMARY

Neutrophils eliminate pathogens efficiently but can inflict severe damage to the host if they over-activate within blood vessels. It is unclear how immunity solves the dilemma of mounting an efficient anti-microbial defense while preserving vascular health. Here, we identify a neutrophil-intrinsic program that enabled both. The gene *Bmal1* regulated expression of the chemokine CXCL2 to induce chemokine receptor CXCR2-dependent diurnal changes in the transcriptional and migratory properties of circulating neutrophils. These diurnal alterations, referred to as neutrophil aging, were antagonized by CXCR4 (C-X-C chemokine receptor type 4) and regulated the outer topology of neutrophils to favor homeostatic egress from blood vessels at night, resulting in boosted anti-microbial activity in tissues. Mice engineered for constitutive neutrophil aging became resistant to infection, but the persistence of intravascular aged neutrophils predisposed them to thrombo-inflammation and death. Thus, diurnal compartmentalization of neutrophils, driven by an internal timer, coordinates immune defense and vascular protection.

INTRODUCTION

The capacity of neutrophils not only to kill pathogens but also to inflict severe damage to tissues suggests the existence of a protective mechanism that balances these opposing functions. Both anti-microbial immunity and vascular inflammation are known to follow circadian patterns (Man et al., 2016; Muller et al., 1989; Scheiermann et al., 2013), suggesting that such mechanisms may be temporally regulated. The nature of this mechanism, however, remains enigmatic.

While studying neutrophils in the steady state, we previously identified a natural phenotypic shift of circulating neutrophils that followed a strict diurnal regime (Casanova-Acebes et al., 2013). Neutrophils released from the bone marrow display high CD62L that is progressively reduced during the day, while surface CXCR4 (C-X-C chemokine receptor type 4) increases prior to their natural egress from blood, a process referred to as clearance (Casanova-Acebes et al., 2013). This process of neutrophil aging has been proposed to be regulated by the gut microbiota and to favor a pro-inflammatory phenotype that predisposes to vascular inflammation (Zhang et al., 2015).

Contrasting with this model of extrinsically driven neutrophil aging, studies have shown that intrinsic programs controlled by the molecular clock also regulate immune cell properties (Druzd et al., 2017; Nguyen et al., 2013; Silver et al., 2012). Because the mechanisms regulating aging and its physiological consequences remain uncertain, we explored whether neutrophils are endowed with an intrinsic program that controls diurnal aging, tunes their anti-microbial functions, and limits vascular inflammation.

In transcriptome analyses of circulating neutrophils performed at different times, we found regulation of clock-related genes and the CXCR2 signaling pathway. Bmal1 (brain and muscle aryl hydrocarbon receptor nuclear translocator [ARNT]-like 1; encoded by *Arntl*) regulated expression of CXCL2 (chemokine [C-X-C motif] ligand 2), a CXCR2 ligand that controlled neutrophil aging in a cell-autonomous manner. Deletion of *Arntl* or *Cxcr2* from neutrophils prevented phenotypic aging, whereas deletion of *Cxcr4*, a negative regulator of CXCR2 signaling, resulted in unrestrained aging. Neutrophil aging disrupted cytoskeletal integrity to specifically prevent rolling and accumulation in inflamed areas without affecting homeostatic migration into naive tissues at night. In turn, this temporal regulation of trafficking regulated diurnal responses to infections, while at the same time removing neutrophils from the bloodstream, thereby protecting vessels from inflammatory injury. This process may underlie the circadian susceptibility of mammals to cardiovascular disease.

RESULTS

A Neutrophil-Intrinsic Timer Drives Diurnal Aging

In a small cohort of healthy volunteers, we found diurnal changes in neutrophil markers similar to those associated with neutrophil aging in mice (Casanova-Acebes et al., 2013), suggesting conservation of this phenomenon across species (Figure S1A). In healthy mice, the number of aged neutrophils in blood follows diurnal patterns with a peak at around zeitgeber time 5 (ZT5, i.e., 5 h after lights on), while non-aged or "fresh" neutrophils predominate at ZT13 (Casanova-Acebes et al., 2013). These diurnal patterns persisted in constant darkness and could be entrained by light shift (Figures S1B and S1C), indicating that neutrophil aging is a bona fide circadian process. To identify genetic programs that were temporally regulated in neutrophils, we compared the transcriptomes of circulating neutrophils purified from wild-type (WT) mice at these two times. We identified changes in over 1,300 genes related to pathways of inflammation, migration, and apoptosis (Figures 1A, 1B, and S1D; Table S1), which suggested modulation of these processes during the day. Given the diurnal pattern of aging, we inspected genes of the molecular clock because they are known to regulate immune rhythms (Man et al., 2016; Scheiermann et al., 2013). Expression of clock-related genes, including *Arntl* (encoding Bmal1) and *Clock*, increased at ZT5, while others, like *Per2*, were decreased at this time (Figures 1B, S1D, and S1E). Transcriptional analyses at multiple times of day revealed circadian oscillations for all these genes in circulating neutrophils (Figure 1C). They also demonstrated reduced expression of *Sell* (encoding CD62L) at ZT5 but no changes for *Cxcr4* (Figure 1B). We also noticed reduced expression of *Cxcr2*, whose expression displayed diurnal patterns (Figure 1D), at ZT5 (Figure 1B). Further, CXCR2 agonists induced phenotypic changes in neutrophils that resembled those seen during natural aging, namely reductions in CXCR2 and CD62L on the cell surface (Figures 1E and S1F).

Guided by the temporal expression patterns of these genes, we predicted that Bmal1 and CXCR2 might be required for diurnal neutrophil aging. Although CXCR4 did not present transcriptional oscillations (Figure 1D), its presence on the cell surface changed diurnally (Figure 1E) (Casanova-Acebes et al., 2013), and this receptor is known to antagonize CXCR2 signaling (Martin et al., 2003), suggesting that CXCR4 might also contribute to aging. Analyses of blood from wild-type animals revealed ligands for both receptors in plasma, with oscillating amounts of CXCL12, and constitutively low amounts of CXCL2 (Figure 1F).

To formally test the possibility that these genes regulated neutrophil aging, we generated mice with neutrophil-specific deficiency in *Arntl*, *Cxcr2*, or *Cxcr4* (herein referred to as $Arntl^{\Delta N}$, $Cxcr2^{\Delta N}$, and $Cxcr4^{\Delta N}$) by using the $hMRP8^{cre}$ driver line, which resulted in robust depletion of the receptors from the surface of neutrophils (Figures S2A and S2B). Immunoblot analysis of the Bmal1 protein confirmed efficient depletion in $Arntl^{\Delta N}$ neutrophils and revealed natural reductions of this protein at ZT13 in wild-type neutrophils and unchanged amounts in $Cxcr2^{\Delta N}$ and $Cxcr4^{\Delta N}$ neutrophils (Figure S2C). We then assessed surface CD62L, a marker reduced during aging (Casanova-Acebes et al., 2013; Uhl et al., 2016; Zhang et al., 2015) (Figure 1E). To ensure that potential alterations were cell-intrinsic, we generated bone marrow transplant chimeras of wild-type and of each of the mutant donors. We found elevated CD62L in circulating neutrophils from $Arntl^{\Delta N}$ and $Cxcr2^{\Delta N}$ donors, suggesting disrupted aging in these mutants (Figure 2A). In contrast, $Cxcr4^{\Delta N}$ neutrophils were low for CD62L, which suggested constitutive aging (Figure 2A). If these alterations were caused by disruption of diurnal aging, we predicted that CD62L would not change over time. Indeed, *in vivo* metabolic pulse and chase of neutrophils with bromodeoxyuridine (BrdU) demonstrated that the temporal changes in CD62L seen in wild-type neutrophils were abrogated in $Arntl^{\Delta N}$ and $Cxcr4^{\Delta N}$ mutants (Figure 2B). To further confirm that these genes regulated the natural dynamics of aging, we measured CD62L through full diurnal cycles in all mutant mice. Although surface CD62L exhibited diurnal oscillations in wild-type neutrophils, all three mutants presented disrupted patterns, and $Arntl^{\Delta N}$ mutants showed a complete loss of rhythmicity (Figure 2C). Accordingly, surface CXCR4 also lost diurnal oscillations in $Arntl^{\Delta N}$ neutrophils (data not shown).

To dissect the antagonistic role of CXCR4 in aging, we pretreated wild-type neutrophils with CXCL12, the main ligand for CXCR4, before exposing them to a CXCR2 agonist. Given the constitutive aging of $Cxcr4^{\Delta N}$ neutrophils, we hypothesized that stimulation through CXCR4 might prevent CXCR2-dependent responses. Indeed, CXCL12 blunted both the reductions in CD62L and the chemotaxis elicited through CXCR2 (Figure S2D). In addition, neutrophils expressing $Cxcr4^{WHIM}$, a hyper-signaling variant of CXCR4 (Balabanian et al., 2012), displayed constitutive elevations in CD62L (Figure S2E). Combined, these data indicated that Bmal1 and CXCR2 promote diurnal aging and that CXCR4 prevents it.

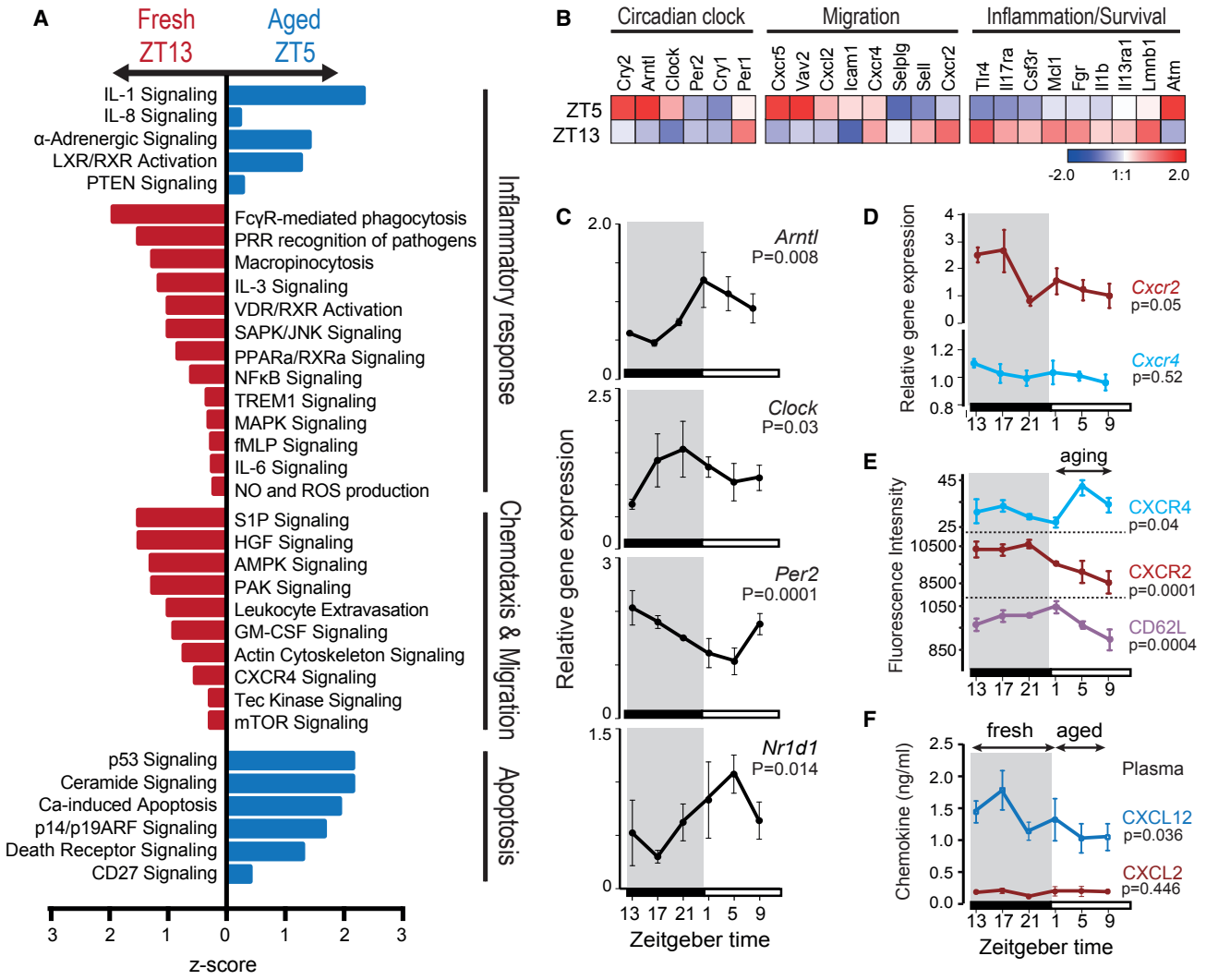

Figure 1. Cell-Intrinsic Rhythms in Circulating Neutrophils, Related to Figure S1

(A) Molecular pathways differentially regulated in circulating wild-type neutrophils at ZT5 versus ZT13. Comparisons are presented as *Z* score values.

(B) Heatmap of selected genes at ZT5 versus ZT13, including genes of the circadian clock and genes encoding proteins related to migration and inflammation. The color scale indicates fold changes of expression for each gene.

(C) Diurnal expression of the indicated clock genes in neutrophils isolated from the circulation of wild-type mice at the indicated zeitgeber times. Shaded areas represent night; n = 2–6 mice per time point.

(D) Diurnal expression of *Cxcr2* and *Cxcr4* in circulating wild-type neutrophils at the indicated times; n = 2–6 mice per time point. The diurnal curves are repeated (dashed lines) to better appreciate the pattern.

(E) Surface CXCR4, CXCR2, and CD62L measured at different diurnal times by flow cytometry; n = 5 mice. Highlighted is the time of aging (ZT1–ZT9), when CD62L and CXCR2 go down and CXCR4 goes up. The diurnal curves are repeated (dashed lines) to better appreciate the pattern.

(F) Diurnal changes of CXCL2 and CXCL12 in the plasma of wild-type mice; n = 5–10 mice per time point. The diurnal curves are repeated (dashed lines) to better appreciate the pattern.

All values are presented as mean ± SEM. p values were determined by amplitude versus zero t test analyses (see Quantification and Statistical Analysis) to test for circadian behavior (C–F).

Aging-Driven Transcriptional Programs

Having identified Bmal1, CXCR2, and CXCR4 as intrinsic regulators of diurnal aging, we used the neutrophil-specific mutant mice as models to examine how programmed diurnal aging impacted neutrophil physiology. We first performed transcriptomic analyses of blood neutrophils extracted from *Arntl*$^{\Delta N}$, *Cxcr2*$^{\Delta N}$, and *Cxcr4*$^{\Delta N}$ mice at ZT5 and compared them with the profiles of wild-type neutrophils at ZT5 and ZT13. Principal component analyses of the five groups revealed that neutrophils that displayed a CD62L^{HI} fresh phenotype (including wild-type at ZT13, *Arntl*$^{\Delta N}$, and *Cxcr2*$^{\Delta N}$) clustered together, whereas those that shared an aged phenotype (wild-type at ZT5 and *Cxcr4*$^{\Delta N}$) separated from the fresh cluster (Figure 2D), and many genes were differentially regulated among the fresh and aged groups (Figure S2F). Consistent with a role in diurnal aging, when we contrasted the transcriptomes of wild-type and mutant mice at

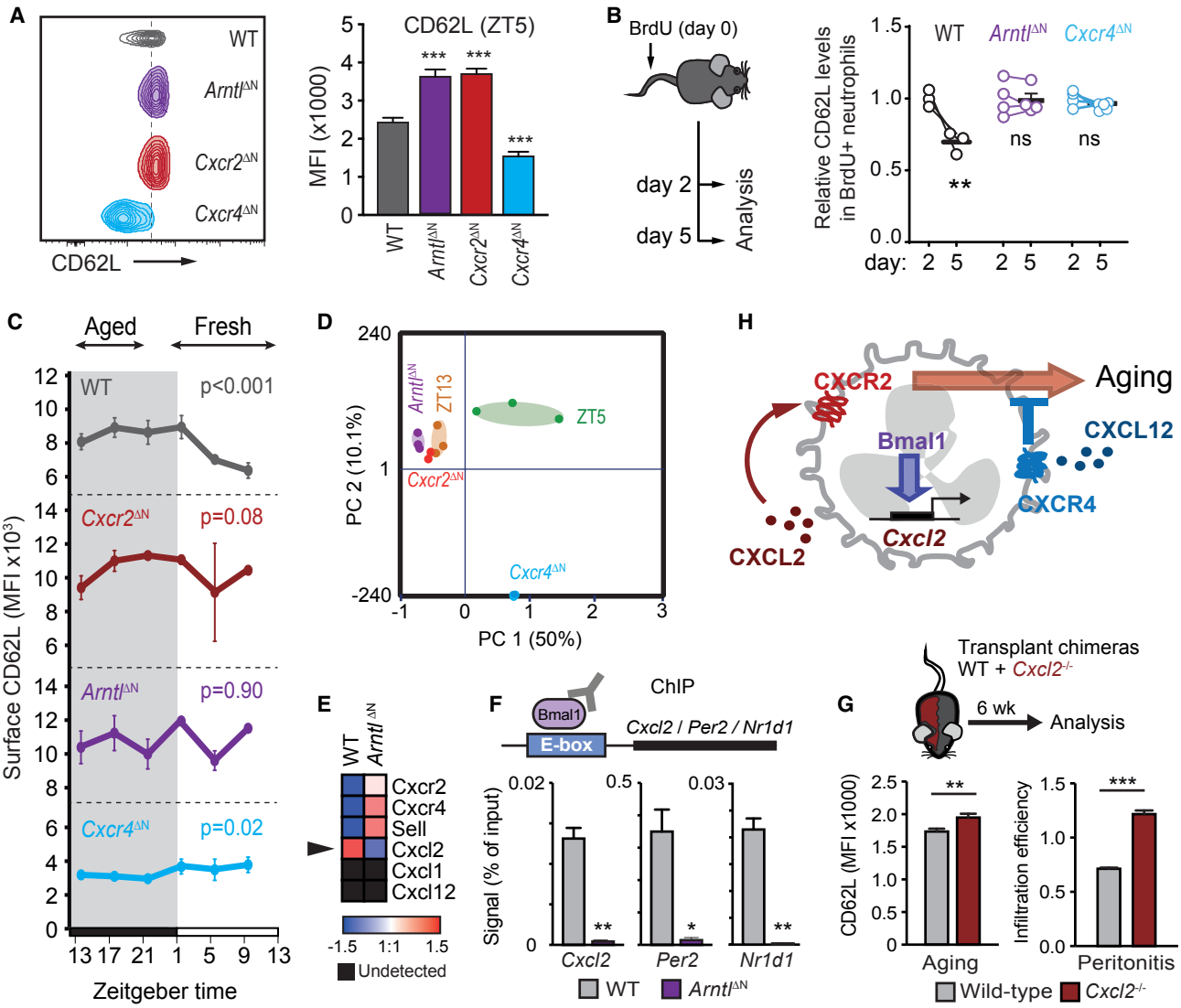

Figure 2. Bma1, CXCR2, and CXCR4 Form a Diurnal Timer in Neutrophils, Related to Figure S2

(A) Surface expression of CD62L in wild-type and mutant neutrophils at ZT5. Cytometric data are from transplant chimeras of wild-type with each mutant. Bars at right show median fluorescence intensity; n = 14–30 mice per group.

(B) *In vivo* BrdU labeling followed by analysis of CD62L in BrdU+ cells 2 and 5 days after injection. Note that as labeled neutrophils enter the bloodstream, they lose CD62L over time in WT mice, but not in *Arntl*$^{\Delta N}$ and *Cxcr4*$^{\Delta N}$ mutants. Data are normalized to day 2 in each group; n = 3–5 mice per group.

(C) Diurnal surface CD62L in circulating neutrophils in wild-type and mutant mice, as determined by flow cytometry. The times when neutrophils are phenotypically fresh or aged are indicated on top; n = 3–10 mice per time point. p values were determined by amplitude versus zero t test analyses.

(D) Principal component analyses of differentially expressed genes in circulating neutrophils from wild-type neutrophils at ZT5 and ZT13, and *Arntl*$^{\Delta N}$, *Cxcr2*$^{\Delta N}$, and *Cxcr4*$^{\Delta N}$ mutants at ZT5.

(E–G) Bmal1-regulated expression of CXCL2 controls aging. (E) Heatmap showing differential expression of aging-related genes in wild-type and *Arntl*$^{\Delta N}$ neutrophils. Expression of Cxcl1 and Cxcl12 was undetectable. Data are from triplicate samples of each group obtained at ZT5. (F) Experimental design and ChIP analyses of Bmal1 binding to E-box-containing promoter regions of *Cxcl2*, *Per2*, and *Nr1d1* in wild-type and *Arntl*$^{\Delta N}$ neutrophils. (G) Experimental setup and phenotype of *Cxcl2*$^{-/-}$ neutrophils in transplantation chimeras. *Cxcl2*$^{-/-}$ neutrophils display elevated CD62L expression and enhanced migration to zymosan-treated peritoneum, both of which are consistent with disrupted aging.

(H) Model of neutrophil aging: CXCR2 signaling drives aging, whereas CXCR4 antagonizes these signals and prevents it. Bmal1 regulates *Cxcl2* expression to promote autocrine aging.

Except where indicated, all values are mean ± SEM. **p < 0.01; ***p < 0.001 as determined by one-way ANOVA (A), upaired t test (B and F), or paired t test (D and G).

both ZT5 and ZT13, we found that the diurnal changes in gene expression of wild-type neutrophils were absent or blunted in *Arntl*$^{\Delta N}$ and *Cxcr4*$^{\Delta N}$ neutrophils (Figure S2G). These findings aligned with the phenotypic data (Figures 2A and 2C) and define diurnal aging as a global transcriptional program of circulating neutrophils that occurs naturally during the day and that could be recapitulated in the mutant mice.

We next focused on genetic programs that consistently changed when independently interrogating the effect of time (ZT5 versus ZT13) and genotype (*Arntl*$^{\Delta N}$ versus *Cxcr4*$^{\Delta N}$). We noticed prominent regulation of the IL-8 (interleukin 8) signaling pathway (a ligand for human CXCR2; Figure S2H), which was in line with our previous results and suggested engagement of CXCR2 during aging. Analyses of our sequencing data revealed that among aging-related genes, only expression of *Cxcl2*, a CXCR2 ligand expressed by neutrophils (Li et al., 2016), was reduced in *Arntl*$^{\Delta N}$ relative to wild-type neutrophils (Figure 2E), suggesting that this chemokine could provide a link between Bmal1 and CXCR2 during aging. Indeed, chromatin immunoprecipitation (ChIP) assays with wild-type neutrophils revealed that Bmal1 bound predicted E-box elements in the promoter regions not only of known target clock genes (*Per2* and *Nr1d1*), but also of the *Cxcl2* gene (Figure 2F). Further analysis of bone marrow chimeras from wild-type and *Cxcl2*$^{-/-}$ donors confirmed that this chemokine was required for neutrophil aging in a cell-autonomous manner (Figure 2G). Consistently, *in vivo* blockade of CXCL2, but not of another CXCR2 ligand (CXCL1), blunted the aging phenotype of wild-type neutrophils without affecting *Cxcr2*$^{\Delta N}$ mutants (Figure S2I). These findings explained the defective aging seen in *Arntl*$^{\Delta N}$ neutrophils (Figure 2A) and revealed that Bmal1-driven production of CXCL2 controlled neutrophil aging through autocrine CXCR2 signaling.

To independently assess the cell-intrinsic nature of aging, we tracked the kinetics of fresh neutrophils transferred into recipient mice at ZT5 (the time of maximal aging). Although host mice became enriched in fresh neutrophils over time, the transferred neutrophils became progressively aged (Figure S2J), further supporting that neutrophil aging is intrinsically driven.

Combined, these findings supported a model whereby diurnal neutrophil aging is driven by Bmal1 through regulation of *Cxcl2* expression. This chemokine in turn signals through CXCR2 to induce phenotypic aging, whereas CXCR4 antagonizes these signals and prevents aging (Figure 2H).

Aging-Regulated Migration of Neutrophils

The transcriptomic analyses additionally identified pathways that changed significantly (-log (p value) > 1.3), including cytokine signaling, activation of nuclear receptors, toll-like receptor signaling, leukocyte extravasation, and actin cytoskeleton signaling (Figures 1A and S2H). Because many of these pathways ultimately regulate the migration of neutrophils into tissues to exert immune functions, we investigated the *in vivo* trafficking patterns associated with neutrophil aging.

We considered two migratory modalities that are relevant in neutrophil physiology: migration into healthy tissues (or clearance, which follows diurnal cycles) (Casanova-Acebes et al., 2018; Scheiermann et al., 2012), and migration into inflamed tissues. We took advantage of our neutrophil-specific mouse models to exclude cell-extrinsic factors influenced by time, such as diurnal changes in adhesion molecules reported on endothelial cells (Scheiermann et al., 2012). In addition, because CXCR2 plays prominent roles in multiple homeostatic and inflammatory scenarios that may not be related to aging, we restricted our subsequent analyses to Bmal1 and CXCR4 mutants as models for fresh and aged neutrophils, respectively.

We first generated parabiotic pairs of wild-type and mutant mice to compare the migration efficiency of fresh (*Arntl*$^{\Delta N}$) and aged (*Cxcr4*$^{\Delta N}$) neutrophils relative to wild-type neutrophils in the same physiological context (Figure 3A). We found that homeostatic clearance of *Arntl*$^{\Delta N}$ neutrophils into multiple tissues of wild-type partners was strongly impaired, whereas it was unaffected for *Cxcr4*$^{\Delta N}$ neutrophils (Figure 3B), indicating that neutrophil aging was required for clearance into tissues.

We next examined the migration of the aging mutant neutrophils into inflamed tissues using zymosan-induced peritonitis in the parabiotic pairs. To our surprise, we found the opposite response: enhanced migration of *Arntl*$^{\Delta N}$ fresh neutrophils and reduced infiltration by *Cxcr4*$^{\Delta N}$ aged neutrophils (Figure 3C). Using an independent model of constitutive aging (mice lacking endothelial selectins, *Selp*; *Sele*$^{-/-}$ mice; Casanova-Acebes et al., 2013), we confirmed that aged neutrophils displayed intact clearance at steady state (Figure S3A) but impaired migration to inflamed tissues (Figure 3B). In contrast, impaired aging of neutrophils expressing the hyper-signaling *Cxcr4*^{WHIM} mutation resulted in enhanced migration to inflamed tissues (Figure S2E). Whole-mount imaging of inflamed cremasteric muscles from transplantation chimeras confirmed the differential capacity of fresh and aged neutrophils to infiltrate inflamed tissues relative to wild-type cells (Figures 3D and 3E), and this became even more prominent when comparing the migration of constitutively aged and fresh neutrophils within the same mouse (Figure 3F). Importantly, these findings aligned with enhanced inflammatory recruitment of wild-type neutrophils when they were phenotypically fresh (ZT13), and this diurnal preference was lost in *Arntl*$^{\Delta N}$ and *Cxcr4*$^{\Delta N}$ mutant mice (Figure S3B). These data reveal that aging instructs a diurnal switch in the migratory preference of neutrophils, from inflammatory to homeostatic.

Surface Topology and Rolling Efficiency Are Regulated during Diurnal Aging

To search for the mechanisms underlying the distinct migratory patterns of fresh and aged neutrophils, we examined the different steps of the recruitment cascade (rolling, adhesion, and extravasation) in the cremasteric microcirculation with intravital microscopy (Figure 4A). We found elevated rolling, adhesion, and extravasation efficiencies of *Arntl*$^{\Delta N}$ neutrophils and significant reductions for *Cxcr4*$^{\Delta N}$ neutrophils (Figure 4B). The defects in the recruitment cascade of *Cxcr4*$^{\Delta N}$ aged neutrophils were independently reproduced in *Selp*; *Sele*$^{-/-}$-derived aged neutrophils (Figure S4A; Video S1). In contrast to rolling, the crawling dynamics of neutrophils on the vessel wall and within tissues (Figures S4B and S4C), as well as the migration to various chemoattractants (Figure S4D), were unaffected by aging. Furthermore, analyses in auto-perfused flow chambers coated with P-selectin alone or together with ICAM-1 (intercellular adhesion molecule 1) and CXCL1 and connected to the circulation of wild-type mice (Figure S4E) revealed elevated rolling efficiencies

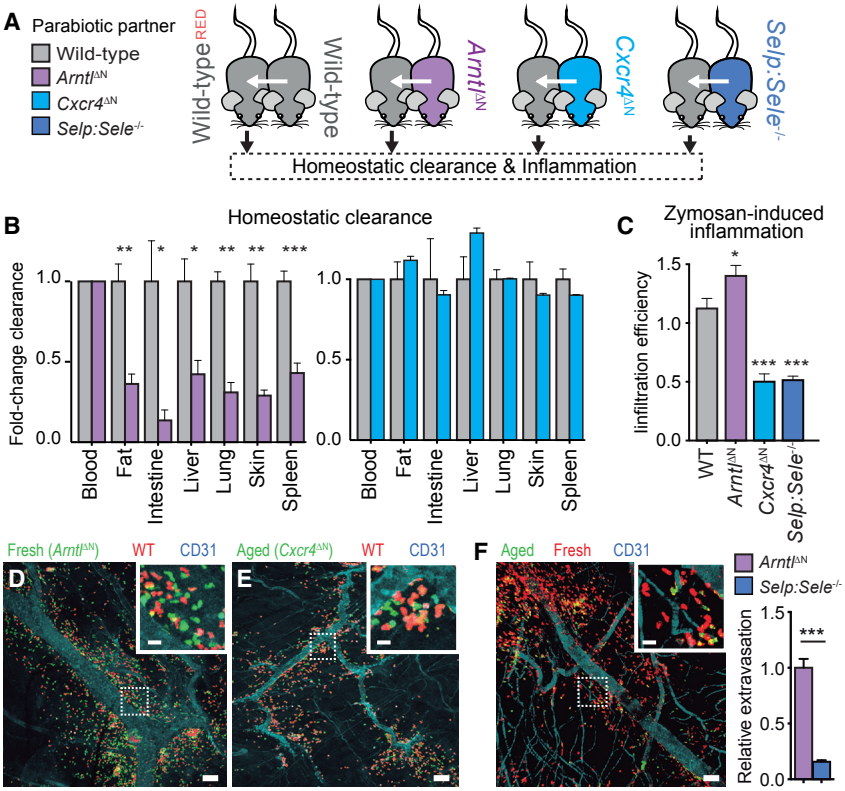

Figure 3. Diurnal Aging Impairs Inflammatory Recruitment but Favors Homeostatic Clearance, Related to Figure S3

(A) Experimental setup to test recruitment in parabiotic pairs. Mutant neutrophils that enter tissues of their wild-type^{RED} partners (expressing DsRed) allowed estimation of their migratory capacity relative to wild-type cells.

(B) Quantification of *Arntl*$^{\Delta N}$ (left) and *Cxcr4*$^{\Delta N}$ (right) neutrophils cleared in multiple tissues of wild-type partners at ZT5; values are adjusted to ratios in blood and normalized to wild-type neutrophils cleared in wild-type partners; n = 3–8 mice.

(C) Infiltration efficiency of control and mutant neutrophils into the peritoneum of wild-type parabiotic partners at ZT5; n = 3–5 mice.

(D and E) Whole-mount staining of TNF-α-treated cremaster muscles from transplant chimeras of wild-type and *Arntl*$^{\Delta N}$ donors (D) or wild-type and *Cxcr4*$^{\Delta N}$ donors (E), showing extravasated neutrophils and vessels. Extravasated neutrophils are quantified in Figure 4B (extravasation).

(F) Whole-mount staining as in (D), comparing constitutively fresh and aged neutrophils in Bmal1$^{\Delta N}$ mice (fresh, red) set in parabiosis with *Selp*; *Sele*$^{-/-;\ GFP}$ (aged, green) partners. The relative infiltration of each partner is quantified in the right bar graph. Scale bars for (D)–(F), 70 μm. Insets scale bars, 10 μm.

All bars show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 as determined by unpaired (B) or paired (C and F) t test analysis.

and subsequent adhesion for neutrophils at ZT13 relative to ZT5 (Figure S4F), indicating that diurnal changes in rolling and adhesion were cell intrinsic. Combined, these data suggested that diurnal aging impaired inflammatory recruitment by specifically targeting rolling, a rate-limiting step during leukocyte recruitment (Ley et al., 2007).

Because rolling is largely mediated by endothelial selectins, the data implied that aging targeted selectin ligands on neutrophils. However, binding analyses using soluble P- and E-selectin antibody chimeras revealed only modest changes in selectin binding, regardless of time of day or genetic background (Figure S4G), suggesting that biosynthesis of selectin ligands was unlikely to cause the loss of rolling during aging. Effective engagement of selectins under flow additionally demands correct topology at the neutrophil's surface to optimize ligand presentation at the tip of microvilli, a type of membrane protrusion that relies on a network of cortical actin (Finger et al., 1996; Simon et al., 2007; von Andrian et al., 1995). Analyses of actin distribution with immunofluorescence staining and of surface topology with scanning electron microscopy revealed dramatic reductions in cortical β-actin in aged neutrophils, which coincided with a reduced number of microvilli both in wild-type mice at ZT5 (Figures 4C and 4D) and in genetically induced aged mice (*Cxcr4*$^{\Delta N}$; Figures S4H and S4I). These data revealed that disruption of the neutrophil's cortical architecture during aging impairs migration to inflamed tissues.

Homeostatic Migration Does Not Require Rolling

These observations, however, failed to explain why rolling-defective aged neutrophils maintained an intact capacity to enter non-inflamed tissues under homeostasis (Figure 3B). To address this issue, we analyzed homeostatic and inflammatory recruitment in control mice and in mice with impaired rolling due to the lack of endothelial selectins (*Selp*; *Sele*$^{-/-}$ mice; Frenette et al., 1996). Although adhesion to inflamed vessels required selectin-mediated rolling as expected, we found that spontaneous adhesion during homeostatic recruitment to the skin occurred even in the absence of rolling (Figure 4E). Video analyses of the dermal microvasculature at times of clearance (ZT9) confirmed that neutrophils arrested suddenly, without the need of a preceding rolling step (Figure 4F; Video S2). Thus, neutrophil aging maintains homeostatic clearance but prevents inflammatory recruitment by disabling selectin-mediated rolling (Figure 4G).

To determine how the differential migratory properties of fresh and aged neutrophils affected tissue injury, we induced ischemic inflammation of the brain by occlusion of the middle cerebral artery. Because the brain is devoid of neutrophils at steady state (Figure S4J), this model allows measuring of the contribution of infiltrating neutrophils to tissue damage (Cuartero et al., 2013; Sreeramkumar et al., 2014). In line with our prediction, brain injury was only exacerbated in *Arntl*$^{\Delta N}$ mice enriched in fresh neutrophils (Figure S4K), suggesting that preferential migration of fresh neutrophils during inflammation contributes to tissue injury.

Diurnal Aging Boosts Anti-microbial Defense

The observations so far raised the possibility that regulation of neutrophil migration during the day might be a primary role of diurnal aging; it could drive compartmentalization of aged

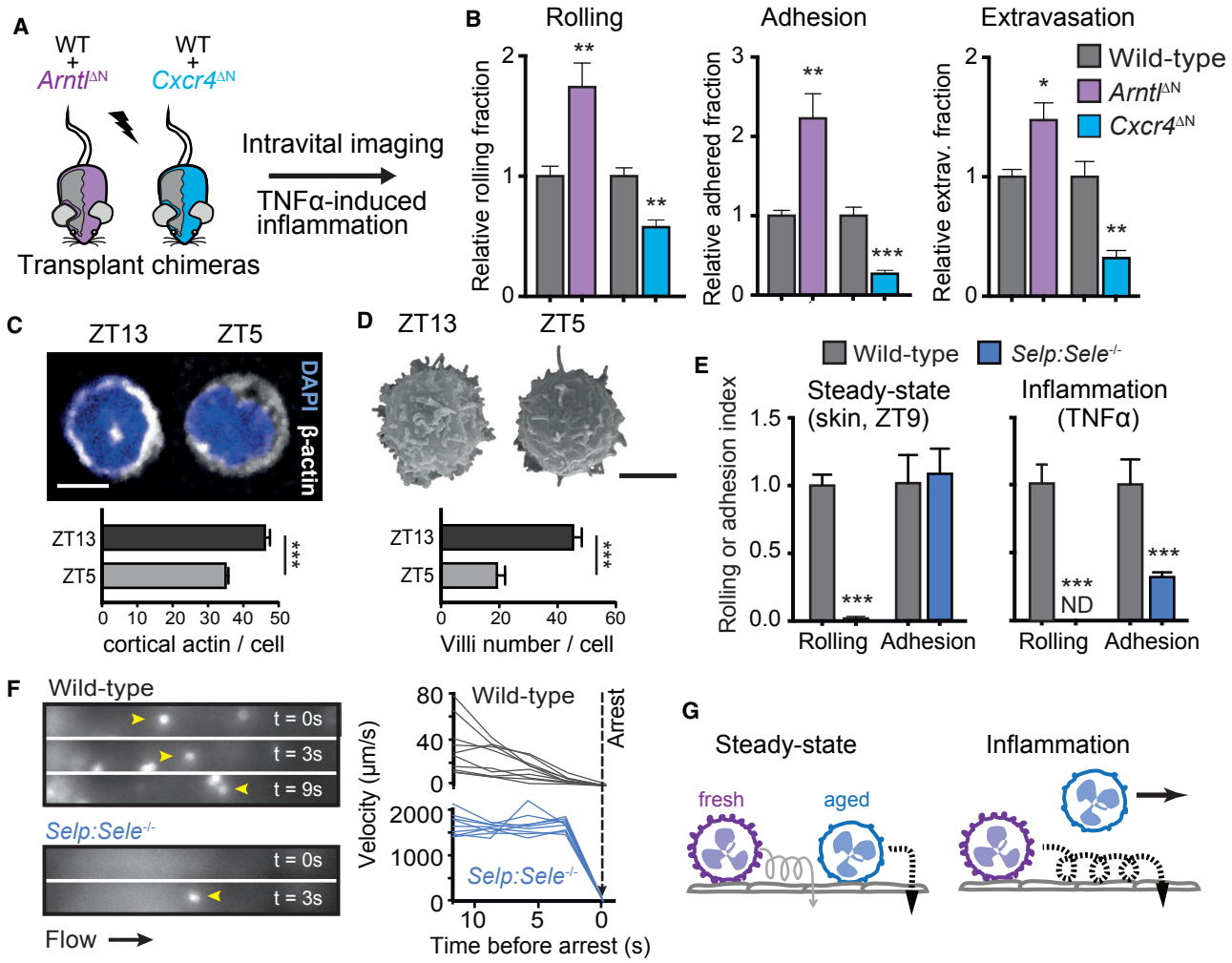

Figure 4. Microvilli Collapse and Impaired Rolling Are Hallmarks of Aged Neutrophils, Related to Figure S4

(A) Strategy for competitive recruitment of neutrophils in bone marrow chimeras, at ZT5.

(B) Relative frequencies of rolling, adherent, and extravasated fresh (*Arntl*$^{\Delta N}$) and aged (*Cxcr4*$^{\Delta N}$) neutrophils, normalized to wild-type controls in chimeric mice; n = 30–61 venules from 5–6 mice.

(C) β-actin staining in wild-type neutrophils at ZT5 and ZT13, and frequency of neutrophils with cortical distribution of actin; n = 324–330 cells per group.

(D) Scanning electron micrographs of wild-type neutrophils at ZT5 and ZT13, and number of microvilli on their surface. Scale bar, 5 μm; n = 23–29 cells per group.

(E) Rolling and adhesion of neutrophils on cremasteric venules after treatment with TNF-α (inflammation), or on naive dermal microvessels at ZT9-13 (steady state) in wild-type or *Selp*;*Sele*$^{-/-}$ mice. n = 50–55 venules from 4–5 mice (steady state) and 25–27 venules from 3–5 mice (inflamed cremaster). ND, none detected.

(F) Kinetics of neutrophils (Ly6G^{+}, yellow arrows) prior to firm arrest on dermal microvessels at steady state. Left, representative sequential intravital frames with neutrophils arresting in the last sequence (reverse arrows). Right, flow or roll dynamics of neutrophils before firm arrest; n = 10 cells shown per group.

(G) Model for the preferential recruitment of fresh and aged neutrophils into inflamed or naive tissues, respectively.

Bars show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 as determined by paired (B) or unpaired t test analysis (C and D) or non-parametric Mann-Whitney test (E).

neutrophils into tissues at night in anticipation of pathogens potentially breaking into tissues, while at the same time reducing their numbers within vessels to minimize injury when the chance of immune activation is higher.

To test this possibility, we analyzed the diurnal dynamics of aged neutrophils in the circulation of wild-type and mutant mice throughout the day. The analyses revealed striking differences: in wild-type mice, aged neutrophils peaked at ZT5 and disappeared at ZT13, whereas in *Arntl*$^{\Delta N}$ mice, they displayed non-oscillating low numbers and *Cxcr4*$^{\Delta N}$ animals presented constitutive elevations in aged neutrophils in blood (Figure 5A). Notably, the absolute number of neutrophils maintained normal oscillations in the blood of *Arntl*$^{\Delta N}$ mutants (Figure S5A), indicating that neutrophil numbers and aging are regulated through different mechanisms. We therefore used these mouse models to determine how aging-driven trafficking regulated immune defense and vascular health.

We first infected mice with *Candida albicans*, using a protocol that allows systemic spread, targets the kidneys, and is controlled by neutrophils (Del Fresno et al., 2018; Lionakis

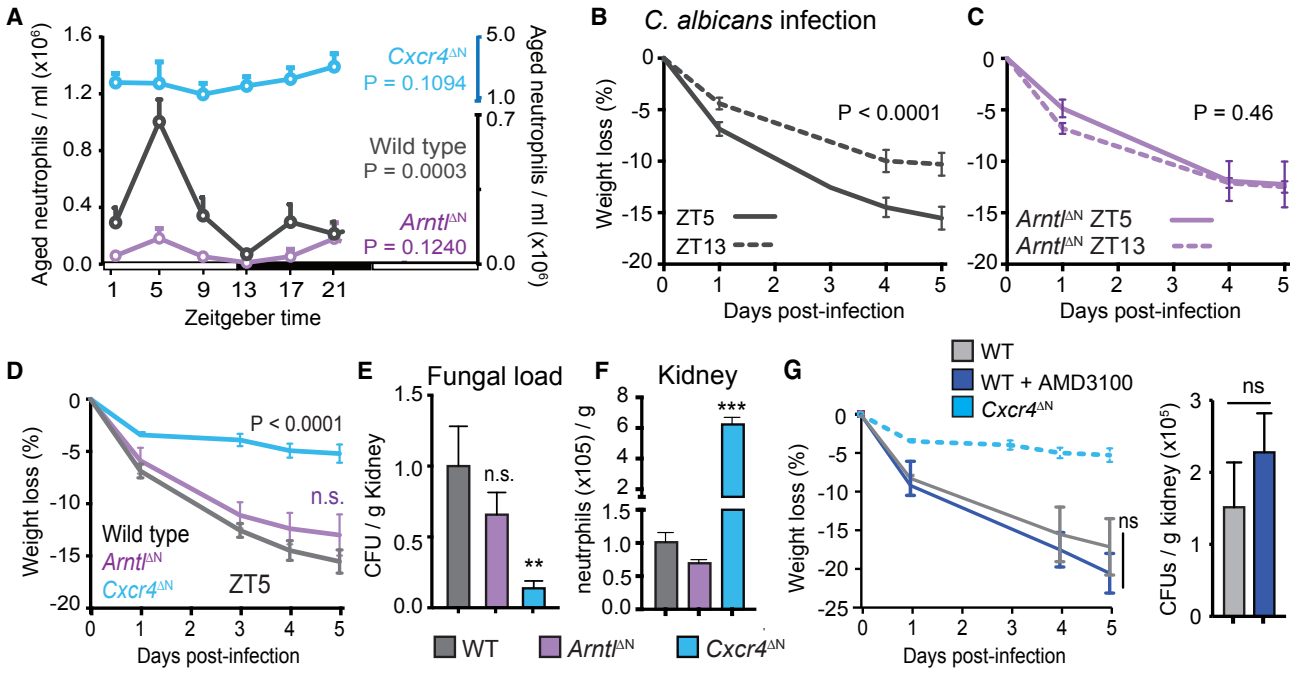

Figure 5. Neutrophil Aging Confers Diurnal Protection against Infection, Related to Figure S5

(A) Diurnal numbers of CD62L^{lo} aged neutrophils in the blood of wild-type, *Arntl*$^{\Delta N}$, and *Cxcr4*$^{\Delta N}$ mice; n = 3–6 mice. See also Figure S5A.

(B and C) Weight loss kinetics of wild-type (B) and *Arntl*$^{\Delta N}$ mice (C) infected with *C. albicans* at ZT5 or ZT13; n = 5–21 mice.

(D) Weight-loss curves of wild-type, *Arntl*$^{\Delta N}$, and *Cxcr4*$^{\Delta N}$ mice infected at ZT5; n = 12–14 mice.

(E) Fungal burden at day 5 in the kidneys from the mice in (D), normalized to WT.

(F) Number of neutrophils in the kidneys of non-infected mice; n = 4 mice per group.

(G) Kinetics of weight loss in control or AMD-treated wild-type mice after systemic *C. albicans* infection at ZT5. The dashed line shows weight loss in *Cxcr4*$^{\Delta N}$ mice as in Figure 4D for reference. Bars at right show fungal burden in kidneys at day 5 post-infection; n = 10 mice.

Data are shown as mean ± SEM. **p < 0.01; ***p < 0.001; n.s., not significant, as determined by amplitude versus zero t test (A), two-way ANOVA (B–D and G), one-way ANOVA with Dunnett's multigroup correction (E and F), and unpaired t test analysis (G).

et al., 2011). Wild-type mice displayed diurnal patterns of response to infection, with increased resistance at ZT13 as defined by reduced weight loss, fungal load in kidneys, and improved survival (Figures 5B, S5B, and S5C). Importantly, the initial time of infection was critical for the long-term immune response because the effect could be seen several days after infection. Resistance to *Candida* at ZT13 coincided with more neutrophils in naive kidneys and fewer in blood (Figures S5D and S5E), suggesting that their presence in tissues at the time of infection conferred protection. Remarkably, the diurnal variation in susceptibility to *Candida* infection was abolished in *Arntl*$^{\Delta N}$ mice (Figure 5C), indicating that neutrophil aging was needed to anticipate the infection. These observations predicted that mice with constitutively aged neutrophils clearing into tissues might perform better against infection. Indeed, *Cxcr4*$^{\Delta N}$ mice had more neutrophils in naive kidneys and manifested remarkable resistance to infection and reduced fungal spread (Figures 5D and 5F). However, because *Cxcr4*$^{\Delta N}$ mice displayed neutrophilia (Figure S5A), this observation could be alternatively explained by elevated numbers rather than by the aging status of neutrophils. To discriminate between these possibilities, we treated wild-type mice with a single injection of the CXCR4 antagonist AMD3100, a treatment that causes acute neutrophilia (Devi et al., 2013) but did not induce aging (Figures S5F and S5G). Despite neutrophil counts that were even higher than those in *Cxcr4*$^{\Delta N}$ mice, AMD3100-treated mice were as susceptible to *Candida* infection as untreated wild-type mice (Figure 5G), indicating that the aging status, rather than the number of neutrophils, conferred protection against *Candida*. In addition, the capacity of fresh (ZT13 or *Arntl*$^{\Delta N}$) and aged (ZT5 and *Cxcr4*$^{\Delta N}$) neutrophils to phagocytose *Candida* conidia to produce reactive oxygen species (Figures S5H–S5J) and to secrete cytokines (Figure S5K) were similar to wild-type cells, which supported the contention that neutrophil migration, rather than other cellular processes, was the relevant process regulated by aging. We obtained evidence of similar diurnal variations in the response to bacterial sepsis, which was also lost in *Arntl*$^{\Delta N}$ mice (Figure S5L), further revealing a general influence of neutrophil aging in responses to infection. Thus, aging-driven clearance of neutrophils into tissues orchestrates anti-microbial defense.

Constitutive Neutrophil Aging Predisposes to Vascular Inflammation

To define whether diurnal neutrophil aging additionally conferred protection to vessels, we used a model of acute myocardial infarction (AMI) induced by ischemia reperfusion of the left anterior descending coronary artery, in which inflammation originates intravascularly without prior neutrophil extravasation (Vinten-Johansen, 2004). Similar to infections, the extent of cardiac damage displayed diurnal variations, with larger infarct sizes at

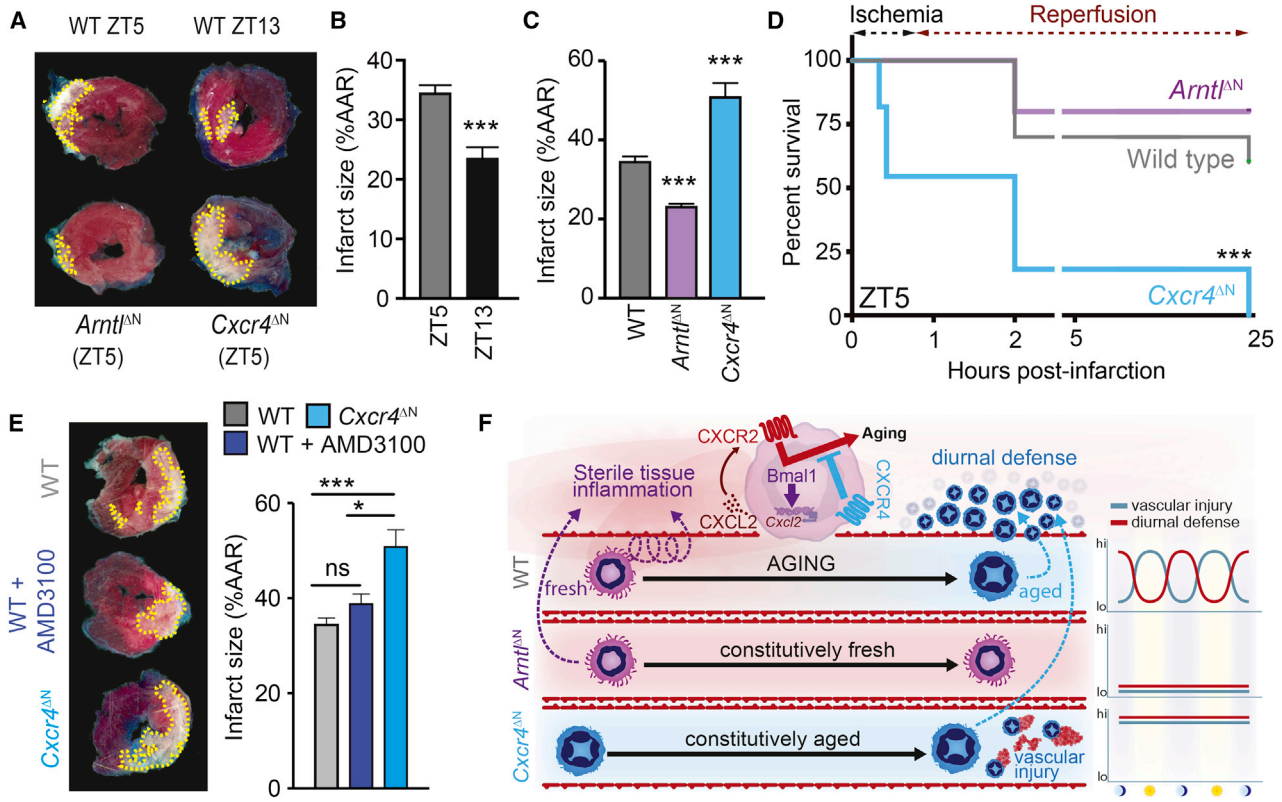

Figure 6. Aged Neutrophils Aggravate Myocardial Infarction, Related to Figure S6

(A) Representative images of hearts from wild-type mice subjected to ischemia reperfusion at ZT5 or ZT13, or the indicated mutant mice at ZT5. Dotted yellow lines highlight areas of dead myocardium; n = 4–8 mice per group from 3 experiments.

(B) Infarct sizes in wild-type mice at different diurnal times, after correction for areas at risk (AAR; see related Figure S5); n = 5–8 mice.

(C) Infarct sizes in wild-type, *Arntl*$^{\Delta N}$, and *Cxcr4*$^{\Delta N}$ mice at ZT5 (see related Figure S5); n = 4–8 mice.

(D) Survival curves of wild-type, *Arntl*$^{\Delta N}$, and *Cxcr4*$^{\Delta N}$ mice subjected to myocardial infarction at ZT5; n = 9–11 mice.

(E) Representative images of hearts from untreated or AMD3100-treated wild-type mice and *Cxcr4*$^{\Delta N}$ mice. Surgeries were performed at ZT5 and dead myocardium is highlighted as in (A). Bars at right show quantification of infarcted areas in the same groups; n = 4–5 mice from one experiment.

Bars show mean ± SEM. *p < 0.05; ***p < 0.001; n.s., not significant, as determined by one-way ANOVA with Dunnett's multigroup correction (C and E), unpaired t test analysis (B), and log-rank test (D).

(F) Molecular regulators and consequences of disrupted neutrophil aging. Defective aging (*Arntl*$^{\Delta N}$) impairs the evening boost in anti-microbial defense but protects from vascular injury; instead constitutive aging (*Cxcr4*$^{\Delta N}$) enhances the response to infections but exacerbates thrombo-inflammation.

ZT5 (Figures 6A, 6B, and S6A). Accordingly, infarct sizes after only 1 h of reperfusion were remarkably larger in *Cxcr4*$^{\Delta N}$ mice and smaller in *Arntl*$^{\Delta N}$ mice (Figure 6C), and this correlated with early death of all *Cxcr4*$^{\Delta N}$ mice (Figure 6D). This dramatic response was not caused by increased numbers of circulating neutrophils in *Cxcr4*$^{\Delta N}$ mice because treatment of wild-type mice with AMD3100 did not aggravate myocardial injury (Figure 6E). Thus, the presence of aged neutrophils in the circulation is detrimental for tissues after vascular ischemia and reperfusion, whereas their diurnal clearance is protective.

We examined potential mechanisms by which aged neutrophils might exacerbate vascular injury in *Cxcr4*$^{\Delta N}$ mice. Using a model of ischemia reperfusion in the cremaster muscle that allows high-resolution live imaging of affected vessels, we found disseminated thrombi in microvessels of *Cxcr4*$^{\Delta N}$ mice (Figure S6C; Video S3). Depletion of neutrophils in *Cxcr4*$^{\Delta N}$ mice prevented thrombi formation and improved survival after infarction (Figures S6B and S6C), indicating that both responses were mediated by neutrophils. Although neutrophil extracellular traps (NETs) can promote thrombosis (Fuchs et al., 2010), they were not responsible for the response of *Cxcr4*$^{\Delta N}$ mice because two different NET inhibitors failed to prevent thrombus formation in reperfused venules (Figure S6D). In addition, endothelial proliferation and apoptosis, as well as vascular permeability, were not affected at baseline across multiple tissues, including hearts, of *Arntl*$^{\Delta N}$ and *Cxcr4*$^{\Delta N}$ mice (Figures S6E–S6G), indicating that aging did not directly compromise basal vascular health prior to the ischemic insult.

Overall, these observations suggest that neutrophil aging is critically driven by an internal program, as we failed to find contributions from other factors, including reactive oxygen species (ROS; data not shown) or the intestinal microbiota (Figures S6H–S6K), both of which had been previously associated with neutrophil senescence or aging, respectively (Harbort et al., 2015; Zhang et al., 2015). In turn, aging controls diurnal compartmentalization of neutrophils into tissues and out of the

circulation, thereby balancing immune protection and vascular protection (Figure 6F; Video S4).

DISCUSSION

Mammalian immunity is not constant in quantity (e.g., number of recruited or mobilized leukocytes) or quality throughout the day, as it adapts to varying diurnal challenges from the environment, including the chance of exposure to infectious pathogens (Man et al., 2016). Likewise, damage to the cardiovascular system, in both humans and model organisms, follows circadian patterns (Muller et al., 1989; Scheiermann et al., 2013). Because neutrophils are major mediators of anti-microbial defense and vascular inflammation, we predicted that the diurnal variations in both processes could be mechanistically explained by the existence of a neutrophil-intrinsic program (or "timer") that regulated their activity through the day. In this study, we identified and characterized this program and revealed that it underlies the circadian susceptibility of mice to infection and vascular inflammation.

We have found that the diurnal program of neutrophils is coordinated by the circadian-related protein Bmal1 in coordination with two chemokine receptors: CXCR2, which drives aging, and CXCR4, which antagonizes it. Multiple functional assays allowed us to confirm that time-of-day differences in wild-type cells could be faithfully recapitulated by the respective mutants: *Arntl*$^{\Delta N}$ cells resembled night (fresh) neutrophils, whereas *Cxcr4*$^{\Delta N}$ mutants behaved similar to daytime (aged) neutrophils. Before release into the bloodstream, maturing neutrophils are retained within the marrow in an environment with high CXCR4 signaling (Eash et al., 2009, 2010), which raises the intriguing possibility that this diurnal timer is inhibited until neutrophils are released into blood. Once in blood, functional analyses of mice in which we disabled each component of this timer revealed that preferential invasion of inflamed or naive tissues is compartmentalized in time. Under steady-state conditions, neutrophils released from the marrow gradually lost their ability to enter inflammatory sites and prepared for clearance into tissues. This migratory switch was intrinsically regulated, but it likely coordinated with extrinsic programs because disruption of rhythms in vascular cells can also affect the diurnal entry of leukocytes in tissues (Scheiermann et al., 2012) and because CXCL12, which is not produced by neutrophils, negatively regulated diurnal aging through CXCR4.

We found that one potential benefit of diurnal infiltration into naive tissues was to optimize immune defense, as demonstrated by the loss of diurnal oscillations in the response against fungal or bacterial infections when *Arntl* was deleted from neutrophils. Removal of *Cxcr4*, the negative regulator of the neutrophil timer, instead caused unrestrained aging and enhanced anti-microbial responses, while at the same time precipitating severe thrombo-inflammatory reactions following ischemia reperfusion. In contrast, *Arntl*$^{\Delta N}$ mutants displayed attenuated damage during myocardial infarction, altogether indicating that an intact neutrophil clock was important to balance anti-microbial defense and cardiovascular inflammation.

Among the various transcriptional pathways activated during aging, we identified those related to leukocyte extravasation and actin cytoskeleton signaling, an observation that allowed us to identify disruption of cortical actin polymerization as a key molecular event linking diurnal aging with alterations in the migratory properties of neutrophils. Although the mechanisms underlying these cytoskeletal changes remain to be elucidated, this observation is consistent with early studies showing disrupted actin polymerization on human CD62L^{lo} neutrophils (Tanji-Matsuba et al., 1998). Diurnal loss of microvilli was particularly relevant because these structures allow presentation of glycoconjugate ligands to endothelial selectins under flow (von Andrian et al., 1995), thus explaining the dramatic loss of rolling and migration of aged neutrophils to inflamed areas. At the same time, loss of microvilli might conceivably enhance the exposure of β2 integrins present on the cell body (Erlandsen et al., 1993) and favor rolling-independent arrest as seen in the naive dermal microvasculature. The fact that a similar behavior of constitutive adhesion in non-inflamed vessels is displayed by patrolling monocytes (Auffray et al., 2007) suggests that this mechanism could be a common property of myeloid leukocytes endowed with homeostatic surveillance roles.

Overall, our findings are consistent with a model in which the oscillatory nature of the aging program enables alert states of neutrophils that are useful to anticipate infections but must be shut down when the risk of infection is low to prevent damage to the vasculature. We note that Bmal1-driven aging of neutrophils may not necessarily adjust to behavioral rhythms because we found that neutrophil aging peaked in the morning in both humans and mice, which are species with opposed activity periods. We therefore propose that a major purpose of aging is to ensure temporal separation of neutrophil-mediated responses within vessels from those in tissues, thereby optimizing defense without compromising vascular health.

The diurnal aging pattern of neutrophils aligns with studies showing temporally gated responses for other leukocyte subsets, including monocytes, macrophages, or T helper-17 (Th17) cells (Nguyen et al., 2013; Silver et al., 2012; Yu et al., 2013), which may be useful to temporally concentrate immune response against specific pathogens in different tissues (Tognini et al., 2017). Different from these other leukocytes, however, the existence of a circadian program in neutrophils was not intuitive because their lifetime in the circulation is generally accepted to be less than one day (Summers et al., 2010), which implies that there cannot be true circadian oscillations of gene expression within a given neutrophil. Further, at present we do not know whether aging is regulated by the transcriptional properties of Bmal1 or by the core circadian clock. For these reasons, we envision this system to function like a cellular timer (rather than a true circadian clock) that resets with every new wave of neutrophils released from the bone marrow. In other words, for short-lived cells such as neutrophils, the clock appears to regulate oscillations on a population scale by acting as a timer at the cellular level.

Given the high prevalence of infections and cardiovascular disease, a final question is whether the identification of a diurnal program in neutrophils could offer therapeutic alternatives for these life-threatening complications. In principle, targeting CXCR2 or CXCR4 with specific agonists might allow pharmacological and transient manipulation of the timer. This "chrono-programming" of neutrophils could allow the generation of phenotypes that promote defense or protect the vasculature, as needed. We expect that manipulation of the timer will not

have detrimental consequences because animals with impaired or enhanced neutrophil aging do not present gross anomalies or spontaneous susceptibility to disease at baseline, at least under specific-pathogen-free conditions (data not shown). Thus, for humans at risk of cardiovascular events, it might be advisable to block aging, whereas immunocompromised patients susceptible to infections might benefit from drugs that promote it. We are currently exploring strategies that exploit the unique temporal properties of neutrophils.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, and four videos and can be found with this article online at https://doi.org/10.1016/j.immuni.2019.01.002.

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AUTHOR CONTRIBUTIONS

J.M.A., C.d.F., M.I.C., M.C.-A., L.A.W., H.H.-E., C.S.-R., J.R., J.A.Q., G.C., J.G.-P., M.G.-P., S.M.-S., M.E., and J.L. performed experiments; C.W., K.B., and F.B. contributed essential reagents; A.Z., O.S., C.L.-R., M.A.M., B.I., D.S., L.N., J.M.A., and A.H. designed and supervised experiments; F.A. and C.M. coordinated the study on humans; A.H. designed and supervised the study. A.H. and J.M.A. wrote the manuscript, which was edited by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CXCR4-APC (Human)	eBioscience	Clone 12G5; RRID: AB_1944349
CD11b-FITC (Human)	eBioscience	Clone ICRF44
CD16-Pacific Blue (Human)	BD	Clone 3G8
CD62L-PE (Human)	BD	Clone DREG56
CD11c-APC (Human)	BD	Clone B-ly6
bio	eBioscience	Clone 1A8
Ly6G-Dylight 450	BioXcell (conjugated in-house)	Clone 1A8; RRID: AB_1107721
Ly6G-Dylight 650	BioXcell (conjugated in-house)	Clone 1A8; RRID: AB_1107721
Ly6G-FITC	eBioscience	Clone 1A8; RRID: AB_2572532
CD45-PerCP-Cy5.5	Biolegend	Clone 30-F11; RRID: AB_893344
CD11b-PE	Tonbo Biosciences	Clone M1/70; RRID: AB_2621746
CD11b-FITC	BD	Clone M1/70; RRID: AB_394774
CXCR2-PerCP-Cy5.5	Biolegend	Clone SA044G4; RRID: AB_2565695
CXCR4-APC	eBioscience	Clone 2B11; RRID: AB_10670877
CD41-PE	eBioscience	Clone MWReg30 RRID: AB_2538354
Ly6C-FITC	Biolegend	Clone HK1.4
CD62L-APC	eBioscience	Clone MEL-14; RRID: AB_469410
CD62L-FITC	eBioscience	Clone MEL-14; RRID: AB_465109
Anti-CXCL1	R&D	MAB453; RRID: AB_2087696
Anti-CXCL2	R&D	MAB452; RRID: AB_2230058
Anti-CXCL12	R&D	MAB350; RRID: AB_2088149
Anti-Ly6G (depleting antibody)	BioXCell	BE0075-1; RRID: AB_1107721
Experimental Models: Organisms/Strains		
WT	Charles River	C57BL/6
$Mrp8^{CRE}$	Passegué et al., 2004	B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw
$Cxcr2^{fl/fl}$	Schloss et al., 2016	C57BL/6-$Cxcr2^{tm1Rmra}$
$Cxcr2^{\Delta N}$	This paper	N/A
$Cxcr4^{fl/fl}$	Nie et al., 2004	B6.129P2-$Cxcr4^{tm2Yzo}$
$Cxcr2^{\Delta N}$	This paper	N/A
$Cxcr2^{WHIM}$	Balabanian et al., 2012	$Cxcr4^{/1013}$
$Arntl^{fl/fl}$	Janich et al., 2011	B6.129S4(Cg)-$Arntl^{tm1Weit}$
$Arntl^{\Delta N}$	This paper	N/A
Selp; $Sele^{-/-}$	Frenette et al., 1996	B6.129S2-$Sele^{tm1Hyn}$ $Selp^{tm1Hyn}$
DsRED	Vintersten et al., 2004	B6.Cg-Tg(CAG-DsRed*MST)1Nagy
$Lyz2^{GFP}$	Faust et al., 2000	B6.129P-$Lyz2^{tm1(EGFP)1.1Graf/Mmmh}$
Candida albicans	Del Fresno et al., 2018	SC5314
Chemicals, Peptides, and Recombinant Proteins		
CXCL12 (recombinant)	R&D	460-SD
CXCL1 (recombinant)	R&D	453-KC
CXCL2 (recombinant)	R&D	452-M2
TNF-α	R&D	410-MT-050
AMD3100	Tocris	Cat# 3299

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primers for qPCR, see Table S3	This paper	N/A
Critical Commercial Assays		
CXCL1 quantification kit	R&D	DY275
CXCL2 quantification kit	R&D	DY276-05
CXCL12 quantification kit	R&D	DSA00
Mouse ProcartaPlex™	Thermo Scientific	PPX-10-MXTZ766
Software and Algorithms		
ImageJ	NIH	Schindelin et al., 2015
Imaris	Bitplane	RRID: SCR_007370
Genesis	TÜ Graz	RRID: SCR_015775
Prism	Graphpad	RRID: SCR_002798
Flowjo vX	Treestar	RRID: SCR_008520

CONTACT FOR REAGENT AND RESOURCE SHARING

Reagents used in this study are readily available from the noted commercial suppliers in the method itself. Further information and requests for other resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andrés Hidalgo (ahidalgo@cnic.es).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All experiments were performed in 7- to 18-week-old male C57BL/6 mice kept in a specific pathogen-free facility at Centro Nacional de Investigaciones Cardiovasculares (CNIC) under a 12 h light and 12 h dark schedule (lights on at 7am, off at 7pm), with water and chow available *ad libitum*. To generate mice with neutrophil-specific deficiency, we crossed *Arntl*$^{fl/fl}$ (kindly provided by S. Benitah) (Janich et al., 2011), *Cxcr4* $^{fl/fl}$ (Nie et al., 2004) or *Cxcr2* $^{fl/f}$ (Schloss et al., 2016) with hMRP8^{CRE} mice (Passegué et al., 2004), *Cxcr4*^{WHIM} mice with a hyper-signalling form of CXCR4 have been described (Balabanian et al., 2012) and were used as donors to generate BM chimeras. *Cxcl2*$^{-/-}$ mice were obtained from Jackson. In some control experiments we crossed *Cxcr2*$^{fl/f}$ mice with the Ly6G^{CRE} mice (Hasenberg et al., 2015). Mice deficient in P an E-selectins (*Selp*; *Sele*$^{-/-}$) have been previously described (Frenette et al., 1996). To obtain reporter mice for intravital microscopy (IVM) studies, we crossed *Selp*; *Sele*$^{-/-}$ mice with transgenic mice expressing DsRed under the control of the β-actin promoter (DsRed^{Tg}; Vintersten et al., 2004) or with the *Lyz2*^{GFP} reporter mouse (*Selp*; *Sele*$^{-/-;\ GFP}$ mice)(Faust et al., 2000). Both were also used in wild-type reporters for some intravital imaging experiments. In control experiments, we confirmed that wild-type mice used as controls yielded a similar phenotype compared with *hMRP8*^{cre} alone, *Arntl*$^{fl/fl}$ and *Cxcr4*$^{/fl}$ mice (data not shown). No specific randomization method was followed in this study. All experimental procedures were approved by the Animal Care and Ethics Committee of CNIC and the regional authorities.

Human Studies

The study comprised blood from 12 healthy volunteers withdrawn at 12am, 4pm, 8pm, 12pm, 4am, and 8am. The study complied with current Spanish legislation on clinical research in humans and was approved by the Ethics Committee for Clinical Research of Hospital Universitario de la Princesa. All volunteers gave written informed consent to participate in the study.

METHOD DETAILS

Analysis of Human Samples

Total blood obtained from 12 healthy volunteers at 12am, 4pm, 8pm, 12pm, 4am and 8am and erythrocytes lysed in hypotonic buffer. Cells were incubated in 100μl PBS buffer containing 2 mM EDTA and 1% BSA (PEB buffer) with the following antibodies: anti-CXCR4-allophycocianin (APC; clone 12G5), anti-CD11b-FITC (clone ICRF44; both from eBiosciences), anti-CD16-pacific blue (clone 3G8), anti-CD62L-phycoerythrin (PE; clone DREG56), anti-CD11c-APC and 7AAD (all from BD Biosciences). Cells were washed and analysed in a Canto flow cytometer at the Hospital de la Princesa, Madrid.

Parabiosis

We followed previously published procedures (Casanova-Acebes et al., 2013). Briefly, anesthetized mice were shaved and matched incisions were made from the olecranon to the knee joint, then olecranon and knee were attached by a single suture from one mouse to the other, using 5-0 polypropylene, and the dorsal and ventral skins were stitched by continuous suture. Analyses were done 4 to 6 weeks after surgery.

Cytometry and Cell Sorting

Cytometric analyses were performed using a Sony SP6800 Spectral Analyzer (Sony Biotechnology, Japan) or a LSRII Fortessa. For human sample cytometry we used a Canto flow cytometer (BD BioSciences). All cell sorting experiments were performed using an FACS Aria cell sorter (BD Biosciences), except the circadian sorting of blood neutrophils for qPCR analysis, which was performed using a Sony SH800S Cell Sorter. In all cases we obtained purities > 95%. All analyses, except for human samples, were done at the Cellomics Unit of the CNIC. All antibodies and streptavidin conjugates used in this study are listed in Table S2.

Whole-Mount Staining of Excised Cremaster Muscles

Excised cremaster muscles were fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were washed 3 times in PBS containing 0.5% Triton-x 100 (PBST) and blocked for 2 h in PBST 25% FBS at room temperature with shaking. Staining of neutrophils was performed using a biotinylated anti-Mrp14 antibody (clone 2B10 kindly provided by Dr. N. Hogg, Cancer Research UK, London) and blood vessels with an anti-CD31 antibody (BD Biosciences) in 10% FBS-PBST overnight at 4°C with shaking. Cremaster muscles were then washed and incubated with secondary antibody (goat anti-rabbit-Alexa 405 or –Alexa 647; Life Technologies) and Alexa-488 conjugated Streptavidin in 10%FBS-PBST for 4h at room temperature. Samples were then washed and mounted in Mowiol 4-88 (Mw 31,000; Sigma). Imaging of whole-mount intestines was performed using a Leica SP8 X confocal microscopy system coupled to a DMI6000 inverted microscope, with 10x (HC PL Fluotar 10x/0.3 Dry) or 63x (HC PL Apo CS2 63x/1.4 OIL) magnification objectives. For in-depth quantification, large Z-stack and panoramic-stitched images were taken with a Nikon A1R confocal system coupled to a Nikon Eclipse-Ti inverted microscope with the following lines: Diode 402nm Argon Laser 457, 476, 488, 514nm Diode 561nm HeNe Laser 642nm using a Plan Apo 10x/0,45 dry objective and the software NIS Elements AR 4.30.02 (Build 1053 LO, 64 bits, Nikon Instruments, Tokyo, Japan) for acquisition of confocal 3D tile-scan images of the whole cremaster muscle, which were afterwards analysed using Imaris (Bitplane, Zurich, Switzerland). All imaging was performed at the Microscopy & Dynamic Imaging Unit of CNIC.

RNA Isolation, Reverse Transcription, and rtPCR

Total RNA was prepared with the RNA Extraction RNeasy Plus Mini-kit (QIAGEN) and RNA was reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative PCR (SYBR-green, Applied Biosystems) assays were performed with an Applied Biosystems 7900HT Fast Real-Time PCR System sequencer detector. Expression was normalized to the expression of the *36b4* housekeeping gene. Primer sequences are listed in the Table S3.

Chromatin Immunoprecipitation (ChIP)

Neutrophils were sorted from the bone marrow as indicated previously and fixed in 0.75% formaldehyde for 10 min at room temperature. Formaldehyde was then quenched with glycine (final concentration 0.26 M) for 5 min. After washing twice with cold PBS, cells were pelleted and frozen at -80°C. Each sample was lysed in 0.25 ml of lysis buffer (1% SDS, 10 Mm EDTA, 50 mM Tris-HCl pH 8, 1 mM PMSF, 5 μg/ml leupeptin-aprotinin, 1 μg/ml pepstatin A, 10mM NaF, 10 mM sodium orthovanadate, and 10 mM β-glycerophosphate) for 30 min with rotation at room temperature. Lysates were sonicated using the Diagenode Bioruptor sonication system (Diagenode, Bioruptor UCD-200TM-EX). Each sample was sonicated for two rounds of six cycles (30s ON and 30s OFF) at the high power setting to obtain DNA fragments between 500 and 1000 bp. After sonication, samples were centrifuged to remove insoluble debris, supernatants were collected and 5% of each sample was separated to use as a measure of chromatin input for normalization. The rest of the sample was diluted 1/10 in ChIP dilution buffer (1% TritonX-100, 20 mM Tris-HCl, pH 8, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 10 mM NaF, 10 mM sodium orthovanadate, and 10 mM β-glycerophosphate) for immunoprecipitation. Samples were precleared with protein A Sepharose beads (GE Healthcare, 17-0780-01) that were previously pre-adsorbed with fish sperm DNA (Roche, 11 467 140 001) and bovine serum albumin (New England Biolabs, Ref. B9001S) for 1 hour at 4°C. Anti-Bmal1 antibody (ChIP Grade [ab3350]) was added to the lysates after removing the preclearing beads and incubated overnight at 4°C. Pre-adsorbed protein A Sepharose beads were then added, incubated for 1 hour at 4°C, and then washed three times with ChIP washing buffer (0.1% SDS, 1% TX-100, 20 mM Tris-HCl, pH 8, 2 mM EDTA, and 150 mM NaCl) and once with final washing buffer (0.1% SDS, 1% TX-100, 20 mM Tris-HCl pH 8, 2 mM EDTA, and 500 mM NaCl). To elute DNA, beads were gently shaken with 200 μl elution buffer (1% SDS and 100 mM $NaHCO_3$) for 45 min at room temperature. To reverse the crosslinking, samples were incubated overnight at 65°C. Then samples were incubated with 3 μl RNase (Roche, 11119915001) 30 min at 37°C prior to the addition of 8 μl of Proteinase K (Roche, 3115828001) for 1 hour at 50°C and DNA was purified by ethanol precipitation. Immunoprecipitated chromatins and their respective inputs before immunoprecipitation were analyzed by RT-qPCR using the primers listed in Table S3. The primers for the Cxcl2, Per2 and Nr1d1 promoter regions were designed in the vicinity of E-box sequences.

Intravital Imaging of the Mouse Skin

For intravital microscopy of the dermal microcirculation, the dorsal side of the ear of anesthetized mice was mounted on a custom-built support, and we acquired images from several venules in 2 minute-long time-lapse videos at 3 s intervals. We used the VIVO system built by 3i (Intelligent Imaging Innovations, Dever, CO) upon an Axio Examiner Z.1 workstation (Zeiss, Oberkochen, Germany) and mounted on a 3D motorized stage (Sutter Instrument, Novato, CA). The system was equipped with a CoolLED pE widefield fluorescence LED light source (CoolLED Ltd. UK) and a quad pass filter cube with Semrock Di01-R405/488/561/635 dichroic and FF01-446/523/600/677 emitter. A plan-apochromat 40x W NA1.0 objective (Zeiss) was used and images were collected with a CoolSnap HQ2 camera (Photometrics, Tucson, AZ). The system was run on a Dell Precision T7500 computer system (Dell Inc., Round Rock, TX) using the SlideBook software (Intelligent Imaging Innovations). Acquisitions were made at ZT5, ZT9 or ZT13 and neutrophils were stained with an AF647-conjugated anti-Ly6G antibody (clone 1A8, BioXcell), while blood vessels were visualized using red fluorescent Dextran (Molecular Probes). In some groups, anti-P and E-selectin antibodies or Rat IgG control antibody (25 μg/mouse) were injected 2h before imaging. Quantification was done using the ImageJ (NIH, Bethesda, MD). Cells were considered adhered if they remained stationary on the venule for over 30 seconds. Vessel diameters were measured using the ImageJ software (Schindelin et al., 2015) to normalize the number of adherent cells.

Intravital Microscopy of the Cremaster Muscle

Intravital microscopy of the cremaster muscle after TNF-α stimulation (R&D Systems, 0.5μg intrascrotal injection) was performed as previously reported (Hidalgo et al., 2009) using the VIVO system indicated above. For confocal IVM, we used laser stacks for 488, 561 and 640nm beams coupled with a confocal scanner (Yokogawa CSUX-A1; Yokogawa, Japan) and images were acquired with 0.5μm Z-intervals. The SlideBook software was used for acquisition and analysis. Ten to twenty venules segments per mouse were analysed 150 to 210 min after TNF-α treatment in multiple fluorescence channels (Cy3/561 for PE, FITC/488 for FITC and Cy5/640 for APC) and bright-field images with 1x1 or 2×2 binning with 3 second interval for 2 min on each field of view. For double staining with PE- and FITC-conjugated antibodies, acquisition was facilitated in single (FITC) and quadrant (PE) filters in order to avoid bleed-through of fluorescent signals between channels. For the visualization of leukocytes, fluorescently labelled anti-Ly6G-APC, anti-Ly6C-FITC and anti-CD62L-APC antibodies were injected intravenously at 1 μg/mouse.

For analysis of rolling and adhered cells to the inflamed endothelium we used the SlideBook software. Counts of rolling or adhered cells in 2-minute captures (captured at 3 second intervals) were normalized using the width of the vessel to allow comparison among all vessels. For adhesion or rolling efficiency indices, these data were compared with the frequency of free-flowing WT and experimental cells in the blood for each mouse, which was obtained from cytometric analysis of blood neutrophils for each parabiont or BM chimeric mouse. Kinetic parameters for crawling neutrophils were calculated using ImageJ, with the help of the Manual Tracking plugin (Fabrice Cordelières, Institut Curie, France) and the Chemotaxis and Migration Tool (Gerhard Trapp and Elias Horn, ibidi GmbH, Germany). Analyses of extravasated neutrophil were performed on large tile-scans of whole-mounted cremaster muscles with Imaris (Bitplane AG, Switzerland). We performed blind automatic counting of extravasated neutrophils by masking out the vessels using $CD31^{+}$ fluorescence (on the BMT WT:$Arntl^{\Delta N}$, WT: $Cxcr4^{\Delta N}$ chimeras and *Selp*; $Sele^{-/-}$: $Arntl^{\Delta N}$ parabionts) or manually delimiting the vessels using brightfield or laminin fluorescence (on *Selp*; $Sele^{-/-}$ with WT parabionts). Kinetic parameters for extravasated neutrophils were obtained in the $DsRed^{Tg}$ with *Selp*; $Sele^{-/-}$-$Lyz2^{GFP}$ parabionts using automatic tracking of cells with Imaris.

Intravital Imaging of Ischemia and Reperfusion Injury

Mouse cremaster were prepared as indicated without TNF-α stimulation. Upon exteriorization of the muscle we intravenously injected fluorescent antibodies to label cellular populations. Ly6G-APC (clone 1A8; BioXcell) for neutrophils, CD41-PE (eBioscience) for platelets, Ly6C-FITC (Biolegend) and CD62L-APC (BD Bioscience). Ischemia was achieved by occlusion of the incoming and outgoing vessels by clamping the tissue connecting the muscle and the animal's body with a 15mm Micro Serrefine clamp (Fine Science Tools, Heidelberg, Germany) for 45 minutes. Reperfusion was achieved by removal of the clamp. In some experiments neutrophils were depleted prior ischemia by injecting an anti-Ly6G-antibody (see below). Recordings were made with 3 second interval for 2 minutes for each field of view. Imaging was performed before and during ischemia, and during reperfusion. Some mice were treated intravenously with 300μg of Cl-amidine (Cayman Chemical Company, Ann Arbor) 1h before imaging, or with 500μg DNAse I (Roche, Basel, Switzerland) immediately before imaging.

Analysis of Neutrophil Clearance in the Steady State

11- to 18-week-old $DsRed^{Tg}$ mice were analysed after 1 month in parabiosis with non-fluorescent $Cxcr2^{\Delta N}$, $Cxcr4^{\Delta N}$, $Arntl^{\Delta N}$ and *Selp*; $Sele^{-/-}$ mice or wild-type controls. The blood of each WT mice was analysed and used to obtain the ratio of neutrophils derived from each partner. Mice were sacrificed with CO_2 and carefully perfused with 30 ml of PBS to remove all blood. Tissues (white adipose tissue or WAT, large intestine, liver, lung, skin and spleen) were extracted and kept in cold PBS (except liver, kept at room temperature in HBSS) and processed immediately. Skin, large intestine (colon), lung and WAT were digested in HBSS with liberase (1U/ml, Roche) and DNAse I (1 mU/ml, Sigma) for 30 min at 37°C. Bone marrow and spleen were mechanically dissociated to prepare single-cell suspensions by flushing and straining, respectively. Enrichment of leukocytes in liver was performed by centrifugation using a 36% Percoll (GE Healthcare, diluted in HBSS) gradient. Colons were pre-incubated with HBSS containing 5mM EDTA for 45 min at 37°C before digestion to remove epithelial cells. Blood counts were analysed in an automated

hemocytometer (Abacus Junior, Diatron; Holliston, USA) and blood red blood cells (RBC) lysed in a hypotonic buffer. Single-cell suspensions from tissues were incubated with fluorescently-conjugated antibodies against CD45, CD11b and Ly6G (BioXcell) and analysed in a Sony SP6800 Spectral Analyzer. DsRed+ Ly6G^{hi} cells and DsRed^{NEG} Ly6G^{HI} cells discriminated host- (DsRedTg) from partner-derived neutrophils. To normalize values between the different parabiotic pairs we corrected the ratios of host versus partner-derived neutrophils in each tissue with the ratios present in blood of each parabiotic pair. Deviations from the original ratio in blood was used to estimate the efficiency of migration of partner-derived neutrophils in each organ. Finally, infiltration efficiencies were normalized to the values obtained for the DsRed^{Tg}:WT pairs, which was set as the control group.

Quantification of Neutrophil Numbers in Tissues

In some experiments, we measured absolute numbers of neutrophils present in tissues (see Neutrophil clearance assays). Truecount beads (Truecount absolute counting tubes, BD) were prepared at a concentration of 10,000 beads per ml of PBS buffer. 300μl of the bead suspension were added to single cell suspensions stained for flow cytometry, and then neutrophil number values were calculated based on the number of beads per tube and corrected by the weight or volume of tissue analysed.

Chemokine Quantification in Plasma

CXCL12, CXCL1 and CXCL2 amounts were measured in plasma samples taken every 4h from WT mice using commercially available ELISA reagents, following the manufacturer's protocol (R&D Systems; Minneapolis; MN).

RNA-Sequencing

Blood neutrophils were FACS sorted using by Ly6G and DAPI labelling, with typical purities > 95%. cDNA amplification from neutrophil RNA (1275pg) and generation of index-tagged sequencing libraries were carried out using the Ovation® Single Cell RNA-Seq System (NuGEN® Technologies, San Carlos CA). Libraries were quantified using a Quant-iTTM dsDNA HS assay with the Q-bit fluorometer (Life Technologies, Carlsbad, California). Average library size and size distribution were determined using a High Sensitivity DNA assay in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Libraries were normalized to 10nM using Tris-Cl 10mM, pH8.5 with 0.1% Tween 20. Libraries were applied to an Illumina flow cell for cluster generation (True Seq SR Cluster Kit V2 cBot) and 61 nt long, single-end reads were generated on a Genome Analyzer IIx, using the TruSeq SBS Kit v5 (Illumina) and following the standard RNA sequencing protocol. Reads were further processed using the CASAVA package (Illumina) to demultiplex reads according to adapter indexes and to produce fastq files. Read quality was determined with the application FastQC (Schageman et al., 2013). The RNA sequencing experiments were performed at the Genomics Unit at CNIC.

Western Blotting

Blood neutrophils were sorted based on Ly6G (clone 1A8) expression. Purified cells were lysed in RIPA buffer containing 50 mM Tris-HCl, pH 8; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1mM PMSF (Sigma) and a protease inhibitor cocktail (Sigma). Proteins from 2x10^5 lysed cells were separated by 10% SDS-PAGE and transferred onto PVDF membrane. Membranes were incubated overnight with antibodies against Bmal1 (Bethyl labs) and β-Actin (Abcam) at 1:1000 dilutions, and then thoroughly washed and incubated with HRP-conjugated anti-rabbit or anti-mouse antibody (1:1000; GE Healthcare Life Sciences). Blots were visualized using the chemiluminescent Luminata Forte Western HRP Substrate (Millipore).

Generation of Transplant Chimeras

To address the aging status of mutants in the same physiological context as wild-type cells, we generated mixed bone marrow chimeras, which has the added advantage of allowing simultaneous staining and analysis of willd-type and mutant cells. Donor BM cells were harvested from DsRed^{Tg} or experimental models by flushing the femur with PBS. Recipient wild-type C57BL/6 mice were lethally irradiated (two 6Gy doses, 3 h apart) before receiving 1 million bone marrow nucleated cells by intravenous injection. For mixed chimeras, equal numbers of experimental and DsRed^{Tg} BM cells were mixed before intravenous injection. Engraftment of recipient animals was assessed 8–10 weeks after transplantation by analysis of the percentage of mutant and DsRed^{Tg} leukocytes in blood by flow cytometry.

Circadian Analysis of Aging Markers and RNA Extraction

Circadian blood samples were extracted every 4 h during 24 h from wild-type or experimental mice, starting at ZT1 (Zeitgeber time, 1 hour after the onset of light, 7:00 at the CNIC's animal facility). For circadian surface marker analysis, RBC were lysed in hypotonic lysis buffer (0.15M KH_4Cl, 0.01M KHCO3 and 0.01M EDTA in water) and incubated 15 minutes with 0.25 μg anti-Ly6G (1A8 clone, BioXcell), -CXCR2 (Biolegend) and -CXCR4 (eBioscience) antibodies, washed and analysed in a Sony SP6800 Spectral Analyser. Analysis was performed using Flowjo vX (Tree Star Inc, Ashland, OR). For circadian RNA assays, blood taken at above circadian time points was lysed for RBCs and sorted using a Sony SH800S Cell Sorter (Sony Biotechnology, Japan) based on viable Ly6G$^+$ cells. RNA was extracted as indicated above.

Analysis of Neutrophil Aging in Light-Dark and Dark-Dark Light Regimes

To check the circadian nature of neutrophil aging we compared twice a day (ZT5 and ZT13) the number of CD62L^{LO} aged neutrophils for 4 consecutive days in WT mice subjected to 12h:12h light: dark cycles, and in mice subjected to constant darkness in a light

cabinet (LFC 3-16 with EF700ET programmer from E. Becker & Co) starting 24h after the onset of the light schedule. We analysed blood neutrophils at the indicated time points by flow cytometry as indicated above.

Entrainment of Neutrophil Aging by Inverted Light Regime

We maintained wild-type mice in 12h: 12h light-dark or dark-light (inverted light cycle) regimes using light-cabinets (Light/ Dark Chamber LT400 from Parkbio). After 3 weeks of this inverted regime we analysed peripheral blood neutrophils every 6 hours for 24 hours by flow cytometry, as indicated above.

Infection with *Candida albicans*

Mice were intravenously infected with $1.5x10^5$ *C. albicans* conidia (SC5314 strain) and monitored daily for weight loss and general health following our institutional guidance. Infections were performed either at ZT5 or ZT13. Kidney fungal burden was determined at day 6 post-infection by plating organ homogenates in serial dilutions on YPD plates (Sigma); colony-forming units (CFUs) were counted after growth for 48 hr at 30°C. Flow cytometry analysis of renal leukocyte infiltrates was performed on cell suspensions obtained from kidney homogenates obtained by digestion with 0.025 mg/ml of Liberase TL (Roche) for 10 min at 37°C and filtered through 40 μm cell strainers (BD PharMingen). Phenotypic analyses of renal leukocyte infiltration were performed by flow cytometry.

Heat-Killed *Candida albicans* (HKC) Phagocytosis Assay

Candida albicans conidia were heat-killed by boiling for 30 minutes. To quantify the phagocytic capacity, BM-sorted neutrophils were stained with 5 μM CFSE and exposed to labelled HKC labelled with 2.5 μM Cell Violet-labelled (both from Molecular probes) at a 30:1 ratio, for 15 min at 37°. After washing, cells were recovered on ice with PBS containing 5 mM EDTA. To remove bound but not internalized HKC, cells were incubated in Trypsin-EDTA (0.25%; Life Technologies) for 15 minutes at 37° prior to analysis by flow cytometry. Cells were fixed in 4% paraformaldehyde and neutrophils engulfing HKC were identified as double-positive cells for Cell Violet and CFSE. Cytospin preparations were also done for microscopic inspection of phagocytosis.

Mouse Model of Acute Myocardial Infarction

Male 8- to 12-week-old mice were subjected to 45 min occlusion of the left anterior descending (LAD) coronary artery followed by 1h reperfusion (for infarct size). For survival experiments, the LAD was reperfused for up to 24h and mice monitored hourly for the first 6h, and at 16, 20 and 24h. The I/R procedure was performed as previously described (García-Prieto et al., 2017). In some experiments mice were depleted of neutrophils as indicated below. Briefly, fully anesthetized animals were intubated and temperature controlled throughout the experiment at 36.5°C to prevent hypothermic cardioprotection. Thoracotomy was then performed and the LAD was ligated with a nylon 8/0 monofilament suture for 45 min. The electrocardiogram was monitored (MP36R, Biopac Systems Inc.) to confirm total coronary artery occlusion (ST-segment elevation) throughout the 45 min ischemia. At the end of the ischemia, the chest was closed and animals were kept with 100% O_2 and analgesized with buprenorphine (subcutaneous injection, 0.1 mg/kg). For quantification of infarct size, mice were re-anesthetized and re-intubated, and the LAD coronary artery was re-occluded by ligating the suture in the same position as the original infarction. Animals were then sacrificed and 1 mL of 1% Evans Blue dye (Sigma) was infused IV to delineate the Area at Risk (AAR: myocardium lacking blood flow, i.e., negative for blue dye staining). The left ventricle (LV) was isolated, cut into transverse slices (5-7 1-mm thick slices per LV), and both sides were imaged. To delineate the infarcted (necrotic) myocardium, slices were incubated in triphenyltetrazolium chloride (TTC, Sigma) at 37 °C for 15 min. The slices were then re-photographed, weighed, and regions negative for Evans Blue staining (AAR) and for TTC (infarcted myocardium) were quantified using ImageJ (NIH, Bethesda, MD). Percentage values for AAR and infarcted myocardium were corrected to mg independently for each slice. Absolute AAR and infarct size were determined as the mg:mg ratio of AAR:LV and infarcted myocardium:AAR, respectively. Outcome assessment was performed blind to condition (mouse type, zeitgeber time or treatment).

Brain Ischemia

To induce brain ischemia (without reperfusion injury) we followed previously described protocols (Sreeramkumar et al., 2014). Mice were anesthetized with isoflurane 1.5%–2% in a mixture of 80% air and 20% oxygen, and body temperature was maintained with a heating pad during the surgical procedure and anaesthesia recovery. Mice were subjected to permanent focal cerebral ischemia (middle cerebral artery occlusion - pMCAO) through the distal occlusion of middle cerebral artery by ligature of the trunk just before its bifurcation between the frontal and parietal branches with a 9-0 suture, in combination with the occlusion of the ipsilateral common carotid artery. Following surgery, individual animals were returned to their cages with free access to water and food. All the groups were performed and quantified in a randomized fashion by investigators blinded to groups. Physiological parameters were not significantly different among the different groups studied. Infarct size was determined by magnetic resonance imaging 48 hours after MCAO using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany). Infarct volume was calculated using the ImageJ software (NIH, USA) from the T2-weighted images. With the observer masked to the experimental conditions, the areas of the infarcted tissue (InfArea), the whole ipsilesional hemisphere (IpsArea) and the whole contralesional hemisphere (ContrArea) were delineated for each slice. Then, infarct volume, expressed as percentage of the hemisphere that is infarcted (%IH) was calculated using the formula: %IH = InfVol/ContrVol*100 where InfVol (Infarcted Tissue Volume) = $\Sigma InfArea_i$ / $SwellingIndex_i$, ContrVol (Contralesional Hemisphere Volume) = $\Sigma ContrArea_i$ and $SwellingIndex_i$ = $IpsArea_i/ContrArea_i$. Neutrophil quantification in brains was performed 48h after surgery. The ipsilateral cortex was dissected, placed in ice-cold PBS and dissociated into a single cell suspension by

mechanical dissociation. Cell suspensions were filtered on 70-μm nylon mesh strainers and centrifuged at 300g for 10 min at room temperature. Pellets were resuspended in 8 mL of 35% Percoll and overlaid on the top of 5 ml HBSS. The gradient was centrifuged at 800*g* for 40 minutes at 4°C and cell pellets resuspended for staining with anti-CD11b-FITC and anti-Ly-6G-PE antibodies (BD Bioscence). Stained cells were analysed in a FACSCalibur flow cytometer with CellQuest software (BD Pharmingen, San Jose, CA) and data were analysed using FlowJo software (Tree Star Inc, Ashland, OR).

Scanning Electron Microscopy

Blood from WT, *Arntl* $^{\Delta N}$ and *Cxcr4*$^{\Delta N}$ was harvested, RBC lysed and leukocytes stained with Ly6G (1A8, BioXcell) before sorting in a FACS Aria sorter (BD Biosciences). Sorted cells were immediately centrifuged and fixed using 4% PFA plus 2.5% glutaraldehyde in PBS for 2h at 4°C. Cells were then dehydrated by serial 5 min incubations in increasing concentrations of ethanol, (30%>50%>70% >80%>90%>100%). Samples were dried in an automated critical point dryer (Leica EM CDP 300) and then coated in a rotary-pumped coating system (Quorum Technologies Q150RS). Imaging was performed at 10kV with a field emission microscope (JEOL 6335F). Critical point drying, coating and imaging of the samples was performed at ICTS National Centre of Electron Microscopy (UCM, Madrid, Spain).

CXCR2 and CXCR4 Cross-Inhibition Assays

Wild-type mice were bled, RBC lysed and leukocytes resuspended in RPMI 1640 (Invitrogen). Some cells were pretreated with CXCL12 (50 ng/ml, R&D Systems) for 5 minutes at 37°C while others were left untreated. Cells were allowed to migrate towards CXCL1 (50ng/ml, R&D Systems) or CXCL2 (50ng/ml, R&D Systems) through 6.5mm transwells with 5μm pore polycarbonate membrane insert (Corning, NY, USA), for 1h at 37°C. Transmigrated cells were collected and stained with anti-Ly6G and anti-CD62L antibodies for cytometric analysis. Migration to only media was also used as a control. Quantification was performed using Truecount beads, as indicated above.

Neutrophil Depletion

In some experiments mice were depleted of neutrophils prior to ischemia/reperfusion. Mice were injected 100μg of anti-Ly6G antibody (1A8 clone; BioXCell; West Lebanon, NH) intraperitoneally for 2 consecutive days resulting in >93% reduction in blood neutrophil counts. Lymphocyte and monocyte counts were not affected by this treatment (Casanova-Acebes et al., 2013).

Zymosan-Induced Peritonitis

Transplantation chimeras or the wild-type partner in parabiotic pairs were treated with zymosan (1mg, intraperitoneal injection, Sigma). After 2 h we took blood samples and obtained the peritoneal lavage for cytometric analyses and cell count. We compared the ratios of neutrophils from each donor in the peritoneum and blood to estimate the migration efficiencies of mutant cells (ratio in peritoneum / ratio in blood). We also compared the migration efficiency of neutrophils in wild-type mice at ZT5 and ZT13. In this case we measured the absolute number of neutrophils in the peritoneal lavage using counting beads (Truecount absolute counting tubes, BD) and normalized migration relative to the absolute number of neutrophils in blood.

For experiments analyzing the dynamics of neutrophil aging during inflammation, we treated wild-type mice with zymosan (1mg, i.p.) and after 24h we analyzed blood neutrophils twice per day (ZT5 and ZT13) for 4 consecutive days. We estimated the absolute number of aged (CD62L^{LO}) neutrophils by flow cytometry as indicated above.

Soluble Selectin Binding Assays

Transplantation chimeras were bled and RBC lysed, then cells washed in RPMI 1640 containing 5% FBS and stained them using E- or P-selectin/human IgM chimeras as reported (Hidalgo et al., 2007). Cells were further incubated for 15 min with a FITC-conjugated anti-human IgM (Jackson Immunoresearch) and anti-Ly6G-APC (BioXcell) antibodies. Control samples contained 5mM EDTA. Cells were analysed in a Sony SP6800 Spectral Analyzer.

Auto-Perfused Flow Chamber Assay

In order to investigate the number of rolling and adherent cells, we used a microflow chamber system (Zarbock et al., 2007). 20 x 200 ⌈m rectangular glass capillaries were filled with P-selectin (50 ⌈g/ml) alone or in combination with ICAM-1 (15 μg/ml) and/or CXCL1 (25 ⌈g/ml)⌈for 2 hr and blocked for 2 hr with 1% casein (Pierce Chemicals, Dallas, TX). One side of the chamber was connected to a PE 10 tubing and inserted into the carotid artery. The other side of the chamber was connected to a PE 50 tubing and used to control the wall shear stress, which was calculated as described (Zarbock et al., 2007). Microscopy was conducted with a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 20, N.A. 0.5). Recordings were taken using an SW40/0.75 objective and a digital camera (Sensicam QE). Capturing on P-selectin was analyzed after 2 minutes and chemokine-induced arrest was analyzed after 6 minutes of perfusion.

Cortical Beta-Actin Quantification

Sorted neutrophils were cytospun onto Superfrost Plus microscope slides (Thermo Scientific, Waltham, USA) with a Shandon Cytospin 4 (Thermo Scientific) for 5 minutes at 500 RPM in medium acceleration. Then cells were fixed with 4% PFA in PBS for 10 minutes and blocked with 5% goat serum, 5% BSA in saline in a humid chamber for 30 minutes. Finally, cells were stained with a rabbit

anti-mouse beta-actin antibody (ab8227, Abcam) and with a secondary goat anti-rabbit antibody conjugated with AF568. Then slides were mounted with Mowiol and captured with a Leica SP8 X confocal microscopy system. Analysis of captured images was performed using Imaris (Bitplane).

ROS Quantification

Red blood cell-lysed blood was plated in RPMI in 96-well polystyrene microplates (Corning Falcon, New York, USA) and stimulated with 50nM of phorbol 12-myristate 13-acetate (PMA) for 1h. Cells were then stained with 5mM Dihydroethidium (DHE, Thermo Fisher, Waltham, USA) for 20 minutes and stained for cytometric analysis.

Chemotaxis Assay

Whole blood was harvested and red blood cells were lysed. Cells were plated in 6.5mm polycarbonate transwells with 5μm pores (Corning, Corning, USA) in RPMI medium. In the bottom well, a single chemokine was added to allow chemotactic migration: 25mg/ml CXCL12 (R&D), 20ng/ml CXCL1 (R&D), 5ng/ml LTB4 (Tocris), 100mM fMLP (Sigma) or 10ng/ml CCL2 (R&D). Transwells were incubated 2h at 37°C and transmigrated cells were harvested from the bottom well and stained for cytometric analysis. The number of transmigrated cells was assessed by the presence of a known number of Truecount beads (BD Biosciences).

Multiplex Cytokine Assay

Mice were i.p. injected with Zymosan as indicated above. After 2 hours peritoneal lavages were collected and neutrophils stained for FACS sorting. One million sorted neutrophils were incubated for 3h in RPMI containing 0.5% BSA and 100,000 heat-killed *C. albicans* conidia. Supernatants were collected and frozen at -80°C until cytokine quantification was performed. CXCL1, IL-10, IL-1β, IL-12, TNF-α, G-CSF, IL-23, CXCL2, IL-6 and CCL2 were measured in neutrophil supernatants using the Mouse ProcartaPlex™ Multiplex Immunoassay (PPX-10-MXTZ766), following the manufacturer's protocol (Thermo Scientific, Waltham, USA). For TGF-β1 detection, samples were activated with HCl and measured using commercial TGF beta-1 Mouse ProcartaPlex™ Simplex Kit (EPX01A-20608-901). Data acquisition was performed on a MagPix instrument (Luminex Inc, Houston, TX) using xPONENT v4.2 software (Luminex) and analyzed with ProcartaPlex Analyst software (v1.0; Thermo Scientific).

AMD3100-Induced Neutrophilia

2.5mg/kg of AMD3100 (Tocris) was injected intraperitoneally into wild-type mice 1h before analysis. Then blood neutrophils were analyzed at ZT5 in an automated hemocytometer (Abacus Junior) and stained for cytometric analysis as previously described. At this time (1h after injection of AMD3100) we subjected control or AMD3100-treated mice to AMI and *Candida albicans* infection, as previously described.

Neutrophil Transfer Experiments

To increase the yield of fresh neutrophils we used AMD3100-treated mice as neutrophil donors (see Figure S5G). To minimize ex vivo manipulation of neutrophils we transferred 200 μl of freshly extracted blood from donor mice by i.v. injection into host wild-type mice at ZT5. Aging markers in host and donor cells were then analyzed 5 minutes and 5 hours after inoculation in the peripheral blood of host mice by flow cytometry as indicated above.

BrdU Labelling

For metabolic labelling with 5-Bromodeoxyuridine, mice were intraperitoneally injected with a single dose of 2.5mg BrdU (BD Biosciences). Blood samples were collected at indicated times and stained for Ly6G, CD62L, CXCR2 and CXCR4, followed by fixation and intracellular labelling of BrdU using an APC-conjugated anti-BrdU antibody as per manufacturer's instructions (BD Biosciences).

In Vivo CXCL1 and CXCL2 Blockade

For in vivo blockade of CXCL1 and CXCL2, mice were injected intraperitoneally twice with 50μg of isotype or monoclonal antibody against CXCL1 (MAB453, R&D) or CXCL2 (MAB452, R&D) the night before the analysis (-17h) and the same day (-5h). Blood from treated mice was harvested and analysed by flow cytometry as previously described. In a set of mice, intravital imaging was performed to analyse neutrophil behaviour in the microvasculature of the cremaster muscle by intravital microscopy as previously described.

Cecal Ligation and Puncture (CLP)-Induced Sepsis

CLP was performed as previously described (Rittirsch et al., 2009). Briefly, the peritoneal cavity of ketamine/xylazine-anesthetised mice was exposed with a small incision and the cecum was exteriorized. 80% of the cecum distal to the ileo-cecal valve was ligated using non-absorbable 7-0 suture. A 23-gauge needle was then used to puncture both walls of the distal end of the cecum, and a small drop of faeces was extruded through the perforation. The ligated and punctured cecum was relocated inside the peritoneal cavity and both peritoneum and skin were closed. Mice were then treated with s.c. injection of Buprenorphine. Control mice (sham) were included with the same procedure but without ligation or puncture.

Evans Blue Vascular Permeability Assay

To address whether the constitutive presence of fresh or aged neutrophils in the mutant mice affected the vascular integrity, we performed vascular permeability assays as previously described (Radu and Chernoff, 2013). In brief, a 0.5% solution of Evans blue in sterile PBS was prepared and 200μl of the solution was i.v. injected into WT or mutant mice. 5 minutes after the transfer mice were sacrificed and tissues extracted and weighted. Then, tissues were submerged in 1ml formamide and incubated at 50°C for 24h. Tissues were removed and the tubes centrifuged for 5 minutes at 645 g. Finally, supernatants were measured for absorbance at 610nm using an xMark Microplate Spectrophotometer (BioRad) plate reader. The vascular permeability test was performed in untreated and LPS-treated (10 mg/kg) mice.

Analysis of Endothelial Proliferation and Apoptosis

We processed tissues from WT or mutant mice as previously described (see section Analysis of neutrophil clearance in the steady-state) to obtain single cell suspensions. One half of the suspension was used to measure apoptosis and the rest to assess proliferation. For proliferation we measured Ki67 by intra-nuclear staining in endothelial cells. Cells were stained with anti-CD45 conjugated with PerCP/Cy5.5 (BioLegend) and anti-CD31-APC (eBioScience) and then fixed and permeabilized using the Fix/Perm and Perm Buffers (eBiosciences) according to manufacturer's instructions. Cells were then stained for 20 minutes at 4°C with an anti-mouse and rat Ki67 antibody labelled with eFluor660 (Thermo Fisher) and analysed by flow cytometry.

For quantification of apoptotic endothelial cells we measured Annexin V binding and DAPI labelling. Cell suspensions were incubated with 1:200 of anti-CD45 and anti-CD31, washed twice in cold PBS and resuspended in Annexin V Binding Buffer (10 mM Hepes, pH adjusted to 7.4 with NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1 million cells per ml. 100μl of this cell suspension ($1x10^5$ cells) was stained with PE-conjugated Annexin V (Invitrogen) for 15 minutes at room temperature in the dark. Finally, 400μl of binding buffer containing DAPI was added to each tube and analyzed by flow cytometry within one hour.

Analysis of Neutrophil Aging in Microbiota-Depleted and Germ-free Mice

For microbiota depletion studies we followed a previously published protocol (Zhang et al., 2015). Briefly, mice were fed with a cocktail of antibiotics (ABX) including ampicillin (1 g/l), neomycin (1 g/l), metronidazol (1 g/l) and vancomycin (1 g/l) in drinking water for 4 weeks prior to analysis by flow cytometry. For germ-free mice we compared by flow cytometry SPF-housed with germ free (GF) mice for markers of aging state (see method of Circadian analysis of aging markers and RNA extraction).

QUANTIFICATION AND STATISTICAL ANALYSIS

Specific quantification protocols are detailed in each method above.

RNA-Sequencing Data Analysis

For data analysis, sequencing adaptor contaminations were removed from reads using Cutadapt and the resulting reads were mapped on the transcriptome (GRCm38 Ensembl gene-build 70) and quantified using RSEM v1.17 (Li and Dewey, 2011). Only genes with at least one count per million in at least 2 samples were considered for statistical analysis. Data were then normalized and differential expression was tested using the Bioconductor package EdgeR (Robinson et al., 2010). Raw and Benjamini-Hochberg adjusted p values were calculated for each of the comparisons of interest. Non-adjusted p values were used to identify overrepresented pathways using Ingenuity Pathway Analysis (IPA, Quiagen, https://www.ingenuity.com/). These results were quantitatively validated by qPCR analyses for a collection of relevant genes, and functionally validated using *in vivo* assays as detailed in the manuscript. K-means clustering, PCA analysis and heatmap representations were produced using the Genesis software (Sturn et al., 2002).

Statistical Analysis

Unless otherwise indicated, data are represented as mean values ± standard error of the mean (SEM). Paired or unpaired t test was used when 2 groups were compared, and comparison of more than two datasets was done using one-way analysis of variance (ANOVA) with Turkey's post-test. Where applicable, normality was estimated using D'Agostino & Pearson or Shapiro-Wilk normality test. Log-rank analysis was used for Kaplan-Meier survival curves. Sample exclusion was not performed unless evident signs of disease were found in a mouse, in which case statistically significant outliers were identified using Grubb's test (ESD method). Comparisons of two-time curves were performed using two-way ANOVA. All statistical analyses were performed using Prism v6 (GraphPad Software, California, USA). A p value below 0.05 was considered statistically significant; non-significant differences (ns) are indicated accordingly.

Amplitude versus Zero Test

For determination of diurnal patterns, we performed COSINOR fitting of circadian curves, using the curve-fitting module of Graphpad Prism with the equation Y = Baseline + Amplitude x cos (Frecuency X + Phaseshift), where Baseline = average of Ymax and Ymin; Amplitude = 0.5 x (Ymax – Ymin), Frecuency=0.2618 ($2*\pi/24$) and Phaseshift= value of X at Ymax. To determine whether a diurnal curve displayed an oscillating pattern we used the COSINOR-calculated amplitudes and compared them with a hypothetical zero-amplitude curve (i.e., with no circadian behaviour) assuming that both curves have identical standard deviations. We finally compared

the two curves' amplitudes using unpaired t test analyses. This analysis gave a better estimation of circadian patterns considering all time points rather than comparing only two times. Through the text this is termed "amplitude versus zero test."

DATA AND SOFTWARE AVAILABILITY

The accession number for the raw data for the RNA sequencing analyses is GEO: GSE102310. Any other pieces of data are available on request. The data are presented in the main manuscript and in the supplementary materials. RNA-seq data are deposited in the Genome Expression Omnibus under accession number GSE86619.