IMMUNOLOGY

Visualizing the function and fate of neutrophils in sterile injury and repair

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Neutrophils have been implicated as harmful cells in a variety of inappropriate inflammatory conditions where they injure the host, leading to the death of the neutrophils and their subsequent phagocytosis by monocytes and macrophages. Here we show that in a fully repairing sterile thermal hepatic injury, neutrophils also penetrate the injury site and perform the critical tasks of dismantling injured vessels and creating channels for new vascular regrowth. Upon completion of these tasks, they neither die at the injury site nor are phagocytosed. Instead, many of these neutrophils reenter the vasculature and have a preprogrammed journey that entails a sojourn in the lungs to up-regulate CXCR4 (C-X-C motif chemokine receptor 4) before entering the bone marrow, where they undergo apoptosis.

Sterile injury is a broad term covering many inflammatory diseases that occur in the absence of microorganisms. Most of these are characterized by an essential inflammatory phase followed by a resolution phase, which leads to homeostasis (*1*). Most studies, however, use models of high-fat diet, smoking, ischemia-reperfusion, toxic drugs, and autoimmune disorders, all of which lack a resolution phase. In these models, neutrophils have been hypothesized to be inappropriately recruited and activated. They are then thought to release a variety of proteases and oxidants, which causes host-tissue injury (*2*, *3*). To date, the therapeutic strategy has been to inhibit the recruitment of neutrophils and thereby allow for repair. However, this simplistic view may be fundamentally flawed inasmuch as neutrophils are also recruited in huge numbers in models of resolving sterile injury, where they may play a critical role in the repair process (*4*). Neutrophils are thought to die at sites of inflammation and then be phagocytosed by monocytes and macrophages (*5*). In zebrafish embryos, neutrophils migrate out of the vasculature to sites of sterile injury but then immediately reenter the vasculature in a process termed reverse migration (*6*). In mammalian systems, there is growing evidence that neutrophils can at least migrate into the subendothelial space adjacent to the basement membrane of postischemic muscle and then migrate back into the vasculature, traveling to the lungs, where they cause injury (*7*, *8*). The function and fate of neutrophils in a sterile injury model that leads to normal healthy repair remains unclear.

In a simple thermal hepatic injury model (~0.02 mm^3), an increase in neutrophil recruitment occurred over the first few hours after ~200 cells died of this insult (Fig. 1A). These neutrophils crawled through the sinusoids from as far away as 600 μm and then elongated and squeezed into the injury site [as previously described in (*9*)]. The area affected by the thermal hepatic injury showed much brighter PECAM-1 (platelet endothelial cell adhesion molecule 1) staining with an antibody administered before injury because of the collapse of the vessels (arrows, Fig. 1B). Sinusoids within the injury site were much narrower when compared to patent intact sinusoids surrounding the injury lesion (Fig. 1C). Fluorescein isothiocyanate (FITC)–albumin, administered intravenously after the injury, flowed through the intact healthy patent sinusoids [movie S1, observed as purple vessels as a result of PECAM-1 (red) and

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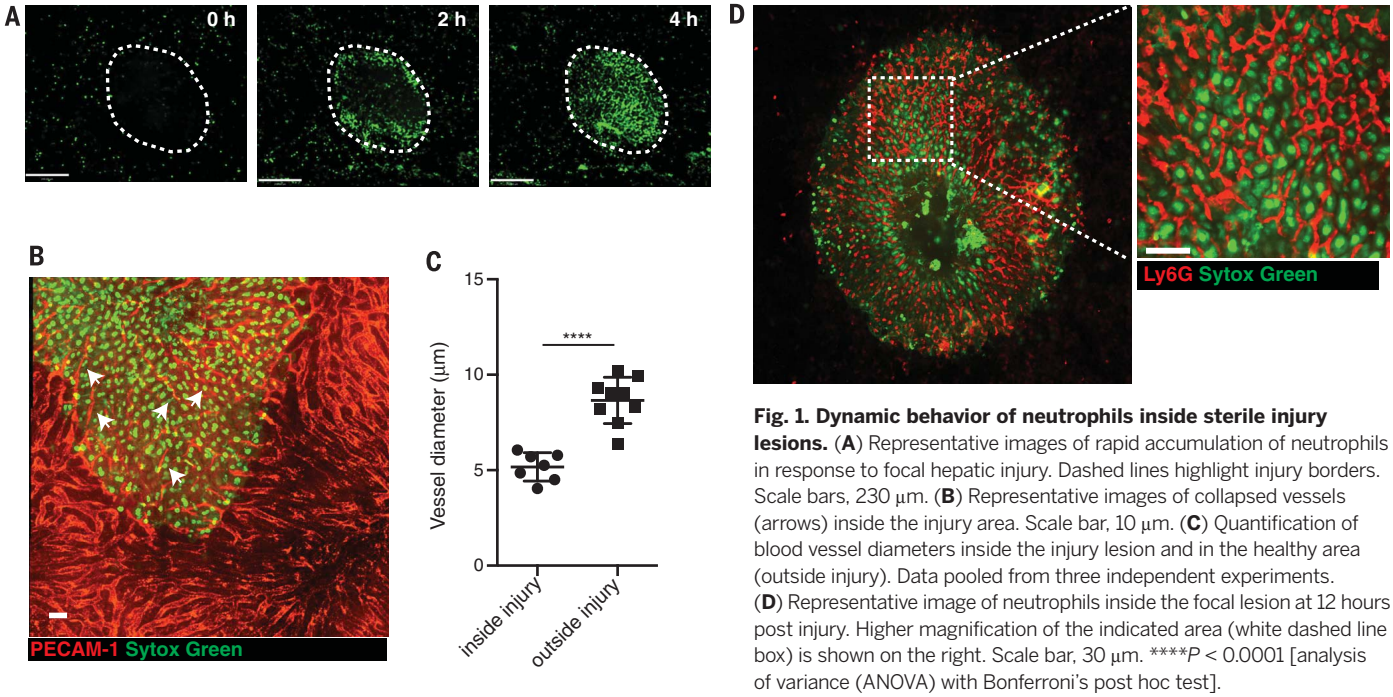

Fig. 1. Dynamic behavior of neutrophils inside sterile injury lesions. (**A**) Representative images of rapid accumulation of neutrophils in response to focal hepatic injury. Dashed lines highlight injury borders. Scale bars, 230 μm. (**B**) Representative images of collapsed vessels (arrows) inside the injury area. Scale bar, 10 μm. (**C**) Quantification of blood vessel diameters inside the injury lesion and in the healthy area (outside injury). Data pooled from three independent experiments. (**D**) Representative image of neutrophils inside the focal lesion at 12 hours post injury. Higher magnification of the indicated area (white dashed line box) is shown on the right. Scale bar, 30 μm. ****$P < 0.0001$ [analysis of variance (ANOVA) with Bonferroni's post hoc test].

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vascular endothelial (VE)–cadherin (blue) costaining]. A small amount of FITC-albumin leaked out of the vessels at the border of the injury (movie S1, the green accumulation at the border of the injury). No FITC-albumin, labeled antibodies, or FITC-dextran moved into the collapsed sinusoids (red) inside the necrotic area, suggesting that vessels lacked patency inside the injury (movie S1).

Neutrophils left the sinusoids at the injury border and surrounded the entire network of collapsed vessels 4 hours post injury (fig. S1 and movie S2). Over the next few hours, neutrophils interacted and dismantled the collapsed vessels (movie S3). In lysozyme M–green fluorescent protein (LysM-GFP) mice, small PECAM-1$^+$ (red) blood vessel particles were present inside GFP$^+$ neutrophils (movie S3). Within 12 hours, the neutrophils had filled the areas that had previously been occupied by the collapsed sinusoids, with many moving back and forth (Fig. 1D and movie S4). Neutrophils also took up small particles of SYTOX Green–stained DNA, although large intact nuclei were not overtly phagocytosed by them (movie S5), consistent with our recent observation that the nuclei of dead cells are dismantled by macrophages within the first few hours (*10*).

Antibody-mediated depletion of neutrophils resulted in far more debris remaining in the injury site at 12 and 24 hours (visualized using SYTOX Green$^+$; Fig. 2, A and B, and fig. S2A). In isotype-control antibody-treated mice, collagen (visualized using second harmonic generation) was deposited in a honeycomb pattern that closely resembled healthy tissue. In the absence of neutrophils, collagen deposition was not observed at 4 days post injury (Fig. 2, C and D). Macroscopic examination of the liver at day 4 revealed that the lesions had disappeared in the presence of neutrophils, whereas without neutrophils, lesions were still visible by eye (fig. S2B). Most notably, neutrophil depletion for a limited period of time (~48 hours) delayed revascularization at 7 days (Fig. 2, E and F). Although this injury eventually healed at 4 weeks and a microscopic examination of the whole liver indicated completely normal vasculature and showed no evidence of SYTOX Green staining, neutrophil depletion resulted in a nonhealing injury area

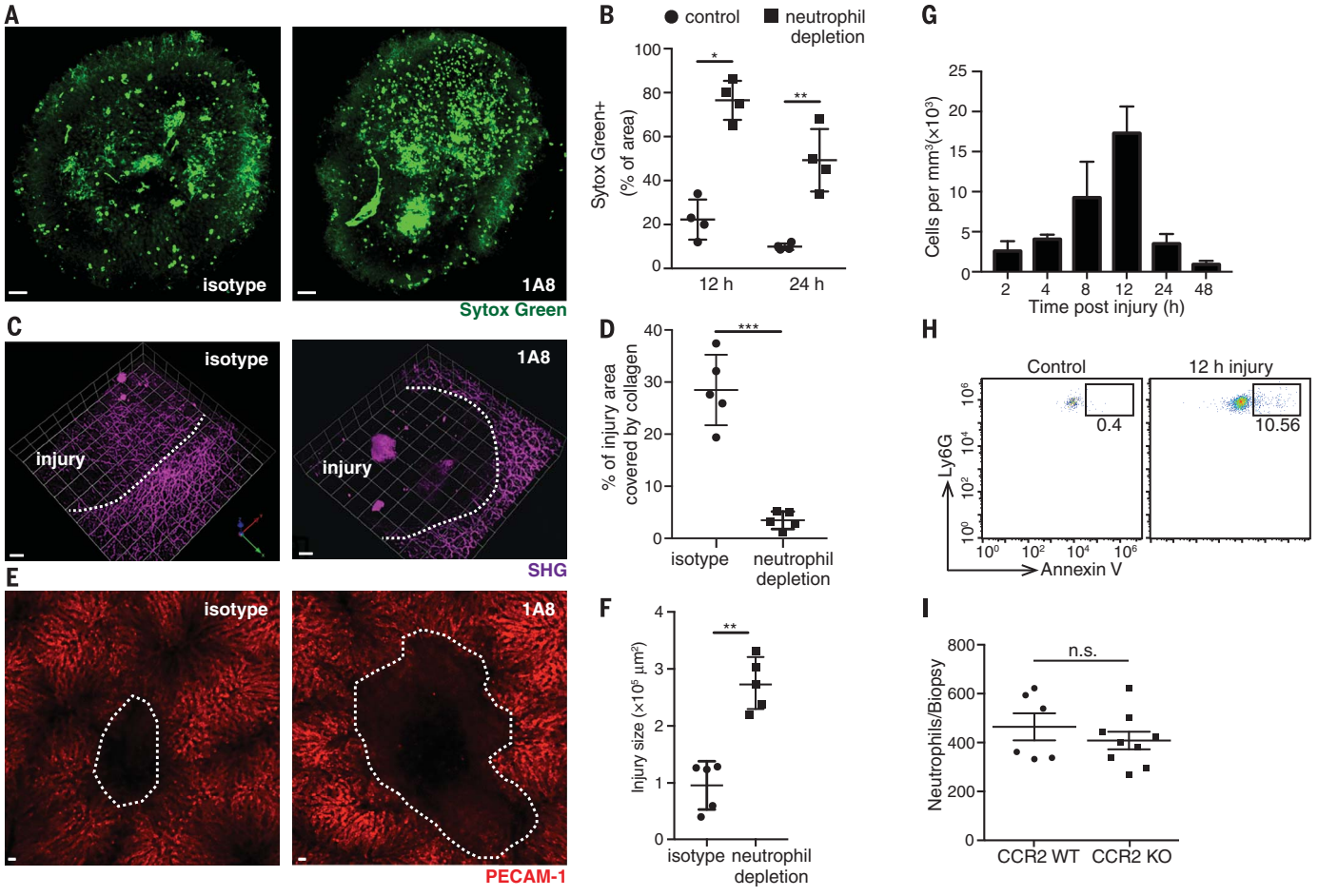

Fig. 2. Neutrophils contribute to tissue repair and are cleared from the injury site independent of monocytes and macrophages. (**A**) Representative images of necrotic cells (SYTOX Green$^+$, green) within lesions 12 hours post injury in isotype control (isotype) or antibody-treated neutrophil-depleted mice (1A8). Data are representative of four independent experiments. Scale bars, 10 µm. (**B**) Quantification of SYTOX Green$^+$ area in isotype control or 1A8 mice at 12 and 24 hours post injury. $n = 4$. (**C** and **D**) Representative images (C) and quantification (D) of collagen deposition into the focal injury area in the liver of isotype control or 1A8 mice at 4 days post injury. Dashed lines highlight injury borders. Scale bars, 20 µm. Each symbol represents one animal in one independent experiment. (**E** and **F**) Representative images (E) and quantification (F) of injury lesion at 7 days post injury in isotype control or 1A8. Mice were intravenously injected with PE (phycoerythrin) anti–PECAM-1 (red) to visualize liver vasculature. Dashed lines highlight injury borders. Scale bars, 10 µm. Each symbol represents one mouse, and data are pooled from two independent experiments, (B) and (F). (**G**) Quantification of neutrophils accumulated within the injury site at indicated time points. $n = 3$ for each time point. (**H**) Flow cytometry analysis for annexin V binding to Ly6G$^+$ cells isolated from injury biopsy (12 hours post injury) or control biopsy (control). Data are representative of three independent experiments. (**I**) Quantification of Ly6G$^+$ neutrophil numbers harvested from injury biopsy from *Ccr2*$^{RFP/+}$ (CCR2 WT) and *Ccr2*$^{RFP/RFP}$ (CCR2 KO) mice. Each symbol represents one mouse. Error bars represent SEM; n.s., not significant. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA with Bonferroni's post hoc test).

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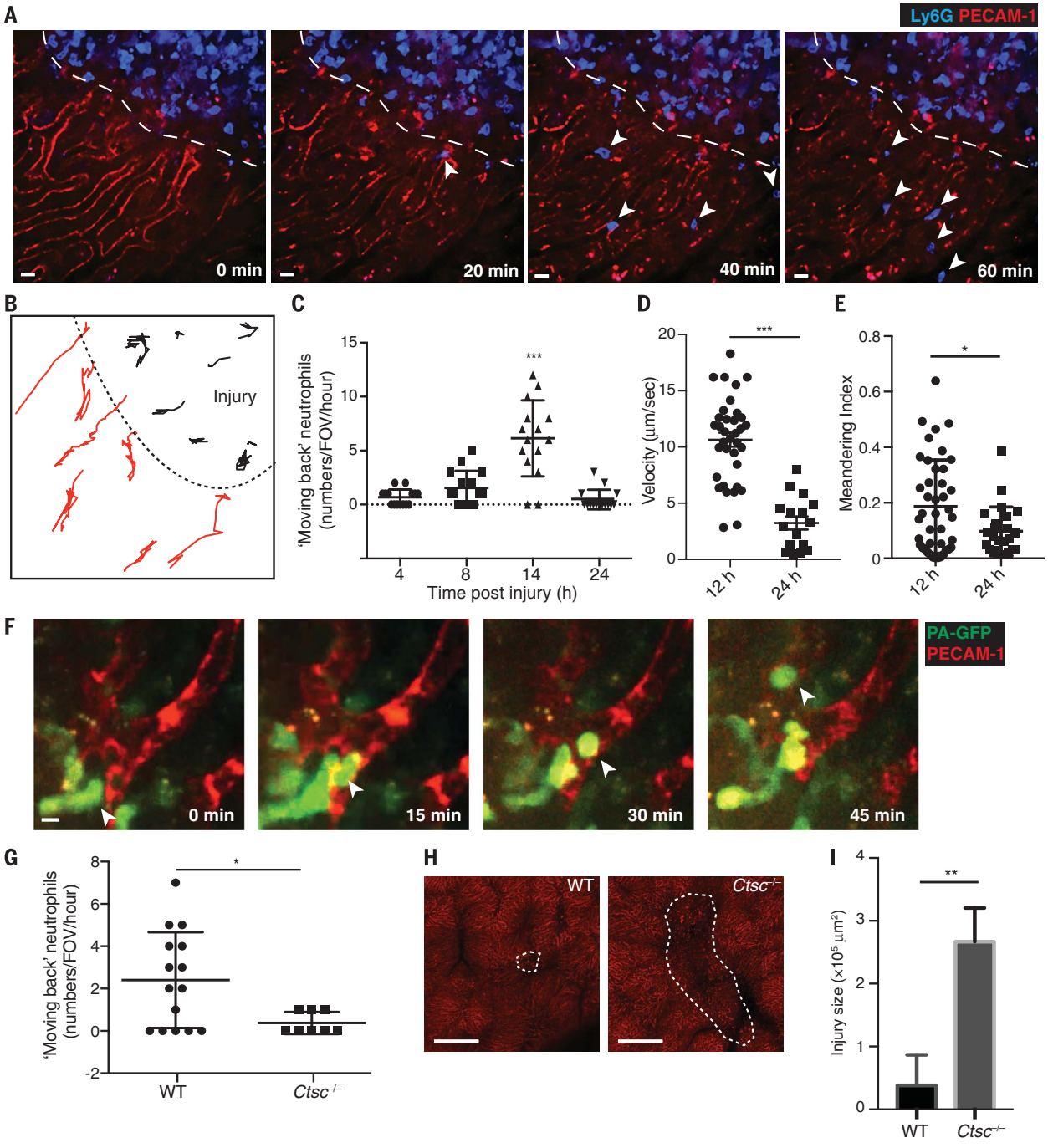

Fig. 3. Reverse migration of neutrophils from inflamed tissue. (**A**) Time-lapse images showing neutrophils (blue) migrating out from the injury lesion (dashed lines highlight injury borders) and migrating into blood vessels (red). Elapsed time is shown in minutes. Scale bars, 10 μm. (**B**) Representative trajectories taken by neutrophils. Tracks were extracted from a 1-hour-duration video taken at 14 hours post injury. Red lines indicate reverse-migrated neutrophils, and black lines indicate neutrophils that remained inside the injury. Dashed line highlights the injury border. (**C**) Quantification of neutrophils that migrated out from the injury lesion at indicated time points post injury. Data were quantified as the number of neutrophils that migrated out from the injury area in one 512-μm by 512-μm field of view (FOV) in a 1-hour-duration video. Each symbol represents one FOV. Data pooled from five independent experiments. ***$P < 0.001$ when compared with the 4-hour group (ANOVA with Bonferroni's post hoc test). (**D** and **E**) Migration velocities (D) and chemotactic index (E) of neutrophils near the injury border at indicated time points post injury. Each symbol represents one cell. $n = 5$ for each time point. (**F**) Time-lapse images showing photoactivated GFP$^+$ cells that migrated into the blood vessels (red) in PA-GFP mice at 12 hours post injury. An area inside the injury that was close to the injury border was photoactivated at 8 hours post injury. Scale bar, 8 μm. (**G**) Quantification of neutrophils that migrated out from the injury lesion at 14 hours post injury in WT and *Ctsc*$^{-/-}$ mice. Each symbol represents one FOV. $n = 5$ for each genotype. (**H**) Representative images of an injury lesion at 7 days post injury in WT or *Ctsc*$^{-/-}$ mice. Mice were intravenously injected with anti–PECAM-1 (red) to visualize liver vasculature. Dashed lines highlight injury borders. Scale bars, 100 μm. (**I**) Quantification of the size of the injury lesion in (H). $n = 3$ (WT) and $n = 4$ (*Ctsc*$^{-/-}$). Data pooled from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (ANOVA with Bonferroni's post hoc test).

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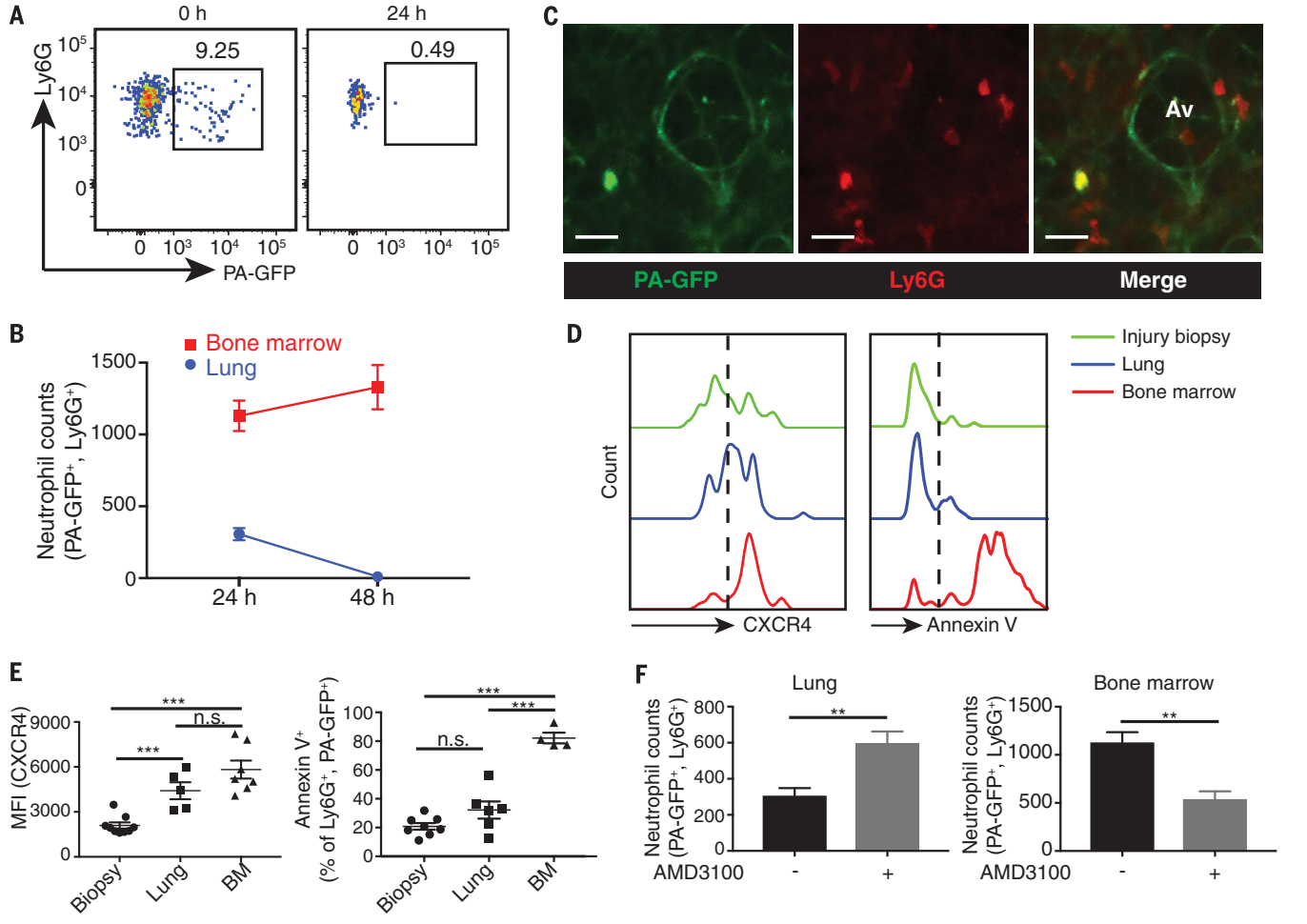

Fig. 4. Reverse migrated neutrophils are arrested in lung and bone marrow. (**A**) Representative flow cytograms of neutrophils from injury biopsy at 0 and 24 hours after photoactivation. (**B**) PA-GFP$^+$ neutrophil counts in lung and bone marrow at indicated time points after injury. Photoactivation was performed at 8 hours post injury. Data are calculated as PA-GFP$^+$ neutrophils in whole lung or in bone marrow from one pair of tibia and femur. $n = 4$. (**C**) Representative image of PA-GFP$^+$ neutrophil (green) in the lung. Photoactivation of the hepatic injury lesion was performed at 8 hours post injury. Ly6G antibody (red) was intravenously administered. Av, alveolar space; scale bars, 50 μm. (**D** and **E**) Expression of CXCR4 and percentage of annexin V on PA-GFP$^+$ neutrophils isolated from indicated tissues. Histograms (D) and independent data (E) are reported. Each data point in (E) is from an independent sample. BM, bone marrow. MFI, mean fluorescence intensity. (**F**) PA-GFP$^+$ neutrophil counts in lung and bone marrow treated with control vehicle or AMD3100 (CXCR4 antagonist) at 24 hours post injury. Data are pooled from three independent experiments. $n = 4$. n.s., not significant. $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA with Bonferroni's post hoc test).

that showed no regrowth of hepatocytes or sinusoids (fig. S2, C and D).

Neutrophil numbers inside the injury site reached peak levels at 12 hours but were reduced more than 90% by 24 hours and were almost entirely absent by 48 hours (Fig. 2G). About 10% of the neutrophils were positive for annexin V, which detects apoptosis, at 12 hours (Fig. 2H). As early as 8 hours post injury, CCR2$^+$ proinflammatory monocytes began to surround the perimeter of the injury site, and some monocytes infiltrated the injury site (fig. S3A). At 12 hours, the number of neutrophils present was an order of magnitude greater than the number of monocytes. Although there were some interactions between neutrophils and monocytes, overt phagocytosis was never observed despite extensive visualization periods (movie S6), suggesting that monocytes were not removing neutrophils. Indeed, the lack of monocyte recruitment in *Ccr2*$^{RFP/RFP}$ [CCR2 knockout (KO)] mice revealed the same rate of clearance of Ly6G$^+$ neutrophils at 24 hours as that in wild-type (WT) mice (Fig. 2I). Although peritoneal macrophages also accumulate at the injury site early post injury, specific depletion of peritoneal macrophages still had no effect on the disappearance of neutrophils from the injury site at 24 and 48 hours (fig. S3B). The number of neutrophils that entered the injury was unaffected after macrophage depletion or in CCR2 KO animals. Although nonprofessional phagocytes such as intestinal epithelial cells have been reported to clear neutrophils (*11*, *12*), we did not observe this phenomenon in our model.

Imaging the border of the injury at several time points from 4 to 24 hours revealed a pronounced egress of Ly6G$^+$ neutrophils from injured to healthy tissue, which predominated at ~14 to 16 hours post injury (Fig. 3, A to C, and fig. S4A). Cells left the injury site and either reentered the healthy vasculature (movie S7) or migrated into healthy tissue through the interstitium (movie S8). Neutrophil tracking revealed increased directional migration at the border of the injury, although the overt migration pattern of neutrophils appeared to be random deeper within the injury (Fig. 3B and fig. S4B). In addition, the velocity of neutrophils was particularly high at 12 hours, and they exhibited a higher chemotactic index compared to that at 24 hours, when fewer neutrophils remained and their migratory phenotype was much less apparent (Fig. 3, D and E). The above results suggest that chemokinesis helps mammalian neutrophils to initially distribute away from the injury site, as is observed in zebrafish embryos (*13*, *14*), but to ultimately chemotax the short distance into the vasculature.

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To confirm that neutrophils were leaving the injury site, we also made use of transgenic mice that express high amounts of photoactivatable-GFP (PA-GFP) in all hematopoietic cell populations (*15*). A small group of cells was photoactivated precisely at the edge of the patent vessels. Most of these GFP^+ cells were $Ly6G^+$ neutrophils (fig. S5). These GFP^+ neutrophils lined up and reentered healthy patent vessels (Fig. 3F and movie S9).

Proteases can mediate reverse migration from the basement membrane back to the vasculature (*8*). Indeed, reducing serine protease activity by using cathepsin C–deficient ($Ctsc^{-/-}$) mice led to a profound reduction in migration of neutrophils back into the vasculature (Fig. 3G and movie S10). The $Ctsc^{-/-}$ neutrophils had no difficulty entering the injury site (fig. S6, A and B), suggesting that the proteases were more essential for their exit. Indeed, more neutrophils remained in the injury at 24 and 48 hours in $Ctsc^{-/-}$ mice (fig. S6C), resulting in delayed revascularization in $Ctsc^{-/-}$ mice as compared to WT mice (Fig. 3, H and I). Thus, the migration of neutrophils out of an injury may also be a mechanism that contributes to the resolution of inflammation.

Previous studies have suggested that reverse-transmigrated neutrophils traffic to the lungs and can cause damage (*7*, *8*). To understand the fate of neutrophils migrating out from the thermal hepatic injury, we generated a mouse in which only $Ly6G^+$ neutrophils express photoactivatable GFP (Ly6G-PA-GFP). The injury area was photoactivated by exposure to ultraviolet light for a short time, activating ~10% of the neutrophils. These GFP^+ neutrophils had all left the site within 24 hours (Fig. 4A) and were detected mainly in two other sites: There were a small number in the lung and a larger number in the bone marrow (Fig. 4B). Few or no GFP^+ neutrophils were observed in spleen or hepatic-draining lymph nodes (fig. S7). Both histology and flow cytometry revealed that there was a transient increase in neutrophil numbers in the lung at 24 hours (but not at 48 hours) after the injury (fig. S8, A and B). However, there was no notable lung injury in sham- or liver-injured mice, as assessed by changes in microvascular permeability (fig. S8C), whereas treatment with our positive control (lipopolysaccharide, LPS) caused a fivefold increase in protein leakage (fig. S8D). Intravital imaging of the few rare photoactivated neutrophils in the lung revealed that they all had a prolonged retention time and adhered firmly to the lung endothelium (Fig. 4C and movie S11). This behavior was restricted only to the neutrophils that had been in the injury site, as the behavior of non-GFP^+ neutrophils in the lung was nearly identical in sham and injured mice; most of these neutrophils tethered and let go or crawled for short durations and returned to the circulation. Only a very small percentage of cells were adherent (fig. S8E and movie S12). By contrast, LPS treatment resulted in most of the neutrophils adhering and crawling very slowly within the vasculature (fig. S8F).

Previous work has suggested that neutrophils migrate to the bone marrow to die (*16*). Rankin and colleagues reported increased CXCR4 on aged neutrophils that allows their recruitment back to bone marrow via CXCL12 (*17*). An upregulation of CXCR4 was observed on photoactivated neutrophils from the lung and bone marrow (Fig. 4, D and E). Almost all photoactivated neutrophils in bone marrow were now annexin V^+, consistent with the elimination of these cells in this tissue (Fig. 4, D and E). Finally, treatment with the CXCR4 antagonist AMD3100 led to an increase of PA-GFP^+ neutrophils in the lung and a decrease in the bone marrow (Fig. 4F), suggesting that injury-derived neutrophil trafficking from the lung to the bone marrow is mediated by CXCR4.

Our study provides in vivo evidence that sterile tissue–infiltrated neutrophils that perform key repair functions do not die at the site of injury. Rather, they migrate back into the circulation as a physiological process and return to the lung, potentially to be deactivated or reprogrammed to then selectively migrate via CXCR4 to the bone marrow. In patients, after severe trauma or hemorrhagic shock, lung injury known as acute respiratory distress syndrome (ARDS) is thought to be the result of the overwhelming recruitment of activated neutrophils into the pulmonary vasculature (*18*). It is conceivable that many of these activated neutrophils have left injured tissue and are simply following a preprogrammed homeostatic path to the lungs that becomes dysregulated in ARDS (*19*). Thus, we contend that most of the neutrophils must leave sterile injury sites for successful resolution and return to the lungs and bone marrow as a form of biologic recycling (*16*), an event that is unlikely to occur in infections.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/358/6359/111/suppl/DC1
Materials and Methods
Figs. S1 to S8
References (*20*–*27*)
Movies S1 to S12

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Imaging the unforeseen fate of neutrophils

Inflammation that results from insults such as ischemia and reperfusion or trauma in the absence of microorganisms is known as "sterile inflammation." Neutrophils are recruited in vast numbers during sterile inflammation and have been thought to play a detrimental role. Wang *et al.* used intravital microscopy to show that neutrophils actually perform helpful tasks such as removing and regenerating thermally damaged blood vessels in the liver (see the P erspective by Garner and de Visser). Moreover, neutrophils neither die nor are phagocytosed. Instead, they return to the circulation in a process called "reverse transmigration," making a pit stop in the lungs, before ending their lives where t hey began—in the bone marrow. Thus, a reconsideration of the use of anti-neutrophil therapies after injury may be warranted.

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