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# Changes in Activation States of Murine Polymorphonuclear Leukocytes (PMN) during Inflammation: a Comparison of Bone Marrow and Peritoneal Exudate PMN

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**To study different activation states in polymorphonuclear leukocytes (PMN) in mice, we compared the function of murine PMN obtained from the bone marrow (BMPMN) with those of PMN obtained by intraperitoneal induction with thioglycolate (TGPMN) or uric acid (UAPMN). When stimulated with chemotactic peptides, e.g., formyl-methionyl-leucyl-phenylalanine (fMLF), WKYMVM, or WKYMVm, the TGPMN and UAPMN showed greatly enhanced generation of reactive oxygen species (ROS) compared with BMPMN, which suggests that exudation to the peritoneum per se induces a primed state in the cells. The WKYMVm peptide was the most potent stimulant of ROS generation, and it desensitized for subsequent stimulation with fMLF or WKYMVM. This desensitization was broken by the addition of cytochalasin B. The TGPMN and UAPMN appeared to be fully primed, since no increase in response was induced by pretreatment with tumor necrosis factor α (TNF-α). In contrast, the BMPMN response was increased 2.5- to 3-fold. The differences in oxidative responses were supported by degranulation studies. Preincubation with TNF-α promoted CR3 expression on BMPMN, and this level of expression was also enhanced by WKYMVm. In contrast, CR3 expression on untreated TGPMN and UAPMN was already similar to that on TNF-α-primed BMPMN and could be only slightly enhanced by TNF-α treatment. Taken together, these results indicate that BMPMN are in a resting state and have the capacity to become primed, while peritoneal exudate PMN are already fully primed upon isolation. These results have major implications for murine neutrophil research and show the importance of defining which PMN subsets to use when investigating murine models.**

Biomedical research today is largely dependent on animal models for elucidating mechanisms of and treatment strategies for human diseases, not least due to the possibility of creating genetically modified animals. However, there is increasing awareness that animal models have certain limitations in terms of mirroring human physiology and pathology.

Studies of polymorphonuclear leukocytes (PMN) have been performed using mice as models for human diseases, such as autoimmune glomerular basement membrane disease (12, 32), bullous pemphigoid (30), chronic granulomatous disease (26), and myeloperoxidase deficiency (1), as well as for biochemical characterization of neutrophil functions (15). However, the number of PMN that can be obtained from murine blood is very limited. Consequently, many researchers use bone marrow or peritoneal exudate preparations to study PMN in mice (18, 22, 31, 37). In humans, the corresponding PMN populations are distinctively different in terms of activation status, since bone marrow PMN are in part immature (11) and circulating cells are primarily resting cells, while exudated PMN have been exposed to several activating factors, such as proinflammatory cytokines, during extravasation and thus exist in a primed state (17, 24). These different basal states are highly relevant to the level of activation that can be achieved by treating these cells with various stimulants. Therefore, the basal activation state of PMN isolated from mice is of the utmost importance when designing and evaluating experiments. Unfortunately, the activation states of different types of murine PMN have not been compared fully.

Some important differences between human and murine neutrophils have been established. Recently, we have demonstrated that the NADPH-oxidase activation of murine peripheral blood neutrophils differs from that of human neutrophils in that agonist-induced activation of murine neutrophils results exclusively in extracellular release of reactive oxygen species (ROS), and activators of intracellular ROS production in human neutrophils do not activate the murine oxidase (9). Furthermore, the most commonly used agonist for human neutrophil NADPH-oxidase activation, the formylated tripeptide formyl-methionyl-leucyl-phenylalanine (fMLF), is a much less potent activator of the murine receptor analogue, although priming of cells to this agonist can be easily accomplished with either bacterial lipopolysaccharide or cytochalasin B (9). It has also been shown that although morphologically mature neutrophils can be identified in human bone marrow, they are in a functionally immature state (11), whereas murine bone marrow contains a large reservoir of functionally competent neutrophils (5).

In this study, we investigated the activation in response to several stimuli of murine NADPH-oxidase activity, complement receptor 3 surface expression, and the chemotaxis of

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TABLE 1. Total cell numbers and PMN purities of cell preparations obtained from peritoneal cavity and bone marrow

| Parameter | Value for indicated cell type[a] | | | | | |
|---|---|---|---|---|---|---|
| | Peritoneal exudate | | | | Bone marrow | |
| | TGPMN | | UAPMN | | Nonisolated | Percoll isolated |
| | 4 h | 18 h | 4 h | 24 h | | |
| Total cell no. ($10^7$ cells/mouse) | 1.16 ± 0.38 | 2.33 ± 0.62 | 0.91 ± 0.20 | 2.15 ± 0.57 | 4.65 ± 0.65 | 0.97 ± 0.31 |
| PMN purity (%) | 88.0 ± 3.1 | 64.7 ± 3.7 | 94.8 ± 1.9 | 78.4 ± 5.3 | 50.5 ± 4.3 | 87.0 ± 3.2 |

[a] Means ± SD (*n*, 6 to 11).

PMN obtained from bone marrow and peritoneal exudates after thioglycolate or urea injection. We show that, similar to human neutrophils, murine PMN can exist in more than one preactivation state. For studies of NADPH-oxidase regulation and degranulation, bone marrow cells appear to be the most suitable, while uric acid administration provides PMN of higher purity than those induced with thioglycolate, and these cells are in a primed and highly responsive state.

## MATERIALS AND METHODS

**Materials.** Percoll and density marker beads were obtained from Amersham Pharmacia (Uppsala, Sweden). May-Grünwald and Giemsa solutions were from HISTOLAB (Göteborg, Sweden). Thioglycolate medium was from Becton Dickinson (Sparks, MD). The hexapeptides Trp-Lys-Tyr-Met-Val-D-Met-$NH_2$ (WKYMVm) and Trp-Lys-Tyr-Met-Val-L-Met-$NH_2$ (WKYMVM) were synthesized and high-pressure liquid chromatography-purified by Alta Bioscience (University of Birmingham, United Kingdom). The peptides were dissolved in dimethyl sulfoxide to 10 mM and stored at −70°C prior to use. Subsequent dilutions were made in Krebs-Ringer phosphate buffer (KRG; pH 7.3), containing glucose (10 mM), $Ca^{2+}$ (1 mM), and $Mg^{2+}$ (1.5 mM). Nuclease-resistant phosphorothioate oligodeoxynucleotides (S-ODNs) were synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden). The nucleotide sequences of ODN 0499 and ODN 0399 are 5′-TCCATGACGTTCCTGATGCT-3′ and 5′-TCCATGAGCTTCCTGATGCT-3′, respectively. These sequences were selected because they are potent proinflammatory and arthritogenic ODNs in mice (4). Cytochalasin B, formylmethionyl-leucyl-phenylalanine (fMLF), luminol, phorbol myristate acetate (PMA), and uric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant murine tumor necrosis factor alpha (TNF-α) was from R&D Systems Europe Ltd. (Abingdon, Oxon, United Kingdom). Horseradish peroxidase (HRP) was from Boehringer Mannheim (Mannheim, Germany). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11b (complement receptor 3) antibody (clone M1/70) and isotype-matched control antibody (clone A95-1) were from BD Pharmingen (San Diego, CA). All other chemicals were commercial products of reagent grade.

**Animals.** Female C57BL/6 mice were purchased from B&K Universal AB (Stockholm, Sweden) and were housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg (Sweden), under standard conditions. Mice of between 6 and 10 weeks of age were used in all of the experiments. The studies were approved by the Ethical Committee for Animal Experimentation, Göteborg, Sweden.

**Preparation of peritoneal exudate PMN.** Inflammation was induced by injecting 1 ml of 3% thioglycolate broth (BD) or 0.1 ml of uric acid solution into the peritoneal cavity of the mouse. The uric acid solution (noncrystalline form) was prepared by mixing with saline (10% [wt/vol]) and sonicated for 10 min. Immediately before inoculation, the milky white precipitated uric acid solution was shaken vigorously. Peritoneal exudate cells were harvested 4, 18, or 24 h after injection by two lavages of the peritoneal cavity with 5 ml of cold phosphate-buffered saline (PBS; total volume, 10 ml). Thioglycolate- and uric acid-induced peritoneal cells (TGPMN and UAPMN, respectively) were then washed by centrifugation at 200 × *g* for 10 min at 4°C, and hypotonic lysis was performed to eliminate red blood cells, regardless the presence of a red pellet. After centrifugation and an additional wash, the cells were resuspended in KRG. The numbers and populations of peritoneal exudate cells were determined by staining the nuclei with Türk reagent and by cytospin centrifugation followed by May-Grünwald-Giemsa staining, respectively. TG injection into the peritoneal cavity gave a yield of 1 to 2 × $10^7$ cells/mouse depending on time of incubation (4 or 18 h) (Table 1). The purity of the thioglycolate-induced PMN was 88% 4 h postinjection and 65% after 18 h (Table 1). For uric acid-induced extravasation to the peritoneum, similar yields were achieved. The levels of purity of the uric acid-induced PMN were 95% and 78%, after 4 h and 24 h, respectively. The remaining cell population was composed of mononuclear cells. Due to their higher levels of purity, peritoneal exudate cells (TGPMN and UAPMN) collected 4 h postinjection were used in the following experiments. The levels of viability of both PMN populations were confirmed as being >95% by trypan blue exclusion.

**Preparation of bone marrow PMN.** Mice were killed by cervical dislocation, the femurs, tibias, and ilia from both hind limbs were removed and freed of soft tissue attachments, and the extreme distal tip of each extremity was cut off. PBS was forced through the bone by using a 1-ml syringe with a 27-gauge needle. After dispersing cell clumps and removing the debris, the bone marrow cells (BMC) were centrifuged at 200 × *g* for 10 min at 4°C, and the pellet was resuspended in PBS or KRG without $Ca^{2+}$ and $Mg^{2+}$ [KRG(−)]. Bone marrow PMN (BMPMN) were isolated according to the procedure described in reference 37 with some modifications. Briefly, BMC suspended in 2 ml of KRG(−) were laid on top of a three-layer Percoll gradient prepared in 15-ml polystyrene tubing by layering 2 ml each of 1.095, 1.085, and 1.070 g/ml Percoll solutions. The density of each Percoll solution was verified using density marker beads. After centrifugation at 500 × *g* for 30 min at 4°C in a swinging bucket rotor, the lowest band (1.085/1.095 g/ml interface) and the upper part of the 1.095 g/ml layer were collected as the PMN fraction. After washing with KRG(−), remaining red blood cells were eliminated by hypotonic lysis. After a final wash with KRG(−), the BMPMN were resuspended in KRG. The cell number and population of BMC or BMPMN were determined as described for the isolation of peritoneal exudate PMN. Before isolation, the BMC (total cell number of 4.7 ± 0.7 × $10^7$ cells/mouse) contained around 50% PMN. Percoll density gradient fractionation raised the fraction of morphologically mature (bands and segmented) PMN from 50% to 87%, and we obtained 1 × $10^7$ BMPMN per mouse (Table 1). The level of BMPMN viability was >95%, as determined by vital staining.

**Preparation of PMN adhesion slides.** For photomicroscopy, noncoated three-well glass slides (Novakemi AB, Enskede, Sweden) were used. Cell suspension (100 μl containing 1 × $10^5$ PMN) was placed in each well, and slides were incubated for 30 min at 37°C in a humidified atmosphere. After incubation, the cells were fixed 4% paraformaldehyde for 30 min at room temperature. The slides were then washed with PBS and water and stained with May-Grünwald-Giemsa stain.

**Measurement of NADPH-oxidase activity.** NADPH-oxidase activity was determined using luminol and HRP-enhanced chemiluminescence (CL) systems that allow for the determination of total (extracellularly released and intracellularly generated) ROS production (14). The CL activity was measured in a six-channel Biolumat LB 9505 apparatus (Berthold Co., Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 400-μl or 450-μl reaction mixture that contained 5 × $10^5$ PMN. The tubes used for measuring total ROS generation contained 2 U HRP (a cell-impermeable peroxidase) and 10 μg/ml luminol (a cell-permeable CL substrate). The tubes were equilibrated in the Biolumat for 10 min at 37°C, after which time the stimulus (50 μl) was added. The light emission was recorded continuously.

When experiments were performed with the combination of a first stimulation (WKYMVm) and a second stimulation (WKYMVm, WKYMVM, fMLF, or cytochalasin B), the interval between the first and second stimulation was 5 min. The PMN were primed with 100 ng/ml (final concentration) of TNF-α or KRG (control) for an equilibration period of 30 min at 37°C, after which period the cells were stimulated and ROS production was determined as described above.

**Flow cytometric analysis.** The degree of degranulation was determined as the level of surface expression of CR3 on PMN before and after stimulation. PMN

($10^6$ cells in 1 ml KRG) were incubated in a microtube for 10 min at 37°C, and TNF-α (final concentration of 100 ng/ml) or WKYMVm ($10^{-7}$ M) was added. The tubes were reincubated for 30 min (TNF-α) or 10 min (WKYMVm) at 37°C. After centrifugation, the supernatants were removed and the cells were fixed in 4% paraformaldehyde for 5 min on ice. The tubes were washed once with fluorescence-associated cell sorter (FACS) buffer (0.1 mM EDTA, 0.02% $NaN_3$ in PBS) and the cells were then incubated with FITC-conjugated rat anti-mouse CD11b. Rat immunoglobulin G2b antibody was used as the isotype-matched, FITC-labeled control. After incubation for 1 h on ice, the cells were washed once in FACS buffer and resuspended in 500 μl FACS buffer before FACS analysis using FACScan (Becton Dickinson, San Jose, CA).

**Chemotaxis assay.** PMN migration was determined using 96-well microplate disposable chemotaxis/cell migration chambers with hydrophobic filters of pore size 3 μm (ChemoTx; Neuro Probe Inc., Gaithersburg, MD). The chemoattractants fMLF and WKYMVm were diluted in KRG that was supplemented with 0.3% (wt/vol) bovine serum albumin, and the solutions were loaded at various concentrations in the lower chambers. Next, PMN suspensions (35 μl of $10^6$ cells/ml) in KRG-bovine serum albumin were placed on top of the filters and the assay system was incubated for 120 min at 37°C. The plate was centrifuged at 350 × *g* for 10 min at 4°C, after which the filter was removed. The numbers of cells that had transmigrated to the lower compartments were determined by counting in representative microscopic fields. All of the conditions were tested in triplicate.

**Statistical analyses.** The data are expressed as means ± standard deviations (SD). Statistical significance was determined by two-tailed unpaired Student's *t* tests. *P* values of <0.05 were considered statistically significant.

## RESULTS

**PMN adhesion to glass.** We first studied the basal activation state of the different subsets of neutrophils (BMPMN, TGPMN, and UAPMN) by simply allowing them to adhere to glass. After a 30-min incubation at 37°C, the majority of the BMPMN had not spread completely across the glass surface and only a few were extending pseudopods (Fig. 1A). These BMPMN were able to spread fully after PMA stimulation (Fig. 1B), which indicates that although they are capable of spreading, they exist in such an inert (resting) state that they need additional stimulating signals to adhere properly. In contrast, large proportions of the TGPMN and UAPMN populations spread sufficiently on the glass surface and extended vast amounts of pseudopods, without additional stimulation (Fig. 1C and D). Thus, the TGPMN and UAPMN appear to be in a more primed/preactivated state than the BMPMN.

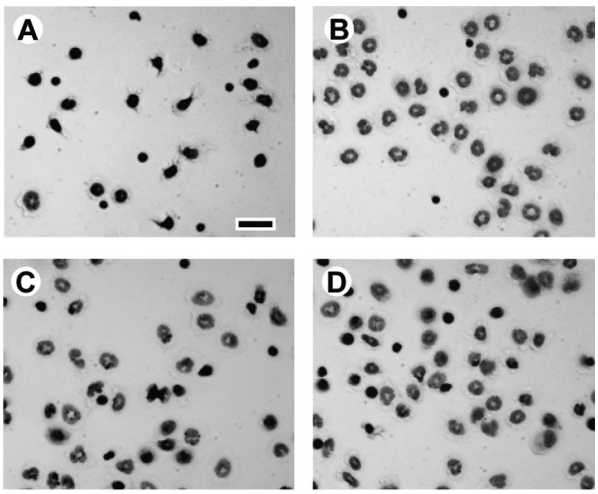


FIG. 1. Adherence of PMN to noncoated glass surface. After 30 min of incubation at 37°C, the cells were fixed with 4% paraformaldehyde and stained with a May-Grünwald-Giemsa stain. (A) Untreated bone marrow PMN. (B) Bone marrow PMN stimulated with PMA ($5 \times 10^{-7}$ M). (C) Untreated thioglycolate-induced peritoneal PMN. (D) Untreated uric acid-induced peritoneal PMN. Bar = 20 μm.

TABLE 2. Reactive oxygen species generation by different types of PMN stimulated with various stimulants[a]

| Stimulant (concn) | Peak value of chemiluminescence ($10^5$ CPM) from: | | |
|---|---|---|---|
| | TGPMN | UAPMN | BMPMN |
| PMA ($5 \times 10^{-7}$ M) | 2,262 ± 715a | 2,237 ± 852a | 1,748 ± 789a |
| WKYMVm ($10^{-7}$ M) | 4,308 ± 554a | 4,092 ± 698a | 762 ± 199b** |
| WKYMVM ($10^{-6}$ M) | 3,799 ± 900a | 3,810 ± 547a | 626 ± 217b** |
| fMLF ($10^{-5}$ M) | 3,511 ± 497a | 3,719 ± 309a | 493 ± 139b** |
| ODN 0499 (10 μM) | 78 ± 32a | 90 ± 27a | 63 ± 18a |
| ODN 0399 (10 μM) | 95 ± 52a | 87 ± 31a | 78 ± 25a |
| Cytochalasin B (5 μg/ml) | 134 ± 22a | 122 ± 33a | 167 ± 15b* |

[a] The data are shown as the means ± SD (*n*, 5 to 7 mice) of peak values of chemiluminescence within 10 min from which the background (nonstimulated PMN responses) has been subtracted. Values in rows having different letters are significantly different (*, $P < 0.05$; **, $P < 0.01$); values with the same letters do not differ significantly.

**PMN NADPH-oxidase activation induced by various stimulants.** The ability of human neutrophil to respond to various stimuli by activation of its superoxide-producing enzyme system, the NADPH-oxidase, depends largely on the activation state of the cell. In order to clarify whether this applies also to murine neutrophils, we investigated the abilities of the different types of murine PMN to respond to well-known stimuli of NADPH-oxidase activation, by using the same molecular mechanisms.

Before the addition of any stimulant, the background levels of chemiluminescence, which corresponded to spontaneous ROS generation by the nonstimulated PMN in response to a temperature increase from 4°C to 37°C, were recorded. The background ROS levels were significantly different between peritoneal exudate PMN and BMPMN (TGPMN versus BMPMN, $P < 0.01$; UAPMN versus BMPMN, $P < 0.01$). Briefly, both TGPMN and UAPMN had values at least fourfold higher than those of BMPMN (mean ± SD [$n = 7$] for TGPMN, 22.0 ± 3.8 Mcpm; UAPMN, 18.5 ± 2.7 Mcpm; BMPMN, 4.5 ± 1.3 Mcpm).

The peak values of chemiluminescence generated from PMN that were directly stimulated with various agonists, and from which the background level was subtracted, are shown in Table 2, and the kinetics of the responses are shown in Fig. 2. The protein kinase C agonist PMA activated all three cell types. In TGPMN and UAPMN, the peak values were observed 1 to 2 min after stimulation with PMA, while the PMA-stimulated BMPMN showed a delay of 1 min (Fig. 2A). This is probably due to differences in the kinetics of PMA diffusion across cell membranes, whereby the activating threshold for PMA is breached somewhat later in BMPMN than in TGPMN and UAPMN.

We and others have shown previously that murine neutrophils respond to chemotactic factors that activate members of the G protein-coupled, seven-transmembrane-spanning receptor family of formyl peptide receptors (FPRs) (9, 22, 23, 29). In this study, the synthetic hexapeptide WKYMVm induced rapid responses in all three types of PMN, with peak times within one minute (Fig. 2B). WKYMVm-stimulated BMPMN gave

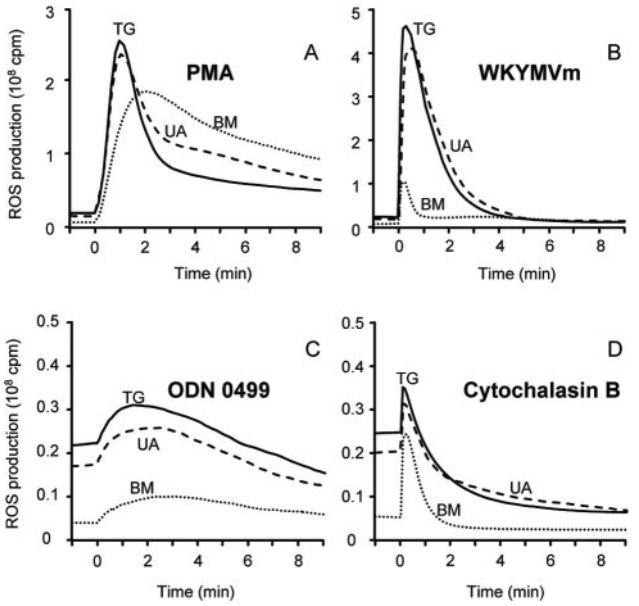


FIG. 2. (A to D) PMN NADPH-oxidase activation induced by the addition of PMA, chemoattractants, S-ODN, or cytochalasin B. PMN ($5 \times 10^5$/0.5 ml) were preincubated for 10 min at 37°C and then stimulated with PMA ($5 \times 10^{-7}$ M), WKYMVm ($10^{-7}$ M), S-ODN 0499 (10 μM), or cytochalasin B (5 μg/ml) at time zero. ROS production was measured and the time course of the response determined. Broken, dotted, and solid lines indicate the kinetics of the chemiluminescence responses obtained from UAPMN, BMPMN, and TGPMN, respectively. Data of representative experiments are shown ($n = 5$).

significantly lower responses than peritoneal exudate PMN ($P < 0.01$), which indicates that the peritoneal cells were primed compared to the BMPMN. Similar responses were seen for WKYMVM- and fMLF-induced PMN, although 10- and 100-fold higher concentrations of WKYMVM and fMLF, respectively, were required to generate a response that was equivalent to the level induced by $10^7$ M WKYMVm (Table 2). These differences in concentration dependencies are in agreement with those seen in previously published studies (9, 23).

Activation of the PMN with oligodeoxynucleotides that are immunostimulatory for mice resulted in oxidase activation for all three types of PMN (Table 2). Both S-ODN 0499, which contains the CpG motif, and the corresponding GpC-containing S-ODN 0399 activated ROS generation in all PMN types, and the responses were gradual and of similar potencies (Fig. 2C; Table 2). There was no difference between the CpG- and GpC-induced responses, in line with previously published data (10).

In human PMN, the cytoskeleton-disrupting agent cytochalasin B does not induce an oxidative burst, but it can increase the response induced by other stimuli (8). However, in murine BMPMN, cytochalasin B induced obvious ROS generation in BMPMN, while TGPMN and UAPMN gave a subsequent lower peak response (Fig. 2D; Table 2).

There was no noticeable response of the PMNs to TNF-α (100 ng/ml), although a negligible increment in the background level was observed (data not shown).

**Desensitization of PMN NADPH-oxidase activation by WKYMVm.** A well-known phenomenon in human PMN activation through G protein-coupled chemotactic receptors is the receptor desensitization effect. The desensitization of chemotactic receptors occurs after stimulation with receptor-specific agonists and prohibits subsequent activation of the same receptor with the same (homologous desensitization) or a different (heterologous desensitization) agonist. We found that the chemotactic receptors of murine PMN were also desensitizable. Once the PMN NADPH-oxidase was activated by WKYMVm, a secondary stimulation with the same concentration of WKYMVm gave no activity. This desensitization of NADPH-oxidase activation was observed in all three types of PMN (Fig. 3A to C). Furthermore, when the secondary dose of WKYMVm was replaced by WKYMVM or fMLF, the cells were heterologously desensitized (Fig. 3D and E). The same pattern of cross-desensitization was evident for all types of PMN (data not shown).

**Reactivation of WKYMVm-desensitized PMN NADPH-oxidase activity by cytochalasin B.** The desensitization of the human FPR family of chemotactic receptors can be broken by cytochalasin B, which inhibits actin polymerization and thereby breaks the cytoskeletal coupling of G protein-coupled receptors (7). In the case of murine PMN, desensitization of the NADPH-oxidase activity induced by WKYMVm was also broken by cytochalasin B, and this was true for all three types of PMN (Fig. 4A to C).

**Priming effect of TNF-α on the cytochalasin B- or WKYMVm-triggered PMN NADPH-oxidase response.** TNF-α is a potent cytokine that has been shown to prime human PMN by receptor upregulation, which leads to the enhancement of NADPH-oxidase activation by a subsequent stimulant (16). BMPMN revealed a marked increase in ROS generation by preincubation with TNF-α followed by cytochalasin B- or WKYMVm-induced stimulation (Fig. 5). In contrast, there was no priming effect or a negligible priming effect of TNF-α on TGPMN, while the UAPMN showed a slight increase in response to TNF-α priming. Thus, TGPMN and UAPMN have partly or entirely lost the capacity to become primed by TNF-α during extravasation into the peritoneum.

**CR3 expression and upregulation on the PMN surface.** Since TNF has been shown to induce priming by degranulation and receptor upregulation in human PMN, we wanted to investigate the state of degranulation (receptor exposure) in the three different types of murine PMN. The marker used was CR3 (CD11b/CD18), which has previously been shown to be upregulated on the surface of activated murine PMN (31, 37). Flow cytometric analysis demonstrated that more than 85% of all three types of PMN were immunoreactive to anti-CD11b antibody (data not shown). The basal level of CD11b surface expression on untreated BMPMN was lower than that on TGPMN or UAPMN (Fig. 6A to C). Treatment of the BMPMN with TNF-α markedly increased CR3 expression, as did treatment of the cells with WKYMVm (Fig. 6A). In contrast, CR3 expression on untreated TGPMN and UAPMN was already at a level similar to that of TNF-α-primed BMPMN, and the increase in expression induced by TNF-α treatment was much lower in TGPMN and UAPMN than that in BMPMN (Fig. 6A to C). Taken together, these results indicate that the TGPMN and UAPMN priming that occurs during extravasation is accompanied by receptor upregulation most probably due to degranulation, which renders these cells inca-

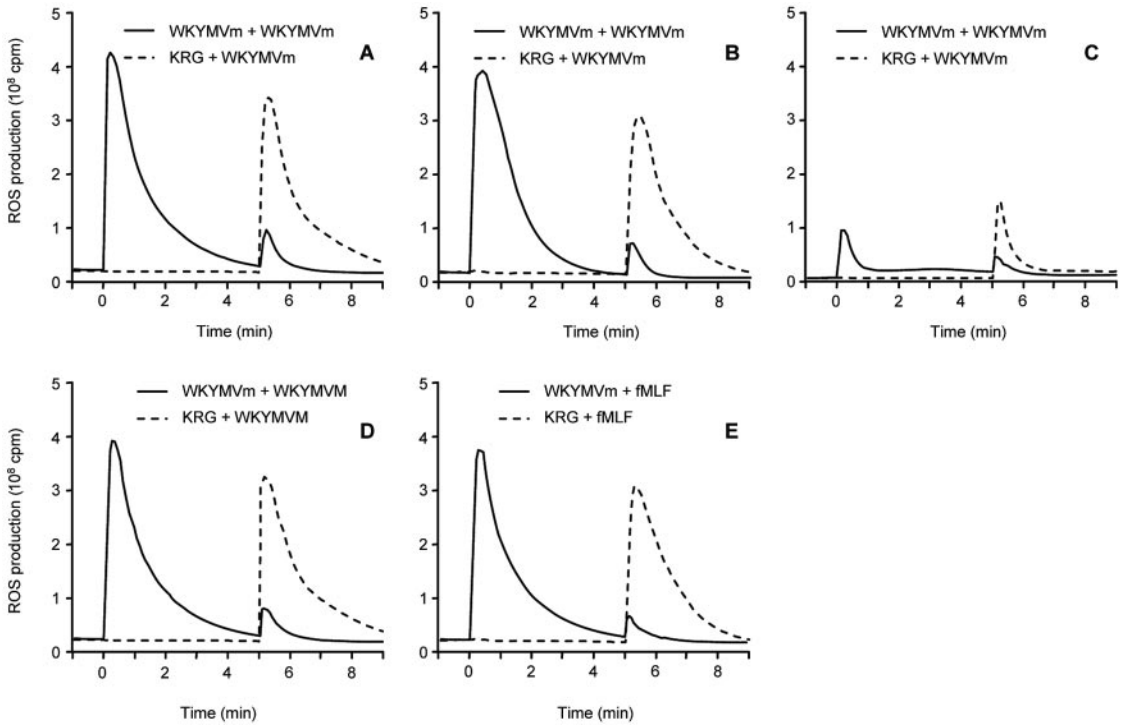


FIG. 3. Desensitization of murine PMN ROS production induced by WKYMVm. Thioglycolate-induced peritoneal PMN (A), uric acid-induced peritoneal PMN (B), and bone marrow PMN (C) were stimulated with WKYMVm ($10^{-7}$ M) at time zero and 5 min later were challenged with WKYMVm ($10^{-7}$ M). Thioglycolate-induced peritoneal PMN were stimulated with WKYMVm ($10^{-7}$ M) at time zero and were challenged with $10^{-6}$ M of WKYMVM (D) or $10^{-5}$ M of fMLF (E) 5 min later. Broken lines indicate control responses without primary stimulation (addition of KRG buffer). ROS production was determined over 10 min, and the curves shown are from a representative experiment ($n = 3$).

pable of responding further to priming agents (e.g., TNF-α). BMPMN, on the other hand, are nonprimed and can be primed by agents such as TNF-α to reach the same priming state as the peritoneal PMNs.

**Chemotactic activity.** Granulocyte chemotaxis depends on the degranulation process and receptor upregulation. Therefore, we investigated the capacities of the three murine PMN types to perform chemotaxis in a chamber system (see Materials and Methods). Although the well-known chemoattractant fMLF is a potent agonist for human neutrophil chemotaxis, it has less potent stimulatory effects on murine PMN (35). Therefore, we used the hexapeptide WKYMVm as the chemoattractant, in addition to fMLF. Both fMLF and WKYMVm induced BMPMN migration with a clearly bell-shaped dose-response curve (Fig. 7A). The maximal response was observed at $10^{-4}$ M and $10^{-8}$ M for fMLF and WKYMVm, respectively, which confirms that WKYMVm possesses a much higher affinity for murine chemotactic receptor(s) than fMLF. To distinguish

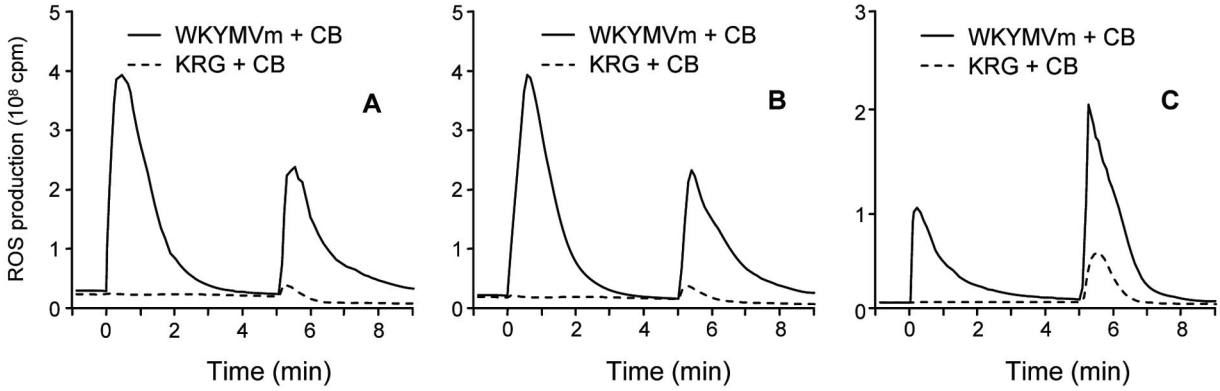


FIG. 4. Reactivation of WKYMVm-desensitized murine PMN ROS production by cytochalasin B. Thioglycolate-induced peritoneal PMN (A), uric acid-induced peritoneal PMN (B), and bone marrow PMN (C) were stimulated with WKYMVm ($10^{-7}$ M) at time zero and were challenged with cytochalasin B (5 μg/ml) 5 min later. Broken lines indicate control responses without primary stimulation (addition of KRG buffer). ROS production was determined over a period of 10 min, and the curves shown are from a representative experiment ($n = 3$).

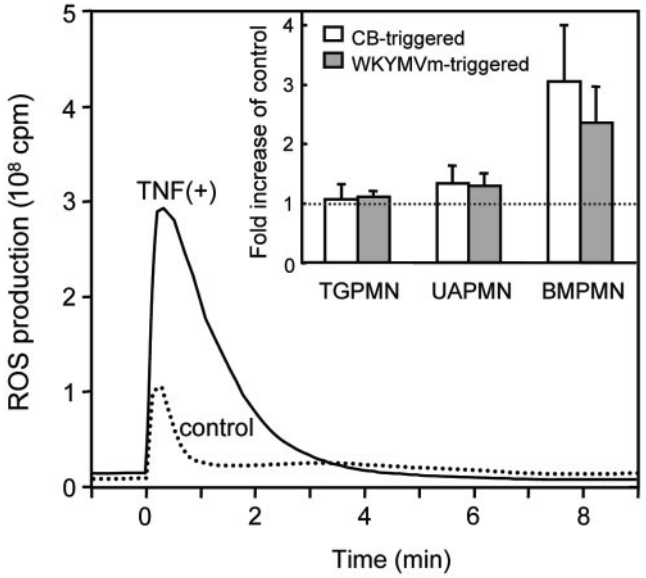


FIG. 5. Priming effect on the WKYMVm- or cytochalasin B-triggered PMN NADPH-oxidase response by TNF-α. BMPMN, TGPMN, and UAPMN were primed with mouse recombinant TNF-α (100 ng/ml) for 30 min at 37°C. The cells were then activated with WKYMVm ($10^{-7}$ M) or cytochalasin B (5 μg/ml) and ROS production was determined in parallel with control cells that were treated in the same way but in the absence of TNF-α. The figure shows the kinetics of representative experiments with WKYMVm-triggered BMPMN, and the inset shows the *n*-fold increase of peak values (mean ± SD; $n = 7$) between the responses of primed and nonprimed cells.

chemotaxis from chemokinesis, equal concentrations of fMLF ($10^{-4}$ M) or WKYMVm ($10^{-8}$ M) were added simultaneously to the upper and lower compartments of the chemotaxis apparatus. In this situation, no PMN migration was observed (data not shown). When the chemotactic activities of BMPMN to fMLF and WKYMVm were compared to those of TGPMN or UAPMN, we found that BMPMN had a much greater capacity for chemotaxis than the peritoneal cells (Fig. 7B). This can be explained by the smaller amount of upregulatable chemoattractant receptors present in the primed peritoneal cells, as they already have degranulated many of their granules, which is reflected as a reduced capacity to further upregulate CR3.

## DISCUSSION

When experimental results obtained from wild-type or transgenic mouse PMN are extrapolated to human neutrophil function, one needs to consider not only those differences that arise from species specificities but also differences that are due to the intraspecies source of the PMN. Understanding the background of the differences between human and murine PMN, as well as between bone marrow and peritoneal PMN responses, will greatly improve the accuracy with which murine models can be applied in medical research.

When choosing PMN isolation models to study, we first attempted to use Percoll fractionation of peripheral (tail vein) blood from mice, as previously described (9). However, it is very difficult to generate sufficient amounts of PMN with high purities from peripheral blood. Boxio et al. have reported that murine bone marrow-derived neutrophils possess functionally similar activities to peripheral blood neutrophils (5), and we have subsequently used bone marrow PMN as our "noninflammatory PMN" model. There are several described techniques to isolate murine bone marrow PMN by using Percoll density gradient fractionation (5, 31, 37), which gives different purities and PMN yields. In this study, we used a three-layer Percoll gradient, as described in Materials and Methods, to obtain BMPMN in high numbers and with high purities.

Thioglycolate-induced intraperitoneal elicitation of neutrophils has been used for a long time to isolate both neutrophils (early extravasation, 4 to 8 h) and mononuclear leukocytes (late extravasation, 24 to 72 h) (22, 25, 28). The purity and number of neutrophils obtained with this irritant after 4 h were reasonable, and we used these cells as a model of "inflammatory PMN." In addition, we employed a newer model that involves the used of uric acid as the irritant. Getting et al. have reported that intraperitoneal injection of monosodium urate crystals causes remarkable peritoneal accumulation of murine PMN at 6 h postinjection and that cellular infiltration into the cavity was sustained for up to 24 h (19). In the present study, we obtained significant numbers of high-purity PMN (95%) 4 h

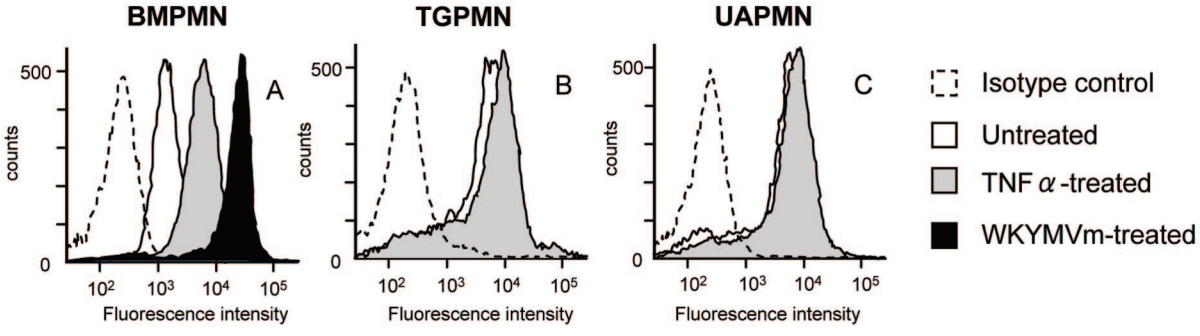


FIG. 6. CR3 upregulation induced by TNF-α or WKYMVm on bone marrow PMN, thioglycolate-induced PMN, and uric acid-induced peritoneal PMN. Untreated PMN (kept on ice) and cells that were stimulated with mouse recombinant TNF-α (100 ng/ml) for 30 min or WKYMVm ($10^{-7}$ M) for 10 min, respectively, at 37°C were fixed with paraformaldehyde, incubated with FITC-conjugated antibodies directed against CR3, and analyzed by flow cytometry. The broken line profiles were obtained with the isotype-matched control antibody. The results are representative of three independent experiments.

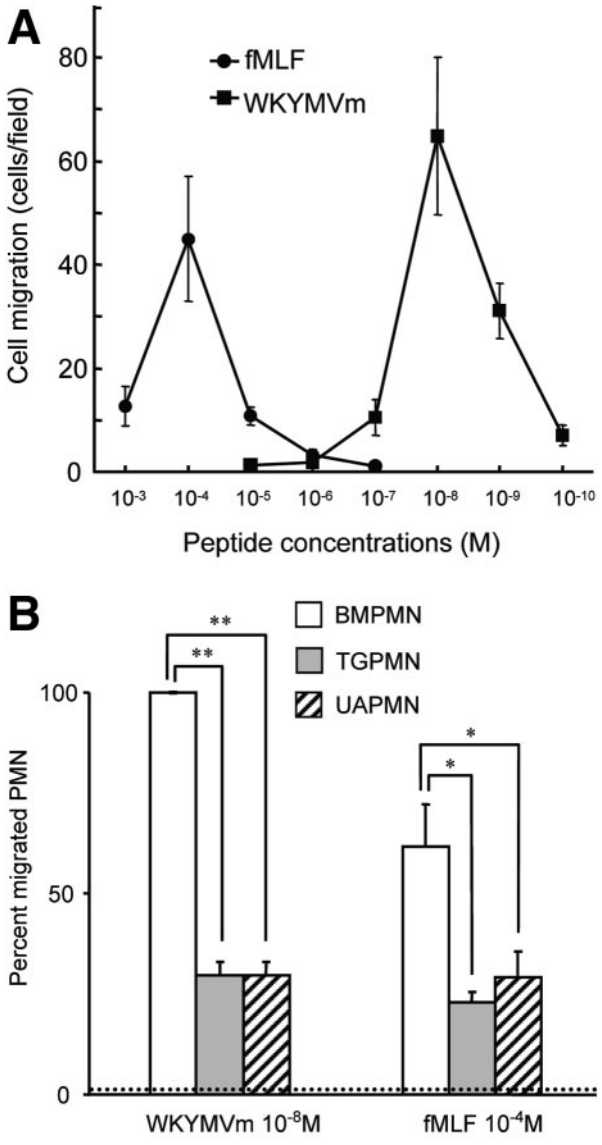


FIG. 7. Chemotaxis of PMN in response to WKYMVm and fMLF. Approximately 35,000 cells were loaded into the top well of a 96-well microchemotaxis chamber. Various concentrations of WKYMVm and fMLF were placed in the bottom wells. The chemotaxis assay was conducted at 37°C for 2 h. Migrated cells were counted microscopically. (A) Chemotaxis of bone marrow PMN in response to different concentrations of WKYMVm (100 pM to 10 μM, closed squares) or fMLF (100 nM to 1 mM, closed circles). The results are shown as means ± standard errors of the means obtained from four or five independent experiments. (B) Chemotaxis of BMPMN, TGPMN, and UAPMN in response to WKYMVm ($10^{-8}$ M) and fMLF ($10^{-4}$ M). Spontaneous transmigration in the absence of chemoattractants was below the dotted line. Data are the means ± standard errors of the means expressed as percentages of the numbers of BMPMN that migrated in response to WKYMVm ($10^{-8}$ M), as obtained from four independent experiments. *, $P < 0.05$; **, $P < 0.01$.

after injection of an emulsion form of uric acid. The injection of uric acid caused a few visible white precipitates in the peritoneal cavity, but this did not affect the isolation and viability of the UAPMN (data not shown). The first indication that peritoneal exudate PMN differed functionally from BMPMN was their propensity to adhere to glass slides. The nonadhesion of BMPMN is not due to a functional incapacity, since the ability to adhere could be elicited with PMA preactivation. Since the level of cell surface adhesion molecule expression is altered in primed PMN (36), the enhancement of adhesive activity for peritoneal exudate PMN as well as PMA-treated BMPMN suggests that these PMN had shifted from a resting to a primed state. Neutrophil NADPH-oxidase activation is known to be regulated by a number of agonists and through various pathways, and much information has been accumulated concerning the priming event in human PMN (36). Furthermore, ROS generated by PMN acts itself as a priming agent, and several pathways have been suggested for this, such as membrane perturbation (27), modulation of kinase activity (6), protein nitration (33), and regulation of nuclear factor-κB (34). Besides, activated phagocytes can regulate the functions of other immunocytes via ROS release (3, 20, 21). We found significantly higher spontaneous ROS production in TGPMN and UAPMN than in BMPMN, which implies that these PMN exist in distinct preactivation states. Furthermore, oxidative responses to several stimulants differed significantly between the types of PMN. Peritoneal exudate PMN had remarkably high responsiveness to chemotactic peptides (fMLF, WKYMVM, and WKYMVm) compared with BMPMN. This can be compared to human exudate neutrophils from skin aseptic chambers, which show an enhanced respiratory burst in response to fMLF compared to peripheral blood PMN; this is probably due to the increased cell surface expression of fMLF receptors (17). We suggest that TGPMN and UAPMN have been exposed to priming agents, such as TNF-α and complement factors, during the inflammatory extravasation process.

It is known that the cytoskeleton-destabilizing agent cytochalasin B is not able to induce NADPH-oxidase activation in human PMN in the absence of an additional stimulant (7). However, we could detect a respiratory burst in murine BMPMN that were induced with cytochalasin B alone. This suggests that cytoskeletal stability or the cytoskeleton interaction with the NADPH-oxidase is different in humans and mice and that cytochalasin B-induced respiratory burst activation in murine BMPMN may occur through a somewhat different pathway. In addition, in the peritoneal exudate PMN, there seemed to be a cytochalasin B-induced response, although it was partially concealed by the high background activity.

In contrast to the FPR family agonists, the phosphorothioate ODNs (S-ODNs), regardless of their CpG content, induced similar responses from the three types of PMN. We have shown previously that the phosphorothioate backbone but not the CpG motif is important for neutrophil activation (10), although the underlying mechanism remains unclear. Our results showing that S-ODN-induced cell stimulation is independent of PMN priming in murine cells indicate that the receptor responsible for ODN activation is probably not affected by the factors that are responsible for priming of the cells.

A common feature of G protein-coupled seven-transmembrane-spanning chemotactic receptors is that they are homologously desensitizable (13), i.e., stimulation of a receptor with an agonist blocks restimulation of the receptor with the same agonist. However, some receptor agonists also desensitize the cells to stimulation with a different agonist (heterologous desensitization), which is most often due to binding (with higher

affinity) to the same receptor. This phenomenon may be used to identify receptors for new ligands. In lipopolysaccharide-primed murine neutrophils, the fMLF-induced response is desensitized by WKYMVM, i.e., these agonists appear to share at least one receptor (9). Here, we take these studies further and show that both resting (bone marrow) and primed (peritoneal) PMN can be desensitized and that complete desensitization to fMLF or WKYMVM stimulation is achieved when WKYMVm is used as the first stimulus. These results suggest that the three peptides share at least one receptor in murine neutrophils. This is in agreement with the results from human neutrophils, whereby cells that are first stimulated with WKYMVm respond to neither fMLF nor WKYMVm (13). It is difficult to perform a rigorous comparison of desensitization events between human and mouse PMN, since the FPR family receptors differ between the species and the affinities of the peptides for each receptor(s) differ markedly. However, our data confirm that the cross-desensitization phenomenon is conserved across species and that WKYMVm is a major ligand for FPR family chemoattactant receptors on murine PMN, just as it is for human PMN.

We have recently demonstrated that human neutrophils desensitized to WKYMVM can be reactivated by cytochalasin B, inducing a robust burst of oxidase activity in deactivated cells (8). In accordance with these results, desensitized murine PMN could be reactivated by cytochalasin B. This was shown for BMPMN, TGPMN, and UAPMN, which indicates that both primed and resting murine PMN possess the ability to become reactivated.

TNF-α has been shown to be a potent priming agent for human neutrophils (36). In murine cells, the enhancement of ROS generation triggered by cytochalasin B or WKYMVm was remarkable in BMPMN preincubated with TNF-α, while peritoneal exudate PMN showed no increased response. These results are reasonable if one considers that the peritoneal exudate PMN are already primed by extravasation and that these cells have reached the fully primed state, as they cannot not be primed further. Bylund and coworkers have suggested two mechanisms for neutrophil priming by TNF-α (8). While the enhanced response to fMLF is explained by the recruitment of new FPRs to the cell surface, the cytochalasin B-sensitive state is achieved through a novel mechanism that transfers G protein-coupled receptors to a primed state, which is fully activated when cytochalasin B uncouples the receptors from the cytoskeleton.

TNF-α priming of human peripheral blood neutrophils exposes higher levels of CR3, which suggests that integrin-storing organelles (secretory vesicles, gelatinase granules, and specific granules) are mobilized by this treatment (8). Through the same mechanism, TNF-α priming is accompanied by an increased number of FPRs on the cell surface (36). In mice, CR3 expression on bone marrow PMN has been shown to be upregulated by TNF-α (31), and casein-induced peritoneal exudate PMN show higher CR3 expression than bone marrow PMN (37). Our results are in agreement with previous studies, in that we show a basal level of CR3 on peritoneal cells that is much higher than on BMPMN and that further upregulation of CR3 expression on peritoneal exudate PMN cannot be induced by cell stimulation, which is in line with the priming results for NADPH-oxidase activation. However, the BMPMN responded with increased CR3 expression to TNF-α or WKYMVm treatment. These observations support the notion that the TNF-α-priming mechanism in murine PMN is similar to that in human neutrophils, i.e., that the primed oxidase response seen after TNF treatment in murine neutrophils is due to receptor upregulation from intracellular stores, which leads to increased exposure of FPR family receptors.

The chemotaxis of the peritoneal exudate PMN was inferior to that of BMPMN, probably because the peritoneal exudate PMN were primed while the BMPMN were not. Migration is dependent on sequential upregulation of chemotactic receptors, and since the peritoneal PMNs have little or no receptor pool remaining (seen as the inability to upregulate CR3) after completing the extravasation process, it is not surprising that they are incapable of responding chemotactically. This is in line with the results obtained for human neutrophils that were treated with high concentrations of recombinant TNF (100 to 10,000 U/ml), which showed inhibition of random movement and of chemotaxis by C5a or fMLF (2). Another potential explanation for the decreased chemotactic capacity is enhanced adhesiveness (38), and considering the increased adhesiveness of peritoneal exudate PMN seen in our study, this mechanism may influence BMPMN chemotaxis.

The PMN response is very finely modulated in order to provide appropriate responses to changes in the environment. One regulatory mechanism used by these cells is the process of priming, and in this study, we have clarified the difference between three murine PMN models with regard to this mechanism. We have clearly demonstrated that peritoneal exudate PMN exist in an activation state distinct from nonactivated BMPMN, whereby the peritoneal PMN are fully primed when isolated, while the BMPMN have a reserve priming capacity. Thus, it is of great importance to choose techniques for murine PMN isolation with great care. For example, BMPMN may be suitable for the analysis of cell physiology, priming, and activation, while peritoneal exudate PMN may be more appropriate for investigations into the pathophysiological functions of PMN at sites of inflammation. These experimental considerations would be expected to lead to a more precise understanding of PMN functions in vivo. Finally, our data were obtained from the C57BL/6 mouse, which is the background strain for a large number of genetically modified mice. Therefore, we hope that our data will contribute to the fundamental understanding of PMN function in gene knockout studies.

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