

# Neutrophil maturation and activation determine anatomic site of clearance from circulation

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Departments of <sup>1</sup>Medicine and <sup>3</sup>Pediatrics, National Jewish Medical  
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**Suratt, Benjamin T., Scott K. Young, Jonathan Lieber, Jerry A. Nick, Peter M. Henson, and G. Scott Worthen.** Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am J Physiol Lung Cell Mol Physiol* 281: L913–L921, 2001.—The long-term disposition of circulating neutrophils and the site of disappearance from circulation remain unclear. We investigated neutrophil localization in mice using <sup>111</sup>In-labeled murine peripheral blood neutrophils, mature bone marrow neutrophils, and peritoneal exudate neutrophils to track in vivo localization of these different cell populations. Infused peripheral neutrophils were found to localize equally between liver and marrow sites by 4 h ( $31.2 \pm 1.9$  vs.  $31.9 \pm 1.8\%$ ), whereas exudate neutrophils predominantly localized to liver ( $42.0 \pm 1.1\%$ ) and marrow-derived neutrophils to the marrow ( $65.9 \pm 6.6\%$ ) where they were found to localize predominantly in the hematopoietic cords. Stimulation of marrow neutrophils before infusion caused a shift in localization from marrow to liver, and subsequent induction of an inflammatory site after infusion and marrow sequestration led to remobilization of infused marrow neutrophils but not of peripheral neutrophils. These results indicate that the marrow participates in removing neutrophils from circulation, with evidence supporting both storage and perhaps disposal functions. Furthermore, models for circulating neutrophil homeostasis should consider that the site of retention is governed by the maturation and activation states of the cell.

bone marrow; liver; lipopolysaccharide; inflammation

AS THE CELL RESPONSIBLE for initial host defense, the neutrophil circulates for only a few hours before leaving the bloodstream. Under normal homeostatic conditions, circulating neutrophil numbers are tightly regulated, and their disappearance has been modeled as a stochastic process, with departure of aging cells into the tissues of the body after a predictable half-life (6, 7). In mammalian systems heretofore described, experimentally infused neutrophils were retained primarily in liver and spleen of normal animals (10, 39, 48, 51), and studies of inflammation have shown migration of labeled cells to inflammatory sites, with eventual disposal in situ (8, 14, 34, 50). Given the potentially

injurious nature of neutrophils, their circulatory regulation is of paramount importance. Recently, the existing paradigms for this regulation have been called into question.

Recent studies have suggested that the bone marrow may also play a role in the sequestration of mature circulating neutrophils in rats (30), a finding previously suggested in humans by nuclear medicine studies of infused neutrophils (48, 53, 58). The bone marrow plays a well-established role in the apoptosis and removal of erythroblasts (9) and B cell clones (41) during hematopoiesis. Recent work in mice suggests that a substantial fraction of developing neutrophils are destroyed in the marrow (35), whereas large numbers of mature neutrophils are removed from the circulation and destroyed in murine marrow during fetal development (44).

These observations suggest the possibility that the bone marrow may participate more actively in the regulation of circulating neutrophils than has previously been suggested. The marrow not only supplies mature neutrophils but can respond to inflammatory conditions through the release of immature neutrophils (26, 28, 32, 52). Although neutrophil disposal in liver, spleen, and sites of local inflammation has been thought to be unidirectional, evidence implicating the bone marrow as a site of neutrophil sequestration raises the possibility that the marrow might sequester and release circulating neutrophils in a reversible fashion or even participate in their disposal at the end of their lifespan.

We have investigated this possibility using three populations of labeled neutrophils: mature circulating neutrophils, morphologically mature but functionally immature neutrophils isolated from marrow, and inflammatory neutrophils isolated after migration to an inflammatory site. The infusion of these cells into recipient mice reveals a potentially important role of marrow in the regulation of not only neutrophil release but of neutrophil circulation as well. Our data raise the possibility that immature neutrophils home in to mar-

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row from which they may again be mobilized in inflammatory settings, whereas sequestration of mature neutrophils in marrow appears to be an irreversible event. Furthermore, the activation state of the neutrophil may dictate the balance of clearance between marrow and liver sites.

## MATERIALS AND METHODS

**Preparation of murine peripheral blood neutrophils.** Mouse peripheral blood neutrophils were isolated by modification of methods previously reported (19) for the purification of rabbit peripheral neutrophils. C57BL/6 mice (Harlan Sprague Dawley) were volume expanded and exsanguinated into a 3.8% citrate solution; the blood was centrifuged at 300 *g* for 20 min. The cell pellet was resuspended in a 6% dextran-0.9% NaCl solution (1:5.25) to a final volume of 150% of the original blood volume and sedimented at unity gravity for 30 min. The leukocyte-rich supernatant was aspirated, washed once in Hanks' balanced salt solution (HBSS), layered on a three-step Percoll (Pharmacia) gradient (78, 66, and 54%) and centrifuged at 1,060 *g* for 30 min. Cytospin samples of the 78–66% interface revealed >90% neutrophils. After lysis with hypotonic saline, typical yields were  $\sim 2\text{--}4 \times 10^5$  peripheral blood neutrophils/mouse. Trypan blue dye exclusion showed the cells to be >97% viable after purification.

**Preparation of morphologically mature murine bone marrow neutrophils.** Femurs and tibias of C57BL/6 mice were dissected, and the marrow was flushed with HBSS and layered on a three-step Percoll gradient (72, 64, and 52%) that was centrifuged at 1,060 *g* for 30 min. Cytospin samples of the 72–64% interface revealed >95% morphologically mature-appearing neutrophils. This method exploits the previously noted correlation between marrow neutrophil density and maturity (3, 40). Typical yields were  $\sim 1\text{--}2 \times 10^7$  neutrophils/mouse, of which >98% were viable. Lipopolysaccharide (LPS)-stimulated marrow neutrophils were prepared by incubating isolated neutrophils in HBSS-1% serum with LPS at concentrations of 0.01, 0.1, and 1  $\mu\text{g/ml}$  for 2 h at 23°C.

**Preparation of murine peritoneal neutrophils.** Mouse peritoneal exudate neutrophils were isolated 4 h after the intraperitoneal injection of 400  $\mu\text{l}$  of thioglycollate solution according to the methods of Savige et al. (49). Cytospin samples revealed nearly pure neutrophils (>90%) with occasional macrophages. Typical yields were  $\sim 2 \times 10^7$  neutrophils/mouse.

**Labeling of neutrophils.** Neutrophils were radiolabeled with  $^{111}\text{In}$ -tropolonate with methods modified from Haslett et al. (19). This method yielded  $3\text{--}20 \times 10^6$  cell-associated counts  $\cdot \text{min}^{-1} \cdot 5 \times 10^6$  neutrophils $^{-1}$  after cells were washed.  $^{111}\text{In}$ -tropolonate was found to be >80% cell associated 6 h after the cells were labeled. A Beckman 7000 gamma counter set to count both 173- and 247-keV peaks of  $^{111}\text{In}$  was used to count radioactivity in all samples. Nuclear labeling of bone marrow neutrophils was performed with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU), which is incorporated into dividing cells. With a protocol modified from Bicknell et al. (4), mice were injected with BrdU (100 mg/kg ip; Sigma) every 12 h for 48 h, after which morphologically mature bone marrow neutrophils were isolated as described in *Preparation of morphologically mature murine bone marrow neutrophils*. BrdU incorporation was found in >85% of cytopun, isolated cells (stained as detailed in *Circulation of BrdU-labeled neutrophils*).

**Circulation of  $^{111}\text{In}$ -labeled neutrophils.** Aliquots of  $5 \times 10^6$  labeled neutrophils in 200  $\mu\text{l}$  of phosphate-buffered saline with 0.1% bovine serum albumin (PBS-BSA) were in-

fused intravenously through the left tail vein of each recipient 4- to 8-wk-old C57BL/6 mouse. Three to seven recipient mice were injected for each time point, subsequently bled (10  $\mu\text{l}$ ) via the right tail vein at the appropriate interval after infusion, and killed by cervical dislocation. Each mouse was dissected, and the lungs, spleen, kidneys, gut, liver, and right femur were removed and washed with 0.9% saline. The remaining mouse carcass was divided into head, tail, thorax, and hindquarters.  $^{111}\text{In}$  content in all tissues is expressed as a percentage of the total counts present in all tissues (the entire mouse). Values for the estimated total blood counts per minute were based on a predicted total blood volume of  $\sim 1.5$  ml for the 4- to 8-wk-old C57BL/6 mouse (2). The marrow content of C57BL/6 mouse femur was estimated to represent 6% of the total marrow content based on the work of Boggs (5). Blood half-life of labeled neutrophils was calculated using the second phase of neutrophil disappearance from the blood (after an initial 60-min redistributive phase) with the methods of Weiblen et al. (58).

**Circulation of BrdU-labeled neutrophils.** Four hours after intravenous infusion of BrdU-labeled neutrophils (performed as in *Circulation of  $^{111}\text{In}$ -labeled neutrophils*), recipient mice were anesthetized with Avertin (tribromoethanol in tert-amyl alcohol, 250 mg/kg ip; Aldrich), the thorax was dissected, the right atrium was punctured, and the left ventricle was cannulated with a 20-gauge butterfly needle attached to a 60-ml syringe. After this, the animals were perfused with cold HBSS and then 10% formalin-PBS (3 ml/min, 10 min each) with a syringe pump (model M22, Harvard Apparatus). The femurs were dissected, soaked in formalin solution overnight, and then placed in 5% EDTA for 3 days at room temperature to decalcify them before paraffin embedding. Five-micrometer sections were stained for BrdU with biotinylated anti-BrdU antibody and streptavidin-horseradish peroxidase (BrdU In-Situ Detection Kit; BD Pharmingen) followed by hematoxylin counterstaining.

**Functional assays.** Nitro blue tetrazolium reduction assays were performed after stimulation with phorbol 12-myristate 13-acetate (PMA; 33 ng/ml) for 30 min at 38°C (16). Superoxide production of PMA (20 ng/ml)-stimulated neutrophil populations was quantitated with the Amplex Red hydrogen peroxide assay (Molecular Probes) according to the methods of Mohanty et al. (36). F-actin polymerization by isolated peripheral, peritoneal exudate, and morphologically mature bone marrow neutrophils in response to stimulation with the murine C-X-C chemokine KC (Molecular Probes) at  $5 \times 10^{-9}$  and  $5 \times 10^{-8}$  M concentrations was performed as previously described (11). After stimulation, filter retention of  $^{111}\text{In}$ -labeled marrow, peripheral, and peritoneal exudate neutrophils was assayed with 6.5- $\mu\text{m}$ -pore polycarbonate filters as previously described (13). Three conditions were assayed for each neutrophil population: unstimulated, stimulated with KC at  $5 \times 10^{-9}$  M, and stimulated with KC at  $5 \times 10^{-8}$  M.

**Chemokine studies.** Migration of labeled neutrophils into the airspaces of mice was determined by bronchoalveolar lavage after the instillation of KC into airways. Mice infused with  $^{111}\text{In}$ -labeled peripheral or marrow neutrophils via tail vein injection were immediately anesthetized with Avertin (250 mg/kg ip), the trachea was cannulated with a 22-gauge metal feeding catheter, and 50  $\mu\text{l}$  of either KC (2  $\mu\text{g}$  in PBS-BSA) or PBS-BSA alone (control) were instilled. At 4 h, the mice were killed by cervical dislocation, the trachea was dissected and cannulated with a 20-gauge catheter with stylette, and 4 serial 0.8-ml lavages with heparinized saline were performed. The lavage cell count was determined by hemacytometer, radioactivity assayed by gamma counter, and cell differential determined by Wright-Giemsa staining.

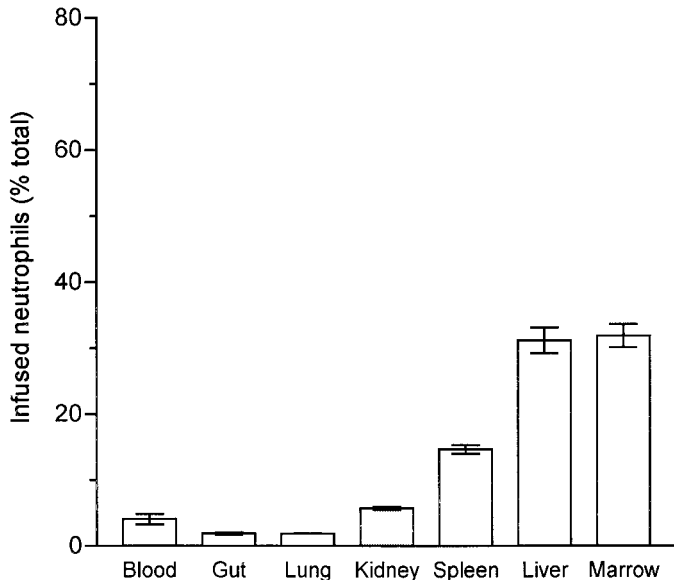


Fig. 1. Localization of labeled peripheral neutrophils 4 h after intravenous infusion into untreated recipient mice. Values are means  $\pm$  SE from 3–7 separate mice.

The data derived are expressed as the percentage of total labeled infused neutrophils present in lavage fluid in both KC-treated and matched control mice, and the ratio of labeled neutrophils in lavage fluid to total labeled neutrophils in the lungs (lavage fluid and parenchyma).

**Neutrophil chemotaxis assays.** A Zigmond chamber (20) was used to assess the chemotaxis of marrow neutrophils and peripheral neutrophils in response to the murine chemokine KC. Bone marrow-derived neutrophils ( $5 \times 10^4$ ) in 50  $\mu$ l of Krebs-Ringer phosphate-buffered dextrose were loaded on glass coverslips and allowed to adhere for 20 min at 37°C before being inverted on a Zigmond chamber slide (Neuro-Probe). The buffer control and chemoattractant chambers were loaded with migration buffer (100  $\mu$ l of Krebs-Ringer phosphate-buffered dextrose with 1% BSA) and 25–100 ng/ml of KC in migration buffer, respectively. The relative morphology, position, orientation, and locomotion of the cells was evaluated with videomicroscopy, and cell tracings were made of each field over time. Mean and peak migratory rates of the neutrophils as well as the mean path length and net displacement of the cells toward the chemoattractant were calculated from these tracings to assess relative chemotaxis and chemokinesis.

**Recirculation of  $^{111}\text{In}$ -labeled neutrophils.** Labeled morphologically mature bone marrow and peripheral neutrophils were prepared and injected into untreated recipient mice as described in *Circulation of  $^{111}\text{In}$ -labeled neutrophils*. Four hours after this, the recipient mice were treated with epinephrine (0.7 mg/kg in 0.3 ml of saline ip), thioglycollate (1 ml ip), or saline (1 ml ip, paired control) and subsequently tail-bled and killed 10 min (epinephrine experiments) or 2 h (thioglycollate experiments) after treatment. Peritoneal lavage was performed on the mice with 7 ml of saline, which was collected and gamma counted, and the counts were normalized to a return volume of 7 ml. The mice were then dissected, and their tissues were gamma counted.

**Statistical analysis.** Analysis of differences in neutrophil localization at measured sites and selected time points was performed by one-way analysis of variance with Tukey's multiple comparison procedure with the use of JMP software

(SAS Institute, Cary, NC). All other comparisons were performed with Student's *t*-test with the use of JMP software.

## RESULTS

**Localization of infused labeled peripheral, exudate, and bone marrow neutrophils.** Infused labeled murine peripheral blood neutrophils were found to localize significantly to both marrow and liver at 4 h ( $31.2 \pm 1.9$  and  $31.9 \pm 1.8\%$ , respectively), having largely departed the blood (Fig. 1). Splenic sequestration was significant but lower, and lung, kidney, and gut localization was minimal at 4 h. To determine whether marrow sequestration might reflect the more immature or, alternatively, the more mature and/or stimulated subsets of peripheral neutrophils in circulation, we examined the behavior of two other populations of neutrophils: morphologically mature bone marrow neutrophils and peritoneal exudate neutrophils. Infused bone marrow neutrophils localized predominantly to the marrow space at 4 h ( $65.9 \pm 6.6\%$ ), with significantly less liver sequestration ( $15.4 \pm 1.0\%$ ; Fig. 2). Inflammatory exudate neutrophils showed modest (but significant) marrow localization after infusion ( $19.8 \pm 0.5\%$ ; Fig. 2); however, the majority of these cells localized to the liver ( $42.0 \pm 1.0\%$ ), and  $11.6 \pm 1.0\%$  appeared to remain sequestered in the lungs at 4 h. Splenic sequestration did not appear to differ significantly among peripheral, marrow, and exudate neutrophils after infusion.

**Functional comparison of isolated mature marrow, peripheral, and exudate neutrophils.** We next examined the functional behavior of each of the isolated

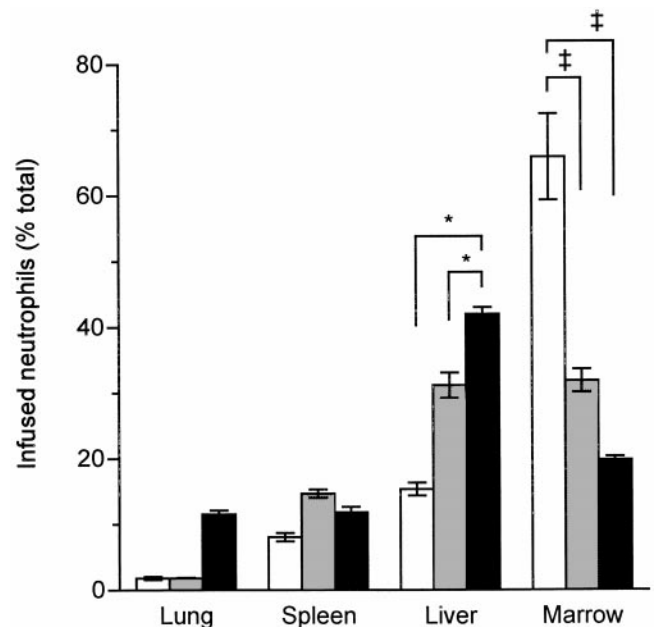


Fig. 2. Localization of bone marrow, peripheral blood, and peritoneal exudate neutrophils 4 h after intravenous infusion. Open bars, bone marrow-derived neutrophils; shaded bars, isolated peripheral neutrophils; solid bars, sterile peritonitis-elicited neutrophils. Values are means  $\pm$  SE from 3–7 separate experiments. Significantly different compared with uptake of morphologically mature bone marrow neutrophils: \* $P < 0.001$ ; ‡ $P < 0.002$ .

neutrophil populations to better characterize the qualities associated with either liver or marrow retention after circulation. Murine bone marrow neutrophils, peripheral neutrophils, and peritoneal exudate neutrophils were found to be morphologically similar with the use of light microscopy and exhibited similar responses to stimulation as assayed by nitro blue tetrazolium reduction (data not shown). Other functional responses were divergent between these three populations, however. All three neutrophil populations demonstrated significant increases in superoxide production in response to *in vitro* stimulation, with peripheral and marrow cells quite similar in their response (Fig. 3). However, stimulation of exudate neutrophils yielded a much more marked increase (nearly eightfold) in superoxide production. Furthermore, although all cells exhibited actin polymerization after *in vitro* stimulation (Table 1), the exudate cells appeared to have already undergone extensive actin assembly, and thus their response to stimulation was markedly attenuated.

The retention of neutrophils within capillary-size pores has been shown to reflect behavior in the pulmonary capillary (13, 59). Consistent with the actin assembly data, exudate cells were retained in 6.5- $\mu$ m pores to a much greater extent than the other two cell populations (Table 1). Furthermore, the response of the marrow neutrophils to stimulation with KC resulted in clear-cut retention approaching that of the peripheral neutrophils, indicating that marrow neutrophils exhibit responsiveness to chemokine stimulation. How-

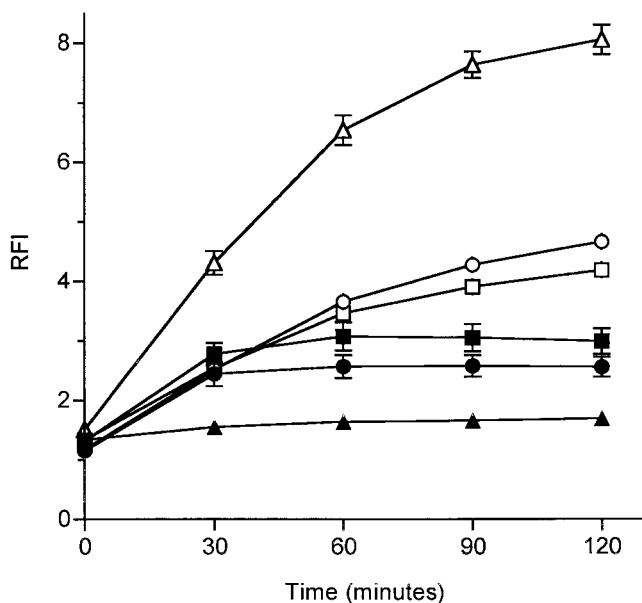


Fig. 3. Superoxide production of 3 neutrophil populations after *ex vivo* stimulation. Superoxide production of isolated bone marrow (circles), peripheral (squares), and peritoneal neutrophils (triangles) over time in the presence (solid symbols) or absence (open symbols) of phorbol 12-myristate 13-acetate (20 ng/ml) is displayed as measured with Amplex Red hydrogen peroxide assay (36). RFI, relative fluorescence index. Each time point is average  $\pm$  SE of peroxide production expressed as multiple of increase over blank wells from 3 separate assay wells.

Table 1. Actin polymerization and filter retention of isolated neutrophils

Condition	Neutrophils		
	Marrow	Peripheral	Peritoneal
<i>Actin polymerization</i>			
Unstimulated KC	1.00	1.00	1.00
$5 \times 10^{-9}$ M	$1.37 \pm 0.20$	$1.50 \pm 0.10$	$1.02 \pm 0.10$
$5 \times 10^{-8}$ M	$1.80 \pm 0.30$	$1.99 \pm 0.10$	$1.15 \pm 0.10$
<i>Filter retention</i>			
Unstimulated KC	$5.07 \pm 2.54$	$12.70 \pm 1.09$	$34.69 \pm 7.04$
$5 \times 10^{-9}$ M	$14.08 \pm 1.92$	$22.52 \pm 7.24$	$43.73 \pm 1.19$
$5 \times 10^{-8}$ M	$13.92 \pm 2.18$	$21.45 \pm 5.83$	$44.04 \pm 4.57$

Values for actin polymerization are averages  $\pm$  SE of fluorescence index from 3 separation preparations of isolated morphologically mature bone marrow, peripheral blood, and peritoneal exudate neutrophils in response to stimulation with the murine C-X-C chemokine KC as measured by the methods of Downey et al. (11). Values for filter retention of labeled neutrophils are averages  $\pm$  SE of percent of total infused neutrophils (13) retained in 65- $\mu$ m filters at baseline and after stimulation with KC from 3 separate samples of isolated cells.

ever, the chemotactic behavior of marrow and peripheral neutrophils was clearly discrepant. The marrow neutrophil chemoattractant response in diffusion chamber experiments was diminished in both chemokinetic and chemotactic elements compared with that in peripheral neutrophils (Fig. 4A), whereas exudate neutrophils have been shown by others to be highly chemotactic (54, 60).

To examine the response of intravenously infused marrow neutrophils to local pulmonary chemokines, the migration of infused labeled cells from the circulation to the lavageable alveolar space in response to the intratracheal instillation of KC was determined. Accumulation of marrow neutrophils, although clearly detectable, was found to be significantly reduced at 4 h compared with that of similarly labeled and infused peripheral neutrophils (Fig. 4B). Likewise, the ratio of labeled neutrophils in lavage fluid to those present in lung parenchyma was substantially greater for peripheral neutrophils than for marrow neutrophils ( $0.172 \pm 0.079$  vs.  $0.061 \pm 0.053$ ). The presence of labeled neutrophils in the lavage fluid of control mice was negligible in both marrow and peripheral neutrophil experiments. These functional characteristics are similar to those of the functionally immature, marrow-released neutrophil population previously described in animal models of systemic inflammation (27, 46, 52).

*Effects of LPS stimulation on neutrophil localization.* To determine whether the marrow sequestration observed for marrow neutrophils could be modified by inflammatory mediators (as might be predicted from our above observations), we exposed marrow neutrophils to LPS before infusion. Marrow neutrophils stimulated with LPS and infused into untreated recipient mice demonstrated a significant transition in subsequent localization from marrow to liver compared with untreated marrow neutrophils (Fig. 5).

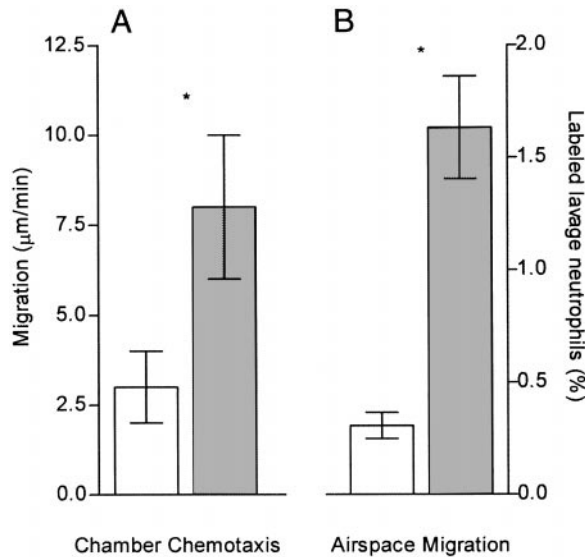


Fig. 4. Chemotactic response of morphologically mature bone marrow (open bars) neutrophils and peripheral blood (shaded bars) neutrophils to ex vivo and in vivo stimulation. *A*: neutrophil chamber chemotaxis in response to KC. Mean rate of migration toward the chemoattractant chamber was measured for both isolated morphologically mature bone marrow and isolated peripheral neutrophils. Data are averages  $\pm$  SE of chemotactic rate from 10–20 cells from 3 separate preparations of isolated cells. *B*: airspace migration of labeled peripheral and morphologically mature bone marrow neutrophils in response to intratracheal instillation of KC. Mice were treated with intratracheal KC, and subsequent airspace accumulation of intravenously infused In-labeled neutrophils was determined by lung lavage 4 h later. Results are averages  $\pm$  SE of percent total infused labeled cells from 4 treated mice. \*Significantly different compared with marrow and peripheral neutrophils,  $P < 0.01$ .

**Kinetics of infused labeled mature marrow neutrophils in the circulation.** Having established that marrow retention was an important fate of both peripheral and marrow neutrophils, we used marrow neutrophils to probe the kinetics and the microanatomic site of marrow retention. Infused labeled mature marrow neutrophils were found to circulate with a half-life of 3.3 h (Fig. 6), which is comparable to data obtained with peripheral neutrophils in rabbits (19, 42). The initial organ retention (determined by dissection and gamma counting) was in the lung, followed by a slow washout (with a half-life of 42 min) as previously described for a variety of mammalian systems (10, 15, 19, 47). Although subsequent liver and spleen sequestration of the neutrophils was significant, the majority of infused neutrophils localized to the bone marrow by 4 h, demonstrating a nearly reciprocal relationship to lung sequestration (Fig. 6). Measured radioactivity in the bone marrow remained constant for at least 20 h.

**Microanatomic localization of infused neutrophils.** To examine the nature of neutrophil localization to the marrow compartment, we used mature marrow neutrophils labeled with BrdU and examined perfusion-fixed marrow of recipient mice 4 h after neutrophil infusion (Fig. 7). This showed labeled cells predominantly in the marrow cords, closely associated with cells of the myelomonocytic lineages. A small but significant population of labeled neutrophils was found to

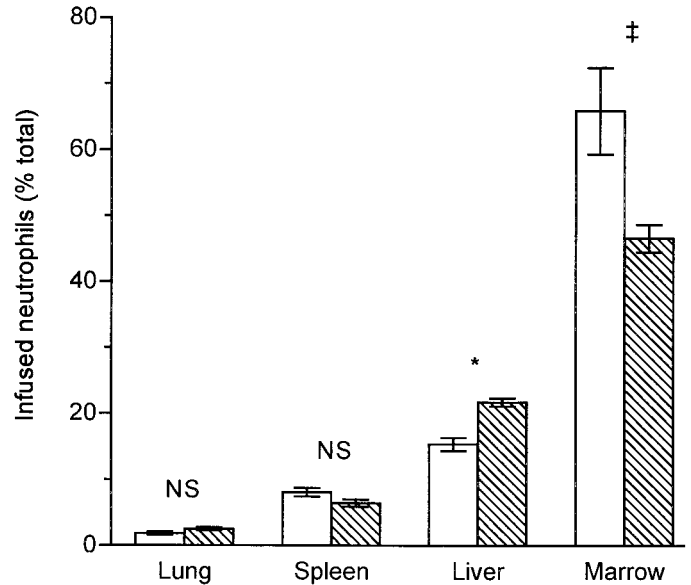


Fig. 5. Effects of stimulation of marrow neutrophils with lipopolysaccharide before infusion (hatched bars) compared with unstimulated neutrophils (open bars). Data are means  $\pm$  SE from 3–7 separate mice. NS, not significant. Significantly different compared with control mice: \* $P = 0.001$ ; ‡ $P = 0.03$ .

be closely approximated to the marrow venous sinusoids, possibly in the process of extravasation to or from the marrow cords.

**Recirculation of marrow-sequestered neutrophils.** To determine whether sequestration of marrow or peripheral neutrophils in the marrow was a reversible event, neutrophils from these two sources were infused, and after 4 h, when neutrophils had localized to marrow, maneuvers designed to mobilize neutrophils were at-

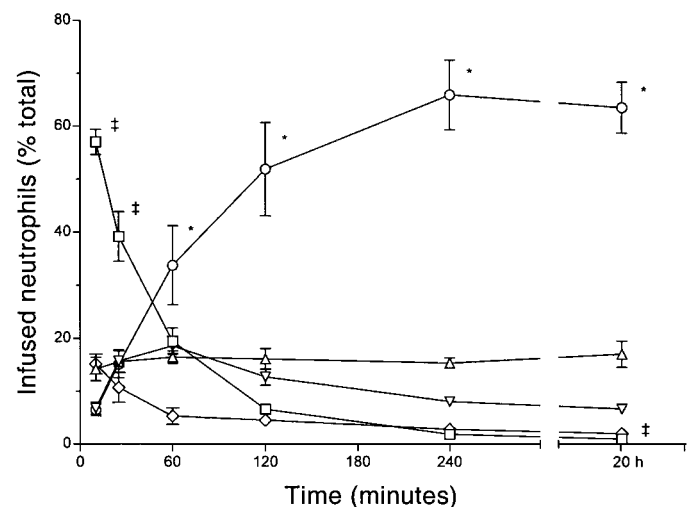
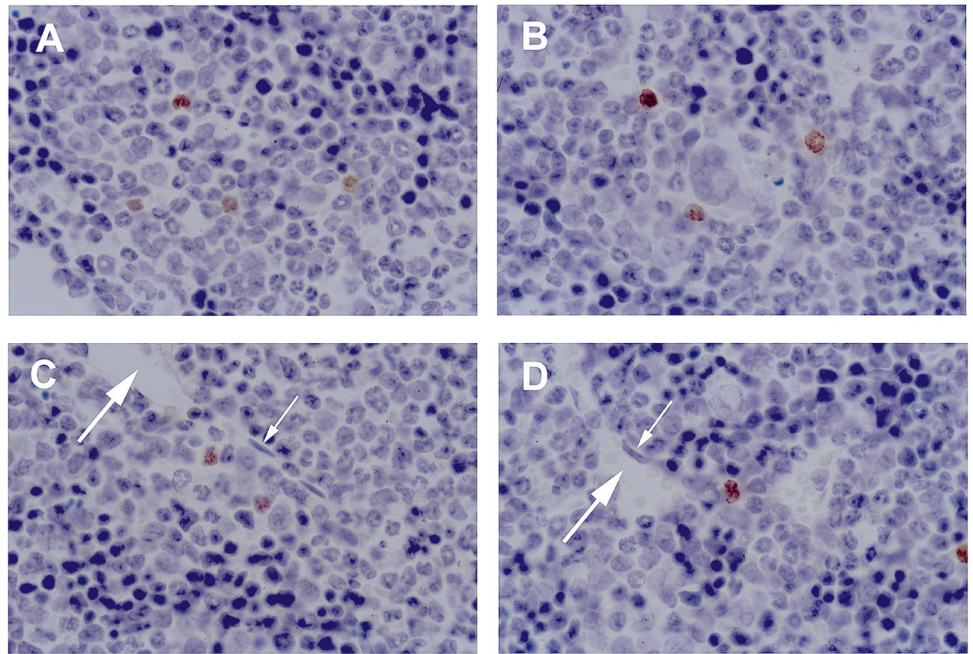


Fig. 6. Morphologically mature marrow neutrophil localization after intravenous infusion. Initial sequestration in lung (□) is followed by a rapid rise in bone marrow uptake (○). Liver uptake (Δ) is fairly constant over time, whereas spleen uptake (▽) slowly decreases. Blood levels (◇) fall rapidly, with a calculated half-life of 3.3 h. Data are means  $\pm$  SE from 3–7 separate experiments. \*Significantly different from lung, liver, and spleen,  $P < 0.01$ . ‡Significantly different from liver, spleen, and marrow,  $P < 0.01$ .

Fig. 7. Homing of infused neutrophils to marrow 4 h after intravenous infusion. Morphologically mature marrow neutrophils labeled with 5-bromo-2'-deoxyuridine (BrdU) were infused intravenously into recipient mice, which were then exsanguinated at 4 h by vascular perfusion and fixed with a formalin solution. Sections of fixed marrow were then stained for BrdU and counterstained with hematoxylin. *A* and *B*: typical distribution of labeled cells (brown), which appear predominantly in the marrow cords. *C* and *D*: marrow venous sinuses (large arrows) lined with typical endothelial cells (small arrows). Several labeled neutrophils appear to be closely approximated to the sinusoids, perhaps in the process of extravasation into or out of the marrow cords. Original magnification,  $\times 250$ .



tempted. Efforts to remobilize marrow-sequestered labeled neutrophils of either type with epinephrine treatment yielded a small but statistically significant increase in circulating blood levels of labeled neutrophils ( $6.06 \pm 0.79$  vs.  $4.19 \pm 0.43\%$ ) but a nonsignificant change in neutrophil localization elsewhere (data not shown). Induction of sterile peritonitis with thioglycollate in animals that had received marrow neutrophils resulted in a shift of labeled cells away from the marrow such that significant increases in blood, lung, and liver levels of infused neutrophils were seen (Fig. 8A). In contrast, no significant change in organ sequestration of infused peripheral neutrophils was seen after thioglycollate treatment (Fig. 8B), suggesting that peripheral neutrophils localized to the marrow did not respond to the induced inflammatory state.

## DISCUSSION

The regulation of neutrophil production and removal is a critical homeostatic mechanism and is central to the development of systemic inflammatory states, but it remains poorly understood. Previous models have suggested the liver and spleen as the primary sites of circulating neutrophil retention and disposal (10, 39, 48, 51). In contrast, in this study, we found that in the mouse, the marrow plays an important role in the removal of neutrophils from the circulation and that a dynamic balance exists between marrow and hepatic sequestration of these cells, suggesting a far more complex system of neutrophil regulation than previously recognized.

Although our data confirm, in mice, the observations of Løvås et al. (30) that circulating neutrophils may localize to the bone marrow in rats, they also suggest a potentially even greater role for marrow sequestration of infused peripheral neutrophils, with a nearly even

distribution of neutrophil localization between the liver and marrow (Fig. 1). We hypothesized that this separation between the two sites might represent the presence of distinct neutrophil subpopulations present in the isolated peripheral neutrophils. Such a heterogeneity of circulating neutrophils has been described previously (17) and is believed to reflect differing degrees of maturation and activation of these cells (25, 57). To investigate this possibility, we examined the behavior of peripheral neutrophils as well as of two isolated neutrophil populations representing distinctly different degrees of maturation and activation: morphologically mature marrow neutrophils and exudate neutrophils from sterile peritonitis.

Functional comparison of these three neutrophil populations demonstrated a spectrum of behavior in response to stimuli that stemmed from differences in development and activation. Peritoneal exudate cells showed a high degree of filter retention (Table 1) and actin polymerization at baseline (data not shown), suggesting that these cells had already undergone cytoskeletal assembly, leading to altered mechanical properties (a hallmark of activation) (59). These cells also showed a substantial increase in superoxide production after stimulation with PMA (Fig. 3), likely because of prior priming as part of the emigration response (12, 18, 29, 60) and deformation during transit (24). In contrast, defining characteristics of the bone marrow neutrophil population included a marked diminution of both chemotactic and chemokinetic functions in diffusion chamber experiments (Fig. 4A) and attenuation of diapedesis into lavageable airspaces of KC-treated mouse lungs in vivo (Fig. 4B). Similar defects have been attributed to the rapidly released marrow neutrophil populations seen in models of sepsis and other systemic inflammatory states (27, 46, 52, 56).

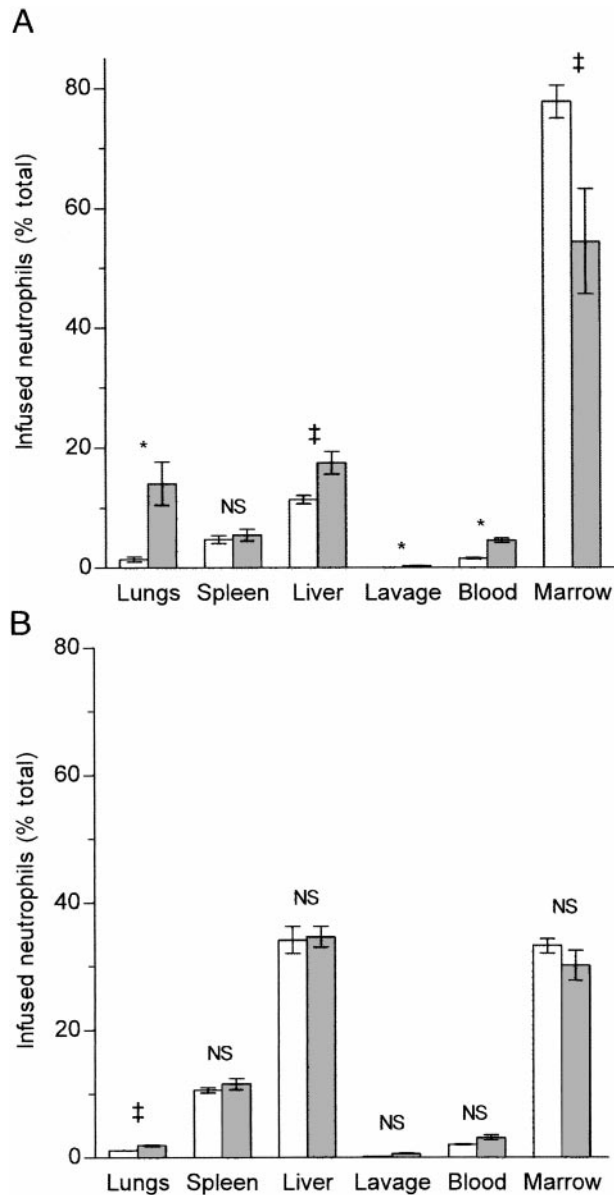


Fig. 8. Effects of inflammation on previously infused and sequestered neutrophils (shaded bars) compared with control neutrophils (open bars). *A*: sterile peritonitis was induced 4 h after infusion of morphologically mature marrow neutrophils and tissue neutrophil localization was determined 2 h later. Note the small accumulation in peritoneal lavage. *B*: similar experiments were performed after the infusion of labeled peripheral neutrophils. Data are means  $\pm$  SE from 2–5 separate mice. Significantly different from uptake in control mice: \* $P \leq 0.01$ ; ‡ $P \leq 0.03$ .

Other functional aspects of the marrow neutrophils, including superoxide production and actin polymerization after stimulation, were remarkably similar to those of isolated peripheral blood neutrophils, suggesting that chemotaxis may not be fully developed even in morphologically mature neutrophils.

When intravenously infused, peritoneal exudate neutrophils sequestered predominantly in the liver after circulation (Fig. 2), as described by others (49), whereas mature marrow neutrophils preferentially homed in to the marrow space. These findings suggest

that different neutrophil populations have distinctly different patterns of removal from the circulation, perhaps determined by the state of maturation or stimulation. The subsequent observation that *ex vivo* exposure of marrow neutrophils to LPS caused a shift in their localization after infusion from marrow to liver (Fig. 5) further suggests that the pattern of sequestration is influenced by the activation state of the neutrophil and that intrinsic neutrophil properties (perhaps cell surface characteristics) may, in part, account for their subsequent localization.

Neutrophil accumulation at an inflammatory site appears to depend on multiple receptor-ligand interactions, prominently involving the selectins and integrins (1). Similarly, neutrophil accumulation in liver and marrow may depend on such interactions. Although several researchers have suggested a role for CD18 in hepatic sequestration of neutrophils during inflammation (22, 37), marrow homing of circulating hematopoietic stem cells appears to require both E- and P-selectins and the  $\beta_1$ -integrins (such as  $\alpha_4\beta_1$ ) (33, 43), which are expressed on neutrophils as well (31) and could perform an analogous function. It remains to be determined, however, which of these receptors might participate in the localization patterns we have described.

Although the role of marrow in the selective release of neutrophils has been extensively investigated (23, 28), its potential as a site for the sequestration of circulating neutrophils has not been explored. The marrow has been demonstrated to be an important site of circulating B cell and erythrocyte retention [the former for eventual recirculation (38), the latter for permanent removal (21)], yet previous models of neutrophil kinetics have emphasized unidirectional transit of neutrophils from the marrow (6). As noted, we found that the majority of both infused marrow-derived and peripheral neutrophils return to and are retained by the bone marrow, sequestering (at least in the case of marrow-derived neutrophils) predominantly within the marrow cords (Fig. 7) and perhaps recapitulating their association with the developing myelomonocytic lineages. Although long-term retention in the marrow space (up to 20 h) might suggest permanent removal from the circulation, the role of such retention in the marrow stroma and perhaps the vasculature cannot be inferred from these methods. We therefore examined the effects of epinephrine administration and the induction of systemic inflammation on previously infused, marrow-retained neutrophils *in vivo*.

Epinephrine treatment 4 h after neutrophil infusion yielded a minute but statistically significant increase in circulating blood levels of labeled neutrophils, but neutrophil localization did not change significantly in the marrow and other sites. These results suggest that epinephrine treatment causes a small population of the previously infused neutrophils to reenter the circulation, possibly by demargination of the few labeled cells remaining in the lung vasculature at 4 h. It would appear then that the vast majority of infused morpho-

logically mature marrow neutrophils are not subject to mobilization by epinephrine. In contrast, when similarly infused and sequestered marrow neutrophils were confronted by a distant inflammatory stimulus in the peritoneum, they not only left the marrow but were recruited to the inflammatory site and the lung. Such lung sequestration is characteristic of the marrow-released neutrophil population described by Kubo et al. (26) and van Eeden et al. (55) and has been implicated in the development of acute lung injury during inflammatory states (45). In contrast to the majority of infused marrow neutrophils, peripheral neutrophils retained in the marrow after circulation were not mobilized by either epinephrine or peritonitis, suggesting that their retention in the marrow might be irreversible and perhaps reflect permanent clearance of the cells.

Taken together, these findings suggest a central role for murine bone marrow not only in the release of circulating neutrophils but in their subsequent retention as well. Intrinsic qualities of the neutrophil appear to define discrete populations that differ in their subsequent site of removal from circulation. Characteristics strongly associated with marrow retention appear to reflect a degree of immaturity and lack of activation. The role of marrow retention in neutrophil regulation appears complex, with both storage (particularly of functionally immature cells) and perhaps permanent removal roles being evidenced. Finally, it is of particular interest to note that the functionally immature marrow neutrophil population described here appears similar to the rapidly marrow-released neutrophils recently implicated in the development of acute respiratory distress syndrome, suggesting the marrow as an important regulator of these potentially injurious cells.

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