

Bone Marrow Megakaryocytes May Predict Therapeutic Response of Severe Thrombocytopenia in Patients with Systemic Lupus Erythematosus

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ABSTRACT. Objective. To analyze the predictive value of megakaryocyte counts in bone marrow (BM-MK) for determining the therapeutic response of severe thrombocytopenia (TP) in patients with systemic lupus erythematosus (SLE).

Methods. Thirty-five patients with SLE with severe TP (platelet count $\leq 50 \times 10^9/l$) from the Peking Union Medical College Hospital admitted between 2007 and 2014 with appreciable bone marrow aspiration results were analyzed retrospectively. The associations between therapeutic response and clinical manifestations, laboratory findings including BM-MK counts, were evaluated.

Results. Seventeen (49%) and 8 (23%) patients achieved a complete response (CR) and a partial response (PR), respectively, and 10 had no response (NR). The BM-MK counts in each group were 102 ± 25 (0–322), 136 ± 48 (2–419), and 28 ± 12 (0–105) per slide, respectively. Significant differences were observed in the counts of BM-MK between patients who achieved a clinical response (CR + PR) and those who did not (NR; $p = 0.007$). Patients in the NR group exhibited fewer BM-MK compared with those in the CR and PR groups ($p = 0.017$ and $p = 0.006$, respectively). A receiver-operation characteristic analysis identified that a cutoff value of BM-MK counts at 20 performed pretty well in discriminating patients with differential responses to immunotherapy, with sensitivity and specificity and area under the curve of 88%, 70%, and 0.798, respectively.

Conclusion. BM-MK count may serve as a good predicting factor for immunotherapeutic response in patients with SLE with severe TP. Patients with BM-MK counts < 20 per slide tend to exhibit poor clinical response. (J Rheumatol First Release May 1 2016; doi:10.3899/jrheum.150829)

Key Indexing Terms:

SYSTEMIC LUPUS ERYTHEMATOSUS
BONE MARROW

THROMBOCYTOPENIA
MEGAKARYOCYTE

Systemic lupus erythematosus (SLE) is a classic, diffuse, connective tissue disease that is characterized by multisystem involvement and a diverse autoantibody spectrum¹. Hematological involvement is very common. About 10%–40% of patients with SLE develop thrombocytopenia (TP) in their disease course^{2,3,4,5,6}. TP is a reliable marker suggesting active disease^{5,7}. TP may also act as an independent

prognostic predictor for end organ damage and higher mortality^{2,8,9}. Refractory TP in SLE is a big challenge that rheumatologists often encounter. Latent risks of fatal bleeding in patients with severe TP justify active immunotherapy. However, the effectiveness of immunotherapy is difficult to predict because of the complicated underlying mechanisms. The presumed mechanisms include platelet destruction and deletion by autoantibodies in the peripheral circulation, inhibition of the development and maturation of megakaryocytes (MK) in bone marrow, consumption of platelets because of thrombotic microangiopathy/thrombotic thrombocytopenic purpura (TMA/TTP), secondary antiphospholipid syndrome (APS), and other miscellaneous causes such as viral infection (e.g., cytomegalovirus, microvirus, Epstein-Barr virus), concomitant hypersplenism, hemophagocytic syndrome (HPS), myelodysplastic syndrome (MDS), aplastic anemia (AA), or myelofibrosis (MF), as well as drug toxicity^{10,11,12,13}. It is essential to discriminate the relevant causes during clinical practice, which necessitates a bone marrow examination to exclude certain hematological disorders. Routine therapeutic interventions for immunological TP include glucocorticoids, immunosuppressive agents [e.g., cyclosporine, tacrolimus (TAC), cyclophos-

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phamide, and mycophenolate mofetil], intravenous immunoglobulin (IVIG), androgens, plasma exchange, rituximab (RTX), and splenectomy^{14,15}. Most physicians and patients prefer pharmacological treatments prior to a splenectomy. However, many patients with SLE with severe TP respond poorly to immunotherapeutic drugs despite careful exclusion of hematological disorders (such as MDS, AA, and MF), TMA/TTP, and hypersplenism. Repeated aggressive treatments may increase the risk of multiple infections and cause considerable economic loss. Therefore, the identification of markers that predict individual responses to immunotherapy is critical. Our retrospective study evaluated whether MK counts in bone marrow (BM-MK) could serve as possible predictive factors for response to immunotherapy.

MATERIALS AND METHODS

Patients with severe TP (platelet count $\leq 50 \times 10^9/l$)⁸ who were admitted to the Peking Union Medical College Hospital between September 2007 and September 2014 and were diagnosed with SLE according to the 1997 American College of Rheumatology SLE classification criteria¹⁶ were screened in our study. Patients with concomitant hematological disorders, such as MDS, HPS, or AA, or patients with TMA/TTP, APS, or hypersplenism were excluded. Altogether, 35 patients with SLE with appreciable bone marrow smear results (satisfactory bone marrow sampling, no dilution, no aggregation, and detailed records of the numbers and categories of BM-MK) at the time of TP occurrence were included. Glucocorticoid had been prescribed no more than 2 weeks before bone marrow biopsy was done. Other treatments such as IVIG, disease-modifying antirheumatic drugs, or RTX were not used until bone marrow biopsy results were received. Their clinical data were collected and analyzed. Since our study was based on a review of medical records obtained for clinical purposes, the requirement for written informed consent was waived. This retrospective study was approved by the Peking Union Medical College Hospital Ethics Committee review board. Patients' information was anonymized and deidentified prior to analysis.

Determination of MK numbers in bone marrow. For all 35 patients, bone marrow were sampled from posterior superior iliac spine, and at least 6 slides of sampled bone marrow smear were prepared with 2 satisfactory slides for Wright-Giemsa staining. Several subtypes of MK can be identified in bone marrow based on morphology, including naked nucleus MK, granulometric MK (GMK), juvenile MK, and platelet-producing MK.

Definition of clinical response. A complete response (CR) was defined as a sustained platelet count equal to or greater than $100 \times 10^9/l$ for at least 4 weeks during 2 months of therapy. A partial response (PR) was defined as an initial platelet count of $20\text{--}50 \times 10^9/l$ and a sustained platelet count between $50\text{--}100 \times 10^9/l$ for at least 4 weeks during 2 months of therapy. Patients with an initial platelet count $< 20 \times 10^9/l$ and a sustained platelet count of $20\text{--}50 \times 10^9/l$ for at least 4 weeks during 2 months of therapy were also defined as achieving a PR. No response (NR) was defined as the failure to meet the criteria for CR or PR^{17,18}. Relapse was defined as a decrease in platelet count to $< 100 \times 10^9/l$ during followup in patients who had obtained a CR or to $< 20 \times 10^9/l$ for patients who had obtained a PR.

Statistical analysis. Statistical analyses were performed using IBM SPSS Statistics software 19 (IBM Corp.). Continuous variables that were normally distributed were compared using ANOVA or the Student *t* test. Non-normally distributed data were compared using nonparametric tests including the Mann-Whitney *U* test or the Kolmogorov-Smirnov test. The chi-square test was used to compare differences between categorical variables. The cutoff value was established using the receiver-operation characteristic (ROC) method with the best sensitivity and specificity. The results are presented as means \pm standard error of mean (SEM). A *p* value < 0.05 was considered significant.

RESULTS

General clinical data. The mean age of the 35 patients with SLE with severe TP was $34.3 \pm \text{SD } 13.6$ years (range 11–77). The female to male ratio was 30:5. Twenty-four patients (68.6%) experienced disease onset with TP, and the other patients had a median disease duration of 11 months (range 1–87) prior to TP. Mucocutaneous bleeding occurred in 28 patients (80.0%), and serious visceral hemorrhage occurred in 4 patients (11.4%). The minimum platelet count during the treatment course ranged from 0 to $46 \times 10^9/l$ (median $4 \times 10^9/l$). The median platelet count was $13 \times 10^9/l$ at the time of bone marrow sampling. Fifteen patients (42.9%) had concurrent leukopenia, and 17 patients (48.6%) had anemia. Thirteen patients (37%) exhibited cytopenia of all 3 cell lineages. Twelve patients (34.3%) had positive anti-dsDNA antibodies, and 14 patients (40.0%) had positive anti-SSA antibodies. The mean SLE Disease Activity Index (SLEDAI) at the time of bone marrow sampling was 6 (range 1–28).

Therapeutic intervention and clinical sequelae. Seventeen (48.6%) and 8 (22.9%) patients achieved CR and PR, respectively, and 10 patients (28.6%) were in the NR group. No significant differences were seen in age, disease duration, clinical manifestations, autoantibody spectrums, or SLEDAI among the 3 groups, except for a relatively higher platelet count before treatment in the CR group (Table 1). Fifteen patients (3 in NR group, 5 in PR group, and 7 in CR group) were examined for platelet-associated immunoglobulin G (PAIgG) titers. Although the NR group seemed to have higher titers of PAIgG, the difference was not convincing because of the small sample size.

All patients received high doses of corticosteroid (equivalent to prednisone $\geq 0.8\text{--}1$ mg/kg) in combination with immunosuppressive agents. Fifteen patients (43%) received combined immunosuppressive therapy. Twenty patients (57%) received pulse methylprednisolone (MP) of $0.5\text{--}1$ g/d for 3 consecutive days. Five of these patients were in the NR group, 7 of these patients were in the PR group, and 8 in the CR group. No differences were observed among the 3 groups regarding the proportion of patients who received pulse MP treatment. Doses of daily steroids were comparable among the 3 groups (CR vs PR vs NR: 80.9 ± 7.1 vs 87.5 ± 8.1 vs 104 ± 14.2 mg/d, *p* = 0.231; Table 2). Nineteen patients (54%) received IVIG infusion (7, 6, and 6 patients in the NR, PR, and CR groups, respectively). Sixteen patients received a vincristine infusion at least once. Five patients received subcutaneous recombinant thrombopoietin (TPO) injections. RTX was administered in 9 patients (2 in the CR group, 4 in the PR group, and 3 in the NR group). More than half of the patients (18 patients, 51%) in this cohort received a platelet pheresis transfusion. A higher percentage of patients in the NR group received cyclosporine/TAC, as well as platelet pheresis transfusion.

Three patients (all in the NR group) died during followup (1–98 mos, median 7 mos). The causes of death were serious

Table 1. Clinical features of 35 patients with SLE and severe TP. Values are % (n) or mean \pm SEM unless otherwise specified.

Clinical Features	Total, n = 35	CR, n = 17	PR, n = 8	NR, n = 10	p
Demographic data					
Sex, female/male, n	30/5	14/3	8/0	8/2	
Age, yrs	34.3 \pm 2.3	32.8 \pm 3.2	32.6 \pm 3.1	38.4 \pm 5.4	0.550
SLE disease courses, mos	64.1 \pm 12.7	49.1 \pm 16.4	95.6 \pm 35.4	64.2 \pm 20.4	0.365
TP disease courses, mos	56.9 \pm 13	47.1 \pm 16.4	84.3 \pm 37.8	51.8 \pm 20.8	0.526
Clinical data					
Arthralgia/arthritis	40.0 (14)	29.4 (5)	62.5 (5)	40.0 (4)	0.300
Nephritis	45.7 (16)	47.1 (8)	25.0 (2)	60.0 (6)	0.341
Neurological disease	22.9 (8)	29.4 (5)	12.5 (1)	20.0 (2)	0.631
Anemia	48.6 (17)	41.2 (7)	37.5 (3)	70.0 (7)	0.282
Leukopenia	42.9 (15)	35.3 (6)	37.5 (3)	60.0 (6)	0.440
Serositis	17.1 (6)	29.4 (5)	0.0 (0)	10.0 (1)	0.157
Mucocutaneous bleeding	80.0 (28)	64.7 (11)	100.0 (8)	90.0 (9)	0.084
Visceral hemorrhage	11.4 (4)	17.6 (3)	12.5 (1)	0.0 (0)	0.388
Other infections	37.1 (13)	35.3 (6)	12.5 (1)	60.0 (6)	0.121
Laboratory index					
Platelet before treatment	19.5 \pm 3.2	29.1 \pm 5.25	6.1 \pm 1.95	13.7 \pm 3.4	0.010
Anti-dsDNA positivity	34.3 (12)	35.3 (6)	50.0 (4)	20.0 (2)	0.419
Anti-dsDNA, IU/ml	333 \pm 65	370 \pm 127	343 \pm 52	205 \pm 25	0.664
Anti-Sm	5.7 (2)	0.0 (0)	12.5 (1)	10.0 (1)	0.864
Anti-SSA	40.0 (14)	41.2 (7)	25.0 (2)	50.0 (5)	0.565
Anti-SSB	8.6 (3)	11.8 (2)	0.0 (0)	10.0 (1)	0.607
Anti-RNP	17.1 (6)	17.6 (3)	37.5 (3)	0.0 (0)	0.118
aPL	28.6 (10)	29.4 (5)	25.0 (2)	30.0 (3)	0.969
Hypocomplementemia	54.3 (19)	58.8 (10)	50.0 (4)	50.0 (5)	0.875
PAIgG, ng/10 ⁷ platelet, n = 15	2400 \pm 754 (151~8155)	1948 \pm 1070 (165~8155)	662 \pm 262 (151~1523)	6351 \pm 1051 (4272~7660)	0.011
SLEDAI	6.09 \pm 1.08	6.35 \pm 1.61	3.50 \pm 0.80	7.70 \pm 2.53	0.562

SEM: standard error of the mean; SLE: systemic lupus erythematosus; TP: thrombocytopenia; CR: complete response; PR: partial response; NR: no response; aPL: antiphospholipid antibodies; PAIgG: platelet-associated immunoglobulin G; SLEDAI: SLE Disease Activity Index.

Table 2. Therapeutic interventions of severe thrombocytopenia among the 3 groups (CR, PR, and NR) in patients with SLE. Values are n (%) or mean \pm SEM unless otherwise specified.

Therapeutic Interventions	CR, n = 17	PR, n = 8	NR, n = 10	p
Pulse MP	8 (47.0)	7 (87.5)	5 (50.0)	0.141
Average daily steroid dose, mg/d	80.9 \pm 7.1	87.5 \pm 8.1	104 \pm 14.2	0.231
IVIG	6 (35.0)	6 (75.0)	7 (70.0)	0.089
HCQ	7 (41.0)	1 (12.5)	5 (50.0)	0.244
TAC/CSA	5 (29.0)	5 (63.0)	8 (80.0)	0.031
CTX	11 (65.0)	2 (25.0)	7 (70.0)	0.108
RTX	2 (12.0)	4 (50.0)	3 (30.0)	0.117
TPO	0 (0.0)	2 (25.0)	3 (30.0)	0.061
Platelet pheresis transfusion	4 (24.0)	5 (63.0)	9 (90.0)	0.003

CR: complete response; PR: partial response; NR: no response; SEM: standard error of the mean; MP: methylprednisolone; IVIG: intravenous immunoglobulin; TAC: tacrolimus; CSA: cyclosporine; RTX: rituximab; TPO: thrombopoietin; CTX: cyclophosphamide.

infections and SLE relapse. Five patients in the CR group and 1 patient in the PR group had a relapse during followup.

BM-MK and therapeutic responses. Bone marrow smears revealed distinctively active proliferation in 2 patients (5.7%), active proliferation in 28 patients (80%), moderate proliferation in 4 patients (11.4%), and reduced proliferation

in 1 patient (2.9%). The BM-MK counts ranged from 0 to 419/slide (median 56/slide). The BM-MK primarily consisted of the GMK subgroup (proportion $76 \pm 22.7\%$, range 0–369/slide, median 42/slide), and this feature was identical among the CR, PR, and NR groups. The BM-MK counts were significantly higher in the CR group (102 ± 25 /slide, 0–

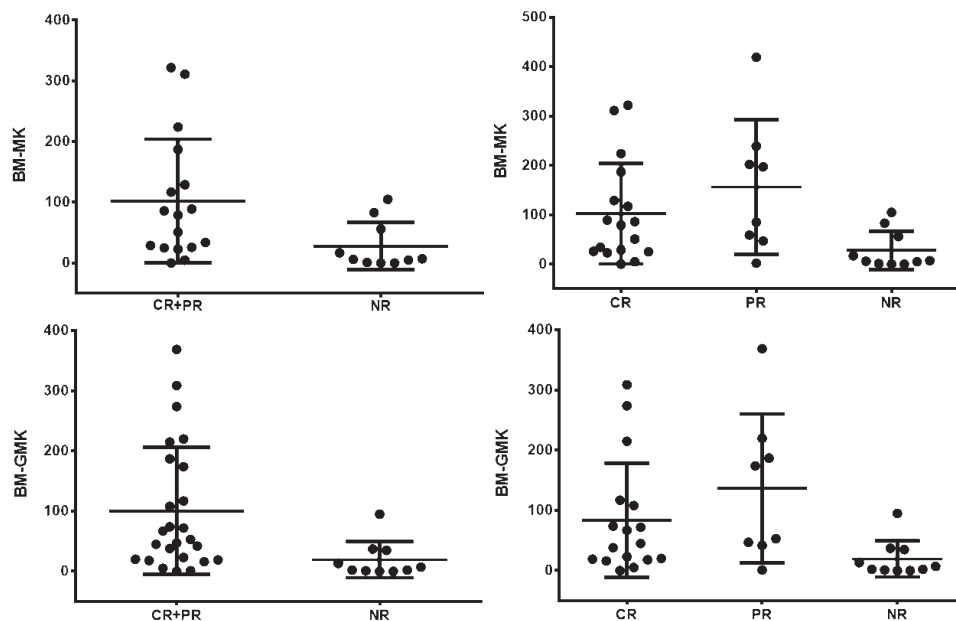


Figure 1. Differential megakaryocyte counts and granulometric megakaryocytes counts in the bone marrow of groups with different clinical responses. BM-MK: megakaryocytes in bone marrow; BM-GMK: granulometric megakaryocytes in bone marrow; CR: complete response; PR: partial response; NR: no response.

322/slide) and the PR group ($136 \pm 48/\text{slide}$, 2–419/slide) compared with the NR group ($28 \pm 12/\text{slide}$, 0–105/slide; $p = 0.017$ and $p = 0.006$, respectively; Figure 1). The GMK counts in bone marrow (BM-GMK) of the 3 groups were 84 ± 23 , 137 ± 44 , and $19 \pm 9.5/\text{slide}$, respectively. BM-GMK counts in patients with clinical responses (CR + PR) were higher than those with no response ($p = 0.003$; Figure 1).

To determine whether the number of BM-MK or BM-GMK could contribute to the prediction of patients' response to treatment, the ROC method was used and the cutoff values of 20/slide for BM-MK and 15/slide for BM-GMK were established, with a sensitivity of 88% and a specificity of 70%. The area under the curve were 0.798 and 0.822, respectively (Supplementary Figure 1, available from the authors on request). The positive predictive value and the negative predictive value were 88% and 70%, respectively. The positive likelihood ratio (LR) was 2.93, and the negative LR was 0.17. When the patients were stratified according to the cutoff value of BM-MK (BM-MK > 20/slide and BM-MK \leq 20/slide), the effective ratios (CR + PR) were 88% and 30%, respectively (Figure 2). The difference was statistically significant ($p = 0.002$). We also performed a logistic, stepwise regression analysis, and demonstrated that patients with BM-MK counts > 20/slide were more likely to exhibit a better clinical response (OR 17.11, 95% CI 2.8–104.8, $p = 0.002$). Average daily dose of steroid immune-suppressants including cyclophosphamide, TAC/cyclosporine, RTX and IVIG, and TPO was not indicative of the clinical responses. Also, in multiple variables logistic stepwise analysis, BM-MK was

the only baseline variable that was associated with clinical responses (OR 1.02, $p = 0.49$); other indices such as SLEDAI, disease course of TP, platelet counts prior to immunotherapy, anti-dsDNA, and hypocomplementemia had no predictive value. One patient repeated bone marrow biopsy 1 month after immunotherapy, and her BM-MK counts before and after treatment were 17 and 9/slide, respectively. This patient was in the NR group with her platelet count continuing to be $< 20 \times 10^9/\text{l}$ (Supplementary Figure 2, available from the authors on request).

DISCUSSION

TP is a common manifestation of patients with SLE, and often predicts poor prognosis^{7,19}. Patients generally receive repeated high-dose steroids and aggressive immune-suppressants to avoid life-threatening hemorrhage. However, a proportion of patients with TP is refractory and may not respond to aggressive immunotherapy, which instead makes the patients vulnerable to severe infections and drug toxicity. Therefore, it is clinically important to identify predicting factors for therapeutic response and avoid unnecessary and inefficient treatment regimens. Platelets are produced by MK, and TPO is a key growth factor released by MK. An effective TPO concentration is crucial for megakaryopoiesis^{20,21}. TPO binds to its receptor, c-Mpl, and stimulates a downstream signaling cascade, which in turn promotes the development and clonal proliferation and maturation of MK, leading to the final release of platelets^{22,23,24}. Numerous autoantibodies were identified in the peripheral circulation

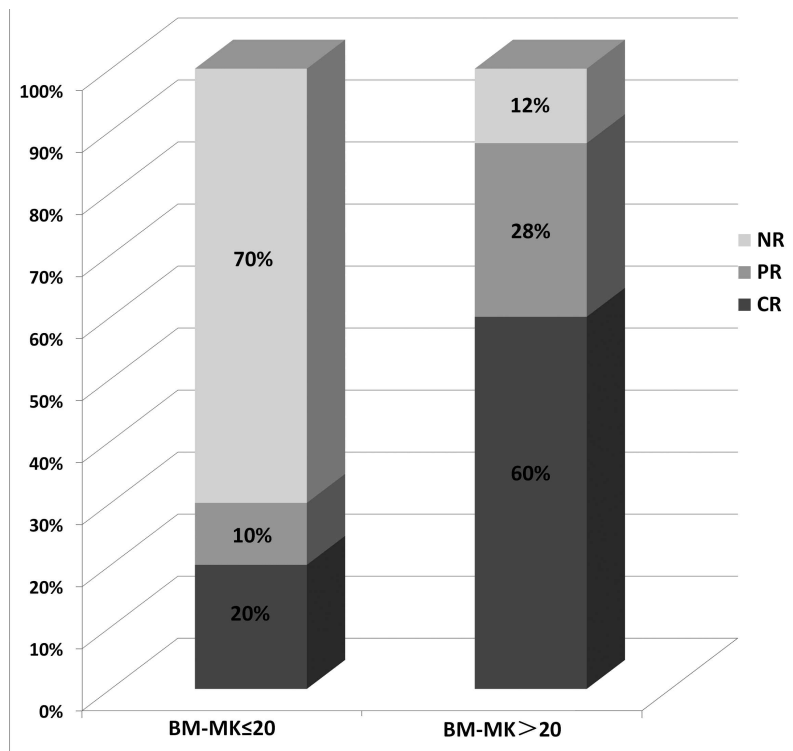


Figure 2. Clinical responses of patients when stratified with megakaryocytes counts in bone marrow (BM-MK counts ≤ 20 vs > 20). CR: complete response; PR: partial response; NR: no response; BM-MK: megakaryocytes in bone marrow.

of patients with SLE that can lead to platelet destruction and MK hypoplasia^{25,26,27}. Autoantibodies that are involved in the pathological mechanisms related to TP include PAIgG, anti-CD40 ligand (anti-CD40L), anti-GPIIB/IIIA, anti-TPO, and anti-c-Mpl antibodies^{28,29,30,31}. PAIgG and anti-CD40L contribute to the peripheral destruction of platelets without affecting the production of platelets in bone marrow. Usually these patients had increased or unaltered BM-MK and exhibited a good response to steroid therapy³⁰. However, anti-TPO and anti-c-Mpl antibodies may be involved in amegakaryocytic TP (AMT)^{25,30,31,32}, which correlates to the inhibition of MK clone formation and maturation and decreased MK in bone marrow^{31,33}. It has been reported that patients with anti-c-Mpl antibodies exhibited AMT and significantly poorer response to steroids and IVIG²⁵. The above findings suggest that changes in BM-MK represent a different mechanism of TP and that BM-MK may act as a predictive marker of therapeutic response. Increased MK counts may indicate a better clinical response to immunotherapy, whereas reduced number of MK suggests a poor response.

Our cohort excluded patients with complex factors, such as TTP, APS, HPS, MDS, and AA. Only patients with appreciable bone marrow smear results and detailed MK information were included because we planned to focus on BM-MK. All patients received high-dose steroids and single or combined immunosuppressive agents, including RTX

treatment in 9 patients. The results showed that patients with positive clinical responses had higher BM-MK counts than those who did not respond, and this difference was statistically significant. Examinations of anti-TPO and anti-c-Mpl antibodies were not routinely performed. Therefore, we did not have the data to evaluate the correlations between reduced BM-MK counts and these autoantibodies. Therapeutic intensity was comparable among the 3 groups with different clinical responses, or even more aggressive therapy was given to patients in the NR group. Consistent with our hypothesis, reduced MK counts in bone marrow were associated with poor response to treatment. Our study also found that the ROC analysis with a cutoff value of 20 BM-MK performed well in discriminating between responders and nonresponders, with sensitivity and specificity of 88% and 70%, respectively. A logistic stepwise regression analysis demonstrated that only stratification based on the number of BM-MK, but not the type of treatment intervention, was indicative of clinical response. Because TPO-receptor agonist drugs such as romiplostim and eltrombopag are promising effective treatments in refractory TP and AMT in patients with SLE^{34,35}, those with severe TP who have significantly decreased BM-MK should receive TPO-receptor agonist treatment more readily.

Our study had limitations. It was retrospective, and only patients with appreciable bone marrow results were included; thus selection bias is possible. The followup time was also

short and varied from patient to patient. We did not stratify patients according to the treatment regimens they received because of the limited sample sizes. TP attributable to AMT in patients with SLE that is resistant to routine therapeutic measures may respond to RTX^{18,36,37}, but the benefits of RTX treatment were not overwhelmingly significant in our study.

Our study suggests that the number of BM-MK may be a good predictor of patients with SLE's response to immunotherapy. Multi-loci sampling of bone marrow may be required for better evaluations because MK counts in bone marrow may not be uniformly distributed.

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