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Neutrophil Priming Occurs in a Sequential Manner and Can Be Visualized in Living Animals by Monitoring IL-1β Promoter Activation

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Rapid enhancement of phagocyte functionality is a hallmark of neutrophil priming. GeneChip analyses unveiled elevated CD54, dectin-2, and IL-1β mRNA expression by neutrophils isolated from inflammatory sites. In fact, CD54 and dectin-2 protein expression was detected on neutrophils recovered from skin, peritoneal, and lung inflammation lesions but not on those in bone marrow or peripheral blood. Neutrophils increased CD54 and dectin-2 mRNA during migration in Boyden chambers and acquired CD54 and dectin-2 surface expression after subsequent exposure to GM-CSF. Neutrophils purified from IL-1β promotor-driven DsRed-transgenic mice acquired DsRed signals during cell migration or exposure to GM-CSF. CD54 and dectin-2 were expressed by DsRed+ (but not DsRed−) neutrophils in GM-CSF-supplemented cultures, and neutrophils recovered from inflammatory sites exhibited strong DsRed signals. The dynamic process of neutrophil priming was studied in chemically induced inflammatory skin lesions by monitoring DsRed expression using confocal microscopy. A majority (>80%) of Ly6G+ neutrophils expressed DsRed, and those DsRed+/Ly6G+ cells exhibited crawling motion with a higher velocity compared with their DsRed−/Ly6G− counterparts. This report unveils motile behaviors of primed neutrophils in living animals. We propose that neutrophil priming occurs in a sequential manner with rapid enhancement of phagocyte functionality, followed by CD54 and dectin-2 mRNA and protein expression, IL-1β promoter activation, and accelerated motility. Not only do these findings provide a new conceptual framework for our understanding of the process of neutrophil priming, they also unveil new insights into the pathophysiology of many inflammatory disorders that are characterized by neutrophil infiltration. The Journal of Immunology, 2015, 194: 1211–1224.

Neutrophils are the most abundant leukocytes in blood circulation and serve as the first line of defense against microbial invasion by extruding neutrophil extracellular traps, engulfing microorganisms, producing reactive oxygen species (ROS), and releasing various enzymes via degranulation (1–3). However, circulating neutrophils exhibit limited antimicrobial activity in the steady-state; they must be preinstructed by microbial or endogenous agents to exert maximal phagocyte functionality, as measured by bacterial uptake and respiratory burst (4, 5). This process, known as “priming,” is a key event whereby neutrophil responsiveness to an activating stimulus is markedly augmented by prior exposure to a priming agent. Although various agents (e.g., microbial products, chemoattractants, and inflammatory cytokines) can induce neutrophil priming, they do not elicit phagocyte functionality on their own unless applied at extremely high concentrations (6). These agents can prime neutrophils in relatively short periods, ranging from several seconds (e.g., ATP) to 120 min (e.g., LPS and GM-CSF) (7–11). Not only do primed neutrophils exhibit markedly enhanced phagocytosis and ROS production upon encountering microorganisms, they change surface phenotype (6, 7, 12). Most of these functional and phenotypic changes occur in the absence of de novo biosynthesis (13–16). For example, inflammatory cytokines augment respiratory burst by phosphorylating NADPH oxidase components (e.g., p47phox) (2, 5, 17, 18). ROS production also can be enhanced via mobilization of flavocytochrome b558 from granules to plasma and phagosomal membranes (14, 19, 20). Exocytosis of secretory vesicles may result in elevated surface expression of fMLF receptor, CD11b, CD35, CD66b, and FcγRs (13–15, 21). Conversely, CD62L surface expression is diminished via enzymatic shedding (22, 23). In essence, neutrophil priming is generally regarded as a rapid process requiring no gene transcription or translation. Interestingly, neutrophils treated in vitro with LPS or G-CSF showed enhanced ROS production, even when tested 24 h after priming (24). Likewise, after in vivo infusion of endotoxin, circulating neutrophils exhibited augmented respiratory burst upon PMA stimulation, and this phenotype was maintained for >24 h (25). These observations imply that neutrophil priming may not necessarily be a rapid and transient process. In the current study, we sought to identify phenotypic and functional changes occurring in a late phase of neutrophil priming.
Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Construction and characterization of pIL1-DsRed transgenic mice were described elsewhere (26). Both male and female animals (10–30 wk old) were used in the experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Toledo and performed in accordance with the National Institutes of Health guidelines.

Abs and flow cytometry

All fluorescently conjugated mAbs were purchased from BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), and Miltenyi Biotec (Auburn, CA). Samples were incubated on ice for 15 min with anti-CD11c/32 mAb (2.4G2) for Fc block and then stained on ice for 30 min with the following Ags: CD11b (M1/70), CD11c (N418), CD48 (HM48-1), CD54 (3E2), dectin-2 (K7a6E7), 7/4 (7/4), Gr-1 (RB6-8C5), and Ly6G (1A8). The samples were analyzed with a FACS-Calibur (BD Biosciences), and only propidium iodide− populations were included in the analyses. The data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Culture media

Cells were cultured in “complete” RPMI medium: RPMI 1640 (HyClone, Logan, UT) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 2 mM l-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 100 μM nonessential amino acids (HyClone), 10 mM HEPES (HyClone), 50 mM 2-ME (Life Technologies, Grand Island, NY), 100 U/ml penicillin (HyClone), 100 μg/ml streptomycin (HyClone), and 0.25 μg/ml amphotericin B (HyClone).

Cell purification

Gr−1/36.7/CD48− neutrophils were sorted from bone marrow (BM) cells isolated from C57BL/6 mice by FACSAria II (BD Biosciences), as described previously (27). We reported recently that peritoneal exudate cell (PEC) samples harvested from thioglycollate-induced peritoneal lesions contained Ly6G/CD11c− neutrophil-dendritic cell hybrids in addition to Ly6G/CD11c+ conventional neutrophils and Ly6G/CD11c+ traditional dendritic cells (28). Thus, the Ly6G/CD11c− population was sorted from PEC samples as “neutrophils” in the current study. DrsRed+/Gr−1/36.7/CD48− neutrophils were sorted from BM cells isolated from pIL1-DsRed reporter mice. In some experiments, those BM neutrophils were cultured (1 × 106 cells/ml) for 16 h in complete RPMI supplemented with 10 ng/ml GM-CSF (R&D Systems, Minneapolis, MN). DsRed+ and DrsRed− populations were then sorted from the cultures. All sorted populations showed >98.5% purity in postsorting analyses.

ROS-production assay

Cells were incubated for 20 min at 37˚C with 200 μM luminol (Acros Organics, Morris Plains, NJ) and 50 μM HRP (Sigma-Aldrich) and then plated onto 96-well plates. After adding IMLF (Sigma-Aldrich) at the final concentrations of 10 or 100 μM, chemiluminescence was immediately measured using the FLUOstar Omega microplate reader (BMG Labtech, Cary, NC).

Bacterial-uptake assay

The Escherichia coli K-12 strain expressing GFP cDNA under control of the lac promoter was used to measure bacterial uptake by neutrophils. After 1 h of incubation at a multiplicity of infection of 10, unbound bacteria were removed by centrifugation over FBS. Samples were then examined for GFP signals with the FACS-Calibur (BD Biosciences), as described (27).

Chemotaxis assay

Chemotaxis assay was performed with 24-well plates with 6.5-mm (3 μm pore size) Transwell inserts (Corning Costar, Tewksbury, MA). After FACS purification, neutrophils were suspended in complete RPMI medium at a density of 1 × 105 cells/ml and added to the upper chamber (100 μl/well). Complete RPMI containing 30 nM rCCL1 and/or rCCL2 (both from PeproTech, Rocky Hill, NJ) was added to the lower chamber (600 μl/well). After a 2-h incubation at 37˚C, “migrated” cells were harvested from the lower chambers. The cells remaining in the upper chambers were harvested as “unmigrated” cells. The samples were examined for cell numbers and viability by FACS, and the level of migration (% migration) was calculated by dividing the number of migrated cells by the total cell numbers recovered from the well (i.e., migrated cells + unmigrated cells).

mRNA-expression analyses

Gene-expression profiles were examined as described previously (27). Total RNA samples were extracted from FACs-purified BM neutrophils with TRIzol reagent (Invitrogen, Grand Island, NY), purified with the RNasey Mini Kit (Qiagen, Valencia, CA), and then hybridized with Affymetrix Mouse Genome 2.0 Arrays. Membrane washing and scanning were described elsewhere (26). The datasets for these BM neutrophils (deposited in the Gene Expression Omnibus under accession number GSE53826, http://www.ncbi.nlm.nih.gov/geo/) were compared with publicly available microarray datasets (GSE24102) for PEC neutrophils purified from case-included acute peritonitis lesions (29). Real-time PCR was performed using the LightCycler II (Roche Diagnostics, Indianapolis, IN) from the first-strand cDNA generated from the total RNA with a Taqman probes and SYBR Green SuperScript III First-Strand Synthesis System (Invitrogen). IL-1α, IL-1β, IL-6, IL-10, IL-17A, and 18s were used as references. The forward and reverse primers were purchased from Qiagen. Other primer pairs were custom prepared by Integrated DNA Technologies using the following sequences: β-actin forward, 5′-TGGTATGGTGGAATGGTCAGAA-3′; β-actin reverse, 5′-TGTGTTGCAAGTCTCTCAGTGT-3′; CD54 forward, 5′-ATGTTTGATGGCCTCTCTGA-3′; CD54 reverse, 5′-AGTTTTATGGCCTCCTGTA-3′; Dectin-2 forward, 5′-ACCCCTGACCTTCTGTGA-3′; Dectin-2 reverse, 5′-TGGAGGGGCCTTGAACCAA-3′; DsRed forward, 5′-GACGGCTCTTCTACATCAAG-3′; and DsRed reverse, 5′-CTTGTGATGTCTCGCCT-3′.

Cytokine screening

A cytokine library was constructed by purchasing a total of 66 recombinant cytokines and chemokines from PeproTech; biological activities were confirmed for many of these cytokines in our previous screening studies (30, 31). The tested cytokines included activin A; activin B; adiponectin; a proliferation-inducing ligand; BM stroma-derived growth factor 20; bone morphogenetic protein 2, 4, 7, and 13; cardiotoxin-1; CXCL1; CXCL2; epidermal growth factor; fibroblast growth factor 1, 2, and 9; FMS-like tyrosine kinase 3 ligand; G-CSF; GM-CSF; insulin-like growth factor 1; IFN-α; IFN-β; IFN-γ; IFN-A2; IL-1α; IL-1β; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-9; IL-10; IL-12; IL-13; IL-15; IL-17A; IL-17F; IL-18; IL-21; IL-23; IL-31; IL-33; keratinocyte growth factor; LIF; LIFR; M-CSF; nerve growth factor; neuropoietin; noggin; platelet-derived growth factor-BB; prolactin; resistin; resistin-like molecule α and β; soluble receptor activator of NF-κB ligand; stem cell factor; thrombopoietin; TGF-β1, -β2, and -β3; TNF-α; TNF ligand superfamily 14; and vascular endothelial growth factor. DsRed+/Gr−1/36.7/CD48− neutrophils purified from the BM of pIL1-DsRed reporter mice were cultured for 24 h in round-bottom 96-well plates (2 × 105 cells/well) in complete RPMI medium supplemented with each of the above cytokines added at 10 ng/ml. To measure baseline levels, some of the above BM neutrophils were cultured as above in the absence of added cytokines. Samples were then analyzed in a semiautomated fashion using a FACS-Calibur equipped with an HTP sampler device (BD Biosciences).

Inflammatory disease models

Acute peritonitis was induced by i.p. injection of 1 ml 3% thioglycollate (BD Biosciences), and PEC samples were collected 24 or 48 h later by washing the peritoneal cavity with 7 ml ice-cold PBS (28). Acute lung inflammation was induced by intratracheal instillation of 40 μl German cockroach frass extracts (1 mg/ml), and bronchoalveolar lavage fluid (BALF) samples were harvested 24 or 48 h later by washing the airway with 1 ml ice-cold phenol red-free HBSS (28). Acute skin inflammation was induced by topical application of 12.5 μl 1.25% oxazolone (OX; Sigma-Aldrich) onto the right ear (26). The treated ears showed significant (p < 0.01) swelling compared with the left ears of the same animals painted with vehicle alone. After 24 or 48 h, the animals were sacrificed to harvest the entire ears. Samples were incubated for 45 min at 37˚C with 0.5% Dispase II (Roche Diagnostics), and the epidermal and dermal compartments were gently separated at the dermo-epidermal junction using a sterile scalpel. The epidermal specimens were incubated at 37˚C with 0.3% trypsin (Worthington, Lakewood, NJ) and 0.1% DNase I (Roche Diagnostics). The dermal specimens were incubated for 60 min at 37˚C with 1000 U/ml collagenase IV (Worthington). The resulting single-cell suspensions were analyzed for surface phenotype and DrsRed expression by FACS (26).

Intravital confocal imaging

Motive behaviors of leukocytes were studied in OX-injected inflammatory skin lesions as described previously (26, 32). Immediately before and 8 h after topical application of OX, pIL1-DsRed mice received an i.v. injection of Alexa Fluor 647− conjugated anti-Ly6G mAb at a low dose (5 μg/animal/injection) to label neutrophils (33). The blood vessels were
visualized by i.v. injection of FITC-conjugated dextran (3 mg/animal, 150 kDa; Sigma-Aldrich). Anesthetized mice were placed onto an imaging stage to mount the tip of the ear, ventral side down, and images of DiRRed* cells and Ly6G* cells were recorded using an Olympus FV1000 confocal microscope (Olympus, Center Valley, PA). Three-dimensional image sets were created by scanning the ear skin from the stratum corneum (the outermost layer readily localizable based on its strong autofluorescence signals) with x, y, z volumes of $317 \times 317 \times 30 \mu m$ (40× objective), $635 \times 635 \times 30 \mu m$ (20× objective), or $1270 \times 1270 \times 50 \mu m$ (10× objective) at 2-μm z-steps. In time-lapse imaging experiments, three-dimensional images were recorded every 2 or 4 min for up to 8 h. Datasets were processed using Metamorph (Universal Imaging, Downingtown, PA) and Photoshop CS2 (Adobe, San Jose, CA) software. Metamorph software also was used to track individual cells, to generate migratory paths, and to calculate the directionality and velocity of migration.

**Statistical analyses**

Microarray analysis was performed in duplicates, and all other experimental analyses were performed with at least triplicate samples. All experiments were repeated at least three times to assess reproducibility. Comparisons between two groups were performed using a two-tailed Student t test, and more than two groups were compared by ANOVA.

**Results**

**Signature genes expressed by fully primed neutrophils recovered from inflammatory sites**

As an experimental approach to study late-phase changes associated with neutrophil priming, we induced acute sterile peritonitis by i.p. injection of thioglycollate. Consistent with previous reports (34, 35), neutrophils purified from PEC samples exhibited typical polymorphonuclear morphology characterized by segmented nuclei, whereas control neutrophils purified from the BM of nontreated mice showed ring-shaped nuclei. Both populations uniformly expressed all tested markers of neutrophils (e.g., Ly6G, Ly6C, 7/4, and CD11b), as we observed previously (27, 28). Augmented ROS production in response to fMLF has been widely used as the “gold standard” to test neutrophil priming (7). An enhanced capacity to phagocytose bacteria is another functional parameter of priming with 10 or 100 μM of fMLF, respectively (16- and 24-h time points in Fig. 2E). To mimic conditions at inflammatory sites, we next exposed the two neutrophil populations to an inflammatory cytokine GM-CSF for up to 40 h. When tested after 16 h of culturing with GM-CSF, migrated neutrophils exhibited significantly augmented ROS production and bacterial uptake. Upon stimulation with 10 or 100 μM of IMLF, migrated cells exhibited higher ROS production compared with unmigrated cells (Fig. 2A). Likewise, migrated cells exhibited more efficient bacterial uptake (Fig. 2B, 2C). To determine whether cell migration was required for these changes, we cultured neutrophils for 2 h with the same two chemokines in conventional culture plates (“no Transwell” in Fig. 2A–C). We observed that simple exposure to CXCL1 and CXCL2 was not sufficient to induce the maximal augmentation of ROS production and bacterial uptake at the levels observed with migrated neutrophils. Thus, we concluded that neutrophils acquire augmented phagocyte functionality during chemotactic migration.

At the end of the 2-h chemotaxis assay, migrated neutrophils (recovered from the lower chambers) also showed elevated mRNA expression of CD54 (~7-fold), dectin-2 (~8-fold), and IL-1β (~5-fold) compared with unmigrated neutrophils (0 h time point in Fig. 2D). Simple exposure to CXCL1 and CXCL2 was not sufficient to produce the same changes. At the protein level, neither CD54 nor dectin-2 was detectable on migrated neutrophils (0 h time point in Fig. 2E). To mimic conditions at inflammatory sites, we next exposed the two neutrophil populations to an inflammatory cytokine GM-CSF for up to 40 h. When tested after 16 h of culturing with GM-CSF, migrated neutrophils exhibited markedly (~50-fold) elevated CD54, dectin-2, and IL-1β mRNA expression compared with their unmigrated counterparts (16-h time point in Fig. 2D). Interestingly, unmigrated neutrophils did not show elevated IL-1β mRNA expression, even after 16 h of exposure to GM-CSF, although they did exhibit slightly elevated CD54 and dectin-2 mRNA expression during this period. Consistent with the mRNA data, surface expression of CD54 and dectin-2 became detectable in the migrated population after 16 and 24 h of exposure to GM-CSF, respectively (16- and 24-h time points in Fig. 2E). However, it should be pointed out that unmigrated neutrophils

**CD54 and dectin-2 expression by neutrophils in a late phase of priming**

We reasoned that neutrophils might acquire CD54 and dectin-2 expression during the process of migration to inflammatory sites and/or after exposure to inflammatory cytokines. To test the first part, we used a standard Boyden chamber model as the simplest model (38). When tested individually, two prototypic neutrophil chemokines, CXCL1 and CXCL2 (39, 40), induced significant migration of BM neutrophils in dose-dependent fashions (Supplemental Fig. 1A). When combined, they induced even more robust migration (Supplemental Fig. 1B), corroborating a previous report (41). Importantly, significant fractions (50–70%) of neutrophils remained “unmigrated” in the upper chambers, even when CXCL1 and/or CXCL2 were added to the lower chambers at the highest tested concentrations or in combination. This implied functional heterogeneity between the migrated neutrophil population and the unmigrated counterpart. Because CXCL1 and CXCL2 were both detected at relatively high levels in various inflammatory lesions (42–45), we chose to add both chemokines to the lower chamber to mimic neutrophil migration under pathogenic conditions. After the 2 h chemotaxis assay, we harvested the migrated cells from the lower chambers and the “unmigrated” cells from the upper chambers and compared their states of priming by measuring ROS production and bacterial uptake. Upon stimulation with 100 μM of IMLF, migrated cells exhibited higher ROS production compared with unmigrated cells (Fig. 2A). Likewise, unmigrated cells exhibited more efficient bacterial uptake (Fig. 2B, 2C). To determine whether cell migration was required for these changes, we cultured neutrophils for 2 h with the same two chemokines in conventional culture plates (“no Transwell” in Fig. 2A–C). We observed that simple exposure to CXCL1 and CXCL2 was not sufficient to induce the maximal augmentation of ROS production and bacterial uptake at the levels observed with migrated neutrophils. Thus, we concluded that neutrophils acquire augmented phagocyte functionality during chemotactic migration.

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FIGURE 1. Signature genes expressed by fully primed neutrophils recruited to inflammatory sites. Gr-1<sup>high</sup>/CD48<sup>−</sup> neutrophils purified from BM and Ly6G+/CD11c<sup>−</sup> neutrophils purified from PECs 24 h after thioglycollate treatment of C57BL/6 mice were compared for ROS production (A), bacterial uptake (B and C), and gene-expression profiles (E). (A) After incubation with luminol and HRP, the cells were stimulated with 10 or 100 μM fMLF, and chemiluminescence was measured immediately thereafter. Data are mean ± SD (n = 3) of relative fluorescence units (RFU). ***p < 0.001 between the peak values of the two populations. (B and C) Cells were incubated for 1 h at 37˚C in the presence or absence of GFP–E. coli (multiplicity of infection = 10) and examined for GFP signals. Data shown are representative graphs (B) and means ± SD (n = 3) of the mean fluorescence intensities (MFI) (C). ***p < 0.001. (D) Gene-expression profiles for our BM neutrophil preparation in (A) were compared with the publicly available dataset for PEC neutrophils harvested from casein-induced peritonitis lesions (GSE24102). Only genes showing >2-fold changes between the two populations are shown in the heat map. (E) BM neutrophils and PEC neutrophils purified from thioglycollate-induced peritonitis lesions (A) were compared for (Figure legend continues)
began to express CD54 and dectin-2 at modest levels at later time points after incubation with GM-CSF (24–40 h time points in Fig. 2E). Moreover, when tested after 16 h of exposure to GM-CSF, migrated and unmigrated neutrophil populations showed robust ROS production and bacterial uptake at comparable levels (Supplemental Fig. 2). We interpreted these data to suggest that neutrophil priming occurs in a sequential manner. Neutrophils first acquire increased phagocyte functionality during chemotactic migration; this process occurs relatively rapidly within 2 h. The migrated neutrophils subsequently acquire CD54 and dectin-2 expression when exposed to GM-CSF; this process occurs more slowly, with a peak at 16–24 h. It should be stated that neutrophil priming also could take place in a migration-independent manner after prolonged exposure to GM-CSF.

Because most neutrophils isolated from BM are relatively young (27, 46), one might argue that the above changes simply reflect differentiation and not priming. To test this, we examined circulating neutrophils, which are considered to be fully differentiated (34). Neutrophils freshly isolated from peripheral blood did not express CD54 or dectin-2 on the surface, but they acquired both when cultured for 16–24 h in the presence of GM-CSF (Fig. 3A). This time-course was somewhat different from that observed for BM neutrophils, especially the unmigrated population, which required 24–40 h of culturing with GM-CSF to acquire CD54 and dectin-2 surface expression. It was reported that BM neutrophils are fully functional, but they are less responsive to IMLF stimulation compared with circulating neutrophils (34). Thus, it is tempting to speculate that BM neutrophils may differ from peripheral blood neutrophils with regard to their state of maturation. Nevertheless, our results identified CD54 and dectin-2 expression as a previously unrecognized phenotypic change occurring during a late phase of neutrophil priming.

**IL-1β promoter activation as a marker of neutrophil priming**

IL-1β was another signature gene that was elevated in neutrophils recovered from inflammatory lesions (Fig. 1E). We recently constructed a transgenic mouse expressing the DsRed reporter gene under control of the mouse IL-1β promoter (pIL1-DsRed) (26). We reasoned that these transgenic mice might allow us to monitor the dynamic process of neutrophil priming. In fact, DsRed fluorescence signals were detected in only a small fraction (3.0 ± 0.9%, n = 3) of neutrophils isolated from BM (Fig. 4A, 4B). In contrast, PEC neutrophils recovered from thioglycollate-induced peritonitis lesions and BALF neutrophils isolated from cockroach allergen–induced inflammatory lung lesions uniformly expressed DsRed at much higher levels. Thus, neutrophils acquire DsRed expression (i.e., IL-1β promoter activation) as they are recruited to inflammatory sites.

We observed previously that pIL1-DsRed mice did not differ significantly from wild-type (WT) mice with regard to the tempo or magnitude of neutrophil recruitment to inflammatory skin lesions (26). Likewise, pIL1-DsRed mice were comparable to WT mice with regard to the numbers of neutrophils recovered from acute peritonitis lesions and inflammatory lung lesions (Supplemental Fig. 3A). Furthermore, neutrophils purified from pIL1-DsRed mice were comparable to those from WT mice with regard to their in vitro migratory behaviors toward CXCL1 and CXCL2 (Supplemental Fig. 3B). A key question then arose whether DsRed expression would occur in association with neutrophil migration. To test this, we allowed BM neutrophils purified from pIL1-DsRed mice to migrate toward CXCL1 and CXCL2 in Boyden chambers. More than 70% of migrated cells expressed DsRed fluorescence signals at high levels, whereas only 25% of unmigrated cells expressed DsRed at much lower levels (Fig. 4C, 4D). To study the requirement for chemokine gradients, we examined DsRed expression by a relatively small population of neutrophils that had migrated into the lower chamber in the absence of added chemokines. Those randomly migrated cells exhibited marked DsRed expression at the same frequency and level as observed in the cells that had migrated toward CXCL1 and CXCL2 (Fig. 4E, 4F). Moreover, simple exposure of neutrophils to the same chemokines in conventional culture plates (without Transwell inserts) failed to increase DsRed expression (Fig. 4E, 4F, lower panels). However, it should be pointed out that DsRed expression also could take place in a migration-independent fashion, with a minor population of unmigrated cells exhibiting modest DsRed signals. Nevertheless, it appears reasonable to conclude that DsRed expression by neutrophils is closely associated with the migratory behavior.

Upon extravasation, neutrophils are exposed to a wide variety of cytokines present in inflammatory sites, including GM-CSF, TNF-α, and IL-1β (1, 2, 47). To study cytokine-dependent regulation of DsRed expression, we cultured BM neutrophils purified from pIL1-DsRed mice for 24 h in the presence of each of 66 different cytokines or chemokines. At the tested concentration (10 ng/ml), GM-CSF and IL-18 were the only two cytokines that increased DsRed expression above the baseline level (mean + 3 SD observed in control cultures treated with vehicle alone) in a reproducible manner (Fig. 5A). When the same cytokines were tested in a 4.5-h screening assay, a classical neutrophil-priming cytokine TNF-α was identified as a hit that elevated DsRed expression above the baseline level. However, TNF-α failed to show DsRed induction in the above 24-h screening assay, consistent with the report that TNF-α elevates IL-1β mRNA expression in neutrophils, but only transiently, with a peak at 1 h (48). GM-CSF triggered DsRed expression in a dose- and time-dependent fashion (Fig. 5B). A small amount (0.03 ng/ml) of GM-CSF was sufficient to induce the maximum DsRed expression, and a significant increase in DsRed signals was detectable within 2 h after exposure to GM-CSF. After a 24–48-h exposure to GM-CSF, >70% of neutrophils exhibited DsRed signals. Only modest DsRed signals were detected in minor populations (10–30%) of the neutrophils freshly isolated from peripheral blood (Fig. 3B, 3C). However, when cultured for 24 h in the presence of GM-CSF, virtually all cells uniformly exhibited DsRed signals at much higher levels. Thus, we concluded that GM-CSF is a potent cytokine triggering neutrophil priming, as assessed by IL-1β promoter activation.

Our data also suggested that DsRed signals might serve as an easy-to-measure indicator of neutrophil priming. To test this, we cultured the BM neutrophils from pIL1-DsRed mice for 16 h in the presence of GM-CSF and then sorted them into DsRed+ and DsRed− populations by FACS. To assess the efficiency of our cell-sorting CD54, dectin-2, and IL-1β mRNA expression by real-time PCR. Expression levels were normalized to β-actin mRNA. Data are the magnitudes of differences (means ± SD, n = 3) between the two neutrophil populations. **p < 0.001. (F–H) Various neutrophil preparations were stained with mAb against CD54 or dectin-2 (yellow graphs) or isotype-matched control IgG (blue lines) and analyzed by FACS. The tested samples included FACS-purified BM neutrophils (F), PEC neutrophils isolated 24 or 48 h after thioglycollate injection (G), and BALF neutrophils isolated 24 or 48 h after intratracheal instillation of cockroach allergen (H). For PEC and BALF neutrophil preparations (G and H), staining profiles were examined within the Ly6G+/CD11c− gated population. Data are representative of three independent experiments.
procedures, we measured DsRed mRNA expression as “quality control.” As expected, the DsRed+ population showed 30-fold higher DsRed mRNA expression compared with the DsRed- population (Fig. 5C). Importantly, DsRed+ neutrophils expressed CD54 and dectin-2 mRNA at much higher (>45-fold) levels compared with their DsRed- counterparts. Furthermore, surface expression of CD54 and dectin-2 was observed only on the DsRed+ population (Fig. 5D). In contrast, DsRed+ and DsRed- populations purified from GM-CSF–supplemented cultures exhibited comparable levels of ROS production after fMLF stimulation (DsRed+ and DsRed- panels in Supplemental Fig. 3C). As an aside, when BM neutrophils purified from WT mice were tested in parallel, they exhibited a similar level of ROS production (WT panels in Supplemental Fig. 3C). Taken together,
our results indicated that DsRed expression could be used as a marker of fully primed neutrophils characterized by CD54 and dectin-2 expression.

**Intravital visualization of neutrophil priming in inflammatory lesions**

We reasoned that one might be able to visualize the process of neutrophil priming in living animals by monitoring DsRed signals. To test this, we recorded images of DsRed+ cells in experimentally induced skin inflammatory lesions under confocal microscopy. We applied the skin sensitizer OX topically on the right ear of pIL1-DsRed mice and vehicle alone on the left ear of the same animal. FITC-conjugated dextran was injected i.v. to demarcate blood vessels. Almost no DsRed+ cells were detectable in the skin of untreated mice, consistent with our previous report (26). A relatively small, but significant, number of DsRed+ cells became detectable 12 h after OX treatment (Fig. 6A, upper right panel). A markedly increased number of DsRed+ cells emerged 24 h after OX treatment, and most of them were found in the extravascular space (Fig. 6A, lower right panel). In contrast, only a few, if any, DsRed+ cells were observed in the control ear treated with vehicle alone (Fig. 6A, left panels).

We reported previously that most of the DsRed+ cells emerging in OX-induced inflammatory skin lesions expressed Gr-1, which is expressed by neutrophils, inflammatory monocytes, and myeloid-derived suppressor cells (26). To further determine the identity of those DsRed+ cells, we tested their surface phenotype more definitively. We harvested ear skin samples 24 h after treatment, separated them into epidermal and dermal compartments, and prepared single-cell suspensions. Very few DsRed+ cells were found in epidermal or dermal cell suspensions prepared from the vehicle-treated ear. In contrast, strong DsRed signals were detected in ∼5% of epidermal cells and ∼15% of dermal cells isolated from the OX-treated ear (Fig. 6C, upper panels). Moreover, CD54 and dectin-2 were both detected on the surface of the Ly6G+ neutrophils (Fig. 6C, upper panels). Most importantly, an overwhelming majority (>90%) of DsRed+ epidermal cells expressed CD11b, Ly6G, and 7/4, indicating that they are mostly primed neutrophils (Fig. 6C, upper panels). Moreover, CD54 and dectin-2 were both detected on the surface of the Ly6G+ neutrophils (Fig. 6C, upper panels).
phil population isolated from the epidermal compartment after OX painting (Supplemental Fig. 4). In contrast, we failed to detect CD54+/Ly6G+ or dectin-2+/Ly6G+ cells in control skin treated with vehicle alone. DsRed+ dermal cells uniformly expressed CD11b and 7/4, but only a portion (∼30%) of them displayed Ly6G (Fig. 6C, lower panels). Compared with Ly6G (known to be expressed exclusively by neutrophils), 7/4 has been detected on neutrophils, as well as on inflammatory monocytes and activated macrophages (49, 50). Thus, we concluded that DsRed expression occurs almost exclusively in fully primed neutrophils in the epidermis, whereas other leukocyte populations also express DsRed in the dermal compartment under inflammatory conditions.

To mark neutrophils in living animals, we used a protocol developed by Yipp and Kubes (33). Briefly, a small amount of Alexa Fluor 647–conjugated anti-Ly6G mAb was administered before and after OX application, and FITC-dextran was injected before imaging to demarcate blood vessels. At 24 h after OX application, numerous DsRed+/Ly6G+ cells were found under confocal microscopy (Fig. 7A). By counting DsRed+ cells and/or Ly6G+ cells in the extravascular space, we estimated that a majority (82.0 ± 1.5%) of the cells were DsRed+/Ly6G+ cells.
examined for DsRed expression by FACS. Each symbol represents the percentage of DsRed+ cells emerging in the culture supplemented with a given cytokine. Baseline levels of the percentage of DsRed+ cells (mean ± 3 SD observed in control cultures without added cytokines) are shown on the top. The cytokines that elevated DsRed expression above the baseline levels are indicated in red. The data are representative of three independent screening experiments with similar results. (B) DsRed+/Gr-1high/CD48+ neutrophils purified from the BM of pIL1-DsRed mice were cultured in 96-well plates for 24 h in the presence of the indicated concentrations of GM-CSF (left panel) or for the indicated periods in the presence of 10 ng/ml GM-CSF (right panel). Then the samples were examined for DsRed expression by FACS (mean ± SD, n = 3). ***p < 0.001 versus baseline without added GM-CSF (left panel) or versus time 0 (right panel). (C) DsRed+/Gr-1high/CD48+ neutrophils purified from the BM of pIL1-DsRed mice were cultured for 16 h in the presence of 10 ng/ml GM-CSF. The DsRed+ population (bottom bar) and DsRed− population (middle bar) sorted from these cultures by FACS were then examined for DsRed, CD54, and dectin-2 mRNA expression by real-time PCR. DsRed+/Gr-1high/CD48+ neutrophils freshly purified from the BM of pIL1-DsRed mice were examined in parallel without culturing to serve as a control (top bar). Data are mean ± SD from triplicate samples. ***p < 0.001. (D) DsRed+/Gr-1high/CD48+ neutrophils purified from the BM of pIL1-DsRed mice were cultured for 40 h in the presence of 10 ng/ml GM-CSF. The DsRed+ population (upper panels) and DsRed− population (lower panels) were examined for surface expression of CD54 and dectin-2. The shaded yellow and red graphs represent staining profiles with mAb against CD54 or dectin-2. Blue lines indicate the staining profiles with isotype-matched control IgG. Data are representative of three independent experiments.

(i.e., primed neutrophils). We also found relatively small numbers of DsRed−/Ly6G+ cells (9.4 ± 2.4%) and DsRed+/Ly6G+ cells (8.6 ± 1.8%); the former population most likely represented resting neutrophils, whereas the latter might have included inflammatory monocytes and activated macrophages. Interestingly, Ly6G+ neutrophils emerging in inflamed skin exhibited typical amoeba-like crawling movement in time-lapse videos (Supplemental Video 1). To compare motile behaviors between the DsRed−/Ly6G+ and DsRed+/Ly6G+ neutrophil populations, we tracked their migratory paths (Fig. 7B). The chemotactic index values reflecting the directionality of migration (51) were relatively low in both DsRed−/Ly6G+ neutrophils (0.23 ± 0.15, n = 169) and DsRed+/Ly6G+ neutrophils (0.21 ± 0.15, n = 51). Likewise, track plot analyses unveiled that both populations exhibited “random” migration after entering the extravascular space (Fig. 7C). Interestingly, the DsRed−/Ly6G+ population exhibited a significantly higher velocity compared with their DsRed+/Ly6G+ counterparts (Fig. 7D). Thus, neutrophil priming is associated with accelerated motility in inflammatory lesions.

To understand the timing of neutrophil priming, we next recorded a series of time-lapse videos starting at different times after OX application (Supplemental Videos 2–4). DsRed−/Ly6G+ cells (i.e., resting neutrophils) became detectable in the extravascular space as early as 8–12 h after OX treatment, and their numbers remained relatively low thereafter (Fig. 7E, Supplemental Videos 3, 4). In contrast, the number of DsRed+/Ly6G+ cells (i.e., primed neutrophils) increased progressively at later time points, starting at 14–16 h after OX application. During this period, we observed that some of the DsRed+/Ly6G+ cells gradually acquired DsRed signals after recruitment to the extravascular space (Fig. 7F, Supplemental Video 5). At 16–20 h, most of the extravascular Ly6G+ neutrophils were scored as “primed” based on DsRed expression. Our data reveal the magnitude, tempo, and location of neutrophil priming occurring at inflammatory lesions, providing new insights into the pathophysiology of many inflammatory disorders that are characterized by neutrophil infiltration.

Discussion
Neutrophil priming is generally viewed as a relatively rapid event during which neutrophils increase their phagocyte functionality (as measured by ROS production and bacterial uptake) via mechanisms requiring no de novo biosynthesis. We report that this early...
event is followed by previously unrecognized changes occurring at transcriptional levels. To recapitulate the essence of our findings, fully primed neutrophils isolated from inflammatory lesions exhibited elevated CD54, dectin-2, and IL-1β mRNA expression, surface expression of CD54 and dectin-2 proteins, and IL-1β promoter activation (as measured by DsRed signals). Interestingly, cell migration was closely associated with enhanced phagocyte functionality, increased CD54 and dectin-2 mRNA expression, and IL-1β promoter activation. In contrast, all of these changes also were inducible by long-term exposure to GM-CSF, which, indeed, was required for surface expression of CD54 and dectin-2. By monitoring DsRed expression by in vivo–labeled Ly6G+ cells in pIL1-DsRed reporter mice, we were able to visualize the dynamic progress of neutrophil priming in living animals. Strikingly, a majority (>80%) of Ly6G+ neutrophils in the extravascular space expressed DsRed signals at inflammatory lesions, and those DsRed+ neutrophils showed a higher velocity compared with their DsRed− counterparts. Thus, we propose that fully primed neutrophils recruited to inflammatory sites are characterized by CD54 and dectin-2 expression, IL-1β promoter activation, and accelerated motility.

Neutrophil priming is known to be associated with elevated surface expression of selected markers (e.g., CD11b, CD35, and CD66b); such changes mediated by exocytosis of the secretory vesicles occur rapidly (within 10–15 min) after exposure to priming agents (14, 21). In contrast, CD54 and dectin-2 expression occurred much more slowly. A modest (up to 10-fold) increase in CD54 and dectin-2 mRNA expression was observed in the neutrophil population recovered from the lower chamber after the 2-h chemotaxis assay. When those migrated neutrophils were exposed to GM-CSF for 16–24 h, they showed a more striking increase in CD54 mRNA (∼100-fold) and dectin-2 mRNA (∼1000-fold) and surface expression of CD54 and dectin-2 proteins. Importantly, CD54 and dectin-2 are both detectable on the surface of neutrophils isolated from experimentally induced inflammatory lesions, indicating that they can serve as markers of fully primed neutrophils.

Molecular interaction between CD54 on endothelial cells and CD11b/CD18 expressed by neutrophils is well known to promote neutrophil extravasation by mediating firm adhesion (52). Although neutrophils are generally regarded to lack CD54 expression, CD54+ neutrophils have been detected in peripheral blood from patients with rheumatoid arthritis, atherosclerosis, and sarcoidosis (53, 54). Some of the neutrophils that have been recruited to the site of inflammation migrate back into the blood circulation (55). Interestingly, neutrophils were shown to acquire CD54 expression during this process of reverse endothelial transmigration (53, 56). Thus, it is tempting to speculate that CD54 on primed neutrophils may facilitate their motility via binding to fibrinogen.

FIGURE 6. Emergence of DsRed+/Ly6G+ neutrophils in chemically induced inflammatory skin lesions. (A–C) pIL1-DsRed mice received topical application of OX (right ear) or vehicle alone (left ear). (A) After 12 or 24 h, the mice were anesthetized and examined for fluorescence signals under confocal microscopy. To demarcate blood vessels, FITC-dextran was injected i.v. immediately before imaging. Data are representative of at least three independent experiments. Scale bar, 100 μm. (B and C) Epidermal and dermal cell suspensions were prepared from ear skin 24 h after topical application of OX or vehicle alone to examine DsRed expression by FACS. (C) Some of the samples were stained with mAb against CD11b, Ly6G, or 7/4 or with control IgG. Data are representative of at least three independent experiments.
FIGURE 7. Visualization of motile activities of DsRed+/Ly6G+ neutrophils in inflammatory skin lesions. (A–F) pIL1-DsRed mice received topical application of OX and i.v. injection of Alexa Fluor 647–conjugated Ly6G mAb immediately before or 8 h after OX application. The mice were anesthetized and examined under confocal microscopy. To demarcate blood vessels, FITC-dextran was injected i.v. immediately before imaging. (A) Static images were recorded at 24 h after OX treatment to show three leukocyte populations in the extravascular space: DsRed+/Ly6G+ cells (asterisks), DsRed−/Ly6G+ cells (triangles), and DsRed+/Ly6G− cells (arrows). Scale bar, 20 μm. (B–D) A time-lapse video (Supplemental Video 1) was recorded every 2 min for 40 min starting at 24 h after OX application. DsRed+/Ly6G+ cells (red) and DsRed−/Ly6G+ cells (blue) were compared for migratory paths (B), track plots (C), and velocity (D) using Metamorph software. Vertical lines in (D) represent the mean velocity. (E) Starting at different time points after OX application, a series of time-lapse movies was recorded (Supplemental Videos 2–4). At the indicated time points, the numbers of DsRed+/Ly6G+ cells and DsRed−/Ly6G+ cells were counted using Metamorph software. Data are cell numbers/mm² (mean ± SD) calculated from three consecutive images. (Figure legend continues)
and other putative ligands (57, 58). Dectin-2 is a C-type lectin known to be expressed almost exclusively by dendritic cells and macrophages and to mediate their fungal recognition (59). Relatively modest expression of dectin-2 was detected on neutrophils recovered from zymosan-induced peritonitis lesions (60). Further studies are required to define the functional roles of CD54 and dectin-2 expressed by neutrophils.

IL-1β is another signature gene that is elevated in primed neutrophils. This finding may not be surprising because neutrophils are known to produce IL-1β mRNA and/or protein in response to various stimuli, including inflammatory cytokines and bacterial products (61). Neutrophils isolated from pIL1-DsRed mice acquired DsRed expression when tested after chemotactic migration toward CXCL1 and CXCL2. Interestingly, DsRed expression was observed after random migration (in the absence of chemokine gradients) but not after exposure to the same chemokines (in the absence of Transwell inserts). This implies that IL-1β promoter activation is closely coupled to the process of cell migration. By testing 66 cytokines and chemokines, we identified GM-CSF as a potent inducer of DsRed expression by neutrophils, and GM-CSF was found to trigger all of the changes associated with neutrophil priming. This implies that IL-1β promoter activation is also inducible by selected cytokines in a migration-independent manner. Importantly, mRNA expression and surface expression of CD54 and dectin-2 occurred almost exclusively within the DsRed+ neutrophil population. Thus, we propose that IL-1β promoter activation represents a previously unrecognized change associated with neutrophil priming. Because DsRed+ neutrophils were found in experimentally induced inflammatory lesions, one might predict the presence of GM-CSF in such lesions. In fact, GM-CSF was detected in OX-induced inflammatory skin lesions (62), thioglycollate-induced peritonitis lesions (63), and cockroach allergen–induced inflammatory lung lesions (63).

Working with pIL1-DsRed reporter mice, we visualized the process of neutrophil priming in living animals under confocal microscopy. The initial wave of small numbers of DsRed+/Ly6G+ neutrophils emerging at inflammatory sites was followed by progressive increases in DsRed+/Ly6G+ neutrophils; this most likely represents in situ neutrophil priming because some of the DsRed+/Ly6G+ cells gained DsRed signals after recruitment into the extravascular space. Although DsRed+/Ly6G+ neutrophils and DsRed+/Ly6G+ neutrophils both exhibited amoeba-like random migration, the former showed a significantly higher velocity. Working with lysosome M promoter-driven GFP transgenic mice (in which GFP is expressed by neutrophils and activated monocytes), Kreisel et al. (64) observed that GFP+ neutrophils were rapidly recruited to experimentally induced lung inflammatory lesions, where they migrated randomly with a mean velocity of 7–9 μm/min. By injecting Alexa Fluor 647–conjugated anti–TNF-α mAb to these animals, Finsterbusch et al. (65) further demonstrated that neutrophils rapidly release TNF-α as they transmigrate through vascular endothelial cells in response to chemoattractants. After in vivo labeling of neutrophils with Alexa Fluor 750–conjugated anti–Gr-1 mAb, Yipp and Kubes (33) observed that Gr-1+ neutrophils exhibited a crawling motion, with a mean velocity of 6 μm/min, in skin lesions infected with Staphylococcus aureus. Working with transgenic mice expressing a cytoplasmic fluorescence resonance energy transfer biosensor, Mizuno et al. (66) demonstrated marked activation of ERK in neutrophils during adhesion to and migration through vascular endothelial cells in inflamed intestine. Because most of the neutrophils found in the extravascular space exhibited DsRed signals, one may assume that TNF-α release and ERK activation most likely occur predominantly in the primed neutrophil population. Our study, together with these intravital-imaging studies, provide new insights into the dynamic behavior and function of neutrophils recruited to inflammatory lesions.

The present study also unveiled significant heterogeneity among neutrophils. Only fractions of neutrophils isolated from BM incorporated GFP-expressing E. coli or migrated toward CXCL1 and CXCL2. Migrated neutrophils, but not unstimulated neutrophils, showed elevated IL-1β mRNA expression (as measured by real-time PCR) and robust IL-1β promoter activation (as measured by DsRed expression). Furthermore, BM neutrophils differed from their peripheral blood counterparts with regard to the kinetics of GM-CSF–induced acquisition of CD54 and dectin-2 surface expression. Our observations are consistent with previous findings in human peripheral blood neutrophils, demonstrating striking heterogeneity in terms of maturity, functionality, and phenotype (67–69). It will be interesting to determine whether full priming, defined by surface expression of CD54 and dectin-2 and IL-1β promoter activation, occurs only in mature neutrophils or most preferentially within a certain subset of neutrophils.

It is equally important to state the limitations of this study. First, the functional significance of CD54 and dectin-2 expression by primed neutrophils remains to be elucidated. Second, we did not address underlying mechanisms for the observed heterogeneity among neutrophils. Third, of the >4000 genes that were significantly elevated in the primed neutrophils, we only studied Icam1, Clec4n, and Il1b. Because this cluster also included several transcription factors, it would be interesting to determine their potential roles in the process of neutrophil priming. Finally, our study was limited to murine neutrophils. In contrast, elevated CD54 mRNA in human neutrophils can be found in a publicly available database. CD54 mRNA expression is markedly (>30-fold) elevated in human peripheral blood neutrophils after in vitro treatment with GM-CSF plus IFN-γ or with LPS alone (GSE22103) (70). Moreover, human neutrophils isolated from the inflamed airway after endotoxin challenge show >5-fold higher CD54 mRNA expression compared with circulating neutrophils (GSE2322) (71). Likewise, human neutrophils were shown to acquire dectin-2 mRNA and protein expression after in vitro stimulation with IL-6 and IL-23 (72). Studies are in progress in our laboratory to determine late-phase changes associated with priming of human neutrophils.

In conclusion, we demonstrated that neutrophil priming occurs in a sequential manner. The changes currently known to occur rapidly and transiently in the absence of de novo biosynthesis are followed by transcription of a relative large number of genes, gradual and sustained expression of CD54 and dectin-2, and accelerated cellular motility. We believe that the current study provides a novel conceptual framework for our understanding of the process of neutrophil priming. Moreover, the pIL1-DsRed reporter system can serve as a powerful tool to study the behavior and function of primed neutrophils in living animals.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Chemotactic migration of neutrophils toward CXCL1 and CXCL2. Gr-1\textsuperscript{high}/CD48\textsuperscript{–} neutrophils purified from the BM of C57BL/6 mice were added to the upper chambers, and CXCL1 and CXCL2 were added to the lower chambers at the indicated concentrations (A) or at 30 nM each individually or in combination (B). After 2 h incubation, the % migration was calculated by dividing the number of cells harvested from the lower chambers by the total cell numbers recovered from the lower and upper chambers. The data shown are a representative dataset (n = 1) from three independent experiments (A) or the means ± SD from triplicate samples (B). ** $P < 0.01$; *** $P < 0.001$. 

![Graphs showing chemotactic migration of neutrophils.](image)
Supplemental Figure 2. Migrated and unmigrated neutrophils exhibit comparable phagocyte functionality when tested after extended exposure to GM-CSF. Gr-1\textsuperscript{high}/CD48\textsuperscript{−} neutrophils purified from the BM of C57BL/6 mice were placed into the upper chambers, and CXCL1 and CXCL2 (30 nM each) were added into the lower chambers. After 2 h incubation, migrated and unmigrated cells were harvested from the lower and upper chambers, respectively. The two neutrophil populations were further cultured with GM-CSF (10 ng/ml) for 16 h and then examined for ROS production (A) and bacterial uptake (B). All the data are representative of at least three independent experiments.
Supplemental Figure 3. Neutrophils in pIL1-DsRed mice are comparable to those in WT mice in migratory behaviors and ROS production. (A) PEC samples were collected 24 h after thioglycollate injection in pIL1-DsRed mice or WT (C57BL/6) mice. BAL samples were harvested 24 h after intratracheal instillation of cockroach allergen in pIL1-DsRed mice or WT mice. The data shown are the numbers of Ly6G+CD11c− neutrophils (means ± SD, n = 3). (B) DsRed−/Gr-1high/CD48− neutrophils purified from the BM of pIL1-DsRed mice or WT mice were plated onto the upper chambers, and two chemokines (CXCL1 and CXCL2) or PBS alone were added to the lower chamber. After 2 h incubation, the % migration (means ± SD, n = 3) was calculated by dividing the number of cells harvested from the lower chambers by the total cell numbers recovered from the lower and upper chambers. (C) DsRed−/Gr-1high/CD48− neutrophils purified from the BM of pIL1-DsRed mice were cultured for 16 h with 10 ng/ml GM-CSF, and DsRed+ and DsRed− populations were then sorted by FACS. DsRed+/Gr-1high/CD48− neutrophils from WT mice were cultured in parallel for 16 h with GM-CSF. The three neutrophil populations were compared for ROS production. The data shown are the means ± SD (n = 3) of RFU.
Supplemental Figure 4. Surface expression of CD54 and dectin-2 by neutrophils recovered from the epidermal compartment of inflamed skin. Epidermal cell suspensions were prepared from the ear skin of C57BL/6 mice 72 h after topical application of OX and examined for surface expression of CD54 and dectin-2 within the Ly6G+/CD11c− gated population by FACS. Blue lines indicate the staining profiles with isotype-matched control IgG. The data shown are representative of three independent experiments.
Supplemental Video 1. Motile behavior of DsRed+ neutrophils in inflammatory skin lesions (24 hours after OX painting). pIL1-DsRed mice received topical application of OX on the ear skin. AF647-Ly6G mAb was i.v. injected immediately before or 8 h after OX application. FITC-dextran was i.v. injected immediately before imaging. Images were recorded every 2 minutes for 40 minutes starting 24 hours after OX application. Bar = 20 µm.

Supplemental Video 2. Motile behavior of DsRed+ neutrophils in inflammatory skin lesions (4 hours after OX painting). pIL1-DsRed mice received topical application of OX on the ear skin. AF647-Ly6G mAb was i.v. injected immediately before OX application. FITC-dextran was i.v. injected immediately before imaging. Images were recorded every 4 minutes for 40 minutes starting 4 hours after OX application. Bar = 100 µm.

Supplemental Video 3. Motile behavior of DsRed+ neutrophils in inflammatory skin lesions (8 hours after OX painting). pIL1-DsRed mice received topical application of OX on the ear skin. AF647-Ly6G mAb was i.v. injected immediately before and 8 hours after OX application. FITC-dextran was i.v. injected immediately before imaging. Images were recorded every 4 minutes for 40 minutes starting 8 hours after OX application. Bar = 100 µm.

Supplemental Video 4. Motile behavior of DsRed+ neutrophils in inflammatory skin lesions (11-19 hours after OX painting). pIL1-DsRed mice received topical application of OX on the ear skin. AF647-Ly6G mAb was i.v. injected immediately before and 8 hours after OX application. FITC-dextran was i.v. injected immediately before imaging. Images were recorded every 4 minutes for 11 hours starting 8 hours after OX application. Bar = 100 µm.

Supplemental Video 5. Acquisition of DsRed signals by Ly6G+ neutrophils. From the time-lapse movie shown in supplemental Video 4, this zoom-in video was created to show the process in which a Ly6G+ cell (indicated with an arrow) acquired DsRed expression in the extravascular space during the observation period (11-14.5 hours after OX painting). Bar = 50 µm.