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Quantitative Dynamics of *in Vivo* Bone Marrow Neutrophil Production and Egress in Response to Injury and Infection

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Abstract—Production rates of blood cells from the bone marrow (BM) can be determined from pool size and residence time in the circulation only during steady state. We describe a method to evaluate changes in BM neutrophil production following severe injury. Male CD-1 mice underwent nonlethal cutaneous burn injury, a lethal burn injury with *Pseudomonas aeruginosa* infection, or sham treatment, and received bromodeoxyuridine (BrdU) to label proliferative cells. Rates of BM neutrophil production and release into the circulation were determined using a mathematical model that integrates BM neutrophil pool size and fraction of BrdU labeled cells as a function of time. Absolute rates could not be quantified without BrdU data for the neutrophil progenitor pool; however, relative rates could be determined. BM neutrophil production and release significantly increased after injury. After nonlethal burn, release transiently exceeded production, causing a temporary decrease in BM neutrophil stores followed by re-establishment of a steady-state BM neutrophil pool similar to sham controls. After lethal burn infection, release always exceeded production, causing complete depletion of BM neutrophils and suppression of BM neutrophil production. This method is generally applicable to estimating production rates of nonproliferating, terminally differentiated cells, arising from a stem cell pool *in vivo*.

Keywords—Neutrophil dysfunction, Cellular proliferation, Hematopoiesis, Bromodeoxyuridine.

INTRODUCTION

Neutrophils are produced during hematopoiesis in the bone marrow (BM), and primarily function in host defense through the phagocytosis and killing of pathogens in tissues. Immunosuppression is often observed in burn and trauma patients, and has partly been attributed to an impairment in bactericidal activity of circulating neutrophils,[1,36] and sometimes low blood neutrophil counts.[3,33] It has been hypothesized that these changes may reflect alterations in the process of hematopoiesis in the bone marrow (BM) leading to the release of immature neutrophils,[33] and in some cases, a reduction in BM neutrophil production.[2,23]

During homeostasis, BM neutrophil production rates *in vivo* have been estimated based on the size and lifespan of the circulating pool of neutrophils.[3] However, this approach is only valid at steady-state, and cannot be used to study the dynamic changes that occur during the systemic inflammatory response, when the circulating pool of neutrophils is rapidly changing due to (a) the massive release of neutrophils stored in the BM, and (b) the sequestration of circulating neutrophils into the microvasculature of the wound as well as several major organ systems.[5]

To investigate the dynamics of cell proliferation and differentiation *in vivo*, DNA labels, such as [^{3}H]-thymidine and the thymidine analog bromodeoxyuridine (BrdU), which are efficiently incorporated into cellular DNA via the salvage pathway, have been used extensively.[12,26] The observed kinetics of the label in a particular cell population depend on several processes, including cell proliferation, differentiation of labeled precursor cells, as well as release of cells from that pool to another pool. These rates can be estimated using population balance models[7,24] as well as continuum mathematical models that assume first order kinetics.[20] More recently, the potential of using mathematical models to estimate *in vivo* cell rates using BrdU and deuterated glucose uptake kinetics to analyze T lymphocyte production has been demonstrated.[8,21,22,28] However, these models cannot be directly applied to the analysis of BM neutrophil development processes because, unlike T cells, the BM neutrophil pool represents a nonproliferating population wherein the DNA label can only be incorporated through the differentiation of prelabeled neutrophil progenitor cells.

In this study, we applied an *in vivo* DNA labeling strategy using BrdU and developed a modeling approach to characterize the population dynamics of the neutrophil pool in the BM of normal mice, and after induction of a systemic inflammatory response by burn injury and burn wound infection. We show that, using fitted parameters

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derived from experimentally observed DNA labeling kinetics and BM composition data in a mathematical model of the BM neutrophil pool, we can provide estimates for neutrophil production rates during nonsteady, rapidly evolving pathological states.

METHODS

Materials

Antibodies, BrdU Flow Kit (cat. #559619), FACSFlow buffer, and streptavidin conjugates were from BD Pharmingen (Franklin Lakes, NJ). Sterile GIBCO™ phosphate buffered saline (PBS) and GIBCO™ Fetal Bovine Serum (FBS) were from Invitrogen (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO). Computer simulations were performed with a Gateway Solo Laptop using Matlab® Version 5.2 (The Mathworks, Natick, MA).

Animal Models of Burn Injury and Infection

All procedures with animals were approved by the Subcommittee on Research Animal Care, Massachusetts General Hospital. Male 22–24 g CD-1 mice (Charles River Laboratories, MA) were housed in a 12-h light/dark cycle and had free access to food and water. Animals were divided into three groups: 1) sham burn control, 2) burn injury, and 3) burns with infection. Neutrophil production in the sham is assumed to be at steady state. Neutrophil production after a 20% burn alone and a 5% burn with infection, representing a moderate inflammatory and severe inflammatory stimulus, respectively, however, is highly unsteady state and cannot be analyzed using techniques that rely on a steady state.[16]

Animals receiving burn injury were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine, and shaved on their back. The animal was placed in a customized mold that exposes 20% of the total body surface area on the dorsum while protecting the head and limbs, and the back of the animal was contacted with boiling water for 7 s.[19,25] The animal was removed from the mold and given 50 mL/kg saline resuscitation intraperitoneally. For the burns with infection model, anesthetized mice were shaved on the abdomen. To prevent injury to internal organs they were then suspended between two needles fixed on a stand by a loose flap of shaved abdominal skin. A small 1 cm^2 full thickness burn (corresponding to approximately 5% of the total body surface area) was performed by pressing two brass blocks heated to 100°C together half a centimeter from the top edge of the exposed abdominal skin for 7 s.[34] The burned skin was injected with 10^5 *Pseudomonas aeruginosa* (UCBPP-PA14 strain), a human clinical isolate. *P. aeruginosa* was grown in Luria broth at 37°C, harvested in midexponential growth, resuspended in 10-mM $MgSO_4$ at 10^6 bacteria/mL, and used within 90 min.[27] The animal was then given 50 mL/kg saline resuscitation intraperitoneally. Control mice were handled similarly to the burn injury group, except that the animals were not burned. After injury, all animals were returned to their cages (3–5 per cage) and allowed free access to food and water.

In Vivo Labeling of Proliferating Cells

Mice received an intraperitoneal (IP) injection of 4 mg of BrdU, administered at the time of injury, followed by 2-mg doses every 12 h until sacrifice. This regime was adapted from previous studies that utilize IP injection of BrdU.[30,35,37]

Flow Cytometry Analysis of Bone Marrow Cells

BM cells were isolated from both femoral bones of each animal by flushing 10-mL calcium-free Hanks buffered saline solution through the femur with a 23-gauge needle and handled on ice. One mL aliquots of the BM isolate containing approximately 10^6 cells were centrifuged at 300g for 5 min, the supernatant was then removed, and cells were resuspended in the stain buffer (PBS + 3% Fetal Bovine Serum (heat inactivated) + 0.09% sodium azide). Cells were centrifuged again (300g, 5 min) and the supernatant decanted and cells were resuspended in the remaining stain buffer.

For immunofluorescence staining of surface molecules, primary antibody constructs were diluted 1:20 by adding 2.5 μL antibody stock to 50 μL of cell suspension that were then incubated for 15 min on ice. The monoclonal antibodies used (BD Pharmingen catalog numbers in brackets) are: R-phycoerythrin (PE)-conjugated rat anti-mouse Ly-6G (cat. #553128), biotin-conjugated anti-mouse CD11b (cat. #553309), and the respective R-PE and biotin conjugated IgG_{2b}, κ isotype controls (cat. #553989 and 553987). One mL of stain buffer was added to the cells after incubation and cells were again centrifuged and the supernatant discarded. Secondary staining with streptavidin-peridinin chorophyll-a protein (cat. #554064) was then performed following procedures similar to that used with biotin-conjugated primary antibodies. Stained cells were resuspended, fixed, and permeabilized in 100-μL Cytofix/Cytoperm Buffer (BD Flow Kit) containing 4% formalin and incubated for 30 min at room temperature. To wash cells, 1 mL of Perm/Wash Buffer (BD Flow Kit) was added to each sample, centrifuged at 300g for 5 min and the supernatant discarded.

For immunofluorescence staining of BrdU, the fixed cells were resuspended with 100-μL Cytoperm Plus Buffer (BD Flow Kit) containing 10% dimethyl sulfoxide and incubated for 10 min on ice before being washed by adding 1-mL Perm/Wash Buffer. After this, samples were refixed with 100-μL Cytofix/Cytoperm Buffer and incubated at room temperature for 5 min and then washed with 1 mL of Perm/Wash Buffer. Cells were then incubated with 0.3 mg/mL DNase (BrdU Flow Kit) in PBS at 37°C for 1 h and then 1-mL Perm/Wash Buffer added and cells were

centrifuged and the supernatant discarded. Finally, cells were resuspended in 50 μL of Perm/Wash buffer containing a 1:50 dilution of FITC-conjugated anti-BrdU antibody (BrdU Flow Kit) and this was incubated for 20 min at room temperature in the dark. Cells were then washed by adding 1 mL of Perm/Wash Buffer, centrifuged at 300g and resuspended in 1 mL of stain buffer.

Stained BM cells were then analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), as described in the BrdU Flow Kit Manual from BD Pharmingen. Samples were analyzed at a data collection rate no greater than 400 events/second and the fraction of neutrophils positive for the BrdU label, defined as the labeling index (LI), was determined using WinList™ 5.0 (Verity Software House, Topsham, ME).

Model Development

Our conceptual model of BM neutrophil production is shown in Fig. 1, and consists of a self-renewing neutrophil progenitor pool (N_P) and a nonproliferative neutrophil maturation pool (N_T). The progenitor pool contains a continuum of developmental stages, from the most primitive stem cells through to proliferating myelocytes, with an overall proliferation rate defined as ν_1. Ideally, the proliferating pool immediately preceding the mature neutrophil pool would be studied, but a clear unambiguous phenotype is not available. Therefore, we consider all precursor cells as a single population of progenitor cells that are eventually destined to become neutrophils. Cells leave the progenitor pool at a rate ν_2 to enter the maturation pool containing neutrophils, which no longer proliferate. The maturation pool can be imagined as a holding tank for cells that migrate into the peripheral blood at a rate ν_3. In order to estimate these rates, we use the DNA label BrdU, and assume that all proliferating cells in the S-phase become labeled instantaneously. Assuming no proliferation within the mature neutrophil pool, BrdU-labeled cells enter this pool solely via differentiation of progenitors. We assume an age-independent probability for neutrophil egress (ν_3) where each cell in that pool has the same probability of entering the circulation.

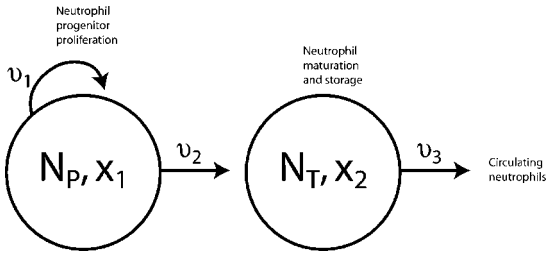

FIGURE 1. Conceptual model for neutrophil production in the bone marrow. See text for explanations.

Prediction of Neutrophil Production Rate at Homeostasis

For definition of all terms refer to *Nomenclature*. At steady state the rate of neutrophil production (ν_2) is equal to the rate of egress (ν_3) such that

$$\frac{dN_T}{dt} = \nu_2(t) - \nu_3(t) = 0 \tag{1}$$

where for the total neutrophil pool we use N_T. When production and egress are equal we can say

$$\nu_1(t) = \nu_2(t) = \nu_3(t) = \nu_{SS} \tag{2}$$

where ν_{SS} is the steady state rate of neutrophil production.

The rate of change of BrdU-labeled cells in the progenitor pool is given by

$$\begin{aligned}\frac{d(x_1 N_P)}{dt} &= 2(1-x_1)\nu_1(t) + x_1\nu_1(t) - x_1\nu_2(t) \\ &= 2\nu_1(t) - \nu_1(t)x_1(t) - \nu_2(t)x_1(t)\end{aligned} \tag{3}$$

where x_1 is the labeled fraction and N_P is the size of the progenitor pool that produces neutrophils. This equation takes account of the fact that unlabeled cells, designated by the fraction $1 - x_1$, generate two labeled cells after mitosis whereas division of previously labeled cells (x_1) generates only one additional labeled cell. What is not apparent in this expression is the age distribution of cells that are labeled or unlabeled. This problem turns out to be nontrivial and would require the development of a population balance model requiring additional assumptions about cell cycle kinetics.[7,24] However, since we are primarily concerned here with the kinetics of the nonproliferating BM neutrophil pool we neglect this structure because in the simulation, regardless of the mechanism, the labeling kinetics is the only information that the population of progenitor cells passes onto the neutrophil pool. Note that Eq. (3) involves two terms for division at the rate of ν_1.

Expanding the left side of Eq. (3) gives

$$x_1\frac{dN_P}{dt} + N_P\frac{dx_1}{dt} = 2\nu_1(t) - \nu_1(t)x_1(t) - \nu_2(t)x_1(t) \tag{4}$$

That when assuming steady state for these cells and applying Eq. (2) reduces to

$$\frac{dx_1}{dt} = \frac{2\nu_{SS}}{N_P}(1 - x_1(t)) \tag{5}$$

where $x_1(0) = s$.

The initial DNA labeling index (s) represents the fraction of progenitor cells (N_P) that is actively undergoing DNA synthesis at the start of BrdU labeling. BrdU is available for labeling cells for as little as 15 min.[38] Thus, experimentally, we observe that one cohort of progenitor cells is labeled (almost) instantaneously at time zero, after which that fraction increases on the basis of new cell entry into S-phase and division of labeled cells.

For the neutrophil maturing pool, the rate of change of the labeled cells is

$$\frac{d(x_2 N_T)}{dt} = x_1(t)\nu_2(t) - x_2(t)\nu_3(t) \quad (6)$$

Implicit in this formulation is an age independent probability of cell egress from the maturing pool of cells, age being the time any single cell has spent in the maturing pool. This treatment is justified and discussed by Nielsen *et al.*[24] Applying the chain rule in Eq. (6) we get

$$x_2\frac{dN_T}{dt} + N_T\frac{dx_2}{dt} = x_1(t)\nu_2(t) - x_2(t)\nu_3(t) \quad (7)$$

At steady state, $dN_T/dt = 0$, and the LI kinetics for mature neutrophils are

$$\frac{dx_2}{dt} = \frac{\nu_{SS}}{N_T}(x_1(t) - x_2(t)) \quad (8)$$

where $x_2(0) = 0$ and $\nu_{SS} = \nu_1 = \nu_2 = \nu_3 =$ constant.

Solving Eqs. (5) and (8) simultaneously using Mathematica 2.1 (Wolfram Research, Champaign, IL) gives

$$x_1 = 1 + e^{\frac{-2\nu_{SS}t}{N_P}}(s-1) \quad (9)$$

$$x_2 = 1 + \frac{N_P(s-1)}{(N_P - 2N_T)e^{\frac{2\nu_{SS}t}{N_P}}} + \frac{(2N_T - N_P s)}{(N_P - 2N_T)e^{\frac{\nu_{SS}t}{N_T}}} \quad (10)$$

Since N_T and x_2 are determined experimentally, this analytical solution can be used to estimate ν_{SS} as a function of N_P and s.

Prediction of Neutrophil Production Rate During Physiological Challenge

During physiological challenge, the rate of neutrophil egress is different from the rate of production, and as a result, the BM pool of neutrophils will change. Since N_T is no longer constant, Eq. (2) no longer applies, and an analytical solution is not feasible, making the use of a numerical approach necessary. Before constructing the numerical model it is convenient to introduce continuous functions to describe experimentally observed kinetics. The change in the total mature neutrophil pool can be described by the empirical function

$$N_T = N_{T0}(1 + e^{-at} - e^{-bt}) \quad (11)$$

For sham control animals, the population is assumed to be at steady state, so in Eq. (11), $a = b = 0$. In the burn injury group, where the mature neutrophil pool is observed to first shrink and then recover to preburn levels, we require that $a > b > 0$. In this way, e^{-bt} is initially greater than e^{-at} with the difference increasing to a maximum before decaying toward zero. In the burn with infection group, where no recovery of the mature neutrophil pool is experimentally observed, we let $a > 0; b = 0$, such that e^{-at} decays to zero while $e^{-bt} = 1$.

Similarly, the observed LI kinetics of the mature neutrophil pool are fitted to the empirical function

$$x_2 = 1 - e^{-ct} \quad (12)$$

where c is a rate constant for the mature neutrophil pool. Since we are not able to solve the nonsteady state case analytically, we propose a convenient model for the incoming LI kinetics (x_1) that is based on Eq. (9).

$$x_1 = \frac{(e^{dt} + s - 1)}{e^{dt}} \quad (13)$$

where d is an empirical specific proliferative rate analogous to $2\nu_{SS}/N_P$ in Eq. (9).

A numerical model was derived on the basis of the preceding differential equations with the general case given below. Starting from a known population of neutrophils, we can predict how the number of neutrophils changes after a small time step Δt into the future with

$$\Delta N_T = \nu_2(t + \Delta t/2)\Delta t - \nu_3(t + \Delta t/2)\Delta t \quad (14)$$

We evaluate ν_2, the neutrophil production rate, and ν_3, the neutrophil egress rate, at $t + \Delta t/2$ to get approximate rates over the interval Δt. Since we have measured N_T over time and can fit a continuous function over time using Eq. (11), we can rewrite Eq. (14) in terms of the neutrophil production rate

$$\nu_2(t + \Delta t/2) = \frac{N_T(t + \Delta t) - N_T(t) + \nu_3(t + \Delta t/2)\Delta t}{\Delta t} \quad (15)$$

Similarly, we can predict the change in DNA-labeled neutrophils a small time step Δt into the future with

$$\Delta(x_2 N_T) = \nu_2(t + \Delta t/2)\cdot x_1(t + \Delta t/2)\Delta t - \nu_3(t + \Delta t/2)\cdot x_2(t + \Delta t/2)\Delta t \quad (16)$$

where x_1 is the simulated progenitor cell labeling kinetics from Eq. (13) and x_2 is the observed neutrophil labeling kinetics fitted to the continuous function Eq. (12). In this way, we can determine the probable rates over time in response to control, burn, and infection conditions with a predicted x_1 kinetic. We do this by substituting Eq. (15) into Eq. (16) and rearranging to solve for ν_3

$$\nu_3(t + \Delta t/2) = \frac{\frac{(N_T(t + \Delta t) - N_T(t))x_1(t + \Delta/2)}{\Delta t} - \frac{\Delta(x_2 N_T)}{\Delta t}}{(x_2(t + \Delta t/2) - x_1(t + \Delta t/2))} \quad (17)$$

where $\Delta(x_2 N_T) = x_2(t + \Delta t)\cdot N_T(t + \Delta t) - x_2(t)\cdot N_T(t)$. Substitution of Eq. (17) back into Eq. (15) gives $\nu_2(t + \Delta t/2)$. Equations (11–17) were encoded in Matlab® (The Mathworks, Natick, MA) subject to the physical constraints that $1 \geq x_1 \geq x_2 0$, $\nu_1 \geq 0$, $\nu_2 \geq 0$, where both x_1 and x_2 are monotonic and increasing functions. The time step size (Δt) was decreased until solutions were independent of the time step and was typically 6 min. By fitting Eqs. (11) and (12) to experimental data, we simulate, using Eqs. (15) and (17), the neutrophil production and

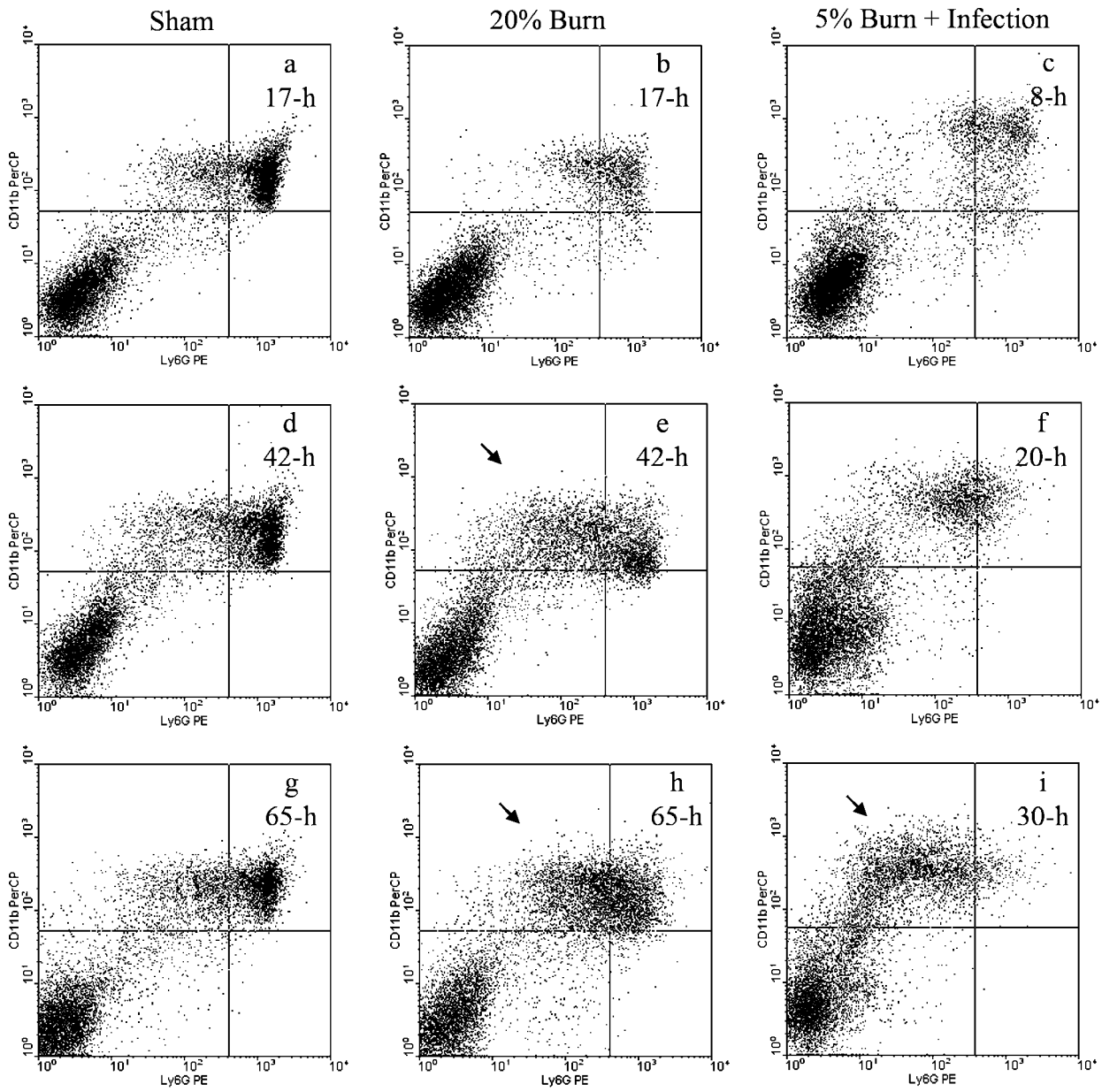

FIGURE 2. Expression of neutrophil surface markers in bone marrow cells. Bone marrow cells were isolated from mice at different times after burns and/or infection and stained for neutrophil surface markers. Mature neutrophils (CD11b$^+$/Ly6G$^+$) are in the upper right hand quadrant. Sham control mice are in left column, 20% burn injured mice in middle column, and 5% burn and infected mice in right column. Arrows point to a CD11b$^+$/Ly6G^{lo} population. Data shown are representative of results for triplicate mice being analyzed at each time point.

egress rates over time for different progenitor kinetics as defined by Eq. (13).

RESULTS

Bone Marrow Population Kinetics

To investigate the effect of severe injury on BM neutrophil production and egress, we measured BM neutrophil content and BrdU uptake kinetics in mice subjected to (a) burn injury covering 20% of the total body surface area, (b) burn injury covering 5% of the total body surface area followed by infection with *P. aeruginosa*, and (c) sham treatment. The progressive effect of these treatments on the composition of the BM cells is depicted in Fig. 2. The CD11b$^+$/Ly6G$^+$ cells in the upper right hand quadrant of each plot characterize the mature neutrophil pool.[17] The average kinetics of the CD11b$^+$/Ly6G$^+$ cells are also represented quantitatively in Fig. 3. In the sham group, we saw a 30% increase in the BM neutrophil pool within the first

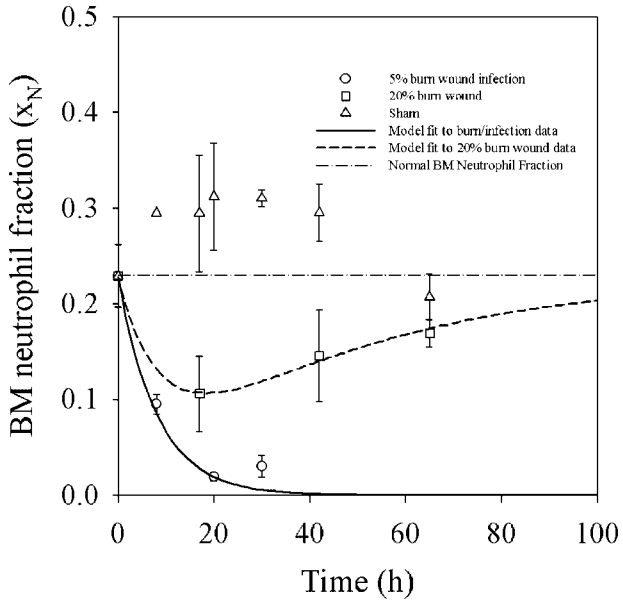

FIGURE 3. Fraction of neutrophils in the bone marrow. Data shown were obtained from Fig. 2 and expressed as the fraction of CD11b$^+$/Ly6G$^+$ cells in the sham control group (triangles), 20% burn group (squares), and 5% burn with infection group (circles). Data shown are averages ± standard error for triplicate animals. The nonlinear fit to Eq. (11) is shown for BM neutrophil composition for the parameter estimation for 20% burn (dashed-dotted line) and 5% burn plus infection (solid line). The normal steady state neutrophil content (dashed-dotted line) is also given.

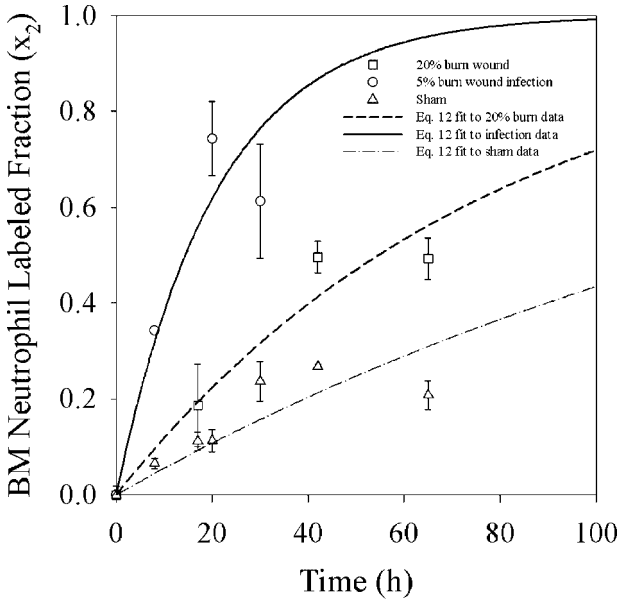

FIGURE 4. Fraction of BrdU labeled neutrophils in the bone marrow. Data shown are the fraction of BrdU$^+$ cells in the CD11b$^+$/Ly6G$^+$ pool for the sham control group (triangles), 20% burn group (squares), and 5% burn with infection group (circles). Data given are averages ± standard error for triplicate animals. The fit to Eq. (12) for the BrdU labeling kinetics used for parameter estimation for 20% burn (dashed line), 5% burn plus infection (solid line), and sham data (dashed-dotted line) are also shown.

8 h after sham treatment (Fig. 3), with no further change up to 65 h after treatment (Figs. 2(a), 2(d), 2(g), and 3). In contrast with the sham control, there was a 50% decrease in the mature neutrophil pool size at the 17-h time point in the burn-only group (Fig. 2(b)), although it progressively returned to normal over time (Figs. 2(e) and 2(h)). In the infected burn group, the mature neutrophil pool size had decreased by 60% by the 8-h time point (Fig. 2(c)), and by ~90% at the 20-h and 30-h time points (Figs. 2(f) and 2(i)). Unlike the burn-only group, the mature neutrophil pool was never replenished in animals with infected burns, and no observations beyond the 30-h time point were made because all animals had died by the 40-h time point. Interestingly, a new pool of CD11b$^+$/Ly6G^{lo} cells appeared in the later time points after burn injury (Figs. 2(e), (h)) or infected burns (Fig. 2(i)), although we could not confirm whether these cells represent newly formed neutrophils.

To investigate the process of neutrophil regeneration (from neutrophil precursors) in response to the various treatments, we next examined the fraction of BrdU-labeled cells in the CD11b$^+$/Ly6G$^+$ pool (Fig. 4). In the sham control group, that fraction increased monotonically and reached a plateau of ~0.25 by 30 h. In the burn group, it increased linearly until the 40-h time point, where it reached a maximum of ~0.5. In the infected burn group, the BrdU-labeled fraction exhibited a rapid increase in the first 20 h to reach a maximal value of ~0.7, and then decreased slightly. Using Eq. (12), we fitted first order rate constants to the data to facilitate comparisons among experimental groups. We found BrdU labeling was two times faster in burned animals vs. sham controls (rate constant (c) ± standard error: 0.013 ± 0.002 h^{-1} vs. 0.006 ± 0.001 h^{-1}), and 8-fold higher in burn and infected animals (0.048 ± 0.01 h^{-1}). We note that BrdU labeling appears to reach a plateau at the final time point (Fig. 3) for all treatments and speculate that this may be indicative of incomplete DNA labeling that may be remedied in the future by more frequent administration of the thymidine analog. Nevertheless, these results show that the turnover rate of the mature neutrophil pool is upregulated by burn injury, and dramatically increased by burns with superimposed infection. Without further analysis, however, it is not possible to determine whether this observation is due to an increase in neutrophil production and/or egress into the circulation.

Steady-State Analysis of Neutrophil Production in Sham Controls

In order to estimate the neutrophil production rate in sham controls (ν_{SS}) using the analytical expression described by Eq. (10), the total neutrophil pool size (N_T) in the BM is required in addition to the BrdU kinetics. To estimate N_T, we multiplied the literature value of 2.55 ×

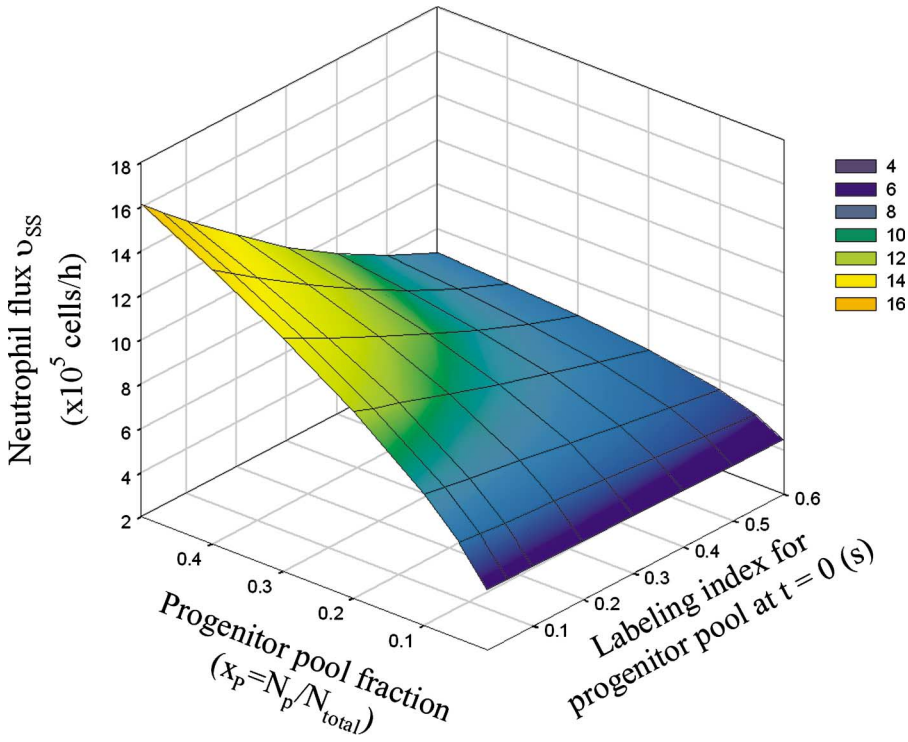

FIGURE 5. Solution space for neutrophil flux predicted by the steady-state population balance model. The neutrophil flux was calculated via nonlinear fitting of Eq. (9) to sham control data for a range of assumed values of the progenitor pool fraction (x_P) and the initial labeling index of the progenitor pool (s).

10^8 cells for the total nucleated BM pool of the mouse[10] by the (initial) neutrophil fraction of total nucleated cells in the marrow, measured to be 0.22 in our experiments (Fig. 3), thus yielding 5.61×10^7 cells. Two parameters (s and N_P) in Eq. (10) are unknown properties of the neutrophil progenitor pool; therefore, we solved Eq. (10) for ν_{SS} using a range of possible values of s and N_P, and the measured neutrophil LI kinetics. The solution space, which is shown in Fig. 5, shows that the neutrophil flux could be in the range of 4–16 $\times$ 10^5 cells/h. However, the size of the progenitor pool represented as a fraction of the total bone marrow pool (x_p) is expected to be less than 0.1 based on the fact that previous studies have indicated that there are as few as 450 granulocyte progenitors per femur per gram body weight in mice.[13] Indeed, assuming one femur is equivalent to 6% of total bone marrow,[10] this represents 1.87×10^5 neutrophil progenitors per 25 g mouse, and dividing by the total nucleated bone marrow pool gives $x_p = 0.0007$. Assuming, therefore, x_p to be between 0 and 0.1, Fig. 5 shows that the flux ν_{SS} is relatively insensitive to s and N_P and equal to approximately 8×10^5 cells/h. By dividing the BM neutrophil pool size by this value, we estimate the neutrophil "residence time" to be 2.9 days being the time available for maturation and storage of an average neutrophil in the BM. This is fairly consistent with known marrow neutrophil transit times of 4–8 days measured for humans.[11]

Analysis of Neutrophil Production Using Unsteady-State Model

In order to facilitate solving the numerical model described by Eqs. (15) and (17), we first fitted the observed kinetics for the BM neutrophil fraction and the BrdU-labeled neutrophil fraction in Figs. 3 and 4 to Eqs. (11) and (12), respectively. Here we assumed that the initial neutrophil pool size, N_{T0}, is the same as the steady-state value of N_T determined above, namely 5.61×10^7 cells. The best-fit parameters a, b, and c are shown in Table 1. The resulting fit captures the time-dependent variations of the data, as shown in Figs. 3 and 4. In addition, solving Eqs. (15) and (17) requires an input for the BrdU-labeled fraction of neutrophil progenitors (x_1). Since we could not measure this value, we simulated x_1 using Eq. (13) and a range of possible values for s and d.

Unsteady-State Analysis of 20% Burn Group

While calculating ν_2 and ν_3 over time, we used an upper limit for s of 0.3. Given that the S phase (when cells take up BrdU) generally accounts for at most 30% of the duration of the cell cycle. A value of $s = 0.3$ implies that 100% of the progenitor cells are proliferating. Thus, our calculations are based on a value of s that is close to its biological upper limit. For $s \leq 0.3$ there was a family of solutions that satisfy both the physical constraints and experimental results. One

TABLE 1. Parameters for fitted curves shown in Figs. 3 and 4.

Treatment group	Rate constant (h^{-1})		
	a [Eq. (11)]	*b* [Eq. (11)]	*c* [Eq. (12)]
Sham control	0	0	0.0057 ± 0.0008
20% burn	0.1098 ± 0.0145	0.0221 ± 0.002	0.0127 ± 0.0016
5% burn + infection	0.123 ± 0.023	0	0.0482 ± 0.0097

representative result for cell rates and corresponding DNA labeling kinetics was calculated using an arbitrary initial labeling index (s) = 0.3 and rate constant (d) = 0.05 (Fig. 6). In the early phase of the response after injury, the rates of neutrophil production and egress were highest initially and rapidly decreasing over time. In addition, during this phase, rates of neutrophil egress exceeded neutrophil production. Later on, neutrophil production rose above the egress rate, and both rates eventually converged toward a constant rate with $\nu_2 = \nu_3 = 7.39 \times 10^5$ cells/h. These results suggest

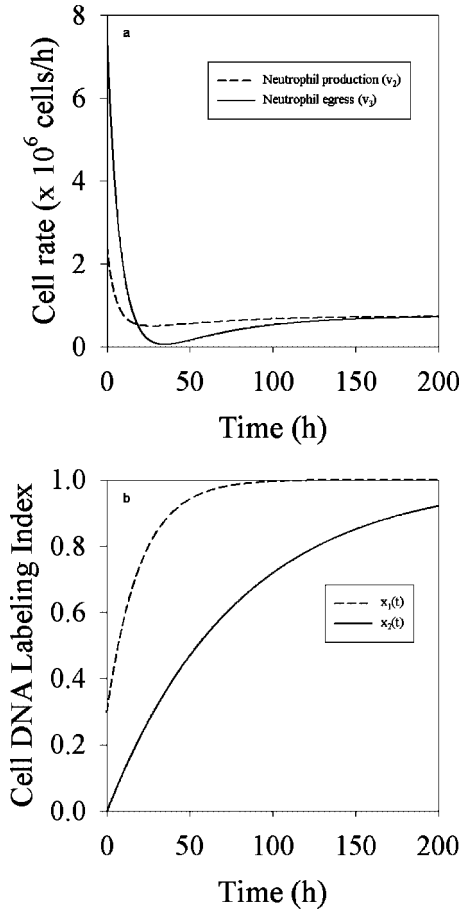

FIGURE 6. Predicted cell rates for the 20% burn group assuming fast progenitor labeling kinetics. (a) Cell rates ν_2 and ν_3 calculated using the numerical model. Both rates converge to 7.4 × 10^5 cells/h. (b) Simulated progenitor BrdU labeling kinetics (x_1) using Eq. (13) with s = 0.3 and d = 0.05.

that there is a net loss of neutrophils from BM early after burn injury, followed by a replenishment of the BM stores, which is consistent with the observed transient decrease in the BM neutrophil pool size described earlier (Fig. 3). Interestingly, this trend was common to *all* 20% burn simulations using Eq. (13) and $0 \le s \le 0.3$. The final rate of 7.39×10^5 new neutrophils per hour was independent of the initial flux and the values of s and d. This rate being very close to the rate of 8×10^5 cells/h for the sham control group estimated above, it may indicate the eventual recovery of neutrophil production in the 20% burn group. While the long-term steady-state behavior was unaffected by the values of s and d in Eq. (13), the initial rate and dynamics of the response were strongly dependent on these parameters. More specifically, decreasing s increased the initial rates ν_2 and ν_3, and decreasing d, representing slower progenitor labeling kinetics, caused the rates of production and egress from the marrow to fluctuate over a longer period of time before reaching steady-state. For example, stable neutrophil production was not established within 200 h when $d = 0.0149$ and $s = 0.3$ (Fig. 7). In this case, the dynamics show an initial high level of both ν_2 and ν_3 followed by a local minimum that precedes an extended hyperproduction and egress with a slow decay toward 7.39×10^5 cells/h. The slowest responses were observed when progenitor labeling kinetics were only slightly faster than the labeling kinetics of the maturing neutrophil pool.

Unsteady-State Analysis of Burn Wound Infection Group

We estimated the rates of neutrophil production and egress for a range of values of s and d. A representative example using $s = 0.3$ and $d = 0.085$ is shown in Fig. 8. We found that ν_2 and ν_3 were highest immediately after injury and decreased in a monotonic fashion toward zero. In addition, ν_3 was greater than ν_2 over all times considered. Although the initial rates and decay kinetics were sensitive to the values of s and d used in the simulation, the rates always converged toward zero. These results suggest that there is a continuous net loss of neutrophils from BM over the entire time course considered, which is consistent with the observed depletion of the BM neutrophil pool size described earlier (Fig. 3). The reduction in neutrophil egress can, therefore, be explained by a combination of the effects of a reduced neutrophil pool size and a diminishing rate of progenitor differentiation.

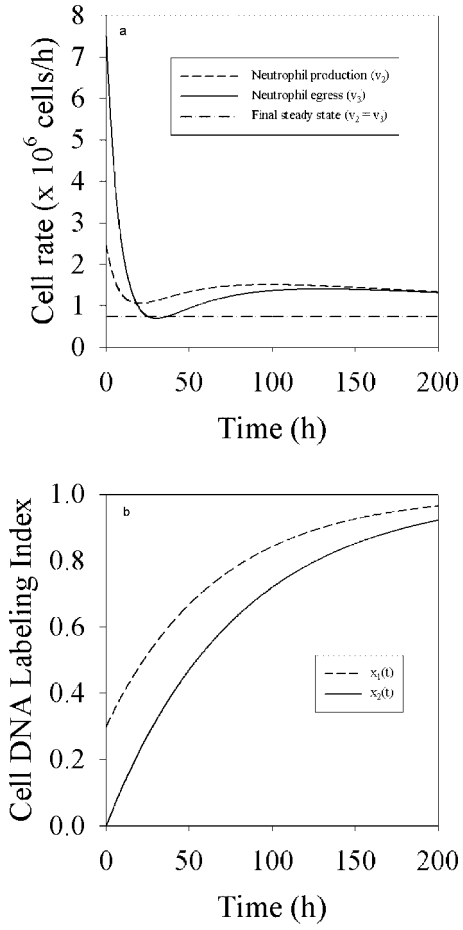

FIGURE 7. Predicted cell rates for the 20% burn group assuming slow progenitor labeling kinetics. (a) Cell rates ν_2 and ν_3 calculated using the numerical model. Rates still converge to 7.4 $\times$ 10^5 cells/h but over a much longer time scale than in Fig. 6. (b) Simulated progenitor BrdU labeling kinetics (x_1) using Eq. (13) with s = 0.3 and d = 0.0149.

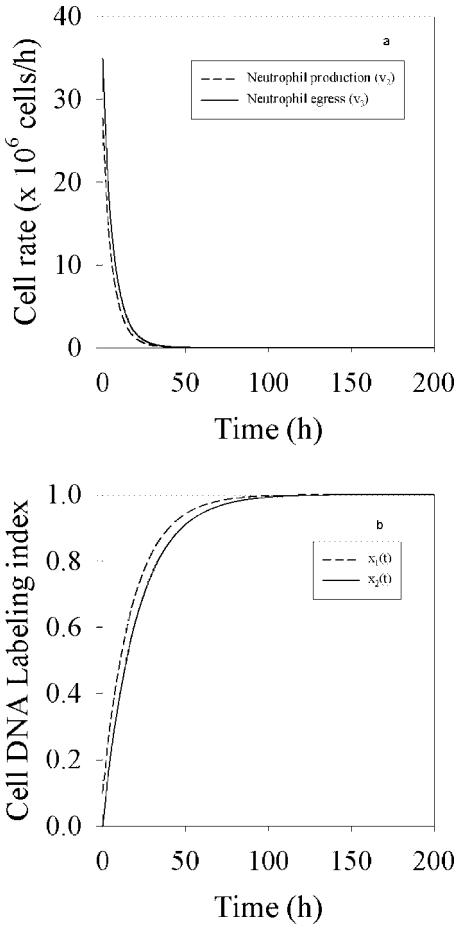

FIGURE 8. Predicted cell rates for the burn wound infection group. (a) Cell rates ν_2 and ν_3 calculated using the numerical model. (b) Simulated progenitor BrdU labeling kinetics (x_1) using Eq. (13) with s = 0.3 and d = 0.085.

DISCUSSION

We have investigated the effects of a nonlethal 20% burn injury and lethal 5% burn wound infection on the process of BM neutrophil differentiation in mice. This required the development of a method to study bone marrow neutrophil production in pathological conditions that evolve rapidly, and therefore where techniques that rely on steady-state analyses will not work. Based on a quantitative analysis of the kinetics of label uptake and pool size of BM neutrophils during continuous BrdU labeling *in vivo*, we estimated the rates of BM neutrophil production and egress into the circulation. We found that, compared to sham controls, the rates of BM neutrophil production and egress were both elevated shortly after injury, with a much higher rate of neutrophil egress relative to production, thereby causing a decrease in the BM neutrophil pool size. In the 20% burn injury group, the rate of egress eventually decreased below the rate of production, which allowed the recovery of the BM neutrophil pool. In addition, both rates eventually reached a steady-state value similar to that found in the sham controls, suggesting a return to homeostasis. In the 5% burn with infection group, the rate of egress was always greater than production and both rates decreased to zero. These results suggest that the BM neutrophil pool acts as a reservoir that allows the rapid release of a large number of neutrophils after severe injury. In addition, BM neutrophil production significantly increases to replenish that pool. This response is transient after nonlethal burn injury; however, a lethal infected burn injury eventually leads to a complete suppression of BM neutrophil production and release.

Using the steady-state model, we estimated the production flux of mature neutrophils to be about 8 $\times$ 10^5 neutrophils/h in the sham controls. Using the unsteady-state model to analyze the 20% burn injury group data, we found that neutrophil egress and production eventually reached a steady-state value of approximately 7.4 $\times$ 10^5 cells/h, which is very close to the sham control value. This may indicate a return to homeostasis, which is consistent with

the observation that these animals generally survive indefinitely from a 20% burn injury. We compared these estimates with that obtained for a typical normal mouse using the previously postulated formula based on the turnover rate of peripheral blood cells:[16]

$$\text{production flux} = \frac{(\text{blood volume}) \cdot (\text{cell concentration})}{\text{lifespan in blood}} \tag{18}$$

Assuming a blood volume of 2 mL, a blood neutrophil concentration of 1×10^6 cells/mL,[29] and using a neutrophil peripheral blood lifespan of 6.7 h,[9] we obtain an estimated flux of 3×10^5 neutrophils/h, which is slightly lower than our estimate, but within the same order of magnitude. Thus, quantitative analysis of the BM neutrophil pool to estimate the neutrophil production flux provides results that are consistent with estimates based on the peripheral neutrophil pool. A similar analysis of peripheral blood cells in animals undergoing systemic inflammation as a result of injury or infection is impractical due to the rapid margination of circulating neutrophils. The flux predicted using Eq. (18) is indeed highly sensitive to the neutrophil lifespan in the blood, a parameter that would be very small, and hence prone to greater error at times when peak margination rates occur. In addition, although Eq. (18) is theoretically valid for instantaneous estimates of production flux during nonsteady state phenomena, it requires measuring the instantaneous lifespan in blood over time, which is currently not possible. Thus, quantitative analysis of the BM neutrophil pool is a more suitable method for estimation of flux in nonsteady state scenarios.

In the unsteady-state model used to analyze the BM neutrophil kinetics following injury, BM neutrophil production (ν_1) and egress (ν_2) were dependent on three other parameters: the kinetics of neutrophil progenitor BrdU labeling (x_1), the kinetics of mature neutrophil BrdU labeling (x_2) and pool size (N_T). Experimentally, it was possible to characterize the kinetics for x_2 and N_T. Thus, our approach was based on first assuming a range of possible neutrophil progenitor BrdU labeling kinetics (x_1) and then searching the solution space for the remaining unknowns that are consistent with the experimental data (x_2 and N_T kinetics) while satisfying physical constraints. Most of our other assumptions rely on conservation of the number of cells and the physical impossibility of labeling more than 100% of the cells or having a negative rate of neutrophil egress from the marrow. One advantage of the model is that it does not require knowledge of the detailed underlying physiological mechanisms that govern progenitor cell proliferation and differentiation, as well as neutrophil egress, and, as such and similar to earlier models,[24] does not include any control structure. Although without measuring the neutrophil progenitor BrdU labeling (x_1) kinetics, it is not possible to exactly determine the BM neutrophil rates solely from the measured neutrophil kinetic data, relative rates (such as the rate of neutrophil egress vs. that of production) could be quantitatively determined.

Literature data have reported that, in the first 24-h after burn injury, there is an accelerated rate of egress of neutrophils from existing pools in bone marrow tissue that is mediated by circulating inflammatory mediators such as complement 5a and granulocyte colony stimulating factor (G-CSF).[15] Consistent with these findings, the model predictions yield greatly elevated rates of neutrophil egress early after injury. We calculated initial rates of egress of 7.5×10^6 cells/h in the 20% burn group and 24×10^7 cells/h in the 5% burn with infection group (Figs. 6(a) and 8(a)), which are respectively 1 and 2.5 orders of magnitude higher than the rates in the sham controls. These results were obtained assuming the biological upper limit of 0.3 for the parameter s, which represents the fraction of progenitor cells that is actively undergoing DNA synthesis, and thus almost immediately labeled with BrdU at the start of the experiment. Using lower values of s yields even higher values for the initial rates of neutrophil production and egress; therefore, the values reported above are lower bound estimates of the model.

Prior studies have suggested that animals receiving a fatal experimentally induced burn wound infection exhibit granulopoiesis suppression.[3] Mice in the burn wound infection group exhibited dramatically elevated levels of neutrophil production and egress shortly after injury. The higher rate of egress compared to production led to a rapid depletion of the BM neutrophil reserve. This is likely to be a response to attempt to control the spread of bacteria in the host in the short term. A similar response, although of lesser magnitude, was observed in the 20% burn group with no infection. In the latter case, there was a recovery of the BM neutrophil pool because the rate of neutrophil egress eventually decreased below the rate of production. In the infected burn group, we found a progressive decrease and eventual suppression of BM neutrophil production. Thus, it is conceivable that elevated BM neutrophil production and egress may be sustainable over a limited period of time. Further studies will be required to determine whether this is the result of a loss of neutrophil progenitors from the BM or inhibition of progenitor proliferation and differentiation.

The model developed here is useful for the analysis of BM neutrophil progenitor kinetics and to study BM neutrophil production and release. In addition, this model could be used to analyze the kinetics of other nonproliferating, terminally differentiated cell populations arising from a stem cell pool, such as single positive $CD4^+$ and $CD8^+$ cells newly produced in the thymus. Extension to these other systems would be achieved as

follows: (1) identify a control space that forms the basis for investigation of cell dynamics (e.g. the thymus); (2) measure changes in both the cell pool size and BrdU labeling state over time; (3) fit composition and labeling kinetic data to Eqs. (11) and (12) prior to using Eqs. (15) and (17) to estimate the rates of cell production and egress over time.

Recent evidence suggests that multiple defects in polymorphonuclear (PMN) cells predispose patients to infections after traumatic injuries. Specifically, a decrease in the expression of or an inability to upregulate CD11b on PMNs correlate with systemic infections.[4] Significant decreases in the percentage and number of PMNs displaying CD11b are observed as patients go from noninfected, to preinfected (3–7 days before infection) and then full infection (two positive blood cultures). We noted a similar shift in neutrophil expression levels in Fig. 2 for the burned and infected mice. Two possible mechanisms have been proposed to explain the reduction: 1) impaired transcription/translation of CD11b, and 2) perturbed mobilization of CD11b from specific intracellular granules.[4] We see a possible third mechanism being related to the kinetics of neutrophil production and release. With increased rates of egress, less mature neutrophils are released sooner than normal and this could account for the decreased CD11b expression levels. The notion that immature neutrophils migrate into the circulation has also been suggested by others,[32] based on reports that circulating neutrophils exhibit decreased deformability,[18] phagocytotic activity,[6,14] chemotaxis, and diapedesis.[31,32] Given a certain rate of neutrophil egress, treatment strategies that increase neutrophil progenitor proliferation rates, for example using specific growth factors, would be expected to increase the BM neutrophil maturation time by increasing the BM storage pool size and possibly result in a faster return to steady-state BM neutrophil production and egress. Evidence shows that administration of G-CSF restores the respiratory burst and normal PMN CD11b expression,[4] strengthening our argument for a role for altering neutrophil kinetics in effective management of burn victims. Thus, further investigation into the effect of severe injury on BM neutrophil progenitor pool dynamics in contributing to neutrophil dysfunction is warranted. For this purpose, identification of reliable cell surface markers specific to neutrophil progenitors that allow flow cytometry analysis of this pool of cells will be necessary. Finally, these results demonstrate the usefulness of mathematical models that integrate both BrdU labeling and composition kinetics to estimate critical cell rates during rapidly evolving pathological states.

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NOMENCLATURE

ν_1 = the net rate of cell birth through division in the progenitor pool (cells/h)
ν_2 = the rate of cell entry into the maturing neutrophil pool from the progenitor pool (cells/h)
ν_3 = the rate of neutrophil egress from the bone marrow into the circulation (cells/h)
x_1 = labeling index of cells entering the maturing neutrophil pool
x_2 = labeling index of mature neutrophils in the bone marrow
x_N = fraction of bone marrow that are mature neutrophils ($CD11b^+/Ly6G^+$)
x_P = fraction of bone marrow that are neutrophil progenitors
N_T = total number of mature neutrophils in the bone marrow of mouse (cells)
N_{T0} = initial steady state neutrophil pool in the bone marrow (cells)
N_P = number of neutrophil progenitor cells in the bone marrow (cells)
s = initial labeling index of neutrophil progenitor cell population
ν_{SS} = steady state neutrophil flux (cells/h)
a = empirical rate of observed neutrophil depletion (Eqs. (11–15)) (h^{-1})
b = empirical rate of observed neutrophil replacement (Eqs. (11–15)) (h^{-1})
c = rate constant for labeling index kinetics (Eqs. (12–17)) (h^{-1})
d = rate constant (Eq. (13) (h^{-1})

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