RESEARCH ARTICLE

Bone marrow infiltration by multiple myeloma causes anemia by reversible disruption of erythropoiesis

Anass Bouchnita,[1] Nathalie Eymard,[1] Tamara K. Moyo,[2] Mark J. Koury,[2]* and Vitaly Volpert[1,3]

Multiple myeloma (MM) infiltrates bone marrow and causes anemia by disrupting erythropoiesis, but the effects of marrow infiltration on anemia are difficult to quantify. Marrow biopsies of newly diagnosed MM patients were analyzed before and after four 28-day cycles of nonerythrotoxic remission induction chemotherapy. Complete blood cell counts and serum paraprotein concentrations were measured at diagnosis and before each chemotherapy cycle. At diagnosis, marrow area infiltrated by myeloma correlated negatively with hemoglobin, erythrocytes, and marrow erythroid cells. After successful chemotherapy, patients with less than 30% myeloma infiltration at diagnosis had no change in these parameters, whereas patients with more than 30% myeloma infiltration at diagnosis increased all three parameters. Clinical data were used to develop mathematical models of the effects of myeloma infiltration on the marrow niches of terminal erythropoiesis, the erythroblastic islands (EBIs). A hybrid discrete-continuous model of erythropoiesis based on EBI structure/function was extended to sections of marrow containing multiple EBIs. In the model, myeloma cells can kill erythroid cells by physically destroying EBIs and by producing proapoptotic cytokines. Following chemotherapy, changes in serum paraproteins as measures of myeloma cells and changes in erythrocyte numbers as measures of marrow erythroid cells allowed modeling of myeloma cell death and erythroid cell recovery, respectively. Simulations of marrow infiltration by myeloma and treatment with nonerythrotoxic chemotherapy demonstrate that myeloma-mediated destruction and subsequent reestablishment of EBIs and expansion of erythroid cell populations in EBIs following chemotherapy provide explanations for anemia development and its therapy-mediated recovery in MM patients.

Am. J. Hematol. 91:371–378, 2016.

Introduction

Diseases that infiltrate bone marrow may cause anemia by disrupting erythropoiesis. However, studies quantifying the contribution of marrow infiltration to the degree of anemia are difficult to perform. Acute myeloid leukemia uniformly infiltrates marrow but, due to their lineage relationship to the leukemic clone, erythroid cells often have intrinsically abnormal proliferation or differentiation that is clinically manifested as myelodysplasia. Myelofibrosis, chronic myeloid leukemia, and lymphoid leukemias may uniformly infiltrate marrow, but splenomegaly in these disorders from extramedullary hematopoiesis or leukemic involvement may confound assessments of erythropoiesis. Marrow infiltration varies widely in patients with chronic infections and metastatic tumors. In multiple myeloma (MM), however, most patients have relatively uniform marrow infiltration by myeloma cells, in addition to variable numbers of macroscopic, lytic bone lesions. Furthermore, erythroid cells have no direct lineage relationship with myeloma cells, and splenic involvement and extramedullary hematopoiesis are extremely rare in MM. Most patients with MM experience anemia sometime during their disease, with the severity of anemia related to stage of disease, proliferative rate of myeloma cells, and amount of myeloma cells in marrow [1,2].

Percentages of myeloma cells, erythroid cells, and other hematopoietic cells can be determined from marrow specimens obtained at diagnosis in untreated MM patients and after those patients complete chemotherapy aimed at achieving remission. Some chemotherapeutic agents kill myeloma cells without killing erythroid cells or other hematopoietic cells. Such nonerythrotoxic chemotherapeutic agents, which allow recovery of marrow erythroid cell populations in the absence of general hematopoietic regeneration, include: immune modulators (thalidomide and lenalidomide), proteasome inhibitors (bortezomib and carfilzomib), and synthetic glucocorticoids (prednisone and dexamethasone) [3]. In most patients, the

Additional Supporting Information may be found in the online version of this article.

[1]Institut Camille Jordan, UMR 5208 CNRS, University Lyon 1, Villeurbanne 69622, France; [2]Division of Hematology/Oncology, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; [3]INRIA Team Dracula, INRIA Antenne Lyon La Doua, Villeurbanne 69603, France, and European Institute of Systems Biology and Medicine, Lyon 69007, France

A. Bouchnita, N. Eymard, and T.K. Moyo contributed equally to this work.

Conflicts of Interest: The clinical data was collected and analyzed by T. Moyo and M. Koury. The data was used to prepare the mathematical models and perform numerical simulations by A. Bouchnita, N. Eymard, and V. Volpert. The manuscript was prepared and written by all five authors. A. Bouchnita, N. Eymard, T. Moyo, M. Koury and V. Volpert declare no conflicts of interest related to this research.

***Correspondence to:** Mark J. Koury; Division of Hematology/Oncology, 777 Preston Research Building, Vanderbilt University, Nashville, TN 37232-6307. E-mail: mark.koury@vanderbilt.edu

Contract grant sponsor: LABEX MILYON (ANR-10-LABX-0070) of Université de Lyon, within the program "Investissements d'Avenir" operated by the French National Research Agency (ANR); Contract grant number: ANR-11-IDEX-0007.
Received for publication: 23 December 2015; **Accepted:** 29 December 2015
Am. J. Hematol. 91:371–378, 2016.
Published online: 9 January 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.24291

concentrations of monoclonal immunoglobulin or immunoglobulin light chain component secreted by myeloma cells can be easily measured in serum, thereby providing an estimate of relative amounts of myeloma in the marrow without performing repeated marrow biopsies during chemotherapy. Similarly, peripheral blood cell counts can be monitored for improvement of anemia during the treatment period.

Erythropoiesis occurs in structural and functional units in the marrow termed erythroblastic islands (EBIs) [4,5]. An EBI consists of a central macrophage surrounded by erythroid cells in various stages of differentiation with more centrally located colony-forming units-erythroid (CFU-Es), their immediate progeny the proerythroblasts (ProEBs) and more peripherally located maturing erythroblasts [6]. Central macrophages and marrow stromal cells produce growth factors required by CFU-Es and ProEBs: stem cell factor (SCF), under normal conditions, and bone morphogenetic protein-4 (BMP4), under erythropoietic stress conditions [7,8]. CFU-E and ProEB populations expand in response to BMP4 and glucocorticoid hormones [8–10]. CFU-E, ProEBs, and their immediate progeny, early-stage basophilic erythroblasts (BasoEBs), require erythropoietin (EPO) to survive [11,12]. EPO is produced in the kidneys, and its concentration varies inversely and exponentially with circulating erythrocyte (RBC) numbers [13]. EPO promotes erythroid progenitor survival by reducing surface expression of FAS, a member of the tumor necrosis factor receptor family that triggers apoptosis after binding FAS ligand (FL) [14,15]. In normal human EBIs, the mature erythroblasts, which enucleate to form reticulocytes, are the major source of FL [14]. After partially maturing in the marrow, reticulocytes enter the blood where they become mature RBCs.

Myeloma cells infiltrating marrow may impair function and structure of EBIs by secreting cytokines. Transforming growth factor-β (TGF-β) secreted by myeloma cells may decrease adhesion and growth of earlier progenitors, thereby decreasing CFU-E numbers [16]. Expression of FL and TNF-related apoptosis-inducing ligand (TRAIL) by myeloma cells may induce apoptosis of EPO-dependent progenitors [17,18]. MM patients may have decreased EPO production due to renal disease from nephrotoxic monoclonal immunoglobulins or light chain components [1]. BMP2 produced by myeloma cells may induce hepcidin [19], which restricts iron required by late-stage erythroblasts for hemoglobin production. A mouse model suggests that myeloma cells may compete with late-stage erythroblasts for iron [20]. A second mechanism by which infiltrating MM can decrease erythropoiesis is physical disruption of EBIs. Total macrophages in bone marrows of patients with myeloma are inversely correlated with the area of marrow infiltrated by myeloma [21]. Although central macrophages of EBIs are a minor percentage of total marrow macrophages, a proportional decrease in the central macrophage population of the marrow would decrease EBI numbers and erythropoietic activity.

Previously developed hybrid discrete-continuous models of erythropoiesis based on the EBI structure and function [22] are used here to study the relationship between marrow infiltration and the degree of anemia in MM. Models are developed and simulations performed using data from newly diagnosed MM patients who were treated uniformly with lenalidomide, bortezomib, and dexamethasone (LBD) chemotherapy [23] to induce remission prior to autologous stem cell transplantation. The mathematical models provide information about the degree of marrow infiltration by MM, its effects on EBI structure/function and the development of anemia, and the potential of nonerythrotoxic therapies to reverse marrow infiltration and improve anemia.

Methods

Clinical data. Laboratory results of MM subjects, who received LBD remission induction therapy, were analyzed after approval by the Vanderbilt Human Research Protection Institutional Review Board. All patients had previously consented to participate in a clinical trial (National Cancer Institute NCT01215344), which was designed to test the effects of autologous stem cell transplant on minimal residual disease in MM patients undergoing initial treatment that included bortezomib. The patients received four consecutive 28-day cycles of LBD to achieve remission of MM prior to stem cell autotransplantation. Each cycle consisted of: lenalidomide 25 mg orally on days 1–14; bortezomib, 1.3 mg/m^2 body surface area intravenously on days 1, 4, 8, 11; dexamethasone 20 mg orally, the day before and day after receiving bortezomib [23]. Bone marrow biopsies were performed at diagnosis and after four cycles of LBD therapy. Complete blood counts, serum monoclonal protein measured by immunoelectrophoresis with immunofixation, quantitative serum immunoglobulin concentration by class, and free κ and λ light chain concentrations were obtained at diagnosis, on day 1 of chemotherapy cycles 2–4, and after completion of cycle 4 of LBD chemotherapy. Patients with elevated serum creatinine, bleeding, iron deficiency, microcytosis (mean cell volume $<$80 fL), macrocytosis (mean cell volume $>$100 fL), or myelofibrosis were excluded. Laboratory results were analyzed from ten male and five female consecutive subjects, ranging from 38 to 69 years old, who met these criteria and completed the LBD protocol.

Percentages of bone marrow areas occupied by hematopoietic [nonadipose] cells and by myeloma cells were determined in diagnostic marrow examinations by the Vanderbilt University Medical Center Hematopathology Service using immunocytochemical staining with anti-syndecan1 (CD138) in decalcified marrow biopsy sections. Differential counts of erythroid, myeloid, and lymphocytic cells in the hematopoietic areas were made using light microscopy of Wright–Giemsa-stained films of marrow that were aspirated when the biopsies were performed [13]. Areas of total bone marrow occupied by erythroid, myeloid or lymphoid cells were calculated by multiplying the respective percentages of these differential counts by the marrow area of hematopoietic cells that was not occupied by myeloma cells.

Mathematical modeling of myeloma infiltration of bone marrow. The previously reported hybrid model of normal erythropoiesis based on the spatial arrangement of cells in EBIs [22] was expanded to study the effects of myeloma cell infiltration on erythropoiesis (see Supporting Information Methods and Supporting Information Fig. 1). In the EBI model, mechanical interactions based on Newton's second law of motion between erythroid cells, each of which is considered as an elastic sphere with an incompressible inner part, displace the more mature cells peripherally after mitoses [22]. Thus, the more mature erythroid stages are peripherally located in EBIs, consistent with described EBI structure [6]. Nascent reticulocytes remain in or near the EBI for various periods of time before entering the blood through marrow vascular sinuses.

In the EBI model, the CFU-E and proerythroblasts can expand by proliferating without differentiation (self-renewal), differentiate with a limited number of cell divisions, or die by apoptosis [22]. These fate decisions of the CFU-E and proerythroblasts are controlled by intracellular regulatory networks that are modeled with ordinary differential equations and extracellular cytokine/hormone concentrations that are modeled with partial differential equations (see "Methods" section of Supporting Information) [22]. In the normal EBI, mature erythroblasts and reticulocytes formed by their enucleation secrete FL, which induces apoptosis of CFU-E and ProEBs. When myeloma cells infiltrate the marrow, they also secrete proapoptotic cytokines FL and TRAIL. Counterbalancing this apoptotic loss, central macrophages produce SCF during normal erythropoiesis and BMP4 during anemia, both of which stimulate proliferation of CFU-E and ProEBs. Plasma provides glucocorticoids and EPO, both of which increase during anemia. Glucocorticoids upregulate self-renewal of CFU-Es and ProEBs, while EPO downregulates their apoptosis by decreasing FAS expression and promotes their differentiation by increasing GATA1, an erythroid transcription factor. When erythroid progenitors die by apoptosis and mature erythroid cells (reticulocytes) exit the bone marrow into the blood, some EBIs can become extinct, with only the central macrophage remaining. Conversely, during severe anemia, EBI size and numbers can increase. New EBIs are assumed to be initiated by burst-forming units-erythroid (BFU-Es), progenitors of CFU-Es, which can circulate in blood and lodge in the marrow where they have some motility [24]. When a BFU-E encounters a potential central macrophage, it divides and gives rise to CFU-Es and Pro-EBs that become physically associated with the macrophage generating an EBI. Supporting Information Figure 2A shows the previously described two-dimensional human EBI model [22], and Supporting Information Figs. 2B and 2C show sections of normal marrow and marrow infiltrated by myeloma, respectively, that contain multiple EBIs. Nonerythropoietic hematopoietic cells, which are not shown in Supporting Information Fig. 2, are situated between the EBIs, and divide or die with a given probability. In the model, these probabilities are chosen such that the nonerythroid hematopoietic cells do not contract or expand their populations.

MM develops from the premalignant, marrow-based plasma cell disorder termed monoclonal gammopathy of uncertain significance (MGUS) [25,26]. Oncogenic events transform MGUS plasma cells into myeloma cells, which have varying rates of proliferation within the marrow space and spread to distant areas of marrow [27–29]. MM patients most often present with relatively uniform marrow infiltration, the degree of which depends upon the growth rate of myeloma cells and the time elapsed after the transformation to myeloma. To determine the effects of myeloma infiltration on marrow erythropoiesis, a small number of myeloma cells are added to the model of normal erythropoiesis in Supporting Information Fig.

 doi:10.1002/ajh.24291

2C. In early stages of myeloma infiltration, EBIs are only slightly affected, and they continue normal erythropoietic function. The myeloma cells, however, will proliferate at a given rate, producing similar myeloma cells after each division. Infiltrating myeloma cells disrupt EBIs by: (1) producing FL or similar cytokines such as TRAIL that induce erythroid cell apoptosis and (2) physical destruction of EBIs. Supporting Information Figure 2C shows both of these processes modeled in two dimensions in a section of bone marrow infiltrated by myeloma cells.

Mathematical modeling of MM patients' responses to chemotherapy. LBD effectively treated MM, and repeated bone marrow biopsies during the four cycles of chemotherapy were not justified in patients who had progressive decreases in serum monoclonal immunoglobulins and free light chains. Therefore, monoclonal immunoglobulin and light chain concentrations were used to determine the relative size of myeloma cell populations in marrow during the course of chemotherapy. In simulations, myeloma cell numbers were determined by differential equations based on changes in paraprotein concentrations at the beginning of each cycle of chemotherapy. Rates of serum paraprotein production per myeloma cell were considered constant and rates of serum paraprotein decay were based on rates reported for each specific protein class (see Supporting Information Methods). RBC numbers served as measures of erythropoiesis.

Like the LBD protocol [23], we modeled four 28-day cycles of treatment, each one consisting of two weeks of drug administration followed by two weeks without any treatment. The drug concentration in the bone marrow was considered as constant during first two weeks of each cycle and zero during the next two weeks. In simulations, achievement of sufficient intracellular drug concentrations that killed myeloma cells was based on concentration and time of drug exposure in the marrow for individual myeloma cells that had constant rates of drug influx and drug degradation (see Supporting Information Methods). Two variations of the LBD protocol that delivered more or less chemotherapy were also simulated (Supporting Information Fig. 4).

Results

Myeloma infiltration of marrow correlates negatively with hemoglobin, RBCs, and marrow erythropoiesis

At diagnosis, marrow biopsies of MM patients demonstrated that the percentage of marrow space infiltrated with myeloma cells correlated negatively with hemoglobin (Hb) levels ($r = -0.73$; $P < 0.05$) and RBC numbers ($r = -0.74$; $P < 0.01$) (Fig. 1A, upper panels). On the other hand, WBC and platelet counts were all within the normal ranges and not correlated with marrow infiltration by myeloma (Fig. 1A, lower panels). Figure 1B, left panel shows that at diagnosis, the percentages of marrow space occupied by myeloma and by erythroid cells were negatively correlated ($r = -0.69$; $P < 0.05$). The half of the group of patients with higher percentages of myeloma infiltration (more than 30%) had fewer marrow erythroid cells and slight to moderate anemias, while those with less than 30% of myeloma infiltration had more marrow erythroid cells and normal or very slightly decreased Hb and RBCs at diagnosis (Fig. 1A, upper panel and 1B, left panel). These results for peripheral blood cells led to examinations of the effects of marrow infiltration on erythropoiesis by comparing results for the group of eight patients with less than 30% infiltration with results for the seven patients with more than 30% infiltration.

Chemotherapy with lenalidomide, bortezomib, and dexamethasone (LBD) that removes infiltrating myeloma cells increases marrow erythroid cells

After completion of LBD chemotherapy, all patients had greatly reduced percentages of marrow space occupied by myeloma. One

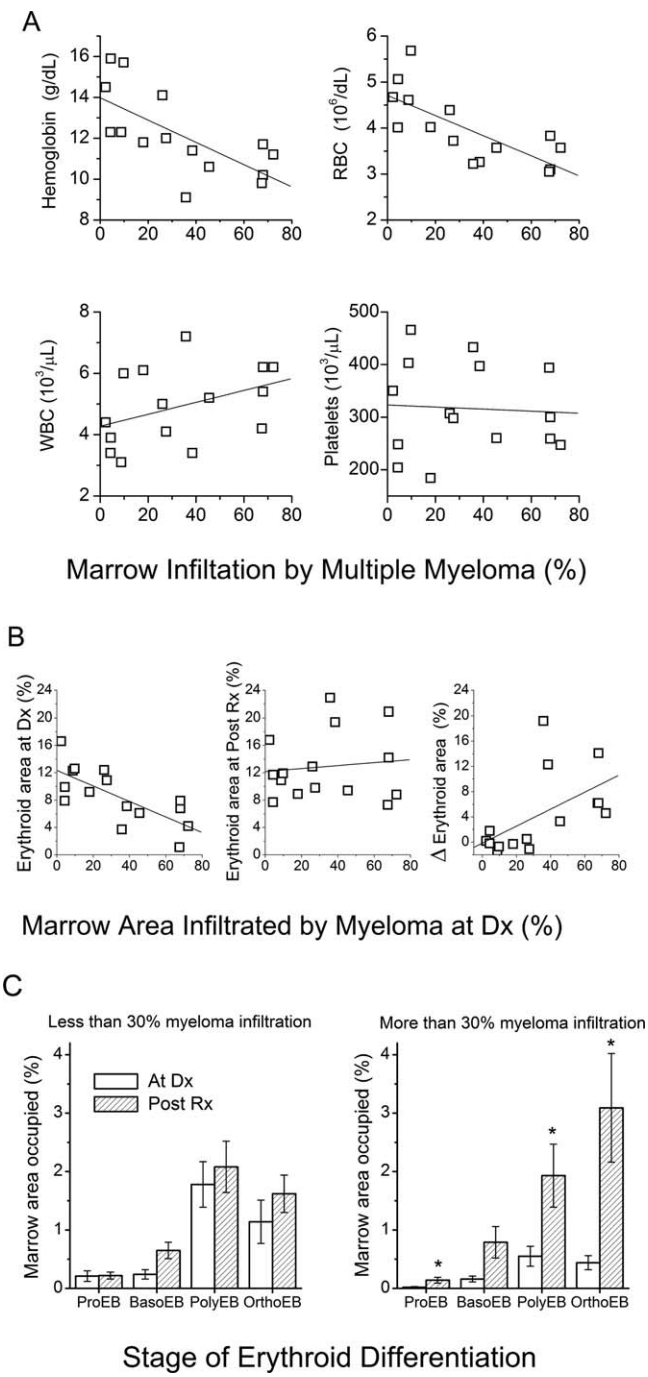

Figure 1. Hemoglobin values, RBCs, and marrow erythroblasts are negatively correlated with marrow area infiltrated by multiple myeloma (MM) at diagnosis, but erythroblasts recover after lenalidomide, bortezomib, and dexamethasone (LBD) chemotherapy. (A) At the time of MM diagnosis, Hb levels ($r = -0.73$; $P < 0.01$) and RBC numbers ($r = -0.74$; $P < 0.01$) in the blood were negatively correlated with the percentages of marrow area occupied by myeloma cells (upper panels). However, leukocyte numbers (WBC; $r = 0.40$, $P = 0.14$) and platelet numbers ($r = -0.06$, $P = 0.84$) at diagnosis were all within normal limits and not correlated with myeloma infiltration (lower panels). (B) In diagnostic bone marrow biopsies, percentages of marrow areas occupied by erythroblasts were negatively correlated with percentages of marrow area occupied by myeloma cells ($r = -0.69$; $P < 0.05$) (left panel). After LBD chemotherapy, percentages of marrow area occupied by erythroid cells no longer correlated with percentages of areas infiltrated by myeloma cells at diagnosis ($r = 0.11$; $P = 0.69$) (middle panel). The changes in percentage of marrow area occupied by erythroblasts between diagnosis and completion of LBD chemotherapy were correlated with the respective percentages of areas infiltrated by myeloma cells at diagnosis ($r = 0.56$; $P < 0.05$) (right panel). (C) After LBD chemotherapy, percentages of marrow occupied by specific erythroblastic stages are not increased in patients with less than 30% myeloma infiltration at diagnosis (left panel), but they are significantly increased in patients with greater than 30% infiltration with myeloma at diagnosis (right panel). Open bars are results from the marrows at diagnosis and hatched bars are results from the marrows after completing remission induction treatment. Data means ± 1 SEM. *statistically significant increases ($P < 0.05$, by Student's t test) in the post-treatment marrows compared to the diagnostic marrows.

patient had a reduction in marrow area occupied by myeloma from 27.5 to 5.3%, whereas all other patients had reduction in marrow areas occupied by myeloma to 2.2% or less. The percentages of marrow area occupied by erythroid cells after LBD chemotherapy were not correlated with the areas infiltrated by myeloma cells at diagnosis (Fig. 1B, middle panel). In Fig. 1B, the right panel shows that the changes in marrow areas occupied by erythroid cells before and after LBD chemotherapy were correlated with areas infiltrated by myeloma cells at diagnosis ($r = 0.56$; $P < 0.05$), indicating that those patients with greater percentages of myeloma infiltration at diagnosis had larger increases in areas of erythroid cells after LBD treatment than those patients with lesser amounts of infiltration at diagnosis. Indeed, eight patients with less than 30% marrow infiltration by myeloma at diagnosis had little change (−1.4% to 1.8%) in the total marrow space occupied by erythroid cells after the treatment (Fig. 1B, right panel). On the other hand, seven patients with more than 30% marrow space infiltrated by myeloma at diagnosis had increases in total marrow space occupied by erythroid cells ranging from 3.4 to 19.2% following LBD treatment. Supporting Information Figure 3 shows photomicrographs of sections of marrow from two patients with greater than 30% marrow infiltration at diagnosis and the same patients' marrows after completion of LBD chemotherapy. In the marrows at diagnosis, the infiltrating myeloma can be seen disrupting the normal marrow structure, whereas the marrows after treatment show restoration of normal marrow structure including foci of erythroblasts as found in EBIs. Analysis of the stages of differentiation of erythroblasts in the marrows at diagnosis and after completion of LBD showed that all stages of differentiation were affected by myeloma infiltration Fig. 1C). Patients with less than 30% myeloma infiltration at diagnosis did not show a significant increase for any stage of erythroid differentiation (Fig. 1C, left panel), whereas those with greater than 30% myeloma infiltration at diagnosis had mean percentages of ProEBs, BasoEBs, and OrthoEBs that were significantly increased after LBD treatment (Fig. 1C, right panel).

Changes in serum monoclonal proteins, hemoglobin values, and RBC numbers during the course of chemotherapy for MM

Between the marrow biopsies at diagnosis and after completion of chemotherapy, the changes in serum paraproteins produced by myeloma cells and RBC numbers in blood were used to model the changes in marrow myeloma and erythroid cell populations. Serum monoclonal immunoglobulin or free light chain concentrations are shown at diagnosis (Dx), on cycle 2 day 1 (C2D1), cycle 3 day 1 (C3D1), cycle 4 day 1 (C4D1), and after completion (Post Rx) of LBD chemotherapy for patients with less than 30% myeloma infiltration of the marrow at diagnosis in Fig. 2A, upper row of panels and for patients with greater than 30% myeloma infiltration in Fig. 2A, lower row of panels. As expected with LBD [23], most patients had large decreases in their serum monoclonal immunoglobulin proteins and free light chains after the first cycle of therapy that were followed by further but smaller decreases after subsequent cycles.

Hb levels (Fig. 2B, upper panels) and RBCs (Fig. 2B, lower panels) are shown at Dx, on C2D1, C3D1, C4D1, and Post Rx. Patients with greater than 30% myeloma infiltration at diagnosis had lower mean Hb values when compared to patients with less than 30% myeloma infiltration at diagnosis (10.6 ± 0.4 g/dL vs. 13.6 ± 0.6 g/dL; $P < 0.001$), and when compared to their own Post Rx values (10.6 ± 0.4 g/dL vs. 12.1 ± 0.5 g/dL; $P < 0.05$). Similarly, patients with greater than 30% myeloma infiltration at diagnosis had lower mean RBCs when compared to patients with less than 30% myeloma infiltration at diagnosis ($3.37 \pm 0.4 \times 10^6$/μL vs. 4.52 ± 0.22 10^6/μL; $P < 0.001$) and when compared to their own Post Rx values ($3.37 \pm 0.4 \times 10^6$/μL vs. $4.09 \pm 0.20 \times 10^6$/μL; $P < 0.05$). Therefore, increased erythropoiesis following LBD treatment in patients with greater than 30% myeloma infiltration at diagnosis increased Hb and RBCs, indicating that myeloma infiltration of marrow played a major role in the development of anemia in these patients, who had no evidence for other common causes of anemia, such as bleeding, renal insufficiency, iron deficiency, vitamin B12 deficiency, myelodysplasia, and myelofibrosis.

Treatment greatly decreased the marrow myeloma infiltration in all patients. However, patients with less than 30% myeloma infiltration at diagnosis had values for marrow erythroid cells, Hbs, and RBCs after treatment that were essentially unchanged from pretreatment baselines, as compared to the patients with greater than 30% marrow myeloma infiltration who had statistically significant increases in all three parameters after treatment. Because the group with less than 30% infiltration at diagnosis had normal or nearly normal marrow erythroid cell, Hb, and RBC values, they had less likelihood of improvement in marrow erythroid cells, Hbs, and RBCs than the group with greater than 30% marrow infiltration.

Modeling myeloma cell death with chemotherapy

For mathematical modeling of the patients' responses to LBD chemotherapy, parameters in differential equations (see Supporting Information) were chosen to fit the clinical data in Fig. 2A. Supporting Information Figure 4 shows application of the mathematical model in simulations comparing the LBD protocol with two variations. The LBD2 protocol intensifies therapy by administering in week one of each cycle the total LBD doses normally given over two weeks. The BD protocol reduces intensity by using two drugs while increasing the number of cycles from four to five within the similar 112-day period. Based on these simulations, LBD2 would be most effective at clearing myeloma from the marrow. However, in practice it would be highly neurotoxic. Less intensified therapy with BD would be less effective than LBD at clearing myeloma from the marrow.

Figure 3 shows results for three virtual patients receiving simulated treatment with the LBD protocol. The three virtual patients differed in the proliferation rate of their respective myeloma cells, with patient A having the highest proliferation rate ($P = 0.617$), patient B having an intermediate rate ($P = 0.616$) and patient C having the lowest ($P = 0.615$). Numbers of myeloma cells and concentrations of monoclonal proteins decrease during drug administration (weeks 1 and 2 of each cycle) and, depending upon proliferation rate, they can increase during weeks 3 and 4 of each cycle, which are without treatment. Responses of specific monoclonal proteins and their respective immunoglobulin class are shown in Fig. 3. Due to stochasticity in cell division, the concentration of monoclonal protein for some patients can be greater in the beginning of the third cycle when compared to the second cycle (Fig. 3A, patient A).

Changes in marrow erythroid cell populations during MM infiltration

Figure 4A depicts simulations using the model of erythroid cell apoptosis and destruction of erythroblastic islands by infiltrating myeloma cells in two virtual patients with greater than 30% marrow infiltration at the time of diagnosis who have complete remission with LBD treatment. At time zero (t_0), myeloma cells were introduced into simulated normal marrow sections. In Fig. 4A, myeloma cell numbers proliferated between t_0 and the time of diagnosis and initiation of LBD chemotherapy, which both occurred on C1D1. Introduction of 28 myeloma cells at t_0 in the marrow section depicted by the black curve in Fig. 4A resulted in more myeloma cells infiltrating the marrow than in the simulation depicted by the gray curve, which had 22 myeloma cells introduced at t_0. Furthermore, the simulation shown by the black curve had three EBIs destroyed at C1D1

 doi:10.1002/ajh.24291

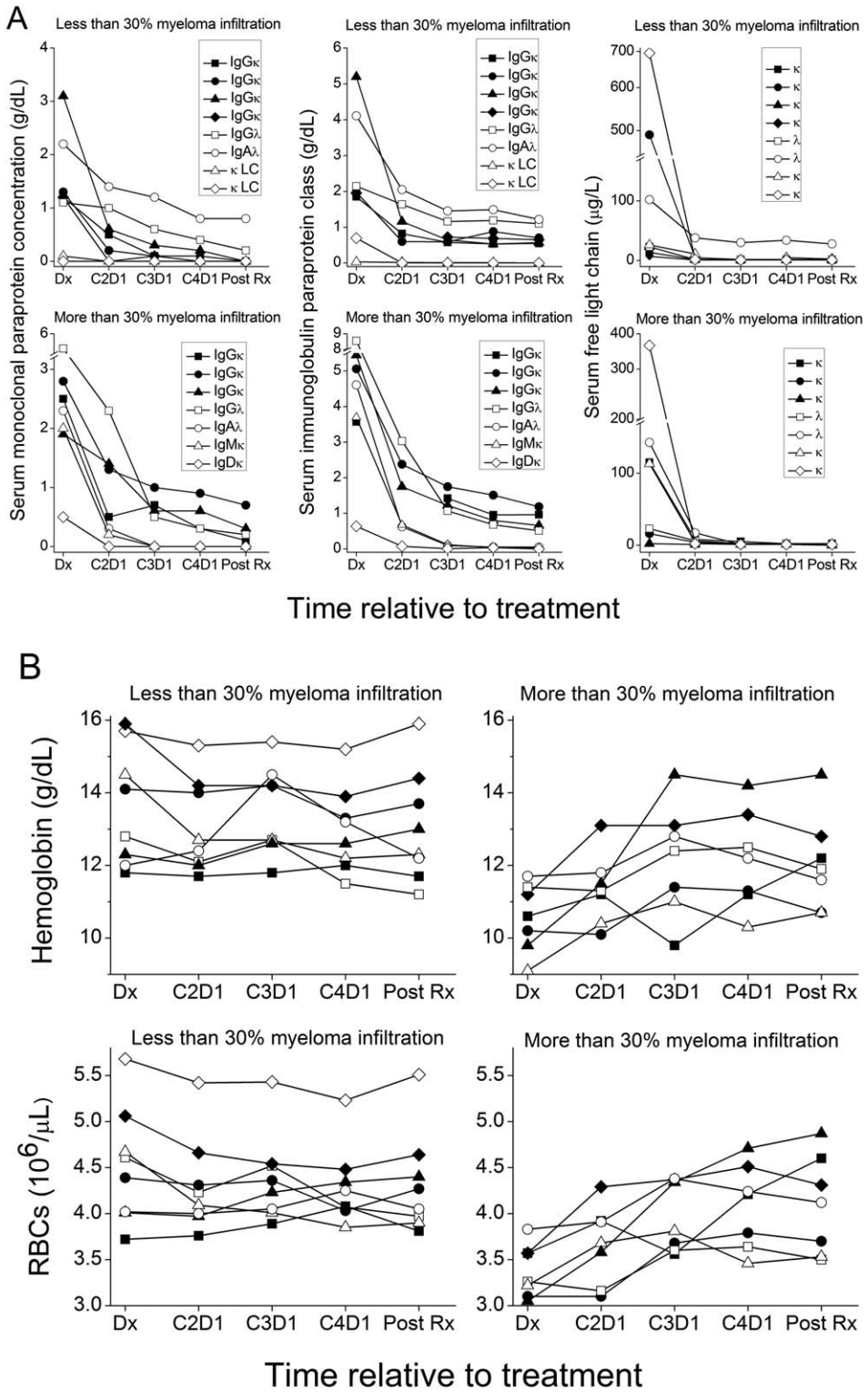

Figure 2. As serum levels of monoclonal paraproteins produced by myeloma cells decline during the LBD treatment of newly diagnosed MM patients, hemoglobin levels and RBC numbers increase in patients with greater than 30% myeloma infiltration at diagnosis. (A) Serum paraprotein values are shown in patients with less than 30% marrow area infiltrated with myeloma at diagnosis (upper panels) and in patients with greater than 30% marrow infiltrated at diagnosis (lower panels). Changes during treatment are shown for serum concentrations of monoclonal immunoglobulin paraproteins measured by electrophoresis with immunofixation (left panels), total immunoglobulin proteins in the class of the respective monoclonal immunoglobulin or light chain (middle panels), and free light chains (right panels) in newly diagnosed patients with MM who were treated with LBD. (B) During treatment with LBD, mean Hb levels did not change in patients with less than 30% myeloma cell infiltration (upper left panel), but increased in patients with greater than 30% myeloma infiltration, who had lower Hb values at diagnosis (upper right panel). Similarly, during treatment with LBD, mean RBC numbers did not change significantly in patients with less than 30% myeloma cell infiltration (lower left panel), but did increase in patients with greater than 30% myeloma infiltration, who had lower mean RBC numbers at diagnosis (lower right panel). In panels A and B, each curve represents a single patient. Times relative to treatment are: Dx, diagnosis; C2D1, cycle 2 day 1; C3D1, cycle 3 day 1; C4D1, cycle 4 day 1: Post Rx, after treatment completed.

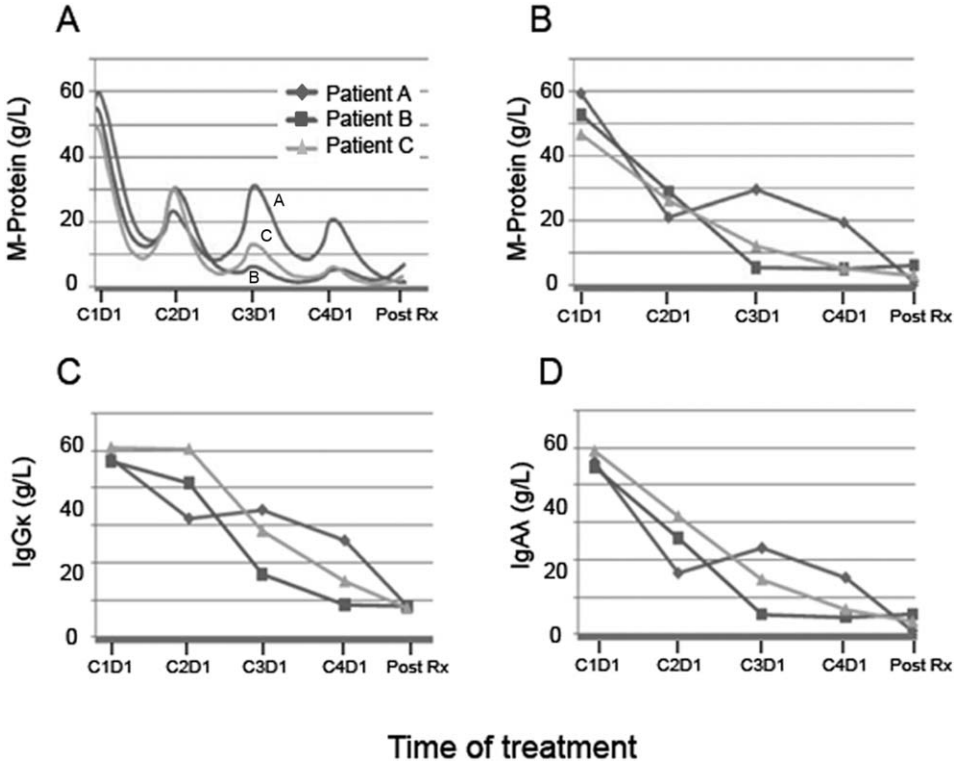

Figure 3. Numeric simulations of monoclonal immunoglobulin concentrations in serum. Monoclonal protein concentrations in three virtual patients as measured (A) daily and (B) on day 1 of each cycle of treatment in numerical simulations of myeloma treatment with protocol LBD (based on data in Fig. 2A). Serum concentrations of (C) immunoglobulin G_κ and (D) immunoglobulin A_λ in numerical simulations for the same three virtual patients as in (A) and (B).

compared with only two EBIs destroyed in the simulation shown by the gray curve. In the erythroid cell recoveries shown in Fig. 4A, the simulation with increased numbers of introduced myeloma cells (black curve) has similar patterns for CFU-E + erythroblasts and reticulocytes as the simulation with fewer introduced myeloma cells (gray curve), except that the black curve recovery was delayed for one cycle of chemotherapy due to greater numbers of residual myeloma cells at C2D1 compared to the gray curve simulation. Marrow reticulocytes were also decreased in both simulations at C1D1 with slightly delayed recovery in the marrow section with more infiltrating myeloma (Fig. 4A). Continual entry of reticulocytes into the blood resulted in no accumulations above baseline as occurred transiently in CFU-E+ erythroblasts in the simulated sections.

Figure 4B–F show marrow sections depicted in two dimensions from the gray curve simulation in Fig. 4A. Prior to the introduction of myeloma cells at time zero (t_0), the marrow section has eight normal EBIs (Fig. 4B). In Fig. 4C, on C1D1, the marrow section has moderate infiltration by myeloma (light blue cells) at left of center and in the lower right, with loss of erythroid cells from nearby EBIs and complete destruction of two EBIs. By C1D15, LBD chemotherapy administered at C1D1 killed a majority of myeloma cells, and one central macrophage has regenerated and interacts with two erythroid progenitors (Fig. 4D). By C2D1, this EBI has been repopulated with erythroid cells and a second central macrophage has regenerated (Fig. 4E). At C3D1, no myeloma cells are present and the EBIs have fully recovered (Fig. 4F), with erythroid cell numbers slightly more than baseline. After 10 days of LBD therapy, the marrow EBIs have returned to normal steady-state (marrow section not shown).

Discussion

The clinical data and mathematical model developed from them are consistent with previous reports of flow cytometry of clinical specimens [2] and a mouse model of myeloma [20] that showed anemia prevalence and decreased marrow erythroid precursor cells are related to the amount of marrow infiltration by myeloma cells. Maintenance of normal WBC and platelets (Fig. 1A) indicates that myeloma infiltration of marrow specifically affects erythropoiesis. Decreased marrow erythroid cells at the stages of differentiation found in EBIs (Fig. 1C) and their prompt recovery after LBD chemotherapy support the model hypotheses that cytokines from myeloma cells induce erythroid cell apoptosis and myeloma cells physically destroy EBIs. FL and TRAIL expressed on the surface and secreted from myeloma cells [17] induce apoptosis of EPO-dependent erythroid cells in the EBIs, thereby reducing EPO-dependent cells and the later stage erythroblasts that develop from them. The negative correlation between myeloma infiltration and erythroid cells ($r = -0.69$) in Fig. 1B, left panel is strikingly similar to the negative correlation between myeloma infiltration and total marrow macrophages ($r = -0.63$) reported by Sadahira et al. [21], suggesting that the physical disruption of EBIs plays a major role in anemia caused by myeloma infiltration. *In vitro*, erythroblasts that have lost physical contact with macrophages proliferate more slowly [30,31], and they have increased apoptotic rates [31].

In Fig. 4, simulations show destroyed EBIs replaced by regenerated macrophages that interact with BFU-Es. Macrophage regeneration from monocyte–macrophage progenitors is completed in less than 1 week in anemic mice that have had macrophages killed by liposomal clodronate [32] or high heme concentrations [33]. *In vitro* coculture experiments of human macrophages and erythroid cells have used macrophages that differentiated from blood monocytes in 1 week [31,34]. BFU-E are considered the source of erythroid cells in EBIs based on results in mice demonstrating that most BFU-Es are not located in EBIs, but most CFU-Es are [32,35]. Thus, BFU-Es appear to differentiate rapidly into CFU-E after they encounter a central macrophage [24]. Erythroid cell regeneration in simulations (Fig.

 doi:10.1002/ajh.24291

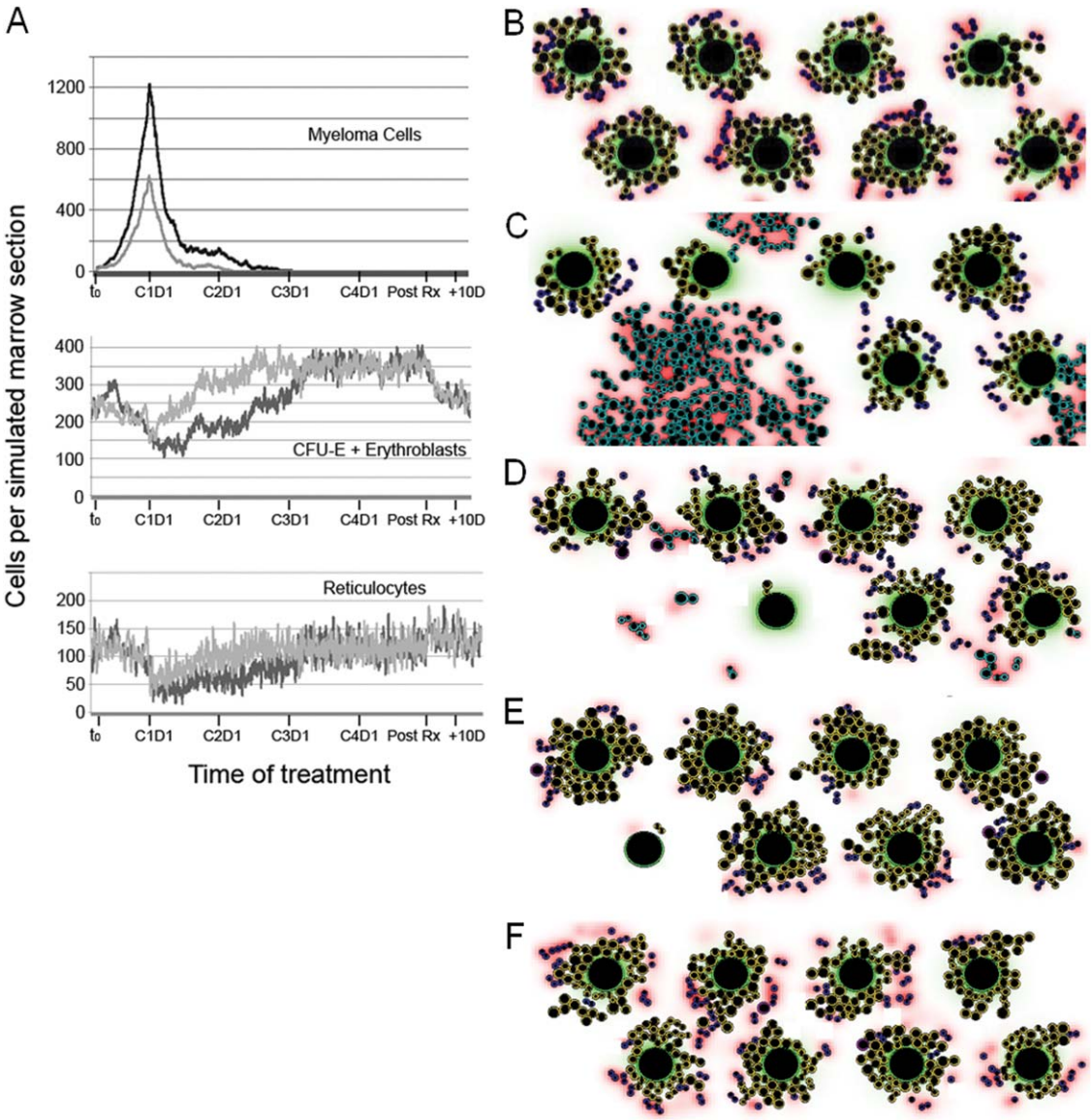

Figure 4. Effects on erythropoiesis of simulated marrow infiltration by multiple myeloma with complete remission following LBD therapy. (A) Simulated numbers of myeloma and erythroid cells in a section of marrow that had eight EBIs prior to the introduction of myeloma cells at time zero (t_0). The results are shown for daily numbers of myeloma cells and erythroid cells in the section, with the gray curves representing a simulation initiated with 22 cells and the black curves representing a simulation initiated with 28 cells at t_0. Diagnosis and initiation of LBD treatment is on day 23 after t_0, and it is designated as C1D1. Four 28-day cycles of LBD therapy are administered. Time points: t_0, C1D1, C2D1, C3D1, C4D1, and day 1 and day 10 after completion of the fourth cycle of therapy (Post Rx and +10D, respectively). Images of the marrow section corresponding to simulation results shown as the gray curves in (A) are shown for the following times: (B) t_0, immediately before myeloma cells are introduced, (C) Dx/C1D1, (D) C1D15, (E) C2D1, and (F) C3D1. Central macrophages are the large central cells in the EBIs. CFU-E and erythroblasts are the yellow cells surrounding the central macrophages. Marrow reticulocytes prior to their entry into the blood are dark blue on the periphery of the EBIs. Myeloma cells are light blue. Black solid circles inside cells show their incompressible parts. Secreted proteins shown extracellularly are green for BMP4 and/or SCF produced by central macrophages and red for FAS ligand produced by mature erythroblasts and reticulocytes within EBIs, and FAS ligand and/or TRAIL produced by infiltrating myeloma cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4A) is based on experiments showing that EBIs depopulated of later stage erythroblasts by hypertransfusion-induced erythropoietic suppression will expand rapidly after EPO administration [6]. Reticulocytes produced by the increased erythroid cells are promptly released into the blood where they increase Hb levels and RBCs in a manner consistent with the clinical data in Fig. 2B.

Elevated levels of EPO decrease FAS expression by CFU-E and ProEBs [15], thereby inhibiting apoptosis of these erythroid progenitors [14]. However, EPO levels are low in MM patients due to inflammatory cytokines and renal toxicity of paraproteins [13], and FAS ligand concentrations are increased due to secretion by myeloma cells [18]. As would be expected, exogenous EPO administration has been shown to improve anemia in patients with myeloma [36]. Our results indicate that a patient with moderately extensive myeloma infiltration of marrow and moderately severe anemia at diagnosis, who has no other identified factor contributing to the anemia, may expect that anemia will improve, if serum paraprotein concentrations decrease following the first cycle of LBD chemotherapy. In such a patient, this expected erythropoietic response would have the potential to reduce not only usage of EPO or other erythropoietic stimulating agents, but it could also decrease RBC transfusions.

The required physical interaction of macrophages and erythroid progenitors for optimal erythropoiesis is likely to have an important role in the increased susceptibility of erythropoiesis compared to

granulopoiesis and thrombopoiesis in marrow infiltrative diseases. The improved erythropoiesis with LBD chemotherapy suggests that the model described here for marrow infiltrated by myeloma may apply to nonerythrotoxic treatments of other marrow-infiltrating hematopoietic malignancies. In this regard, dexamethasone and lenalidomide have been reported to promote in vitro growth of human BFU-E and CFU-E, respectively, including when cocultured with macrophages [34]. Furthermore, thalidomide, a drug with similar activity as lenalidomide, decreased erythroblast production of FAS, TRAIL, and their respective receptors in myeloma patients [37]. Nonerythrotoxic therapies of other marrow infiltrative diseases may have a similar pattern of erythropoietic recovery, but will require longer recovery periods without the salutary effects on erythroid cell growth produced by dexamethasone and lenalidomide. Thus, the model may apply to the nonerythrotoxic therapy of chronic myeloid leukemia with imatinib, in which reduction of marrow infiltration by excess myeloid cells has been associated with increases in erythroblasts [38]. Similarly, rituximab, a nonerythrotoxic treatment, can decrease chronic lymphocytic leukemia cells that are inversely correlated with Hb when infiltrating the marrow [39].

Acknowledgment

The authors thank Dr. Madan Jagasia for his helpful discussions and identification of subjects in the NCT01215344 clinical trial as the source of data reported here and used in developing the model.

References

1. Beguin Y, Yerna M, Loo M, et al. Erythropoiesis in multiple myeloma: defective red cell production due to inappropriate erythropoietin production. Br J Haematol 1992;82:648–653.
2. Fossa A, Brandhorst D, Myklebust JH, et al. Relation between S-phase fraction of myeloma cells and anemia in patients with multiple myeloma. Exp Hematol 1999;27:1621–1626.
3. Ocio EM, Richardson PG, Rajkumar SV, et al. New drugs and novel mechanisms of action in multiple myeloma in 2013: A report from the International Myeloma Working Group (IMWG). Leukemia 2014;28:525–542.
4. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. Blood 2008;112:470–478.
5. Manwani D, Bieker JJ. The erythroblastic island. Curr Top Dev Biol 2008;82:23–53.
6. Mohandas N, Prenant M. Three-dimensional model of bone marrow. Blood 1978;51:633–643.
7. Muta K, Krantz SB, Bondurant MC, Dai CH. Stem cell factor retards differentiation of normal human erythroid progenitor cells while stimulating proliferation. Blood 1995;86:572–580.
8. Xiang J, Wu DC, Chen Y, Paulson RF. *In vitro* culture of stress erythroid progenitors identifies distinct progenitor populations and analogous human progenitors. Blood 2015;125:1803–1812.
9. Panzenbock B, Bartunek P, Mapara MY, Zenke M. Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells *in vitro*. Blood 1998;92:3658–3668.
10. von Lindern M, Zauner W, Mellitzer G, et al. The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors *in vitro*. Blood 1999;94:550–559.
11. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 1990;248:378–381.
12. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 1995;83:59–67.
13. Koury MJ. Abnormal erythropoiesis and the pathophysiology of chronic anemia. Blood Rev 2014;28:49–66.
14. De Maria R, Testa U, Luchetti L, et al. Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood 1999;93:796–803.
15. Liu Y, Pop R, Sadegh C, et al. Suppression of Fas-FasL coexpression by erythropoietin mediates erythroblast expansion during the erythropoietic stress response *in vivo*. Blood 2006;108:123–133.
16. Bruns I, Cadeddu RP, Brueckmann I, et al. Multiple myeloma-related deregulation of bone marrow-derived CD34(+) hematopoietic stem and progenitor cells. Blood 2012;120:2620–2630.
17. Silvestris F, Cafforio P, Tucci M, Dammacco F. Negative regulation of erythroblast maturation by Fas-L(+)/TRAIL(+) highly malignant plasma cells: A major pathogenetic mechanism of anemia in multiple myeloma. Blood 2002;99:1305–1313.
18. Silvestris F, Tucci M, Cafforio P, Dammacco F. Fas-L up-regulation by highly malignant myeloma plasma cells: role in the pathogenesis of anemia and disease progression. Blood 2001;97:1155–1164.
19. Maes K, Nemeth E, Roodman GD, et al. In anemia of multiple myeloma, hepcidin is induced by increased bone morphogenetic protein 2. Blood 2010;116:3635–3644.
20. Bordini J, Bertilaccio MT, Ponzoni M, et al. Erythroblast apoptosis and microenvironmental iron restriction trigger anemia in the VK*MYC model of multiple myeloma. Haematologica 2015;100:834–841.
21. Sadahira Y, Wada H, Manabe T, Yawata Y. Immunohistochemical assessment of human bone marrow macrophages in hematologic disorders. Pathol Int 1999;49:626–632.
22. Eymard N, Bessonov N, Gandrillon O, et al. The role of spatial organization of cells in erythropoiesis. J Math Biol 2015;70:71–97.
23. Richardson PG, Weller E, Lonial S, et al. Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. Blood 2010;116:679–686.
24. Koury MJ. Tracking erythroid progenitor cells in times of need and times of plenty. Exp Hematol 2015, doi:10.1016/j.exphem.2015.10.007 (in press).
25. Landgren O, Kyle RA, Pfeiffer RM, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: A prospective study. Blood 2009;113:5412–5417.
26. Weiss BM, Abadie J, Verma P, et al. A monoclonal gammopathy precedes multiple myeloma in most patients. Blood 2009;113:5418–5422.
27. Anderson KC, Carrasco RD. Pathogenesis of myeloma. Annu Rev Pathol 2011;6:249–274.
28. Kuehl WM, Bergsagel PL. Molecular pathogenesis of multiple myeloma and its premalignant precursor. J Clin Invest 2012;122:3456–3463.
29. Bianchi G, Munshi NC. Pathogenesis beyond the cancer clone(s) in multiple myeloma. Blood 2015;125:3049–3058.
30. Rhodes MM, Kopsombut P, Bondurant MC, et al. Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin. Blood 2008;111:1700–1708.
31. Ramos P, Casu C, Gardenghi S, et al. Macrophages support pathological erythropoiesis in polycythemia vera and beta-thalassemia. Nat Med 2013;19:437–445.
32. Sadahira Y, Yasuda T, Yoshino T, et al. Impaired splenic erythropoiesis in phlebotomized mice injected with CL2MDP-liposome: An experimental model for studying the role of stromal macrophages in erythropoiesis. J Leukoc Biol 2000;68:464–470.
33. Haldar M, Kohyama M, So AY, et al. Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. Cell 2014;156:1223–1234.
34. Narla A, Dutt S, McAuley JR, et al. Dexamethasone and lenalidomide have distinct functional effects on erythropoiesis. Blood 2011;118:2296–2304.
35. Sadahira Y, Mori M. Role of the macrophage in erythropoiesis. Pathol Int 1999;49:841–848.
36. Cazzola M, Messinger D, Battistel V, et al. Recombinant human erythropoietin in the anemia associated with multiple myeloma or non-Hodgkin's lymphoma: Dose finding and identification of predictors of response. Blood 1995;86:4446–4453.
37. Grzasko N, Dmoszynska A, Hus M, Soroka-Wojtaszko M. Stimulation of erythropoiesis by thalidomide in multiple myeloma patients: its influence on FasL, TRAIL and their receptors on erythroblasts. Haematologica 2006;91:386–389.
38. Hasserjian RP, Boecklin F, Parker S, et al. ST1571 (imatinib mesylate) reduces bone marrow cellularity and normalizes morphologic features irrespective of cytogenetic response. Am J Clin Pathol 2002;117:360–367.
39. Tsopra OA, Ziros PG, Lagadinou ED, et al. Disease-related anemia in chronic lymphocytic leukemia is not due to intrinsic defects of erythroid precursors: A possible pathogenetic role for tumor necrosis factor-alpha. Acta Haematol 2009;121:187–195.